

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). De-
10 spite evidence that the inclusion of coupled carbon and nutrient cycles can reduce
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 framework for predicting leaf and whole plant acclimation re-
37 sponds to environmental change. The theory, which unifies optimal coordination
38 (Chen et al. 1993; Maire et al. 2012) and least-cost (Wright et al. 2003) theories,
39 posits that plants optimize photosynthetic processes by minimizing the summed
40 cost of nutrient and water use (i.e., β). The summed cost of nutrient and water
41 use is predicted to be positively correlated with the ratio of intercellular CO₂ to
42 atmospheric CO₂ (leaf C_i:C_a). Leaf C_i:C_a is determined by factors that influ-
43 ence leaf nutrient demand, such as CO₂, temperature, vapor pressure deficit, and
44 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 [DWS: a ton of passive voice through here]

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

72 dynamically change across environments and in species with different nutrient
73 acquisition strategies.

74 [DWS: Sort of fuzzy on what source of variation and “change” is... eco-
75 typic? plastic?]

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

86 In this dissertation, I use a combination of greenhouse, field manipula-
87 tion, environmental gradient, and growth chamber experiments to quantify leaf
88 and whole plant acclimation responses across various climatic and edaphic con-
89 ditions and [DWS; species represnting?] different nutrient acquisition strategies.
90 Together, these experiments evaluate patterns expected from photosynthetic least-
91 cost theory and test mechanisms predicted to drive responses expected from the-
92 ory. The empirical data collected in these experiments provide important infor-
93 mation needed to refine existing optimality models that include photosynthetic
94 least-cost frameworks, and could help determine whether such models are suitable
95 for implementing in next-generation terrestrial biosphere models. While theory

96 suggests that plants acclimate across environments by minimizing the summed
97 cost of nutrients relative to water, I chose to isolate effects of soil nitrogen avail-
98 ability on costs of nitrogen acquisition relative to water for the sake of brevity. I
99 acknowledge that patterns expected from theory may be modified by other nu-
100 trients (e.g., phosphorus) or other edaphic characteristics (Smith et al. 2019;
101 Paillassa et al. 2020; Westerband et al. 2023), and, though not included here,
102 should also be investigated.

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

118 Across experiments, I find strong and consistent support for patterns ex-
119 pected from photosynthetic least-cost theory, showing that shifts in edaphic char-

120 characteristics predictably alter β , and that shifts in β facilitate changes in leaf
121 nitrogen-water use tradeoffs and leaf nitrogen-photosynthesis relationships. I also
122 show that costs of nitrogen acquisition vary in species with different nitrogen
123 acquisition strategies. Finally, I show strong evidence suggesting that leaf accli-
124 mation responses to elevated CO₂ are decoupled from soil nitrogen availability and
125 inoculation with symbiotic nitrogen-fixing bacteria. It is my hope that these ex-
126 periments will encourage future iterations of optimality models that adopt photo-
127 synthetic least-cost frameworks to consider frameworks for implementing dynamic
128 β values across soil resource availability gradients and in species with different nu-
129 trient acquisition strategies.

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

138

Chapter 2

139

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

142 Perkowski EA, EF Waring, NG Smith, "Root mass carbon costs to acquire nitro-
143 gen are determined by nitrogen and light availability in two species with different
144 nitrogen acquisition strategies", *Journal of Experimental Botany*, 2021, Volume
145 72, Issue 15, Pages 5766-5776, by permission of Oxford University Press

146 2.1 Introduction

147 Carbon and nitrogen cycles are tightly coupled in terrestrial ecosystems. This
148 tight coupling influences photosynthesis (Walker et al. 2014; Rogers et al. 2017),
149 net primary productivity (LeBauer and Treseder 2008; Thomas et al. 2013), de-
150 composition (Cornwell et al. 2008; Bonan et al. 2013; Sulman et al. 2019), and
151 plant resource competition (Gill and Finzi 2016; Xu-Ri and Prentice 2017). Ter-
152 restrial biosphere models are beginning to include connected carbon and nitrogen
153 cycles to improve the realism of their simulations (Fisher et al. 2010; Brzostek
154 et al. 2014; Wieder et al. 2015; Shi et al. 2016; Zhu et al. 2019). Simula-
155 tions from these models indicate that coupling carbon and nitrogen cycles can
156 drastically influence future biosphere-atmosphere feedbacks under global change,
157 such as elevated carbon dioxide or nitrogen deposition (Thornton et al. 2007;
158 Goll et al. 2012; Wieder et al. 2015; Wieder et al. 2019). Nonetheless, there
159 are still limitations in our quantitative understanding of connected carbon and
160 nitrogen dynamics (Thomas et al. 2015; Meyerholt et al. 2016; Rogers et al.
161 2017; Exbrayat et al. 2018; Shi et al. 2019), forcing models to make potentially
162 unreliable assumptions.

163 Plant nitrogen acquisition is a process in terrestrial ecosystems by which
164 carbon and nitrogen are tightly coupled (Vitousek and Howarth 1991; Delaire
165 et al. 2005; Brzostek et al. 2014) [dWS: redundant with above]. Plants must
166 allocate photosynthetically derived [DWS: as opposed to?] carbon belowground
167 to produce and maintain root systems or exchange with symbiotic soil microbes in
168 order to acquire nitrogen (Högberg et al. 2008; Högberg et al. 2010). Thus, plants
169 have an inherent carbon cost associated with acquiring nitrogen, which can include
170 both direct energetic costs associated with nitrogen acquisition and indirect costs
171 associated with building structures that support nitrogen acquisition (Gutschick
172 1981; Rastetter et al. 2001; Vitousek et al. 2002; Menge et al. 2008). [DWS: You
173 make this sound more complicated than it is. It is just good old allocation]. Model
174 simulations (Fisher et al. 2010; Brzostek et al. 2014; Shi et al. 2016; Allen et al.
175 2020) and meta-analyses (Terrer et al. 2018) suggest that these carbon costs vary
176 between species, particularly those with different nitrogen acquisition strategies.
177 For example, simulations using iterations of the Fixation and Uptake of Nitrogen
178 (FUN) model indicate that species that acquire nitrogen from non-symbiotic active
179 uptake pathways (e.g. mass flow) generally have larger carbon costs to acquire
180 nitrogen than species that acquire nitrogen through symbiotic associations with
181 nitrogen-fixing bacteria (Brzostek et al. 2014; Allen et al. 2020).

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

187 nitrogen acquisition is through symbiotic active uptake, then nitrogen availabil-
188 ity may incur additional carbon costs to acquire nitrogen if it causes microbial
189 symbionts to shift toward parasitism along the parasitism–mutualism continuum
190 (Johnson et al. 1997; Hoek et al. 2016; Friel and Friesen 2019) or if it reduces
191 the nitrogen acquisition capacity of a microbial symbiont (van Diepen et al. 2007;
192 Soudzilovskaia et al. 2015; Muñoz et al. 2016). Species may respond to shifts in
193 soil nitrogen availability by switching their primary mode of nitrogen acquisition
194 to a strategy with lower carbon costs to acquire nitrogen in order to maximize
195 the magnitude of nitrogen acquired from a belowground carbon investment and
196 outcompete other individuals for soil resources (Rastetter et al. 2001; Menge et al.
197 2008).

198 Environmental conditions that affect demand to acquire nitrogen to sup-
199 port new and existing tissues could also be a source of variance in plant carbon
200 costs to acquire nitrogen [DWS: Awkward sentence with too many prepositions.
201 Watch out for this throughout]. For example, an increase in plant nitrogen de-
202 mand could increase carbon costs to acquire nitrogen if this increases the carbon
203 that must be allocated belowground to acquire a proportional amount of nitrogen
204 (Kulmatiski et al. 2017; Noyce et al. 2019). This could be driven by a temporary
205 state of diminishing return associated with investing carbon toward building and
206 maintaining structures that are necessary to support enhanced nitrogen uptake,
207 such as fine roots (Matamala and Schlesinger 2000; Norby et al. 2004; Arndal
208 et al. 2018), mycorrhizal hyphae (Saleh et al. 2020), or root nodules (Parvin et al.
209 2020). Alternatively, if the environmental factor that increases plant nitrogen de-
210 mand causes nitrogen to become more limiting in the system (e.g. atmospheric

211 CO₂) (Luo et al. 2004; LeBauer and Treseder 2008; Vitousek et al. 2010; Liang
212 et al. 2016), species might switch their primary mode of nitrogen acquisition to
213 a strategy with lower relative carbon costs to acquire nitrogen in order to gain a
214 competitive advantage over species with either different or more limited modes of
215 nitrogen acquisition (Ainsworth and Long 2005; Taylor and Menge 2018).

216 Using a plant economics approach, I examined the influence of plant ni-
217 trogen demand and soil nitrogen availability on plant carbon costs to acquire
218 nitrogen. This was done by growing a species capable of forming associations
219 with nitrogen-fixing bacteria (*Glycine max* L. (Merr)) and a species not capable
220 of forming these associations (*Gossypium hirsutum* L.) under four levels of light
221 availability (plant nitrogen demand proxy) and four levels of soil nitrogen fertil-
222 ization (soil nitrogen availability proxy) in a full-factorial, controlled greenhouse
223 experiment. [DWS: these species come out of nowhere? Why these two? Seems
224 random. I mean they are both rosids but otherwise? What can one learn by just
225 growing two random species out of hundreds of thousands? You need to justify
226 this or it is a huge surprise..]

227 I used this experimental set-up to test the following hypotheses:

228 1. An increase in plant nitrogen demand due to increasing light availability will
229 increase carbon costs to acquire nitrogen through a proportionally larger
230 increase in belowground carbon than whole-plant nitrogen acquisition. This
231 will be the result of an increased investment of carbon toward belowground
232 structures that support enhanced nitrogen uptake, but at a lower nitrogen
233 return.

234 2. An increase in soil nitrogen availability will decrease carbon costs to acquire
235 nitrogen as a result of increased per root nitrogen uptake in *G. hirsutum*.
236 However, soil nitrogen availability will not affect carbon costs to acquire
237 nitrogen in *G. max* because of the already high return of nitrogen supplied
238 through nitrogen fixation.

239 2.2 Methods

240 2.2.1 *Experiment setup*

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

251 Three blocks were set up in the greenhouse, each containing four light
252 treatments created using shade cloth that reduced incoming radiation by either 0
253 (full sun), 30, 50, or 80%. Two weeks post-germination, individuals were randomly
254 placed in the four light treatments in each block. Individuals received one of four
255 nitrogen fertilization doses as 100mL of a modified Hoagland solution (Hoagland
256 and Arnon 1950) equivalent to either 0, 70, 210, or 630 ppm N twice per week

257 within each light treatment. Nitrogen fertilization doses were received as topical
258 agents to the soil surface. Each Hoagland solution was modified to keep concen-
259 trations of other macro- and micronutrients equivalent (Table A1). Plants were
260 routinely well watered to eliminate water stress.

261 2.2.2 *Plant measurements and calculations*

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

268 All harvested material was dried, weighed, and ground by organ type.
269 Carbon and nitrogen content (g g^{-1}) was determined by subsampling from ground
270 and homogenized biomass of each organ type using an elemental analyzer (Costech
271 4010; Costech, Inc., Valencia, CA, USA). I scaled these values to total leaf, stem,
272 and root carbon and nitrogen biomass (g) by multiplying dry biomass of each
273 organ type by carbon or nitrogen content of each corresponding organ type. Whole
274 plant nitrogen biomass (g) was calculated as the sum of total leaf (g), stem (g),
275 and root (g) nitrogen biomass. Root nodule carbon biomass was not included in
276 the calculation of root carbon biomass; however, relative plant investment toward
277 root or root nodule standing stock was estimated as the ratio of root biomass to
278 root nodule biomass (g g^{-1}), following similar metrics to those adopted by Dovrat
279 et al. (2018) and Dovrat et al. (2020).

280 Carbon costs to acquire nitrogen (N_{cost} ; gC gN⁻¹) were estimated as the
281 ratio of total root carbon biomass (C_{bg} ; gC) to whole-plant nitrogen biomass
282 (N_{wp} ; gN). This calculation quantifies the relationship between carbon spent on
283 nitrogen acquisition and whole plant nitrogen acquisition by using root carbon
284 biomass as a proxy for estimating the magnitude of carbon allocated toward ni-
285 trogen acquisition. This calculation therefore assumes that the magnitude of root
286 carbon standing stock is proportional to carbon transferred to root nodules or my-
287 corrhizae, or lost through root exudation or turnover. The assumption has been
288 supported in species that associate with ectomycorrhizal fungi (Hobbie 2006; Hob-
289 bie and Hobbie 2008), but is less clear in species that acquire nitrogen through
290 non-symbiotic active uptake or symbiotic nitrogen fixation. It is also unclear
291 whether relationships between root carbon standing stock and carbon transfer to
292 root nodules are similar in magnitude to carbon lost through exudation or when
293 allocated toward other active uptake pathways. Thus, because of the way mea-
294 surements were calculated, proximal values of carbon costs to acquire nitrogen are
295 underestimates.

296 2.2.3 *Statistical analyses*

297 I explored the effects of light and nitrogen availability on carbon costs to acquire
298 nitrogen using separate linear mixed-effects models for each species. Models in-
299 cluded shade cover, nitrogen fertilization, and interactions between shade cover
300 and nitrogen fertilization as continuous fixed effects, and also included block as a
301 random intercept term. Three separate models for each species were built with
302 this independent variable structure for three different dependent variables: (i)

303 carbon costs to acquire nitrogen (gC gN^{-1}); (ii) whole plant nitrogen biomass
304 (denominator of carbon cost to acquire nitrogen; gN); and (iii) belowground car-
305 bon biomass (numerator of carbon cost to acquire nitrogen; gC). I constructed two
306 additional models for *G. max* with the same model structure described above to
307 investigate the effects of light availability and nitrogen fertilization on root nodule
308 biomass (g) and the ratio of root nodule biomass to root biomass (unitless).

309 I used Shapiro–Wilk tests of normality to determine whether species spe-
310 cific linear mixed-effects model residuals followed a normal distribution. Zero
311 models satisfied residual normality assumptions when models were fit using un-
312 transformed data (Shapiro–Wilk: $p < 0.05$ in all cases). I attempted to satisfy
313 residual normality assumptions by first fitting models using dependent variables
314 that were natural-log transformed. If residual normality assumptions were still
315 not met (Shapiro–Wilk: $p < 0.05$), then models were fit using dependent variables
316 that were square root transformed. All residual normality assumptions were satis-
317 fied when models were fit with either a natural-log or square root transformation
318 (Shapiro–Wilk: $p > 0.05$ in all cases). Specifically, I natural-log transformed *G.*
319 *hirsutum* carbon costs to acquire nitrogen and *G. hirsutum* whole-plant nitrogen
320 biomass. I also square root transformed *G. max* carbon costs to acquire nitrogen,
321 *G. max* whole-plant nitrogen biomass, root carbon biomass in both species, *G.*
322 *max* root nodule biomass, and the *G. max* ratio of root nodule biomass to root
323 biomass. I used the ‘lmer’ function in the ‘lme4’ R package (Bates et al. 2015) to
324 fit each model and the ‘Anova’ function in the ‘car’ R package (Fox and Weisberg
325 2019) to calculate Wald’s χ^2 to determine the significance ($\alpha = 0.05$) of each fixed
326 effect coefficient. Finally, I used the ‘emmeans’ R package (Lenth 2019) to conduct

327 post-hoc comparisons of our treatment combinations using Tukey's tests. Degrees
328 of freedom for all Tukey's tests were approximated using the Kenward–Roger ap-
329 proach (Kenward and Roger 1997). All analyses and plots were conducted in R
330 version 4.0.1 (R Core Team 2021).

331 2.3 Results

332 2.3.1 *Carbon costs to acquire nitrogen*

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

337 Carbon costs to acquire nitrogen in *G. max* also increased with increasing
338 light availability ($p<0.001$, Table 2.1; Fig. 2.1) and decreased with increasing
339 nitrogen fertilization ($p<0.001$; Table 2.1; Fig. 2.1). There was no interaction
340 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

341 *Significance determined using Wald's χ^2 tests ($p=0.05$). P -values less than 0.05 are in bold and p -values between
 342 0.05 and 0.1 are italicized. Negative coefficients for light treatments indicate a positive effect of increasing light
 343 availability on all response variables, as light availability is treated as percent shade cover in all linear mixed-effects
 344 models.

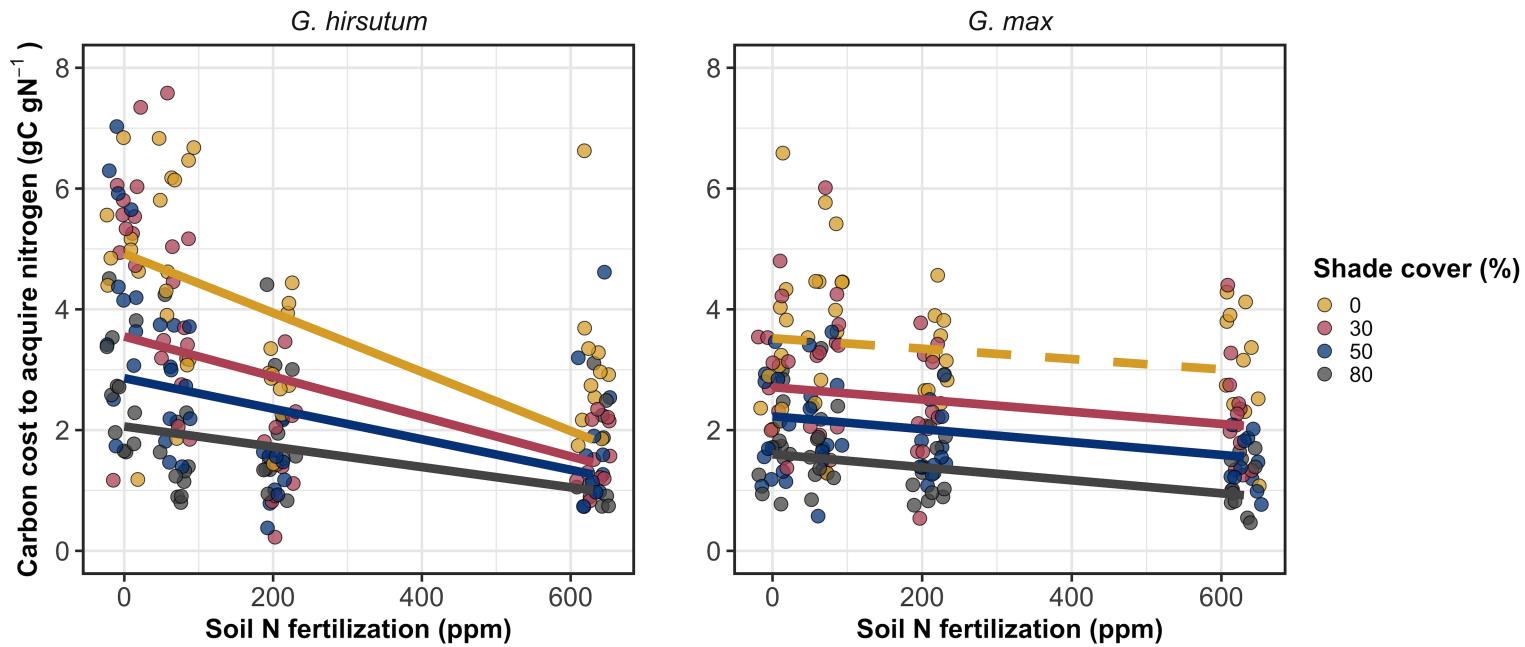


Figure 2.1. Relationships between soil nutrient fertilization and light availability on carbon costs to acquire nitrogen in *G. hirsutum* and *G. max*. Nitrogen fertilization treatments are represented on the x-axis. Shade cover treatments are represented through colored points and trendlines. Trendlines 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.

345 2.3.2 *Whole plant nitrogen biomass*

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

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

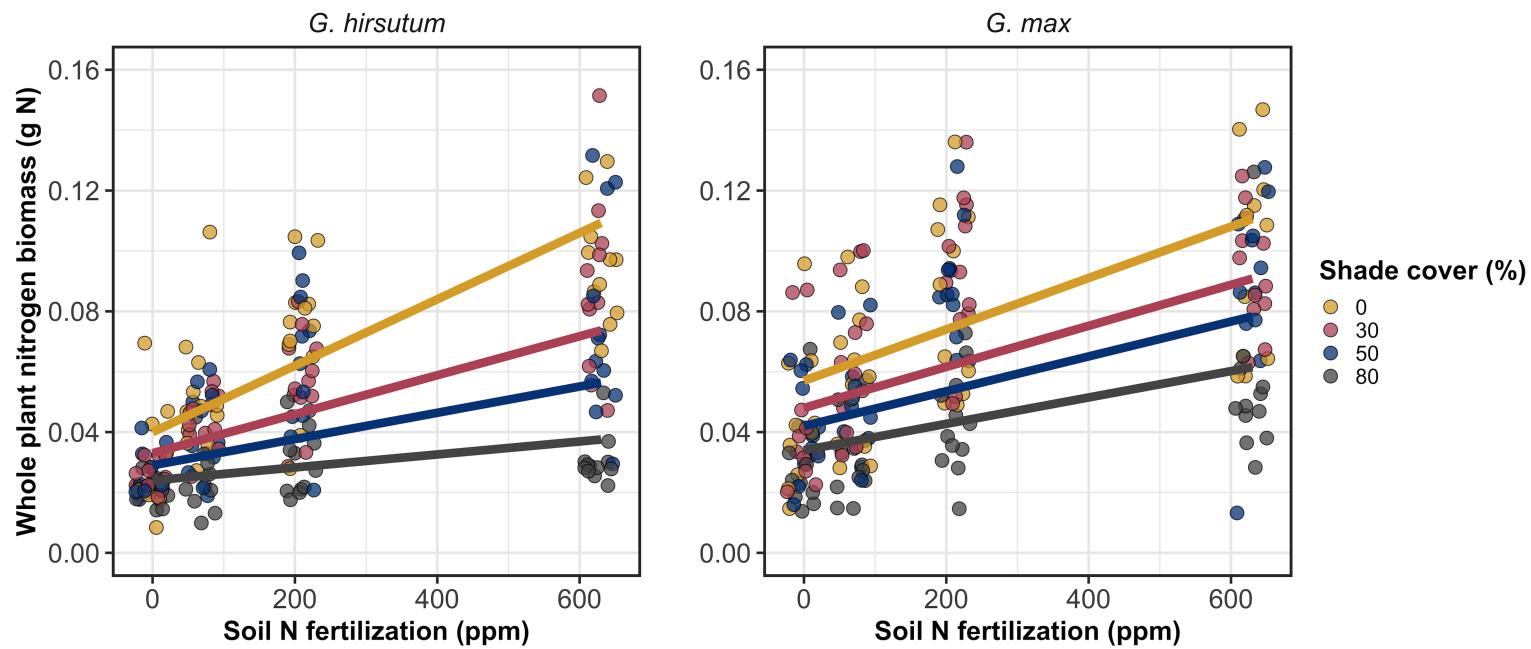


Figure 2.2. Relationships between soil nutrient fertilization and light availability on whole-plant nitrogen biomass in *G. hirsutum* and *G. max*. Whole-plant nitrogen biomass is the denominator of the carbon cost to acquire nitrogen calculation. Nitrogen fertilization treatments are represented on 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.

353 2.3.3 *Root carbon biomass*

354 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).

364 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).

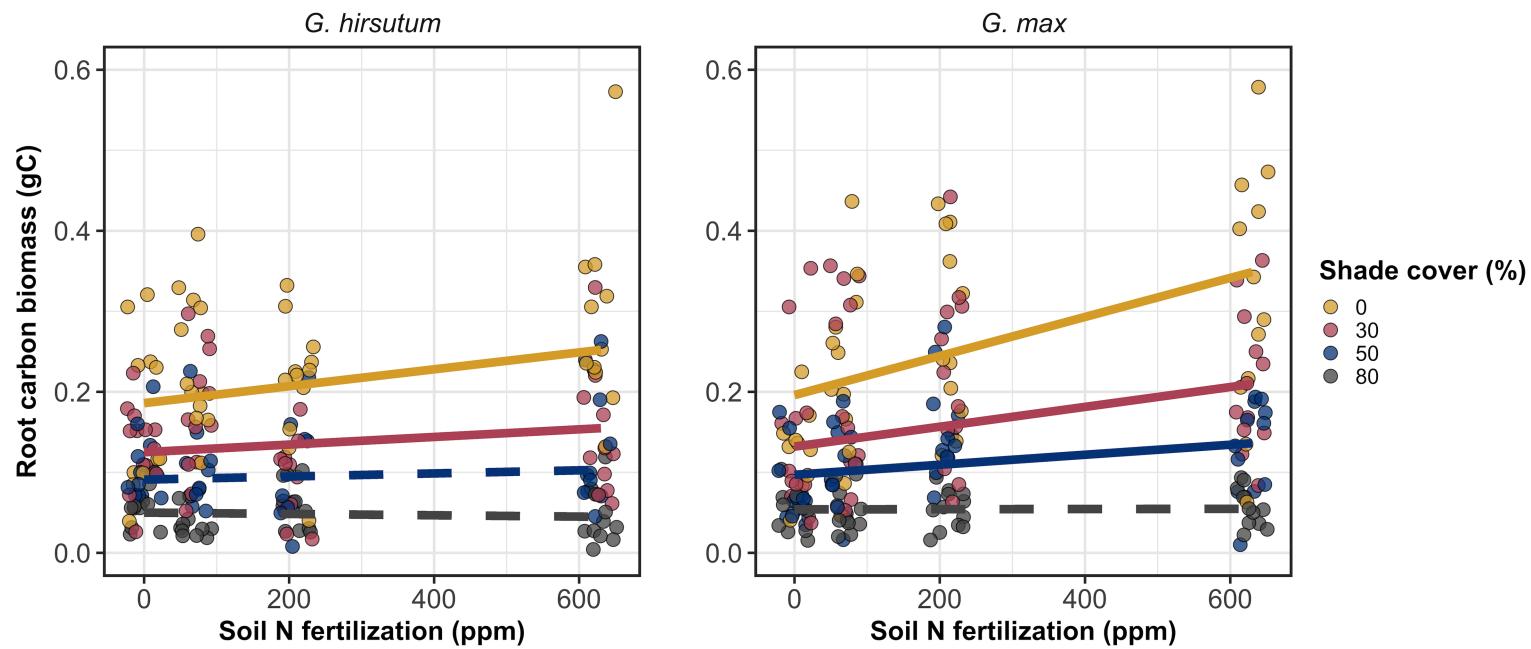


Figure 2.3. Relationships between soil nutrient fertilization and light availability on root carbon biomass in *G. hirsutum* and *G. max*. Root carbon biomass is the numerator of the carbon cost to acquire nitrogen calculation. Nitrogen fertilization treatments are represented on the x-axis. Colored 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.

371 2.3.4 *Root nodule biomass*

372 Root nodule biomass in *G. max* increased with increasing light availability ($p <$
373 0.001; Table 2.2; Fig. 2.4a) and decreased with increasing nitrogen fertilization
374 ($p < 0.001$; Table 2.2; Fig. 2.4a). There was no interaction between nitrogen
375 fertilization and light availability ($p = 0.133$; Table 2.2; Fig. 2.4a). The ratio of
376 root nodule biomass to root biomass did not change in response to light availability
377 ($p = 0.481$; Table 2.2; Fig. 2.4b) but decreased with increasing nitrogen fertilization
378 ($p < 0.001$; Table 2.2; Fig. 2.4b). There was no interaction between nitrogen
379 fertilization and light availability on the ratio of root nodule biomass to root
380 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

381 *Significance determined using Wald's χ^2 tests ($\alpha=0.05$). *P*-values less than 0.05 are in bold. Negative coefficients for
 382 light treatments indicate a positive effect of increasing light availability on all response variables, as light availability
 383 is treated as percent shade cover in all linear mixed-effects models. Root nodule biomass and nodule biomass: root
 384 biomass models were only constructed for *G. max* because *G. hirsutum* was not inoculated with *B. japonicum* and
 385 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

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

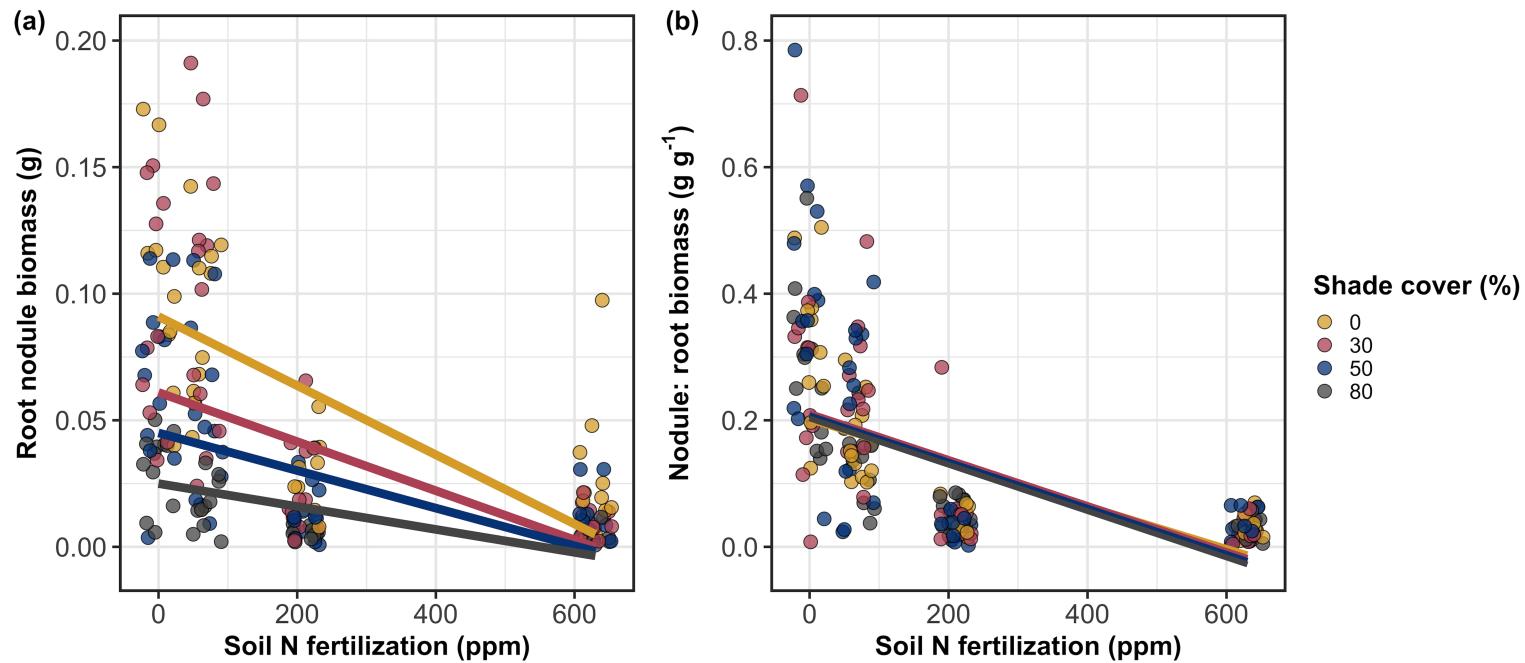


Figure 2.4. Effects of shade cover and nitrogen fertilization on root nodule biomass (a) and the ratio of root nodule biomass to root biomass (b) in *G. max*. Nitrogen fertilization treatments are represented on the x-axis. Shade cover treatments are represented through colored points and trendlines. 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.

390 2.4 Discussion

391 In this chapter, I determined the effects of light availability and soil nitrogen
392 fertilization on root mass carbon costs to acquire nitrogen in *G. hirsutum* and *G.*
393 *max*. In support of my hypotheses, I found that carbon costs to acquire nitrogen
394 generally increased with increasing light availability and decreased with increasing
395 soil nitrogen fertilization in both species. These findings suggest that carbon costs
396 to acquire nitrogen are determined by factors that influence plant nitrogen demand
397 and soil nitrogen availability. In contrast to my second hypothesis, root nodulation
398 data suggested that *G. max* and *G. hirsutum* achieved similar directional carbon
399 cost responses to nitrogen fertilization despite a likely shift in *G. max* allocation
400 from nodulation to root biomass along the nitrogen fertilization gradient.

401 2.4.1 *Carbon costs to acquire nitrogen increase with light availability and*
402 *decrease with fertilization*

403 Both *G. max* and *G. hirsutum* experienced an increase in carbon costs to ac-
404 quire nitrogen due to increasing light availability. These patterns were driven by
405 a larger increase in root carbon biomass than whole-plant nitrogen biomass. In-
406 creases in root carbon biomass due to factors that increase plant nitrogen demand
407 are a commonly observed pattern, as carbon allocated belowground provides sub-
408 strate needed to produce and maintain structures that satisfy aboveground plant
409 nitrogen demand (Nadelhoffer and Raich 1992; Giardina et al. 2005; Raich et al.
410 2014). Findings suggest that plants allocate relatively more carbon for acquiring
411 nitrogen when demand increases over short temporal scales, which may cause a
412 temporary state of diminishing return due to asynchrony between belowground

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

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

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

446 2.4.2 *Modeling implications*

447 Carbon costs to acquire nitrogen are subsumed [DWS: ?] in the general discussion
448 of economic analogies to plant resource uptake (Bloom et al. 1985; Rastetter et al.
449 2001; Vitousek et al. 2002; Phillips et al. 2013; Terrer et al. 2018; Henneron et al.
450 2020). Despite this, terrestrial biosphere models rarely include costs of nitrogen
451 acquisition to predict plant nitrogen uptake. There is currently one plant resource
452 uptake model, FUN [DWS: is this an acronym? Why caps?], that quantitatively
453 predicts carbon costs to acquire nitrogen within a framework for predicting plant
454 nitrogen uptake for different nitrogen acquisition strategies (Fisher et al. 2010;
455 Brzostek et al. 2014). Iterations of FUN are currently coupled to two terrestrial
456 biosphere models: the Community Land Model 5.0 and the Joint UK Land En-
457 vironment Simulator (Clark et al. 2011; Shi et al. 2016; Lawrence et al. 2019).
458 Recent work suggests that coupling FUN to CLM 5.0 caused a large overpredic-
459 tion of plant nitrogen uptake associated with nitrogen fixation (Davies-Barnard

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

465 These findings broadly [DWS: ? what would broad vs narrow support
466 mean here?] support the FUN formulation of carbon costs to acquire nitrogen in
467 response to soil nitrogen availability. FUN calculates carbon costs to acquire ni-
468 trogen based on the sum of carbon costs to acquire nitrogen via nitrogen fixation,
469 mycorrhizal active uptake, non-mycorrhizal active uptake, and retranslocation
470 (Fisher et al. 2010; Brzostek et al. 2014). Carbon costs to acquire nitrogen via
471 mycorrhizal or non-mycorrhizal active uptake pathways are derived as a function
472 of nitrogen availability, root biomass, and two parameterized values based on ni-
473 trogen acquisition strategy (Brzostek et al. 2014). Due to this, FUN simulates
474 a net decrease in carbon costs to acquire nitrogen with increasing nitrogen avail-
475 ability for mycorrhizal and non-mycorrhizal active uptake pathways, assuming
476 constant root biomass. This was a pattern I observed in *G. hirsutum* regardless
477 of light availability. In contrast, FUN would not simulate a net change in carbon
478 costs to acquire nitrogen via nitrogen fixation due to nitrogen availability. This
479 is because carbon costs to acquire nitrogen via nitrogen fixation are derived from
480 a well established function of soil temperature, which is independent of soil ni-
481 trogen availability (Houlton et al. 2008; Fisher et al. 2010). I observed a net
482 reduction in carbon costs to acquire nitrogen in *G. max*, except when individu-
483 als were grown under 0% shade cover. While a net reduction of carbon costs in

484 response to nitrogen fertilization runs counter to nitrogen fixation carbon costs
485 simulated by FUN, these patterns were likely because *G. max* individuals switched
486 their primary mode of nitrogen acquisition from symbiotic nitrogen fixation to a
487 non-symbiotic active uptake pathway.

488 2.4.3 *Study limitations*

489 The metric used in this study to determine carbon costs to acquire nitrogen has
490 several limitations. Most notably, this metric uses root carbon biomass as a
491 proxy for estimating the amount of carbon spent on nitrogen acquisition. While
492 [although?] it is true that most carbon allocated belowground has at least an
493 indirect structural role in acquiring soil resources, it remains unclear whether this
494 assumption holds true for species that acquire nitrogen via symbiotic nitrogen fix-
495 ation. I also cannot quantify carbon lost through root exudates or root turnover,
496 which may increase due to factors that increase plant nitrogen demand (Tingey
497 et al. 2000; Phillips et al. 2011), and can increase the magnitude of available
498 nitrogen from soil organic matter through priming effects on soil microbial com-
499 munities (Uselman et al. 2000; Bengtson et al. 2012). It is also not clear whether
500 these assumptions hold under all environmental conditions, such as those that
501 shift belowground carbon allocation toward a different mode of nitrogen acqui-
502 sition (Taylor and Menge 2018; Friel and Friesen 2019) or between species with
503 different acquisition strategies. In this study, increasing soil nitrogen fertiliza-
504 tion increased carbon investment to roots relative to carbon transferred to root
505 nodules. By assuming that carbon allocated to root carbon was proportional to
506 carbon allocated to root nodules across all treatment combinations, these observed

507 responses to soil nitrogen fertilization were likely to be overestimated in *G. max*.
508 I encourage future research to quantify these carbon fates independently.

509 [DWS: also you looked at two species out of hundreds of thousands of
510 possible species! It is anecdotal]

511 Researchers conducting pot experiments must carefully choose pot volume
512 to minimize the likelihood of growth limitations induced by pot volume (Poorter
513 et al. 2012). Poorter et al. (2012) indicate that researchers are likely to avoid
514 growth limitations associated with pot volume if measurements are collected when
515 the plant biomass:pot volume ratio is less than 1 g L^{-1} . In this experiment, all
516 treatment combinations in both species had biomass:pot volume ratios less than
517 1 g L^{-1} except for *G. max* and *G. hirsutum* that were grown under 0% shade
518 cover and had received 630 ppm N. Specifically, *G. max* and *G. hirsutum* had
519 average respective biomass:pot volume ratios of $1.24 \pm 0.07 \text{ g L}^{-1}$ and 1.34 ± 0.13
520 g L^{-1} , when grown under 0% shade cover and received 630 ppm N (Table A2,
521 A3; Fig. A1). If growth in this treatment combination was limited by pot vol-
522 ume, then individuals may have had larger carbon costs to acquire nitrogen than
523 would be expected if they were grown in larger pots. This pot volume induced
524 growth limitation could cause a reduction in per-root nitrogen uptake associated
525 with more densely packed roots, which could reduce the positive effect of nitro-
526 gen fertilization on whole-plant nitrogen biomass relative to root carbon biomass
527 (Poorter et al. 2012).

528 Growth limitation associated with pot volume [DWS: Can you write this
529 directly? Eg: “small pots limited growth?” You litter your writing with these ver-
530 bose and awkward sentences/] provides a possible explanation for the marginally

531 insignificant effect of increasing nitrogen fertilization on *G. max* carbon costs to
532 acquire nitrogen when grown under 0% shade cover. This is because the regres-
533 sion line describing the relationship between carbon costs to acquire nitrogen and
534 nitrogen fertilization in *G. max* grown under 0% shade cover would have flattened
535 if growth limitation had caused larger than expected carbon costs to acquire ni-
536 trogen in the 0% shade cover, 630 ppm N treatment combination. This may have
537 been exacerbated by the fact that *G. max* likely shifted relative carbon allocation
538 from nitrogen fixation to soil nitrogen acquisition, which could have increased the
539 negative effect of more densely packed roots on nitrogen uptake. These patterns
540 could have also occurred in *G. hirsutum* grown under 0% shade cover; however,
541 there was no change in the effect of nitrogen fertilization on *G. hirsutum* carbon
542 costs to acquire nitrogen grown under 0% shade cover relative to other shade
543 cover treatments. Regardless, the possibility of growth limitation due to pot vol-
544 ume suggests that effects of increasing nitrogen fertilization on carbon costs to
545 acquire nitrogen in both species grown under 0% shade cover could have been un-
546 derestimated. Follow-up studies using a similar experimental design with a larger
547 pot volume would be necessary in order to determine whether these patterns were
548 impacted by pot volume-induced growth limitation.

549 2.4.4 *Conclusions*

550 In conclusion, this chapter provides empirical evidence that carbon costs to ac-
551 quire nitrogen are influenced by light availability and soil nitrogen fertilization
552 in a species capable of acquiring nitrogen via symbiotic nitrogen fixation and a
553 species not capable of forming such associations. We show that carbon costs to

554 acquire nitrogen generally increase with increasing light availability and decrease
555 with increasing nitrogen fertilization. This chapter provides important empirical
556 data needed to evaluate the formulation of carbon costs to acquire nitrogen in
557 terrestrial biosphere models, particularly carbon costs to acquire nitrogen that
558 are associated with symbiotic nitrogen fixation. Findings broadly support the
559 general formulation of these carbon costs in the FUN biogeochemical model in
560 response to shifts in nitrogen availability. However, there is a need for future
561 studies to explicitly quantify carbon costs to acquire nitrogen under different en-
562 vironmental contexts, over longer temporal scales, and using larger selections of
563 phylogenetically related species. In addition, I suggest that future studies mini-
564 mize the limitations associated with the metric used here by explicitly measuring
565 belowground carbon fates independently.

566

Chapter 3

567 Soil nitrogen availability modifies leaf nitrogen economies in mature
568 temperate deciduous forests: a direct test of photosynthetic least-cost
569 theory

570 3.1 Introduction

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

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

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

603 Recent work calls the generality of relationships between soil nutrient avail-
604 ability, leaf nutrient content, and photosynthetic capacity into question, suggest-
605 ing instead that leaf nutrients and photosynthetic capacity are better predicted as
606 an integrated product of aboveground climate, leaf traits, and soil nutrient avail-
607 ability, rather than soil nutrient availability alone (Dong et al. 2017; Dong et al.
608 2020; Dong et al. 2022; Firn et al. 2019; Smith et al. 2019; Peng et al. 2021).
609 It has been reasoned that this result is because plants allocate added nutrients to
610 growth and storage rather than alterations in leaf chemistry (Smith et al. 2019),
611 perhaps as a result of nutrient limitation of primary productivity (LeBauer and
612 Treseder 2008; Fay et al. 2015). Additionally, recent work suggests that relation-
613 ships between leaf nutrient content and photosynthesis vary across environments,

614 and that the proportion of leaf nutrient content allocated to photosynthetic tis-
615 sue varies over space and time with plant acclimation and adaptation responses
616 to light availability, vapor pressure deficit, soil pH, soil nutrient availability, and
617 environmental factors that influence leaf mass per area (Pons and Pearcy 1994;
618 Niinemets and Tenhunen 1997; Evans and Poorter 2001; Hikosaka and Shigeno
619 2009; Ghimire et al. 2017; Onoda et al. 2017; Luo et al. 2021). The use of linear
620 relationships between leaf nutrient content and V_{cmax} to predict photosynthetic
621 capacity, as commonly used in terrestrial biosphere models (Rogers 2014), is not
622 capable of detecting such responses.

623 Photosynthetic least-cost theory provides an alternative framework for un-
624 derstanding relationships between soil nutrient availability, leaf nutrient content,
625 and photosynthetic capacity (Harrison et al. 2021). Leveraging a two-input mi-
626 croeconomics approach (Wright et al. 2003), the theory posits that plants accli-
627 mate to a given environment by optimizing leaf photosynthesis rates at the lowest
628 summed cost of using nutrients and water (Prentice et al. 2014; Wang et al. 2017;
629 Smith et al. 2019; Paillassa et al. 2020). Across resource availability gradients,
630 the theory predicts that optimal photosynthetic rates can be achieved by trading
631 less efficient use of a resource that is less costly to acquire (or more abundant)
632 for more efficient use of a resource more costly to acquire (or less abundant). For
633 example, an increase in soil nutrient availability should reduce the cost of acquir-
634 ing and using nutrients (Bae et al. 2015; Eastman et al. 2021; Perkowski et al.
635 2021), which could increase leaf nutrient investments in photosynthetic proteins to
636 allow similar photosynthetic rates to be achieved with higher nutrient use (lower
637 nutrient use efficiency) but lower water use (greater water use efficiency). The

638 theory suggests similar tradeoffs in response to increasing soil pH (Paillassa et al.
639 2020), specifically, that increasing soil pH should reduce the cost of acquiring soil
640 nutrients due to an increase in plant-available nutrient concentration (Paillassa
641 et al. 2020; Dong et al. 2022). The theory is also capable of reconciling dynamic
642 leaf nutrient-photosynthesis relationships at global scales (Luo et al. 2021).

643 Patterns expected from photosynthetic least-cost theory have recently re-
644 ceived empirical support both in global environmental gradient (Smith et al.
645 2019; Paillassa et al. 2020; Luo et al. 2021; Querejeta et al. 2022; Wester-
646 band et al. 2023) and local manipulative invasion (Bialic-Murphy et al. 2021)
647 studies. However, nutrient addition experiments that directly examine nutrient-
648 water use tradeoffs expected from the theory are rare (but see Guerrieri et al.
649 2011), and only global gradient studies testing the theory have considered soil pH
650 in their analyses. As a result, there is a need to use nutrient addition and soil pH
651 manipulation experiments to test mechanisms driving responses predicted by the
652 theory.

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

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

668 3.2 Methods

669 3.2.1 *Study site description*

670 I conducted this study in summer 2019 at three stands located within a 20-km ra-
671 dius of Ithaca, NY, USA (42.444 °N, 76.502 °W). All stands contain mature,
672 closed-canopy forests dominated by deciduous tree species. Stands contained
673 abundant sugar maple (*Acer saccharum* Marshall), American beech (*Fagus gran-*
674 *difolia* Ehrh.), and white ash (*Fraxinus americana* L.), accounting for 43%, 15%,
675 and 17% of the total aboveground biomass across the three stands, respectively,
676 with less frequent red maple (*Acer rubrum* L.; 9% of total aboveground biomass)
677 and red oak occurrences (*Quercus rubra* L.; 10% of total aboveground biomass).
678 Soils at each site were broadly classified as a channery silt loam Inceptisols using
679 the USDA NRCS Web Soil Survey data product (Soil Survey Staff 2022). Between
680 2006 and 2020, study sites averaged 972 mm of precipitation per year and had an
681 average temperature of 7.9 °C per a weather station located near the Cornell Uni-
682 versity campus (42.449 °N, 76.449 °W) part of the NOAA NCEI Global Historical
683 Climatology Network (Menne et al. 2012).

684 3.2.2 *Experimental design*

685 Four 40 m x 40 m plots were set up at each site in 2009, each with an additional
686 10 m buffer along plot perimeters (60 m x 60 m total). The plots were set up as a
687 nitrogen-by-pH field manipulation experiment, with one each of four treatments
688 at each site. Two nitrogen treatments were applied, both at $50 \text{ kg N ha}^{-1} \text{ yr}^{-1}$, as
689 either sodium nitrate (NaNO_3) to raise soil pH, or ammonium sulfate ($(\text{NH}_4)_2\text{SO}_4$)
690 to acidify; an elemental sulfur treatment was selected to acidify without nitrogen,
691 applied at the same rate of S addition ($57 \text{ kg S ha}^{-1} \text{ yr}^{-1}$); and control plots
692 received no additions. All amendments were added in pelletized form using hand-
693 held fertilizer spreaders to both the main plots and buffers. Amendments were
694 divided into three equal doses distributed across the growing season from 2011-
695 2017 and added as a single dose from 2018 onward. During 2019, plots were
696 fertilized during the week of May 20.

697 3.2.3 *Leaf gas exchange and trait measurements*

698 I sampled one leaf each from 6 to 10 individuals per plot between June 25 and July
699 12, 2019 for gas exchange measurements (Table B1). Leaves were collected from
700 deciduous broadleaf trees represented across all sites and plots and were replicated
701 in efforts to mimic the species abundance of each plot at each site. I attempted
702 to collect leaves from the upper canopy to reduce differential shading effects on
703 leaf physiology. Leaves were accessed by pulling down small branches using an
704 arborist's slingshot and weighted beanbag attached to a throw line. Branches
705 were immediately recut under deionized water and remained submerged to reduce
706 stomatal closure and avoid xylem embolism, as done in Smith and Dukes (2018),

707 until gas exchange data were collected.

708 Randomly selected leaves with little to no visible external damage were
709 attached to a Li-COR LI-6800 (Li-COR Bioscience, Lincoln, Nebraska, USA)
710 portable photosynthesis machine to measure net photosynthesis (A_{net} ; $\mu\text{mol m}^{-2}$
711 s^{-1}), stomatal conductance (g_{sw} ; $\text{mol m}^{-2} \text{s}^{-1}$), and intercellular CO_2 concentra-
712 tion (C_i ; $\mu\text{mol mol}^{-1}$) at different reference CO_2 concentrations (C_a ; $\mu\text{mol mol}^{-1}$)
713 concentrations (i.e., an A_{net}/C_i curve) under saturating light conditions (2,000
714 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Reference CO_2 concentrations followed the sequence: 400, 300,
715 200, 100, 50, 400, 400, 600, 800, 1000, 1200, 1500, and 2000 $\mu\text{mol mol}^{-1} \text{CO}_2$. Leaf
716 temperatures were not controlled in the cuvette and ranged from 21.8 °C to 31.7
717 °C (mean±SD: 27.2±2.2 °C). A linear and second order log-polynomial nonlinear
718 regression suggested no effect of temperature on stomatal conductance measured
719 at 400 $\mu\text{mol mol}^{-1} \text{CO}_2$ or net photosynthesis measured at 400 $\mu\text{mol mol}^{-1} \text{CO}_2$
720 (Table B2, B3; Fig. B1). All A_{net}/C_i curves were generated within one hour of
721 branch severance.

722 Leaf morphological and chemical traits were collected on the same leaf used
723 to generate each A_{net}/C_i curve. Images of each leaf were taken using a flat-bed
724 scanner to determine fresh leaf area using the ‘LeafArea’ R package (Katabuchi
725 2015), which automates leaf area calculations using ImageJ software (Schneider
726 et al. 2012). Each leaf was dried at 65°C for at least 48 hours, weighed, and
727 ground using a Retsch MM200 ball mill grinder (Verder Scientific, Inc., Newtown,
728 PA, USA) until homogenized. Leaf mass per unit leaf area (M_{area} , g m^{-2}) was
729 calculated as the ratio of dry leaf biomass to fresh leaf area. Using a subsample
730 of ground and homogenized leaf biomass, leaf nitrogen content (N_{mass} ; gN g^{-1})

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

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

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

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

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

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

749 3.2.4 A_{net}/C_i curve-fitting and parameter estimation

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

758 I determined Michaelis-Menten coefficients for Rubisco affinity to CO₂ (K_c ;
759 $\mu\text{mol mol}^{-1}$) and O₂ (K_o ; $\mu\text{mol mol}^{-1}$), and the CO₂ compensation point (Γ^* ;
760 $\mu\text{mol mol}^{-1}$) using leaf temperature and equations described in Medlyn et al.
761 (2002) and derived in Bernacchi et al. (2001). Specifically, K_c and K_o were
762 calculated as:

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

763 and

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

764 while Γ^* was calculated as:

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

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

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

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

768 nius equation (Kattge and Knorr 2007):

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

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

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

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

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

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

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

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

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

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

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

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

779 and

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

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

784 3.2.5 *Proportion of leaf nitrogen allocated to photosynthesis and structure*

785 I used equations from Niinemets and Tenhunen (1997) to estimate the proportion
786 of leaf nitrogen content allocated to Rubisco and bioenergetics. The proportion of
787 leaf nitrogen allocated to Rubisco ($\rho_{rubisco}$; gN gN $^{-1}$) was calculated as a function
788 of V_{cmax25} and N_{area} :

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

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

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

794 where N_b is the amount of nitrogen in cytochrome f, set to 0.12407 gN (μ mol
795 cytochrome f) $^{-1}$ assuming a constant 1: 1: 1.2 cytochrome f: ferredoxin NADP
796 reductase: coupling factor molar ratio (Evans and Seemann 1989; Niinemets and
797 Tenhunen 1997), and J_{mc} is the capacity of electron transport per cytochrome f,

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

799 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
800 is an underestimate of the proportion of leaf nitrogen allocated to photosynthetic
801 tissue because it does not include nitrogen allocated to light harvesting proteins.
802 This leaf nitrogen pool was not included because I did not perform chlorophyll
803 extractions on focal leaves. However, the proportion of leaf nitrogen content al-
804 located to light harvesting proteins tends to be small relative to ρ_{rubisco} and ρ_{bioe} ,
805 and may scale with changes in ρ_{rubisco} and ρ_{bioe} (Niinemets and Tenhunen 1997).

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

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

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

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

811 3.2.6 *Tradeoffs between nitrogen and water use*

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

816 availability and pH as intrinsic water use efficiency measured from gas exchange
817 ($A_{\text{net}}/g_{\text{sw}}$). Tradeoffs between nitrogen and water use were determined by cal-
818 culating the ratio of N_{area} to χ ($N_{\text{area}}:\chi$; gN m⁻²) and V_{cmax25} to χ ($V_{\text{cmax25}}:\chi$;
819 $\mu\text{mol m}^{-2} \text{ s}^{-1}$). This approach is similar to tradeoff calculations in which nitrogen-
820 water use tradeoffs are measured as the ratio of N_{area} or V_{cmax25} to g_{sw} (Paillassa
821 et al. 2020; Bialic-Murphy et al. 2021). In this chapter, I quantify these rela-
822 tionships using χ in lieu of g_{sw} because g_{sw} rapidly changes with environmental
823 conditions and therefore may have been altered by recent tree branch severance
824 and/or placement in the cuvette.

825 3.2.7 *Soil nitrogen availability and pH*

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

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

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

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

863 3.2.8 *Statistical analyses*

864 I built two separate series of linear mixed-effects models to explore effects of soil
865 nitrogen availability, soil pH, species, and leaf nitrogen content on leaf physiolog-
866 ical traits. In the first series of linear mixed-effects models, I explored the effect
867 of soil nitrogen availability, soil pH, and species on leaf nitrogen content, leaf
868 photosynthesis, stomatal conductance, and nitrogen-water use tradeoffs. Models
869 included plot-level soil nitrogen availability and plot-level soil pH as continuous
870 fixed effects, species as a categorical fixed effect, and site as a categorical ran-
871 dom intercept term. Interaction terms between fixed effects were not included
872 due to the small number of experimental plots. I built a series of separate mod-
873 els with this independent variable structure to quantify individual effects of soil
874 nitrogen availability, soil pH, and species on N_{area} , M_{area} , N_{mass} , A_{net} , V_{cmax25} ,
875 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
876 $V_{\text{cmax25}}:\chi$.

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

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

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

892 In the first series of models, models for N_{area} , M_{area} , N_{mass} , V_{cmax25} , J_{max25} ,
893 χ , $N_{\text{area}}:\chi$, and $V_{\text{cmax25}}:\chi$, ρ_{rubisco} , $\rho_{\text{bioenergetics}}$, ρ_{photo} , $\rho_{\text{structure}}$ satisfied residual
894 normality assumptions without data transformations (Shapiro-Wilk: $p>0.05$ in
895 all cases). The model for $J_{\text{max25}}:V_{\text{cmax25}}$ satisfied residual normality assumptions
896 with a natural log data transformation, while models for A_{net} and PNUE each
897 satisfied residual normality assumptions with square root data transformations.
898 In the second series of models, models for V_{cmax25} , J_{max25} , χ , and $V_{\text{cmax25}}:\chi$ satis-
899 fied residual normality assumptions without data transformations (Shapiro-Wilk:
900 $p>0.05$ in all cases). The model for $J_{\text{max25}}:V_{\text{cmax25}}$ required a natural log data
901 transformation and the model for A_{net} required a square root data transformation
902 (Shapiro-Wilk: $p>0.05$ in both cases).

903 In all models, I used the ‘lmer’ function in the ‘lme4’ R package (Bates
904 et al. 2015) to fit each model and the ‘Anova’ function in the ‘car’ R package
905 (Fox and Weisberg 2019) to calculate Type II Wald’s χ^2 and determine the signif-
906 icance level ($\alpha=0.05$) of each fixed effect coefficient. Finally, I used the ‘emmeans’
907 R package (Lenth, 2019) to conduct post-hoc comparisons using Tukey’s tests,
908 where degrees of freedom were approximated using the Kenward-Roger approach
909 (Kenward and Roger 1997). All analyses and plots were conducted in R version
910 4.1.1 (R Core Team 2021). All figure regression lines and associated 95% confi-

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

913 3.3 Results

914 3.3.1 *Leaf nitrogen content*

915 Increasing soil nitrogen availability generally increased N_{area} (Table 3.1; Fig. 3.1a).
916 This pattern was driven by an increase in N_{mass} (Table 3.1; Fig. 3.1c) and a
917 marginal increase in M_{area} (Table 3.1; Fig. 3.1e) with increasing soil nitrogen
918 availability. There was no effect of soil pH on N_{area} , N_{mass} , or M_{area} (Table 3.1);
919 however, I also observed strong differences in N_{area} (Fig. 3.1b), N_{mass} (Fig. 3.1d),
920 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

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

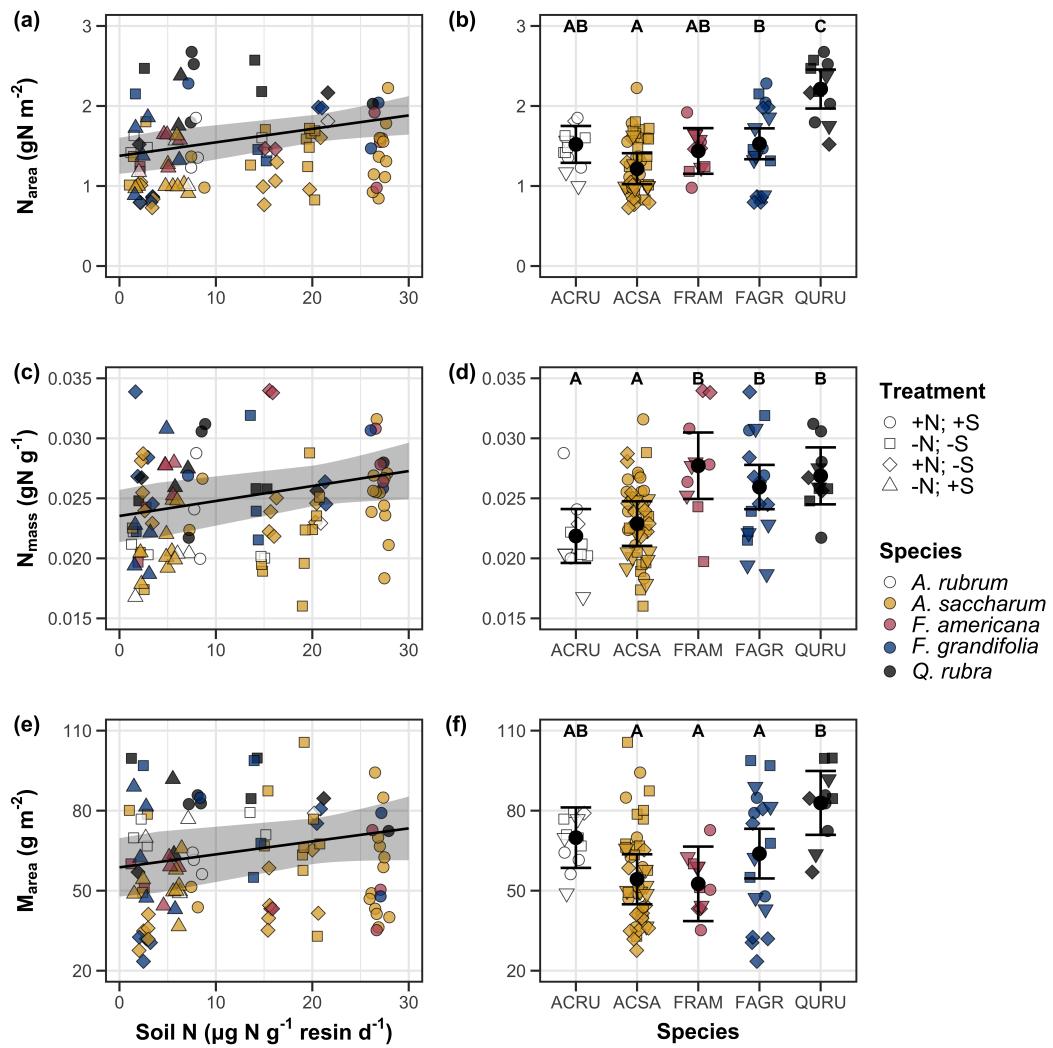


Figure 3.1. Effects of soil N availability and species on leaf nitrogen content per unit leaf area (a-b), leaf nitrogen content per unit leaf biomass (c-d), and leaf mass per unit leaf area (e-f). Soil nitrogen availability is represented on the x-axis in the left column of panels, while species is represented 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 ($p < 0.05$).

922 3.3.2 *Net photosynthesis and leaf biochemistry*

923 Increasing soil nitrogen availability generally had no effect on A_{net} , V_{cmax25} , J_{max25} ,
924 or $J_{\text{max25}}:V_{\text{cmax25}}$ (Table 3.2, Figs. 3.2a, 3.2d, 3.2g). I also observed strong species
925 effects on all measured leaf photosynthetic traits (Table 3.2; Figs. 3.2b, 3.2e, 3.2h).
926 Increasing soil pH had a marginal negative effect on A_{net} , but had no effect on
927 V_{cmax25} , J_{max25} , or $J_{\text{max25}}:V_{\text{cmax25}}$ (Table 3.2). There was a weak positive effect of
928 increasing N_{area} on A_{net} (Fig. 3.2c), but quite strong positive effects of increasing
929 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

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

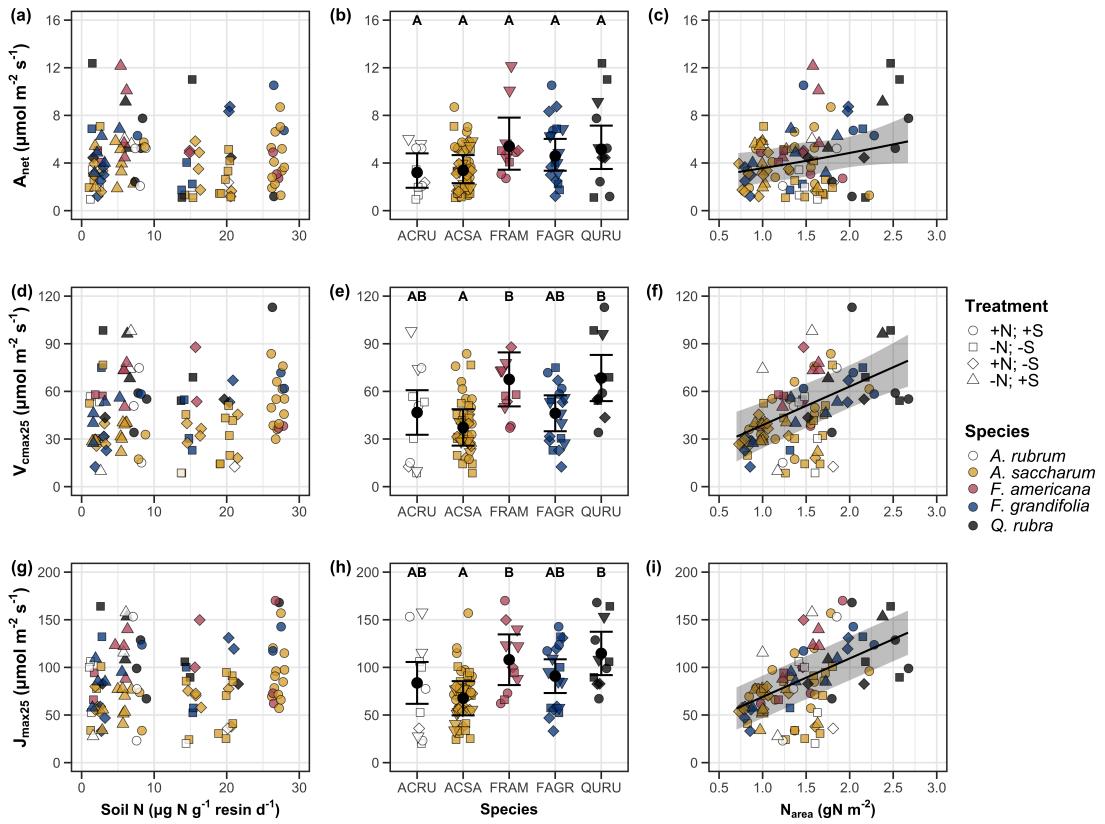


Figure 3.2. Effects of soil nitrogen availability (left column of panels), species (middle column of panels), and leaf nitrogen content per unit leaf area (right column of panels) on net photosynthesis (a-c), maximum Rubisco carboxylation rate (d-f), and maximum RuBP regeneration rate (g-i). Soil nitrogen 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.

935 3.3.3 *Leaf nitrogen allocation*

936 Neither soil nitrogen availability nor soil pH affected the proportion of leaf nitrogen
937 allocated to Rubisco or bioenergetics (Table 3.3; Fig. 3.3a, Fig. 3.3c). There was
938 also no effect of soil nitrogen availability or soil pH on the proportion of leaf
939 nitrogen allocated to photosynthesis (Table 3.3; Fig. 3.3f). I found no effect of
940 soil nitrogen availability or soil pH on the proportion of leaf nitrogen allocated to
941 structure (Table 3.3; Fig 3.3g). Species varied in the proportion of leaf nitrogen
942 allocated to Rubisco, photosynthesis, and structure (Fig 3.3b, Fig. 3.3f, Fig 3.3h),
943 with no detectable species effect on the proportion of leaf nitrogen allocated to
944 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

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

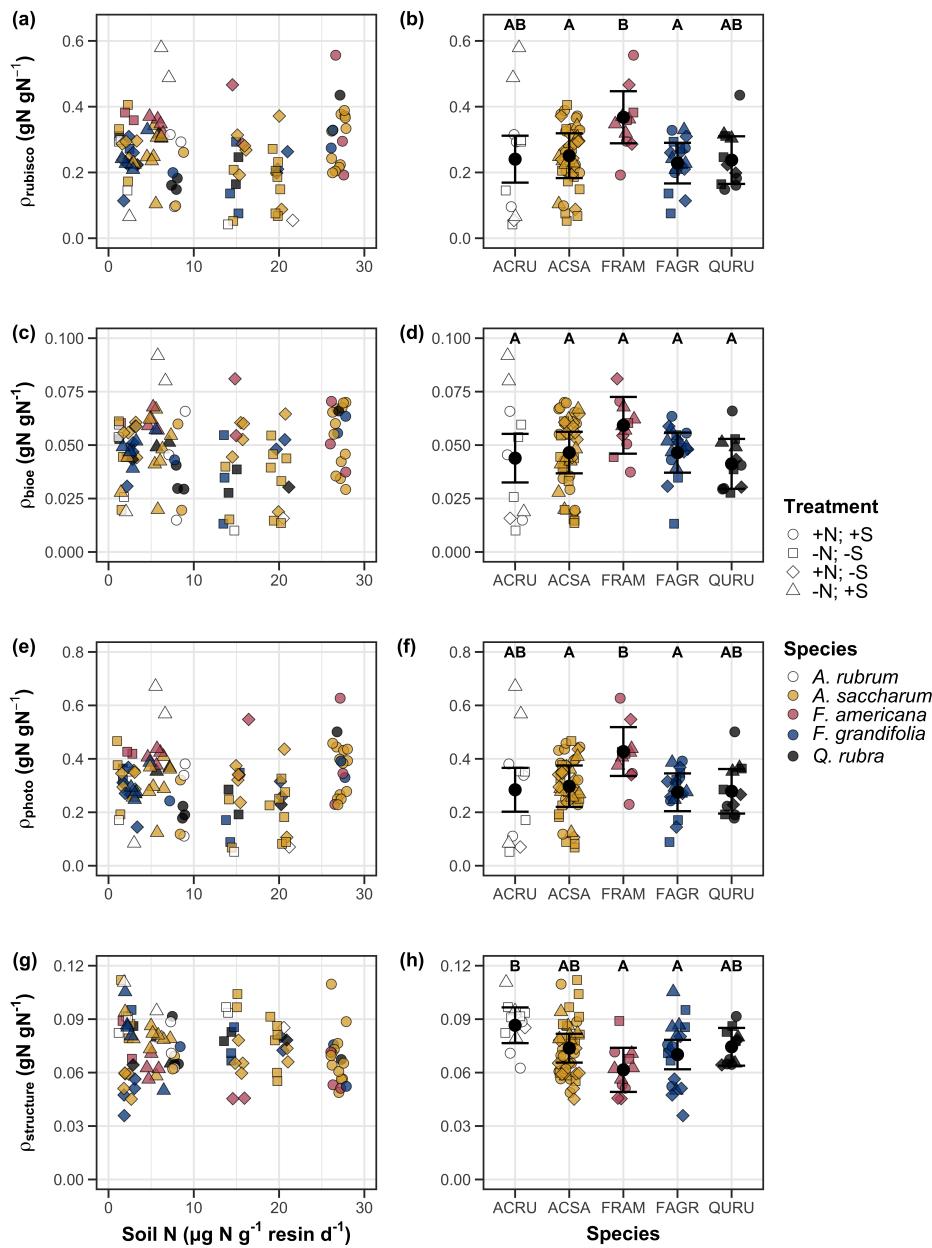


Figure 3.3. Effects of soil nitrogen availability and species on the proportion of leaf nitrogen content allocated to Rubisco (a-b), bioenergetics (c-d), photosynthesis (e-f), and structure (g-h). Soil nitrogen availability is represented on the x-axis in the left column of panels and species are represented 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.

946 3.3.4 *Tradeoffs between nitrogen and water use*

947 Although soil nitrogen availability did not affect χ (Table 3.4; Fig. 3.4a), increasing
948 soil nitrogen availability decreased PNUE (Table 3.4; Fig. 3.4d) and increased
949 the ratio of $N_{\text{area}}:\chi$ (Table 3.4; Fig. 3.4f). Specifically, this response yielded a
950 26% reduction in PNUE and 37% stimulation in $N_{\text{area}}:\chi$ across the soil nitrogen
951 availability gradient. There was no apparent effect of soil nitrogen availability on
952 $V_{\text{cmax25}}:\chi$ (Table 3.4; Fig. 3.4h). Increasing soil pH had a weak marginal nega-
953 tive effect on PNUE, but did not influence χ , $N_{\text{area}}:\chi$, or $V_{\text{cmax25}}:\chi$ (Table 3.4). I
954 observed differences in χ (Fig. 3.4b), PNUE (Fig. 3.4e), $N_{\text{area}}:\chi$ (Fig. 3.4g), and
955 $V_{\text{cmax25}}:\chi$ (Fig. 3.4i) between species (Table 3.4). Finally, increasing N_{area} had a
956 strong negative effect on χ (Table 3.4; Fig. 3.4c) and a strong positive effect on
957 $V_{\text{cmax25}}:\chi$ (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 ($V_{\text{cmax25}}:\chi$; $\mu\text{mol m}^{-2} \text{ s}^{-1}$)^{*}

	df	χ		PNUE				$N_{\text{area}}:\chi$		
		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	-	-	-	-	-	-

	df	$V_{\text{cmax25}}:\chi$		
		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

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

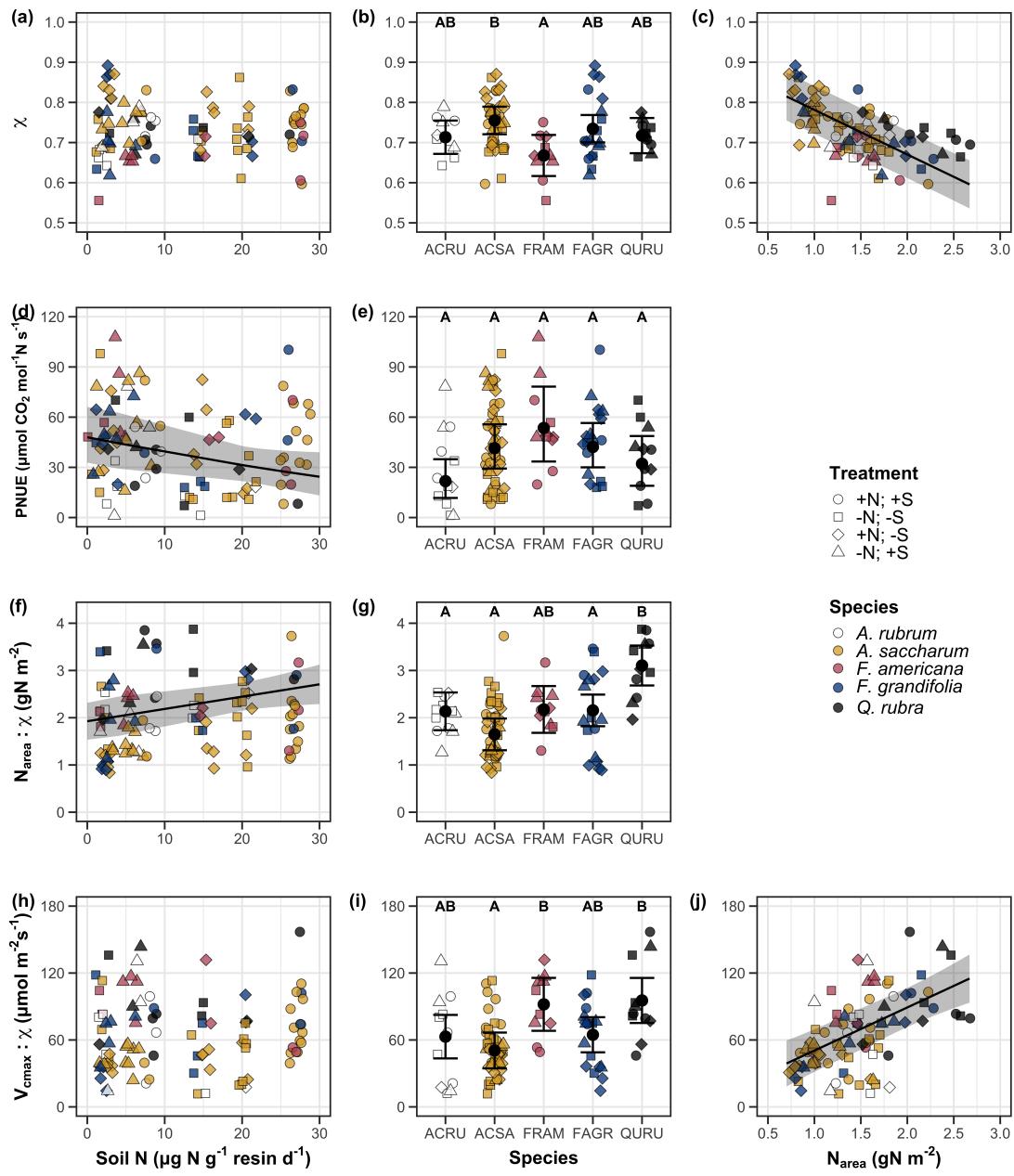


Figure 3.4. Effects of soil nitrogen availability and species on the proportion of leaf nitrogen content allocated to Rubisco (a-b), bioenergetics (c-d), photosynthesis (Rubisco + bioenergetics; e-f), and structure (g-h). Soil nitrogen availability is represented on the x-axis in the left column of panels 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.

963 3.4 Discussion

964 Photosynthetic least-cost theory provides an explanation for understanding rela-
965 tionships between soil nutrient availability, leaf nutrient allocation, and photosyn-
966 thetic capacity. The theory suggests that plants acclimate to a given environment
967 by optimizing leaf photosynthesis rates at the lowest summed cost of using nu-
968 trients and water (Prentice et al. 2014; Wang et al. 2017; Smith et al. 2019;
969 Paillassa et al. 2020). The theory predicts that an increase in soil nutrient avail-
970 ability should allow similar photosynthesis rates to be achieved with increased leaf
971 nutrient content and photosynthetic capacity (i.e., V_{cmax25} and J_{max25}) at lower
972 leaf $C_i:C_a$ (χ), resulting in an increase in water use efficiency, decrease in nutri-
973 ent use efficiency, and increase in both leaf nutrient content and photosynthetic
974 capacity per unit χ . The theory predicts similar leaf responses to increasing soil
975 pH under acidic conditions, presumably due to generally faster nutrient cycle dy-
976 namics and consequent reductions in the cost of acquiring nutrients relative to
977 water with increasing soil pH (Wang et al. 2017; Paillassa et al. 2020; Dong et al.
978 2020).

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

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

992 3.4.1 *Soil nitrogen availability modifies tradeoffs between nitrogen and water use*
993 In support of expected least-cost outcomes and past environmental gradient stud-
994 ies (Dong et al. 2017; Paillassa et al. 2020), increasing soil nitrogen availability
995 was associated with increased leaf nitrogen content. Soil nitrogen availability had
996 smaller impacts on measures of net photosynthesis and χ , which led to reductions
997 in PNUE and increases in leaf nitrogen content per unit χ , as expected from the-
998 ory. Photosynthetic least-cost theory suggests that reductions in PNUE should
999 be driven by an increase in the proportion of leaf nitrogen allocated to photosyn-
1000 thetic tissue, a pattern that should allow plants to achieve optimal photosynthetic
1001 rates with greater photosynthetic capacity to make better use of available light.
1002 Contrasting theory predictions, I found no effect of soil nitrogen availability on
1003 photosynthetic capacity. However, photosynthetic capacity did tend to increase
1004 with increasing soil nitrogen availability ($p<0.20$; Table 3.2) resulting in no effect
1005 of soil nitrogen availability on the relative fraction of leaf nitrogen allocated to
1006 photosynthesis, Rubisco, or bioenergetics. These lines of evidence support the
1007 idea that trees use additional nitrogen to support increased leaf nitrogen alloca-
1008 tion toward photosynthetic tissue and enhance photosynthetic capacity (Wright
1009 et al. 2003).

1010 Soil nitrogen availability had a stronger effect on leaf nitrogen than photo-
1011 synthetic capacity. This pattern suggests that additional plant nitrogen up-
1012 take due to increased soil nitrogen availability was also being used to support
1013 non-photosynthetic nitrogen pools, possibly to structural tissue or stress-induced
1014 amino acid and polyamine synthesis (Minocha et al. 2000; Onoda et al. 2004;
1015 Bubier et al. 2011). While I found no change in the proportion of leaf nitrogen
1016 allocated to leaf structural tissue, the overall stimulation in leaf nitrogen content
1017 with increasing soil nitrogen availability suggests an increase in the net amount of
1018 nitrogen invested in leaf structural tissue along the nitrogen availability gradient.
1019 Importantly, leaf nitrogen allocated to structure was calculated using an empiri-
1020 cal relationship between M_{area} and the amount of leaf nitrogen allocated to cell
1021 walls (Onoda et al. 2017). As the generality of relationships between M_{area} and
1022 the amount of leaf nitrogen allocated to cell walls has been called into question
1023 (Harrison et al. 2009), future work should consider explicitly measuring nitrogen
1024 allocation to cell wall tissue and stress-induced amino acid synthesis to confirm
1025 these patterns.

1026 In opposition to patterns expected from least-cost theory, increasing soil
1027 nitrogen availability had no apparent effect on χ . Interestingly, despite the null
1028 effect of soil nitrogen availability on χ , I observed a strong negative effect of
1029 increasing N_{area} on χ , consistent with the nitrogen-water use tradeoffs expected
1030 from theory. The null response of χ to increasing soil nitrogen availability may
1031 have been due to a lack of water limitation in the system, given that the area
1032 received approximately 20% more precipitation (1167 mm) during the 12-month
1033 period leading up to our measurement period than normally expected (972 mm).

1034 However, droughts can and do occur in temperate forests of the northeastern
1035 United States (Sweet et al. 2017), so the observed increase in leaf nitrogen content
1036 with increasing soil nitrogen availability could be a strategy that allows trees to
1037 hedge bets against drier than normal growing seasons (Onoda et al. 2004; Onoda
1038 et al. 2017; Hallik et al. 2009). As was suggested in Paillassa et al. (2020),
1039 and more recently by Querejeta et al. (2022), negative effects of soil nitrogen
1040 availability on χ may increase with increasing aridity. This strategy would be
1041 especially advantageous if it allows individuals growing in arid regions to maintain
1042 carbon assimilation rates with reduced water loss. Future work should attempt to
1043 quantify interactive roles of climate and soil nitrogen availability on nitrogen-water
1044 use tradeoffs, which could be done using coordinated and multifactor nutrient
1045 (Borer et al. 2014) and water (Knapp et al. 2017) manipulation experiments
1046 across broad climatic gradients.

1047 3.4.2 *Soil pH did not modify tradeoffs between nitrogen and water usage*
1048 While the primary purpose of this study was to examine the role of soil nitrogen
1049 availability on nitrogen-water use tradeoffs, this experiment manipulated both
1050 soil nitrogen and pH, thus providing an opportunity to isolate the these variables
1051 [DWS: example edit]. Previous correlational studies along environmental gradi-
1052 ents have identified soil pH as a particularly important factor that can modify
1053 tradeoffs between nutrient and water use (Smith et al. 2019; Paillassa et al. 2020;
1054 Westerband et al. 2023) and the proportion of leaf nitrogen allocated to photosyn-
1055 thesis (Luo et al. 2021). Such studies implied that these patterns may be driven
1056 by reductions in the cost of acquiring nutrients relative to water with increasing

1057 pH, which may be exacerbated in acidic soils.

1058 Consistent with theory (Wright et al. 2003; Prentice et al. 2014), results
1059 indicate that increasing soil pH was negatively associated with PNUE. However,
1060 there was no effect of soil pH on leaf nitrogen content, χ , or leaf nitrogen content
1061 per unit χ , most likely because the experimental nitrogen additions increased soil
1062 nitrogen supply while both increasing (sodium nitrate) and decreasing (ammo-
1063 nium sulfate) soil pH. These results suggest that soil pH did not play a major
1064 role in modifying expected photosynthetic least-cost theory patterns, contrasting
1065 findings from Paillassa et al. (2020) and other gradient studies that note positive
1066 effects of increasing soil pH on leaf nitrogen content, Rubisco carboxylation, and
1067 χ (Viet et al. 2013; Cornwell et al. 2018; Luo et al. 2021). Instead, null responses
1068 to soil pH show that leaf photosynthetic parameters depend more on soil nitrogen
1069 availability than pH per se, and that inferences from gradient studies might be
1070 confounding covariation between nitrogen availability and soil acidity.

1071 3.4.3 *Species identity explains a large amount of variation in leaf and whole*
1072 *plant traits*

1073 Species [species identity?] generally explained a larger amount of variation in
1074 measured leaf traits than soil nitrogen availability or soil pH. Interspecific vari-
1075 ation is an important factor to consider when deducing mechanisms that drive
1076 photosynthetic least-cost theory, particularly for species that form distinct myc-
1077 orrhizal associations or have different photosynthetic pathways, growth forms, or
1078 leaf habit (Espelta et al. 2005; Adams et al. 2016; Bialic-Murphy et al. 2021;
1079 Scott and Smith 2022). The need to consider species may also be important when

1080 comparing nutrient-water use tradeoffs in early and late successional species, or in
1081 species with different resource economic strategies (Abrams and Mostoller 1995;
1082 Ellsworth and Reich 1996; Wright et al. 2004; Reich 2014; Onoda et al. 2017;
1083 Ziegler et al. 2020).

1084 [DWS: implications for chapter 2?]

1085 A strength of the study design and sampling effort is that it controls for
1086 many species differences that should modify nitrogen-water use tradeoffs expected
1087 from theory. All tree species measured in this study shared the leaf habit of de-
1088 ciduous broadleaves, were growing in forests of similar successional stage, but
1089 differed in mycorrhizal association and consequent resource economic strategies.
1090 As stands tended to be dominated by trees that associate with arbuscular myc-
1091 orrhizae (*Fraxinus* and both *Acer* species made up roughly 70% of total above-
1092 ground biomass across stands), ecosystem biogeochemical cycle dynamics may be
1093 more closely aligned to the inorganic nutrient economy proposed in Phillips et al.
1094 (2013), which may promote stronger nitrogen-water use tradeoffs in tree species
1095 that associate with arbuscular mycorrhizae. This result was not observed here,
1096 as photosynthetic properties varied as much within as across the two mycorrhizal
1097 associations represented.

1098 3.4.4 *Implications for photosynthetic least-cost theory model development*

1099 In the field, soil nutrient availability is heterogeneous across time and space (Ta-
1100 ble B4). Unaccounted within-plot heterogeneity may have contributed to the low
1101 amount of variation explained by soil nitrogen availability in statistical models,
1102 as resin bags are a coarse surrogate for soil nitrogen availability. Despite this, I

1103 still observed evidence for nutrient-water use tradeoffs, suggesting that observed
1104 responses reported here may be an underestimate toward the net effect of soil ni-
1105 trogen availability on these tradeoffs. While I urge caution in the interpretation of
1106 these results, they do provide a promising baseline for future studies investigating
1107 patterns expected from photosynthetic least-cost theory at finer spatiotemporal
1108 resolutions.

1109 The general stronger relationship between leaf nitrogen content and photo-
1110 synthetic parameters versus between leaf nitrogen content and soil nitrogen avail-
1111 ability suggests that leaf nitrogen content is more directly tied to photosynthesis
1112 than soil nitrogen availability. While this could be due to the high spatiotemporal
1113 heterogeneity of soil nitrogen availability, principles from photosynthetic least-
1114 cost theory suggest that leaf nitrogen content is the downstream product of leaf
1115 nutrient demand to build and maintain photosynthetic machinery, which is set by
1116 aboveground environmental conditions such as light availability, CO₂, tempera-
1117 ture, or vapor pressure deficit (Smith et al. 2019; Paillassa et al. 2020; Peng et al.
1118 2021; Westerband et al. 2023). The stronger relationship between leaf nitrogen
1119 and photosynthetic parameters, paired with the strong negative relationship be-
1120 tween leaf nitrogen and χ , could indicate a relatively stronger effect of climate on
1121 leaf nitrogen-photosynthesis relationships than soil resource availability. However,
1122 the short distance between plots and across sites limited my ability to test this
1123 mechanism.

1124 Variation in soil pH affected least cost responses less than variations in soil
1125 nitrogen availability, in part because experimental treatments directly increased
1126 soil nitrogen and affected soil pH in opposite directions. While soil pH has been

1127 shown to drive nitrogen-water tradeoffs in global gradient analyses (Viet et al.
1128 2013; Paillassa et al. 2020), these responses may be due to covariations between
1129 soil pH and nutrient cycling rather than a role of pH per se. The direct manipula-
1130 tions of soil pH and soil nitrogen availability in this study partly disentangle these
1131 factors and show that variation in nitrogen availability matters more for least-cost
1132 tradeoffs than pH alone.

1133 3.4.5 *Conclusions*

1134 Increasing soil nitrogen availability generally increased leaf nitrogen content (both
1135 area- and mass-based), but did not significantly influence χ . This shift in leaf ni-
1136 trogen led to a reduction in PNUE, and an increase in leaf nitrogen per unit
1137 χ with increasing soil nitrogen availability. Despite null effects of soil nitrogen
1138 availability on χ , I observed a strong negative relationship between leaf nitrogen
1139 content and χ . These results provide empirical support for the nutrient-water use
1140 tradeoffs expected from photosynthetic least-cost theory in response to increas-
1141 ing soil nutrient availability, but suggest that all tenets of the theory may not
1142 hold in every environment. These results experimentally test previous work sug-
1143 gesting that leaf nitrogen-water economies vary across gradients of soil nutrient
1144 availability and pH, and show that variations in nutrient availability matter more
1145 for determining variation in leaf photosynthetic traits than soil pH.

1146

Chapter 4

1147 The relative cost of resource use for photosynthesis drives variance in
1148 leaf nitrogen content across a climate and soil resource availability
1149 gradient

1150 4.1 Introduction

1151 Terrestrial biosphere models, which comprise the land surface component of Earth
1152 system models, are sensitive to the formulation of photosynthetic processes (Knorr
1153 and Heimann 2001; Ziehn et al. 2011; Booth et al. 2012; Walker et al. 2021).
1154 This is because photosynthesis is the largest carbon flux between the atmosphere
1155 and terrestrial biosphere (IPCC 2021), and is constrained by ecosystem carbon
1156 and nutrient cycles (Hungate et al. 2003; LeBauer and Treseder 2008; Fay et al.
1157 2015). Many terrestrial biosphere models formulate photosynthesis by parame-
1158 terizing photosynthetic capacity within plant functional groups through empiri-
1159 cal linear relationships between area-based leaf nitrogen content (N_{area}) and the
1160 maximum carboxylation rate of Ribulose-1,5-bisphosphate carboxylase/oxygenase
1161 (V_{cmax}) (Kattge et al. 2009; Rogers 2014; Rogers et al. 2017). Models are also
1162 beginning to include connected carbon-nitrogen cycles (Wieder et al. 2015; Shi
1163 et al. 2016; Davies-Barnard et al. 2020; Braghieri et al. 2022), which allows leaf
1164 photosynthesis to be predicted directly through changes in N_{area} and indirectly
1165 through changes in soil nitrogen availability (e.g., LPJ-GUESS, CLM5.0) (Smith
1166 et al. 2014; Lawrence et al. 2019). Despite recent model developments, open
1167 questions remain regarding the generality of ecological relationships between soil
1168 nitrogen availability, leaf nitrogen content, and leaf photosynthesis across edaphic
1169 and climatic gradients.

1170 Empirical support for positive relationships between soil nitrogen availabil-
1171 ity and N_{area} is abundant (Firn et al. 2019; Liang et al. 2020), and is a result
1172 often attributed to the high nitrogen cost of building and maintaining Rubisco
1173 (Evans 1989; Evans and Seemann 1989; Onoda et al. 2004; Walker et al. 2014;
1174 Onoda et al. 2017; Dong et al. 2020). Such patterns imply that positive relation-
1175 ships between soil nitrogen availability and N_{area} should increase leaf photosyn-
1176 thesis and photosynthetic capacity by increasing the maximum rate of Rubisco
1177 carboxylation through increased investments to Rubisco construction and mainte-
1178 nance. This integrated N_{area} -photosynthesis response to soil nitrogen availability
1179 has been observed both in manipulative experiments and across environmental
1180 gradients (Field and Mooney 1986; Evans 1989; Walker et al. 2014; Li et al.
1181 2020), and is thought to be driven by ecosystem nitrogen limitation, which lim-
1182 its primary productivity globally (LeBauer and Treseder 2008; Fay et al. 2015).
1183 However, this response is not consistently observed, as recent studies note variable
1184 N_{area} -photosynthesis relationships across edaphic and climatic gradients (Liang
1185 et al. 2020; Luo et al. 2021) and that aboveground growing conditions (e.g., light
1186 availability, temperature, vapor pressure deficit) or species identity traits (e.g.,
1187 photosynthetic pathway, nitrogen acquisition strategy) may be more important
1188 for explaining variance in N_{area} and photosynthetic capacity across environmental
1189 gradients (Adams et al. 2016; Dong et al. 2017; Smith et al. 2019; Dong et al.
1190 2020; Peng et al. 2021; Dong et al. 2022; Westerband et al. 2023).

1191 One hypothesized mechanism to explain variance in N_{area} across environ-
1192 mental gradients has been proposed via photosynthetic least-cost theory (Wright
1193 et al. 2003; Prentice et al. 2014; Paillassa et al. 2020; Harrison et al. 2021).

1194 The theory predicts that plants acclimate to environments by optimizing photo-
1195 synthetic assimilation rates at the lowest summed cost of nitrogen and water use
1196 (Wright et al. 2003; Prentice et al. 2014). In a given environment, the theory
1197 suggests that nitrogen and water use can be substituted for each other to maintain
1198 the lowest summed cost of resource use, such that optimal photosynthetic rates
1199 are achieved with less efficient use of the more abundant and less costly resource
1200 to acquire in exchange for more efficient use of the less abundant and more costly
1201 resource to acquire.

1202 Photosynthetic least-cost theory predicts that, all else equal, an increase in
1203 soil nitrogen availability should decrease the cost of acquiring and using nitrogen
1204 relative to water (a ratio referred to herein as β), resulting in optimal photosyn-
1205 thetic rates achieved with greater N_{area} at lower stomatal conductance and lower
1206 leaf $C_i:C_a$ (Wright et al. 2003; Prentice et al. 2014; Paillassa et al. 2020). Alter-
1207 natively, an increase in soil moisture should reduce costs of water acquisition and
1208 use, increasing β (Lavergne et al. 2020), stomatal conductance, and leaf $C_i:C_a$, re-
1209 sulting in optimal photosynthetic rates achieved with decreased N_{area} . The theory
1210 also predicts variability in stomatal conductance and N_{area} in response to climatic
1211 factors, suggesting that the optimal response to increased vapor pressure deficit
1212 should be a reduction in stomatal conductance and leaf $C_i:C_a$ that is counter-
1213 balanced by an increase in N_{area} to support the greater photosynthetic capacity
1214 needed to maintain high assimilation at lower conductance (Grossiord et al. 2020;
1215 Dong et al. 2020; López et al. 2021; Westerband et al. 2023).

1216 Leaf nitrogen allocation responses to changing climates or soil resource
1217 availability may also depend on their mode of nutrient acquisition or photo-

1218 synthetic pathway. For example, species that form associations with symbiotic
1219 nitrogen-fixing bacteria (referred as “N-fixing species” from this point forward)
1220 should, in theory, have access to less finite nitrogen supply than species not capa-
1221 ble of forming such associations (referred as “non-fixing species” from this point
1222 forward), which may result in lower β values in N-fixing species than non-fixing
1223 species. This result was previously shown in a greenhouse experiment, where a
1224 leguminous species generally had lower costs of nitrogen acquisition compared to a
1225 non-leguminous species, although these differences were generally stronger under
1226 increased nitrogen limitation (Perkowski et al. 2021). Lower β values could be an
1227 explanation for why N-fixing species commonly have greater leaf nitrogen content
1228 than non-fixing species (Adams et al. 2016; Dong et al. 2017).

1229 Similarly, leaf nitrogen allocation patterns across environmental gradients
1230 may be dependent on photosynthetic pathway. Lower leaf $C_i:C_a$ values in C₄
1231 species suggests that C₄ species should have lower β values than C₃ species (Scott
1232 and Smith 2022), a pattern that could be the result of increased costs associated
1233 with water acquisition and use or reduced costs of nitrogen acquisition and use
1234 relative to C₃ species. Theory predicts that this response in C₄ species will cause
1235 C₄ species to have higher leaf nitrogen content on average compared to C₃ species,
1236 though ample evidence exists documenting general lower leaf nitrogen content in
1237 C₄ species (Schmitt and Edwards 1981; Sage and Pearcy 1987; Ghannoum et al.
1238 2011). No study to date has directly quantified β in C₄ species aside from the
1239 initial parameterization of β in an optimality model for C₄ species (Scott and
1240 Smith 2022) using a global dataset of leaf $\delta^{13}\text{C}$ values (Cornwell et al. 2018).

1241 While photosynthetic least-cost theory provides a unified framework for

1242 understanding integrated effects of climate and soil resource availability on N_{area} ,
1243 empirical tests of the theory are sparse. Previous work shows that increasing
1244 soil nitrogen availability decreases costs of acquiring nutrients (Bae et al. 2015;
1245 Perkowski et al. 2021; Lu et al. 2022), which can induce predictable nutrient-
1246 water use tradeoffs expected from the theory across broad environmental gradients
1247 (Paillassa et al. 2020; Querejeta et al. 2022; Westerband et al. 2023) and in
1248 manipulation experiments (Balic-Murphy et al. 2021). Additionally, increasing
1249 vapor pressure deficit has been shown to have a positive effect on N_{area} , which is
1250 commonly associated with reduced leaf $C_i:C_a$ (Dong et al. 2017; Dong et al. 2020;
1251 Firn et al. 2019; López et al. 2021).

1252 Despite evidence for patterns expected from photosynthetic least-cost the-
1253 ory, studies have been restricted to exploring these patterns in C₃ species and,
1254 while variance in N_{area} across environmental gradients has been shown to be driven
1255 by strong negative relationships with leaf $C_i:C_a$ (Dong et al. 2017; Paillassa et al.
1256 2020; Westerband et al. 2023), no study has explicitly investigated effects of soil
1257 resource availability or species identity on N_{area} using β as a direct predictor of
1258 leaf $C_i:C_a$. Furthermore, as N_{area} can be broken down into structural (leaf mass
1259 per area; M_{area} ; g m⁻²) and metabolic (mass-based leaf nitrogen content; N_{mass} ;
1260 gN g⁻¹) components (Dong et al. 2017), no study has investigated which compo-
1261 nent of N_{area} drives the hypothesized response of N_{area} to leaf $C_i:C_a$, which limits
1262 our ability to assess whether changes in N_{area} across environmental gradients are
1263 driven by changes in leaf morphology (i.e. M_{area}), leaf stoichiometry (i.e. N_{mass}),
1264 or both.

1265 In this study, I measured N_{area} , N_{mass} , M_{area} , leaf $\delta^{13}\text{C}$ -derived estimates

1266 of leaf $C_i:C_a$, and leaf $\delta^{13}\text{C}$ -derived estimates of β in 504 individuals spanning
1267 52 species scattered across 24 grassland sites in Texas, USA. The state of Texas
1268 contains a diverse climatic gradient, indicated by 2006-2020 mean annual precipi-
1269 tation totals ranging from 204 to 1803 mm and 2006-2020 mean annual tempera-
1270 ture ranging from 11.8° to 24.6°C within state boundaries (Fig. 4.1). Variability
1271 in soil nitrogen availability and soil moisture was expected across sites, owing to
1272 differences in soil texture and aboveground climate that would drive differential
1273 rates of water retention and nitrogen transformations to plant-available nitrogen
1274 substrate. I leveraged the expected climatic and soil resource variability across
1275 sites to test the following hypotheses:

- 1276 1. Soil nitrogen availability will decrease β through a reduction in costs of
1277 nitrogen acquisition and use, while soil moisture will increase β through a
1278 reduction in costs of water acquisition and use. Following previous results, I
1279 expected that N-fixing species would have lower β values and that C_4 species
1280 would have lower β values.
- 1281 2. Leaf $C_i:C_a$ will be positively related to β , a pattern that will result in a
1282 negative indirect effect of increasing soil nitrogen availability on leaf $C_i:C_a$,
1283 a positive indirect effect of increasing soil moisture on leaf $C_i:C_a$, and lower
1284 leaf $C_i:C_a$ in both N-fixing species and C_4 species. I expected that leaf
1285 $C_i:C_a$ would be negatively related to vapor pressure deficit, as increasing
1286 atmospheric dryness would cause plants to close stomata to minimize water
1287 loss.
- 1288 3. N_{area} will be negatively related to leaf $C_i:C_a$. This response will result in an
1289 indirect positive and negative effect of increasing soil nitrogen availability

1290 and soil moisture, respectively, on N_{area} , and larger N_{area} values in N-fixing
1291 species. While theory predicts that lower β values in C₄ species should
1292 yield larger N_{area} in C₄ species, I expected that C₄ species would have lower
1293 N_{area} than C₃ species due to greater nitrogen use efficiency in C₄ species.
1294 Additionally, I expected vapor pressure deficit to increase N_{area} , a pattern
1295 that would be directly mediated through the reduction in leaf $C_i:C_a$ with
1296 increasing vapor pressure deficit.

1297 4.2 Methods

1298 4.2.1 *Site descriptions and sampling methodology*

1299 Leaf and soil samples were collected from 24 open canopy grassland sites scattered
1300 across central and eastern Texas in summer 2020 and summer 2021 (Fig. 4.1).
1301 Twelve sites were visited between June and July 2020 and 14 sites (11 unique from
1302 2020) were visited between May and June 2021 (Table 4.1). Sites were chosen to
1303 maximize precipitation and edaphic variability across sites (Table 4.1). No site
1304 with personally communicated or anecdotal evidence of grazing or disturbance
1305 (e.g., mowing, feral hog activity, etc.) was used. Leaf material was collected
1306 from three individuals each of the five most abundant species at random locations
1307 at each site, only selecting species that were broadly classified as graminoid or
1308 forb/herb growth habits per the USDA PLANTS database (USDA NRCS 2022).
1309 All collected leaves were fully expanded with no visible herbivory or other external
1310 damage and also free from shading by nearby shrubs or trees. Five soil samples
1311 were collected from 0-15 cm below the soil surface at each site near the leaf
1312 collection sample locations. Soil samples were mixed together by hand to create

1313 one composite soil sample per site.

1314 4.2.2 *Leaf trait measurements*

1315 Images of each leaf were taken immediately following each site visit using a flat-

1316 bed scanner. Fresh leaf area was determined from each image using the ‘LeafArea’

1317 R package (Katabuchi 2015), which automates leaf area calculations using ImageJ

1318 software (Schneider et al. 2012). Each leaf was dried at 65°C for at least 48 hours

1319 to a constant mass, weighed, and manually ground in a mortar and pestle until

1320 homogenized. Leaf mass per area (M_{area} ; g m⁻²) was calculated as the ratio of

1321 dry leaf biomass to fresh leaf area. Subsamples of dried and homogenized leaf

1322 tissue were used to measure leaf nitrogen content (N_{mass} ; gN g⁻¹) through ele-

1323 mental combustion analysis (Costech-4010, Costech Instruments, Valencia, CA).

1324 Leaf nitrogen content per unit leaf area (N_{area} ; gN m⁻²) was calculated as the

1325 product of N_{mass} and M_{area} .

1326 Subsamples of dried and homogenized leaf tissue were sent to the University

1327 of California-Davis Stable Isotope Facility to determine leaf $\delta^{13}\text{C}$. Leaf $\delta^{13}\text{C}$ values

1328 were determined using an elemental analyzer (PDZ Europa ANCA-GSL; Sercon

1329 Ltd., Chestshire, UK) interfaced to an isotope ratio mass spectrometer (PDZ

1330 Europa 20-20 Isotope Ratio Mass Spectrometer, Sercon Ltd., Chestshire, UK).

1331 I used leaf $\delta^{13}\text{C}$ values (‰; relative to Vienna Pee Dee Belemnite international

1332 reference standard) to estimate the ratio of intercellular (C_i) to extracellular (C_a)

1333 CO₂ ratio (leaf $C_i:C_a$; unitless) following the approach of Farquhar et al. (1989)

1334 described in Cernusak et al. (2013). Specifically, I derived leaf $C_i:C_a$ as:

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

1335 where $\Delta^{13}C$ represents the relative difference between leaf $\delta^{13}\text{C}$ (\textperthousand) and air $\delta^{13}\text{C}$

1336 (\textperthousand), calculated as:

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

1337 $\delta^{13}\text{C}_{air}$, which is commonly assumed to be $-8\text{\textperthousand}$ (Keeling et al. 1979; Farquhar

1338 et al. 1989), was calculated as a function of calendar year t using an empirical

1339 equation derived in Feng (1999):

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

1340 Using this equation, $\delta^{13}\text{C}_{air}$ values were set to $-9.04\text{\textperthousand}$ and $-9.09\text{\textperthousand}$ for 2020 and

1341 2021, respectively. The parameter a represents the fractionation between ^{12}C

1342 and ^{13}C due to diffusion in air, assumed to be $4.4\text{\textperthousand}$, while b represents the

1343 fractionation caused by Rubisco carboxylation, assumed to be $27\text{\textperthousand}$ (Farquhar

1344 et al. 1989). For C_4 species, b in Eqn. 4.1 was set to $6.3\text{\textperthousand}$, and was derived from:

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

1345 Where c was set to $-5.7\text{\textperthousand}$ and d was set to $30\text{\textperthousand}$ (Farquhar et al. 1989). ϕ , which

1346 is the bundle sheath leakiness term, was set to 0.4. All leaf $C_i:C_a$ values less than

1347 0.1 and greater than 0.95 were assumed to be incorrect and removed from the

1348 analysis.

1349 I derived the unit cost of resource use (β) using leaf $C_i:C_a$ and site climate

1350 data using equations first described in Prentice et al. (2014) and simplified in

1351 Lavergne et al. (2020):

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

1352 where η^* is the viscosity of water relative to 25°C, calculated using elevation and

1353 mean air temperature of the seven days leading up to each site visit following equa-

1354 tions in Huber et al. (2009). VPD (Pa) was set to the mean vapor pressure deficit

1355 of the seven days leading up to each site visit, C_a represents atmospheric CO₂

1356 concentration, arbitrarily set to 420 μmol mol⁻¹ CO₂. K_m (Pa) is the Michaelis-

1357 Menten coefficient for Rubisco affinity to CO₂ and O₂, calculated as:

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

1358 where K_c (Pa) and K_o (Pa) are the Michaelis-Menten coefficients for Rubisco

1359 affinity to CO₂ and O₂, respectively, and O_i is the intercellular O₂ concentration.

1360 Γ^* (Pa) is the CO₂ compensation point in the absence of dark respiration. K_c , K_o ,

1361 and Γ^* were determined using equations described in Medlyn et al. (2002) and

1362 derived in Bernacchi et al. (2001), invoking an elevation correction for atmospheric

1363 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

1364 * Rows are arranged by longitude to visualize precipitation variability across sites

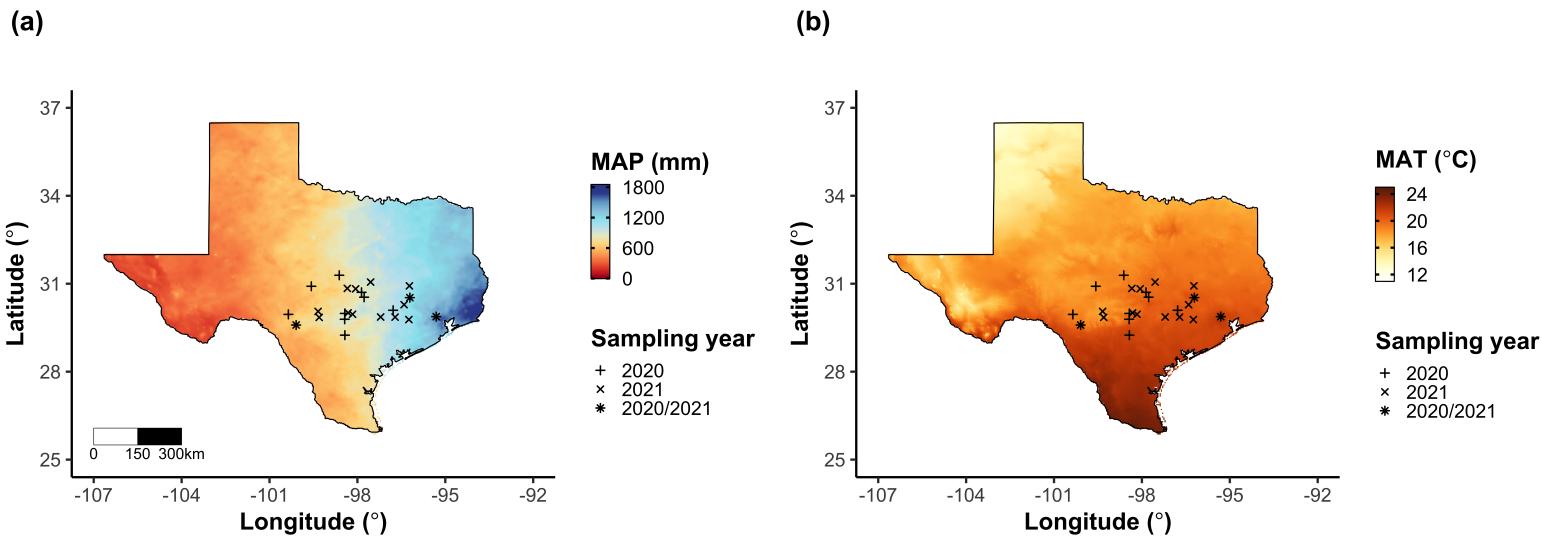


Figure 4.1. Site locations along 2006-2020 mean annual precipitation (a) and mean annual temperature (b) gradients in Texas, USA. Precipitation and temperature data were plotted using PRISM data at a 4-km grid resolution and are masked to include only grid cells that occur within the Texas state boundary 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).

1365 4.2.3 *Site climate data*

1366 I used the Parameter elevation Regressions on Independent Slopes Model (PRISM)
1367 (Daly et al. 2008) climate product to access gridded daily temperature and precip-
1368 itation data for the coterminous United States at a 4-km grid resolution between
1369 January 1, 2006 and July 31, 2021 (PRISM Climate Group, Oregon State Uni-
1370 versity, <https://prism.oregonstate.edu>, data created 4 Feb 2014, accessed 24
1371 Mar 2022). Mean daily air temperature, mean daily vapor pressure deficit, and
1372 total daily precipitation data were extracted from the grid cell that contained the
1373 latitude and longitude of each property using the ‘extract’ function in the ‘terra’
1374 R package (Hijmans 2022). PRISM data were used in lieu of local weather sta-
1375 tion data because several rural sites did not have a local weather station present
1376 within a 20-km radius of the site. Daily site climate data were used to estimate
1377 mean annual precipitation and mean annual temperature for each site between
1378 2006 and 2020 (Table 4.1). I calculated total precipitation and mean daily vapor
1379 pressure deficit for the prior 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 60, and 90
1380 days leading up to each site visit. Temperature was not included in any analy-
1381 sis due to the close range in mean annual temperature between sites (mean±SD:
1382 $19.8 \pm 0.9^\circ\text{C}$; Table 4.1).

1383 4.2.4 *Site edaphic characteristics*

1384 Composted soil samples were sent to the Texas A&M Soil, Water and Forage
1385 Laboratory to quantify soil nitrate concentration ($\text{NO}_3\text{-N}$; ppm). Soil $\text{NO}_3\text{-N}$
1386 was determined by extracting composite soil samples in 1 M KCl, measuring
1387 absorbance values of extracts at 520 nm using the end product of a $\text{NO}_3\text{-N}$ to

1388 NO₂-N cadmium reduction reaction (Kachurina et al. 2000; Keeney and Nelson
1389 1983). Soil texture data from 0-15 cm below the soil surface were accessed using
1390 the SoilGrids2.0 data product (Poggio et al. 2021) through the ‘fetchSoilGrids’
1391 function in the ‘soilDB’ R package (Beaudette et al. 2022). I used SoilGrids2.0
1392 to access soil texture data in lieu of analyses using the composite soil sample due
1393 to a lack of soil material from some sites after sending samples for soil NO₃-N.

1394 Soil moisture was not measured in the field, but was estimated using the
1395 ‘Simple Process-Led Algorithms for Simulating Habitats’ model (SPLASH) (Davis
1396 et al. 2017). This model, derived from the STASH model (Cramer and Prentice
1397 1988), spins up a bucket model using Priestley-Taylor equations (Priestley and
1398 Taylor 1972) to calculate daily soil moisture (W_n ; mm) as a function of the previous
1399 day’s soil moisture (W_{n-1} ; mm), daily precipitation (P_n ; mm), condensation (C_n ;
1400 mm), actual evapotranspiration (E_n^a ; mm), and runoff (RO; mm):

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

1401 Models were spun up by equilibrating the previous day’s soil moisture using succes-
1402 sive model iterations with daily mean air temperature, daily precipitation total,
1403 the number of daily sunlight hours, and latitude as model inputs (Davis et al.
1404 2017). Daily sunlight hours were estimated for each day at each site using the
1405 ‘getSunlightTimes’ function in the ‘suncalc’ R package, which estimated sunrise
1406 and sunset times of each property using date and site coordinates (Thieurmel and
1407 Elmarhraoui 2019). Water holding capacity (mm), or bucket size, was estimated
1408 as a function of soil texture using pedotransfer equations explained in Saxton and

1409 Rawls (2006), as done in Stocker et al. (2020) and Bloomfield et al. (2023). A
1410 summary of these equations is included in Appendix C.1.

1411 Daily soil moisture outputs from the SPLASH model for each site were
1412 used to calculate mean daily soil moisture for the prior 1, 2, 3, 4, 5, 6, 7, 8, 9,
1413 10, 15, 20, 25, 30, 60, and 90 days leading up to each site visit. Mean daily
1414 soil moisture values were then expressed as a fraction of water holding capacity
1415 to normalize across sites with different bucket depths, as done in Stocker et al.
1416 (2018). Site water holding capacity values are referenced in Table 4.1.

1417 4.2.5 *Plant functional group assignments*

1418 Plant functional group was assigned to each species and used as the primary de-
1419 scriptor of species identity. Specifically, plant functional groups were assigned
1420 based on photosynthetic pathway (C_3 , C_4) and ability to form associations with
1421 symbiotic nitrogen-fixing bacteria (N-fixer, non-fixer). The ability to form asso-
1422 ciations with symbiotic nitrogen-fixing bacteria was assigned based on whether
1423 species were in the *Fabaceae* family, and photosynthetic pathway of each species
1424 was determined from past literature and confirmed through leaf $\delta^{13}C$ values. I
1425 chose these plant functional groups based on *a priori* hypotheses regarding the
1426 functional role of nitrogen fixation and photosynthetic pathway on the sensitivity
1427 of plant nitrogen uptake and leaf nitrogen allocation to soil nitrogen availability
1428 and aboveground growing conditions. These plant functional group classifications
1429 resulted in three distinct plant functional groups within our dataset: C_3 N-fixers
1430 (n=53), C_3 non-fixers (n=334), and C_4 non-fixers (n=117).

1431 4.2.6 *Data analysis*

1432 All analyses and plotting were conducted in R version 4.1.1 (R Core Team 2021).

1433 I constructed a series of separate linear mixed-effects models to investigate en-

1434 vironmental drivers of β , leaf $C_i:C_a$, N_{area} , N_{mass} , and M_{area} , followed by a path

1435 analysis using a piecewise structural equation model to investigate direct and

1436 indirect effects of climate and soil resource availability on N_{area} .

1437 To explore environmental drivers of β , I built a linear mixed-effects model

1438 that included soil moisture, soil nitrogen availability, and plant functional group

1439 as fixed effect coefficients. Species were designated as a random intercept term.

1440 Interaction coefficients between all possible combinations of the three fixed effect

1441 coefficients were also included. β was natural log transformed to linearize data.

1442 I used an information-theoretic model selection approach to determine whether

1443 90-, 60-, 30-, 20-, 15-, 10-, 9-, 8-, 7-, 6-, 5-, 4-, 3-, 2-, or 1-day mean daily soil

1444 moisture conferred the best model fit for β . To do this, I constructed 16 separate

1445 linear mixed-effects models where log-transformed β was included as the response

1446 variable and each soil moisture time step was separately included as a single

1447 continuous fixed effect. Species were included as a random intercept term for all

1448 models. I used corrected Akaike Information Criterion (AICc) to select the soil

1449 moisture timescale that conferred the best model fit, indicated by the model with

1450 the lowest AICc score (Table C3; Fig. C1).

1451 To explore environmental drivers of leaf $C_i:C_a$, I constructed a second lin-

1452 ear mixed effects model that included vapor pressure deficit, soil moisture, soil

1453 nitrogen availability, and plant functional group as fixed effect coefficients. Two-

1454 way interactions between plant functional group and vapor pressure deficit, soil

1455 nitrogen availability, or soil moisture were included as additional fixed effect coef-
1456 ficients, in addition to a three-way interaction between soil moisture, soil nitrogen
1457 availability, and plant functional group. Species were included as a random inter-
1458 cept term. I used an information-theoretic model selection approach to determine
1459 whether 90-, 60-, 30-, 20-, 15-, 10-, 9-, 8-, 7-, 6-, 5-, 4-, 3-, 2-, or 1-day mean daily
1460 vapor pressure deficit conferred the best model fit for leaf $C_i:C_a$ using the same
1461 approach explained above for the soil moisture effect on β . The soil moisture
1462 timescale was set to the same timescale that conferred the best fit for β .

1463 To explore environmental drivers of N_{area} , N_{mass} , and M_{area} , I constructed
1464 a linear mixed effects model for each trait, including leaf $C_i:C_a$, soil nitrogen
1465 availability, soil moisture, and plant functional group as fixed effect coefficients
1466 for each model. Two-way interactions between plant functional group and β , leaf
1467 $C_i:C_a$, soil nitrogen availability, or soil moisture were included as additional fixed
1468 effect coefficients, in addition to a three-way interaction between soil nitrogen
1469 availability, soil moisture, and plant functional group. Species were included as a
1470 random intercept term, with the soil moisture timescale set to the same timescale
1471 that conferred the best fit for β .

1472 In all linear mixed-effects models explained above, including those to select
1473 relevant timescales, I used the ‘lmer’ function in the ‘lme4’ R package (Bates et al.
1474 2015) to fit each model and the ‘Anova’ function in the ‘car’ R package (Fox and
1475 Weisberg 2019) to calculate Type II Wald’s χ^2 and determine the significance
1476 level ($\alpha=0.05$) of each fixed effect coefficient. I used the ‘emmeans’ R package
1477 (Lenth 2019) to conduct post-hoc comparisons using Tukey’s tests, where degrees
1478 of freedom were approximated using the Kenward-Roger approach (Kenward and

1479 Roger 1997). Trendlines and error ribbons for all plots were drawn using a series
1480 of ‘emmeans’ outputs across the range in plotted x-axis values.

1481 Finally, I conducted a path analysis using a piecewise structural equation
1482 model to examine direct and indirect pathways that determined variance in N_{area} .
1483 Six separate linear mixed effects models were loaded into the piecewise structural
1484 equation model. Models were constructed per *a priori* hypotheses following pat-
1485 terns expected from photosynthetic least-cost theory. The first model regressed
1486 N_{area} against N_{mass} and M_{area} . The second model regressed M_{area} against leaf
1487 $C_i:C_a$ and soil nitrogen availability. The third model regressed N_{mass} against
1488 leaf $C_i:C_a$ and M_{area} (Dong et al. 2017; Dong et al. 2020). The fourth model re-
1489 gressed leaf $C_i:C_a$ against β and vapor pressure deficit. The fifth model regressed β
1490 against soil nitrogen availability, soil moisture, ability to associate with symbiotic
1491 nitrogen-fixing bacteria, and photosynthetic pathway. The sixth model regressed
1492 soil nitrogen availability against soil moisture. All models included the relevant
1493 timescale selected in the individual linear mixed effect models explained above.
1494 Models included species as a random intercept term, were built using the ‘lme’
1495 function in the ‘nlme’ R package (Pinheiro and Bates 2022), and subsequently
1496 loaded into the piecewise structural equation model using the ‘psem’ function in
1497 the ‘piecewiseSEM’ R package (Lefcheck 2016).

1498 4.3 Results

1499 4.3.1 *Cost to acquire nitrogen relative to water*

1500 Model selection indicated that 90-day mean soil moisture conferred the best model

1501 fit for β (AICc=1387.54; Table C3; Fig. C1).

1502 Increasing soil nitrogen availability generally decreased β ($p<0.001$; Table

1503 4.2; Fig. 4.2a), a pattern driven by a negative effect of increasing soil nitrogen on β

1504 in C₃ non-fixers (Tukey: $p=0.005$) and C₃ N-fixers (Tukey: $p=0.035$) despite a null

1505 effect of increasing soil nitrogen on β in C₄ non-fixers (Tukey: $p=0.856$). There

1506 was no effect of soil moisture on β ($p=0.872$; Table 4.2; Fig. 4.2b). A functional

1507 group effect ($p<0.001$; Table 4.2) indicated that C₄ non-fixers generally had lower

1508 β values than both C₃ N-fixers and C₃ non-fixers (Tukey: $p<0.001$ in both cases),

1509 while β values in C₃ N-fixers did not differ from C₃ non-fixers (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

1510 *Significance determined using Type II Wald χ^2 tests ($\alpha=0.05$). P-values<0.05
1511 are in bold. Model coefficients are expressed on the natural-log scale and are only
1512 included for continuous fixed effects. Key: df=degrees of freedom; χ^2 =Wald Type
1513 II chi-square test statistic

1514 [DWS: exponential notation not used correctly in these tables. Looks

1515 copied from a graphing calculator output]

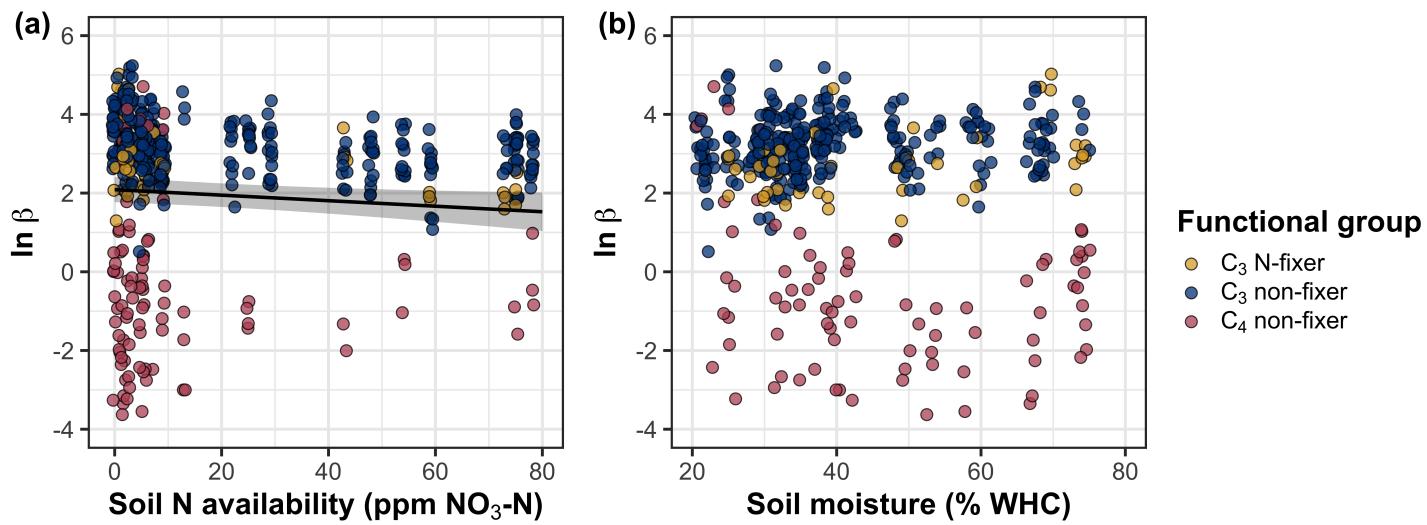


Figure 4.2. Effects of soil nitrogen availability (a) and soil moisture (b) on the cost of acquiring and using nitrogen (β ; unitless). Soil nitrogen availability is represented on the x-axis in (a), soil moisture is represented on the x-axis in (b) as a percent of site water holding capacity, and natural-log transformed β is represented on the y-axis for both panels. Yellow points represent C₃ N-fixers, blue points represent C₃ non-fixers, and red points represent C₄ non-fixers. 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.

1516 4.3.2 *Leaf C_i:C_a*

1517 Model selection indicated that 4-day mean vapor pressure deficit was the timescale

1518 that conferred the best model fit for leaf $C_i:C_a$ (AICc=-755.81; Table C3; Fig. C1).

1519 Model results revealed that increasing vapor pressure deficit generally de-

1520 creased leaf $C_i:C_a$ ($p<0.001$; Table 4.3; Fig. 4.3a). There was no effect of soil mois-

1521 ture ($p=0.549$; Table 4.3; Fig. 4.3b) or soil nitrogen availability ($p=0.549$; Table

1522 4.3; Fig. 4.3c) on leaf $C_i:C_a$. A strong plant functional group effect ($p<0.001$; Ta-

1523 ble 4.3) indicated that C₄ non-fixers had lower leaf $C_i:C_a$ than C₃ N-fixers and C₃

1524 non-fixers (Tukey: $p<0.001$ in both cases), with no difference between C₃ N-fixers

1525 and C₃ non-fixers (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

1526 *Significance determined using Type II Wald χ^2 tests ($\alpha=0.05$). *P*-values less
1527 than 0.05 are in bold and *p*-values where $0.05 < p < 0.1$ are italicized. Leaf $C_i:C_a$
1528 was not transformed prior to model fitting, so model coefficients are reported
1529 on the response scale. Model coefficients are only included for continuous fixed
1530 effects. Key: df=degrees of freedom; χ^2 =Wald Type II chi-square test statistic

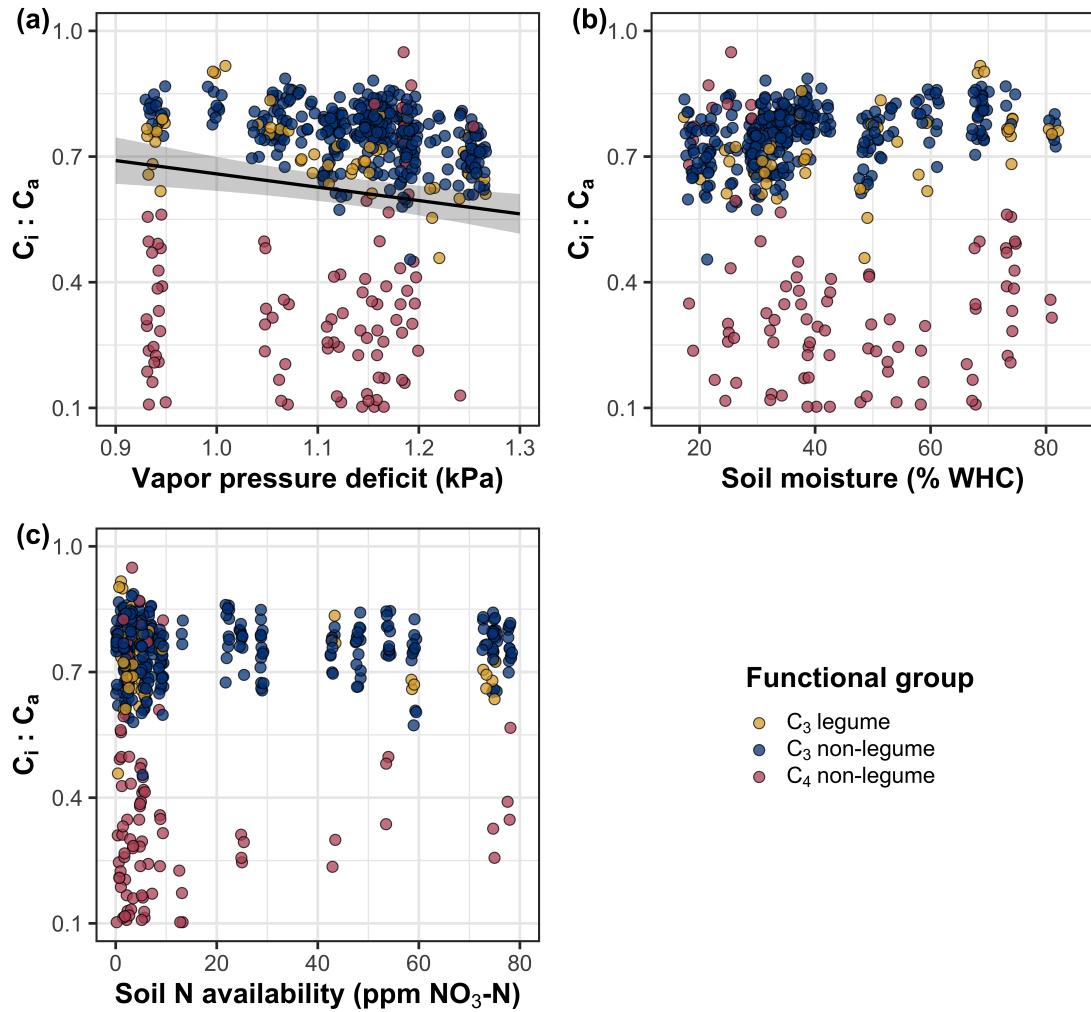


Figure 4.3. Effects of 4-day mean vapor pressure deficit (a), 90-day soil moisture (per water holding capacity; b), and soil nitrogen availability (c) on leaf $C_i:C_a$. Shading and trendlines are as explained in Figure 4.2. Points are jittered for visibility. Variably colored trendlines are only included 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.

1531 4.3.3 *Leaf nitrogen content*

1532 An interaction between leaf $C_i:C_a$ and plant functional group ($p<0.001$; Table
1533 4.4) revealed that the negative effect of increasing leaf $C_i:C_a$ on N_{area} ($p<0.001$;
1534 Table 4.4) was driven by a negative effect of increasing leaf $C_i:C_a$ on N_{area} in
1535 C_3 non-fixers and C_3 N-fixers (Tukey: $p<0.001$ in both cases), but not C_4 non-
1536 fixers (Tukey: $p=0.786$; Fig. 4.4a). A marginal interaction between soil nitrogen
1537 availability and plant functional group ($p=0.057$; Table 4.4) indicated that the
1538 positive effect of increasing soil nitrogen ($p=0.007$; Table 4.4) was only apparent
1539 in C_3 N-fixers (Tukey: $p<0.001$; Table 4.4; Fig. 4.4d), but not C_3 non-fixers
1540 (Tukey: $p=0.329$) or C_4 non-fixers (Tukey: $p=0.682$). Increasing soil moisture
1541 increased N_{area} ($p=0.011$, Table 4.4). A plant functional group effect ($p<0.001$;
1542 Table 4.4) indicated that C_4 non-fixers had lower N_{area} compared to C_3 N-fixers
1543 and C_3 non-fixers (Tukey: $p<0.001$ in both cases), while C_3 N-fixers had lower
1544 N_{area} compared to C_3 non-fixers (Tukey: $p=0.024$).

1545 Leaf $C_i:C_a$ had no effect on N_{mass} ($p=0.455$; Table 4.4; Fig. 4.4b). Increas-
1546 ing soil nitrogen availability and soil moisture each had a positive effect on N_{mass}
1547 ($p<0.001$ in both cases; Table 4.4; Fig. 4.4h). A plant functional group effect
1548 ($p<0.001$; Table 4.4) indicated that C_4 non-fixers had lower N_{mass} compared to
1549 C_3 N-fixers and C_3 non-fixers (Tukey: $p=0.001$ in both cases), while N_{mass} did
1550 not differ between C_3 N-fixers and C_3 non-fixers (Tukey: $p=0.323$).

1551 Variance in M_{area} was driven by a three-way interaction between soil nitro-
1552 gen availability, soil moisture, and plant functional group ($p=0.018$; Table 4.4).
1553 This interaction indicated that increasing soil moisture increased the positive effect
1554 of increasing soil nitrogen availability on M_{area} in C_3 N-fixers (Tukey: $p=0.028$)

1555 but did not modify the negative effect of increasing soil nitrogen availability on
1556 M_{area} in C₄ non-fixers (Tukey: $p=0.806$) or C₃ non-fixers (Tukey: $p=0.998$). There
1557 was otherwise no effect of soil moisture on M_{area} ($p=0.436$; Table 4.4). An inter-
1558 action between leaf $C_i:C_a$ and plant functional group ($p<0.001$; Table 4.4; Fig.
1559 4.4c) indicated that the negative effect of increasing leaf $C_i:C_a$ on M_{area} ($p<0.001$;
1560 Table 4.4) was driven by a negative effect of increasing leaf $C_i:C_a$ on M_{area} in
1561 C₃ N-fixers (Tukey: $p<0.001$) and C₃ non-fixers(Tukey: $p=0.003$), but not C₄
1562 non-fixers (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	0.036
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	<0.001
N*PFT	1	-	5.713	0.057	-	1.286	0.526	-	19.405	<0.001
SM ₉₀ *PFT	1	-	3.487	0.175	-	0.889	0.641	-	2.998	0.223
SM ₉₀ *N*PFT	1	-	3.523	0.172	-	0.161	0.923	-	7.996	0.018

97

1563 *Significance determined using Type II Wald χ^2 tests ($\alpha=0.05$). P-values less than 0.05 are in bold and p-values where $0.05 < p < 0.1$ are italicized. Coefficients are reported on the natural-log scale for all traits and are only included for continuous fixed effects. Key: df=degrees of freedom; χ^2 =Wald Type II chi-square test statistic

1564

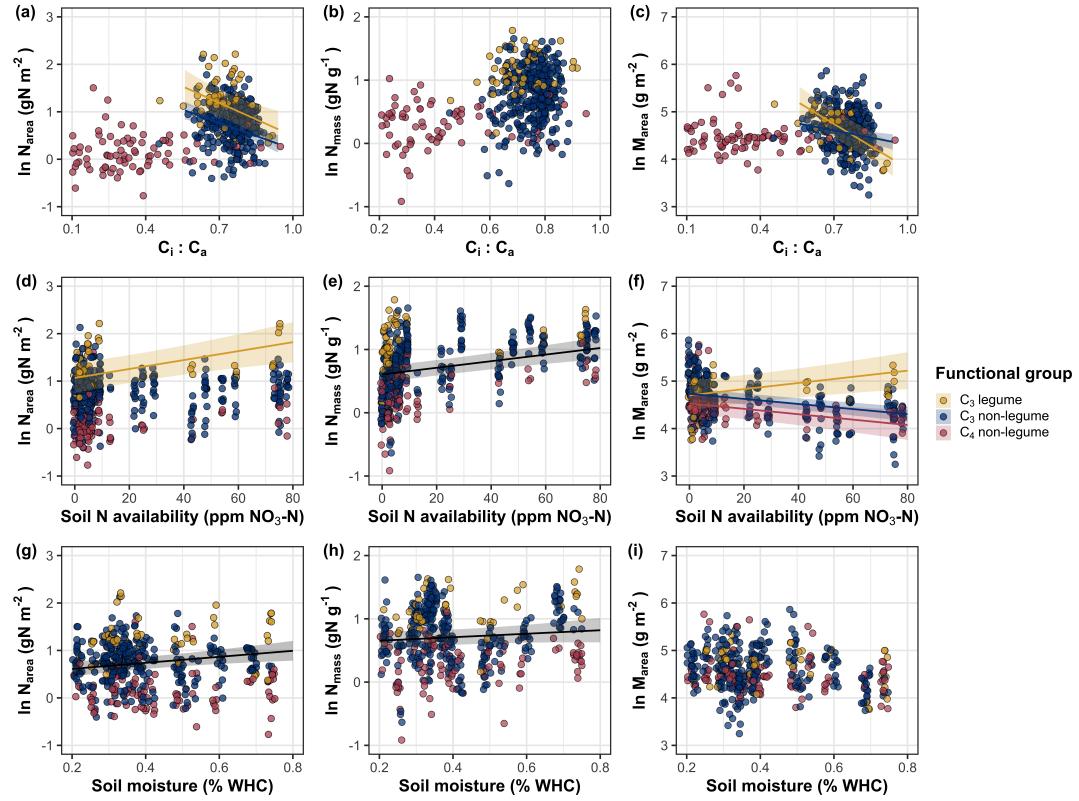


Figure 4.4. Effects of leaf $C_i:C_a$ (a-c), soil nitrogen availability (d-f), and soil moisture (g-i) on leaf nitrogen content per unit leaf area (a, d, g), leaf nitrogen content per unit leaf biomass (b, e, h), and leaf mass per area (c, f, i). Yellow points and trendlines indicate C₃ N-fixers, blue points and trendlines indicate C₃ non-fixers, and red points and trendlines indicate C₄ non-fixers. 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.

1566 4.3.4 *Structural equation model*

1567 The piecewise structural equation model explained 89%, 55%, 56%, 82%, and
1568 38% of variance in N_{area} , N_{mass} , M_{area} , leaf $C_i:C_a$, and β , respectively (Table
1569 4.5; Fig. 4.5). Increasing N_{mass} and M_{area} were each positively related to N_{area}
1570 ($p<0.001$ in both cases; Table 4.5; Fig. 4.5). N_{mass} increased with increasing
1571 soil nitrogen availability ($p<0.001$; Table 4.5) and leaf $C_i:C_a$ ($p=0.040$; Table
1572 4.5), and was generally larger in N-fixing species ($p<0.001$; Table 4.5), but was
1573 negatively related to increasing M_{area} ($p<0.001$; Table 4.5). M_{area} decreased with
1574 increasing leaf $C_i:C_a$ and soil nitrogen availability ($p<0.001$ in both cases; Table
1575 4.5). Leaf $C_i:C_a$ declined with increasing vapor pressure deficit, but was positively
1576 related to β ($p<0.001$ in both cases; Table 4.5). β decreased with increasing soil
1577 nitrogen availability and was higher in C₃ species ($p<0.001$ in both cases; Table
1578 4.5), but did not change with soil moisture ($p=0.895$; Table 4.5) or with ability
1579 to acquire nitrogen via symbiotic nitrogen fixation ($p=0.519$; Table 4.5). Finally,
1580 soil nitrogen availability was positively associated with increasing soil moisture
1581 ($p=0.003$; 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} ($R^2_c=0.55$)		
Leaf $C_i:C_a$	0.113	0.040
M_{area}	-0.201	<0.001
Soil N	0.246	<0.001
N-fixing ability	0.326	<0.001
M_{area} ($R^2_c=0.56$)		
Leaf $C_i:C_a$	-0.224	<0.001
Soil N	-0.199	<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

1582 *Coefficients are standardized across the structural equation model. *P*-values less
1583 than 0.05 are noted in bold. Positive coefficients for photosynthetic pathway
1584 indicate generally larger values in C₃ species, while positive coefficients for N-
1585 fixing ability indicate generally larger values in N-fixing species. Key: df=degrees
1586 of freedom; χ^2 =Wald Type II chi-square test statistic; R^2_c =conditional R² value;
1587 N_{mass} =leaf nitrogen content per unit leaf biomass (gN g⁻¹); M_{area} =leaf mass per
1588 unit leaf biomass (g m⁻²); β =cost of acquiring nitrogen relative to water (unitless);
1589 VPD_4 =4-day mean vapor pressure deficit (kPa); SM_{90} =90-day mean soil moisture
1590 (mm)

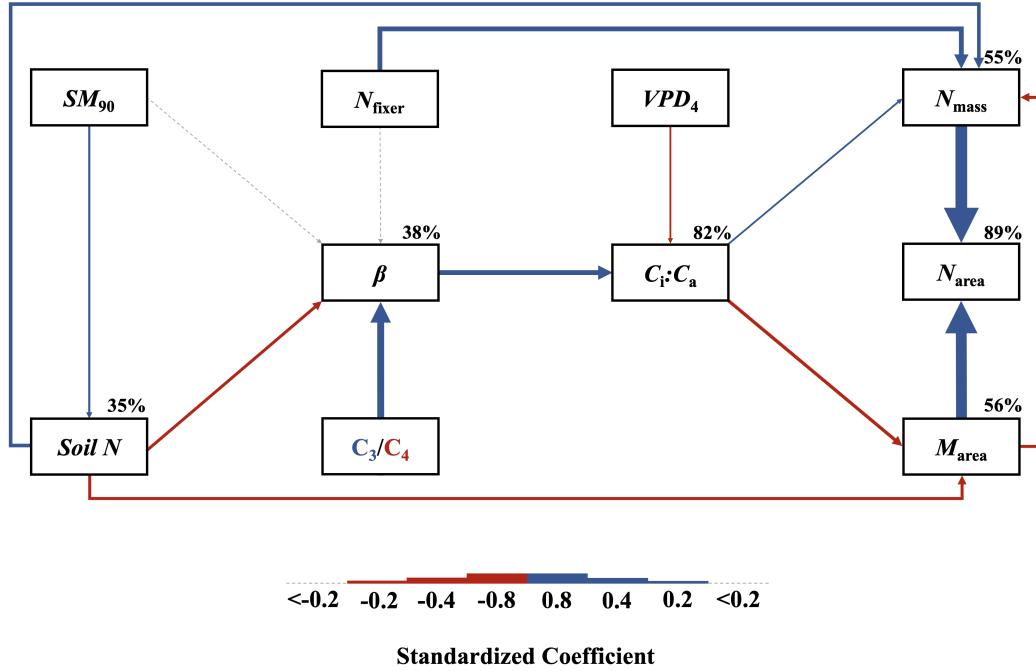


Figure 4.5. Structural equation model results exploring drivers of N_{area} . Boxes indicate measured edaphic factors, climatic factors, and leaf traits. Solid arrows indicate bivariate relationships where $p < 0.05$, while dashed arrows indicate relationships where $p > 0.05$. Positive model coefficients 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 C₃ 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 R² values for each response variable reported on the top right of each box.

1591 4.4 Discussion

1592 In this study, direct and indirect effects of edaphic and climatic characteristics on
1593 N_{area} and components of N_{area} (N_{mass} and M_{area}) were quantified in 504 individuals
1594 spanning across a soil resource availability and climate gradient in Texas, USA.
1595 Consistent patterns emerged in support of those expected from photosynthetic
1596 least-cost theory, a result driven by a strong direct negative relationship between
1597 leaf $C_i:C_a$ and N_{area} mediated through changes in M_{area} . In further support of
1598 patterns expected from theory, increasing soil nitrogen availability had a nega-
1599 tive effect on β , resulting in an indirect stimulation in N_{area} mediated through
1600 a positive relationship between β and $C_i:C_a$. Increasing vapor pressure deficit
1601 also indirectly increased N_{area} through a direct negative effect of increasing vapor
1602 pressure deficit on leaf $C_i:C_a$, following hypotheses and patterns expected from
1603 theory. Interestingly, a positive association between soil moisture and N_{area} was
1604 driven by covariance between soil moisture and soil nitrogen availability and was
1605 not associated with a direct effect of soil moisture on β . Overall, results provide
1606 strong and consistent support for patterns expected from photosynthetic least-cost
1607 theory, showing that both soil resource availability and climate drive variance in
1608 N_{area} through changes in leaf $C_i:C_a$.

1609 4.4.1 *Negative effects of leaf $C_i:C_a$ on N_{area} are driven by reductions in M_{area} ,*
1610 *not N_{mass}*

1611 The negative response of N_{area} to increasing leaf $C_i:C_a$ is consistent with pre-
1612 vious environmental gradient (Dong et al. 2017; Querejeta et al. 2022) and
1613 manipulation experiments (3.4c), showing strong support for the nitrogen-water

1614 use tradeoffs expected from photosynthetic least cost theory (Wright et al. 2003;
1615 Prentice et al. 2014). Negative effects of increasing leaf $C_i:C_a$ on N_{area} were driven
1616 by negative effect of increasing leaf $C_i:C_a$ on M_{area} coupled with a weak positive
1617 effect of increasing leaf $C_i:C_a$ on N_{mass} , suggesting that changes in N_{area} across
1618 the environmental gradient were driven more strongly by changes in leaf morphol-
1619 ogy than leaf chemistry. Interestingly, the negative relationship between M_{area}
1620 and N_{mass} suggested that stimulations in N_{mass} were often associated with larger,
1621 thinner leaves (i.e., lower M_{area}). These results are consistent with patterns re-
1622 ported from previous studies indicating that variance in N_{area} is driven by changes
1623 in M_{area} across environmental gradients, and that part of this response is due to
1624 negative covariance between M_{area} and N_{mass} (Dong et al. 2017; Dong et al. 2020).
1625 Negative covariance between M_{area} and N_{mass} could be a response associated with
1626 tradeoffs between leaf longevity and leaf productivity (Wright et al. 2004; Dong
1627 et al. 2017; Dong et al. 2022; Querejeta et al. 2022; Wang et al. 2023).

1628 The negative relationship between leaf $C_i:C_a$ and M_{area} could be indicative
1629 of tradeoffs between leaf longevity and leaf productivity. Tradeoffs between leaf
1630 longevity and leaf productivity are commonly observed and are included in a
1631 continuum of coordinated leaf traits that position individuals along a fast- or
1632 slow-growing leaf economics spectrum (Wright et al. 2003; Onoda et al. 2004;
1633 Reich 2014; Onoda et al. 2017; Wang et al. 2023). Negative relationships between
1634 leaf $C_i:C_a$ and M_{area} indicate that increased stomatal conductance and reduced
1635 water use efficiency were associated with thinner, larger leaves (i.e., lower M_{area}).
1636 Combined with the negative covariance between M_{area} and N_{mass} mentioned above,
1637 these responses may have allowed individuals to maximize light interception and

1638 productivity by exploiting high light environments at the expense of increased
1639 water loss and decreased water-use efficiency. This strategy may be especially
1640 advantageous for fast-growing species in open canopy systems. In this study, C₃
1641 N-fixers and C₃ non-fixers dominated the dataset (77% of total sampling effort),
1642 of which 23% (17% of total sampling effort) were classified as annual species with
1643 short growing seasons. We observed no effect of leaf $C_i:C_a$ on N_{area} or M_{area} in C₄
1644 non-fixers, which made up 23% of the sampling effort and were generally classified
1645 as warm season graminoid species with slower growth rates and longer growing
1646 seasons. These patterns indicate that stronger tradeoffs between nitrogen and
1647 water use may be more apparent in fast-growing species with high demand for
1648 building and maintaining productive leaf tissues.

1649 4.4.2 *Soil nitrogen availability increases N_{area} through changes in β*
1650 The structural equation model indicated multiple pathways where increasing soil
1651 nitrogen availability increased N_{area} . First, N_{area} increased with increasing soil
1652 nitrogen availability due to larger positive direct effects of increasing soil nitrogen
1653 availability on N_{mass} than the corresponding negative direct effect of increasing
1654 soil nitrogen availability on M_{area} . These patterns corroborate those observed in
1655 the individual linear mixed effect models and previous work. Second, soil nitrogen
1656 availability increased N_{area} indirectly through reductions in β , which increased leaf
1657 $C_i:C_a$ and stimulated N_{area} through a stronger negative effect of increasing leaf
1658 $C_i:C_a$ on M_{area} than corresponding positive effect of increasing leaf $C_i:C_a$ on N_{mass} .
1659 Reductions in β with increasing soil nitrogen availability were likely driven by re-
1660 ductions in the cost of acquiring and using nitrogen, following patterns observed

1661 in previous experiments (Bae et al. 2015; Eastman et al. 2021; Perkowski et al.
1662 2021; Lu et al. 2022). These pathways indicate that soil nitrogen availability can
1663 have direct positive effects on N_{area} by increasing leaf nitrogen concentration, fol-
1664 lowing previous work (Firn et al. 2019; Liang et al. 2020), or can alternatively have
1665 indirect positive effects on N_{area} through changes in leaf morphology associated
1666 with a reduction in the cost of acquiring nitrogen, following patterns expected
1667 from photosynthetic least-cost theory. Results reported here indicate that pho-
1668 tosynthetic least-cost frameworks are capable of detecting predictable variance in
1669 N_{area} and tradeoffs between nitrogen and water use across soil nitrogen availability
1670 gradients.

1671 4.4.3 *Soil moisture increases N_{area} by facilitating increases in soil nitrogen
1672 availability*

1673 Increasing soil moisture had a positive effect on N_{area} , though this response was
1674 associated with a null effect of soil moisture on β . These results contrast patterns
1675 expected from theory, where increasing soil moisture is expected to indirectly
1676 decrease N_{area} through an increase in β due to a reduction in costs associated
1677 with water acquisition and use (Wright et al. 2003; Prentice et al. 2014; Lavergne
1678 et al. 2020). Interestingly, structural equation model results revealed a strong
1679 positive association between soil moisture and soil nitrogen availability, indicating
1680 an indirect positive effect of increasing soil moisture on N_{area} mediated by the
1681 negative effect of increasing soil nitrogen availability on β . In Texan grasslands,
1682 productivity and nutrient uptake are often co-limited by precipitation and nutrient
1683 availability (Yahdjian et al. 2011; Wang et al. 2017). Thus, increases in soil
1684 moisture may have facilitated more favorable and productive environments for
1685 soil microbial communities (Reichman et al. 1966; Stark and Firestone 1995;

1686 Paul et al. 2003), or alternatively greater nitrogen mobility in soil solution. As
1687 discussed above, the positive indirect response of N_{area} to increasing soil nitrogen
1688 availability as mediated through reductions in β follow patterns expected from
1689 theory.

1690 4.4.4 *Indirect effects of climate on N_{area} are mediated through changes in leaf*
1691 $C_i:C_a$ *and β*

1692 In support of hypotheses and patterns expected from theory, increasing vapor
1693 pressure deficit indirectly increased N_{area} , mediated through the negative effect
1694 of increasing vapor pressure deficit on leaf $C_i:C_a$. These responses are consistent
1695 with previous work noting strong reductions in stomatal conductance with increas-
1696 ing vapor pressure deficit (Oren et al. 1999; Novick et al. 2016; Sulman et al.
1697 2016; Grossiord et al. 2020; López et al. 2021), a response that allows plants
1698 to minimize water loss as a result of high atmospheric water demand. Results
1699 also support findings from previous experiments across environmental gradients,
1700 where increasing vapor pressure deficit generally increases N_{area} at lower stomatal
1701 conductance across environmental gradients (Dong et al. 2017; Dong et al. 2022;
1702 Paillassa et al. 2020; Westerband et al. 2023). The increase in N_{area} with increas-
1703 ing vapor pressure deficit could allow plants to maximize photosynthetic capacity
1704 under reduced stomatal conductance (Dong et al. 2022), though this pattern con-
1705 trasts previous work suggesting that long-term increases in vapor pressure deficit
1706 are associated with increased plant mortality, reduced net primary productivity,
1707 and perhaps reductions in net photosynthesis rates over time due to prolonged
1708 stomatal closure (Eamus et al. 2013; Yuan et al. 2019; Grossiord et al. 2020).
1709 Importantly, such negative effects of increasing vapor pressure deficit often occur
1710 along much broader timescales compared to the timescale used here. Responses

1711 observed here suggest that variance in N_{area} across environmental gradients is
1712 a deterministic acclimation response to changing aboveground climate, allowing
1713 plants to satisfy demand to build and maintain photosynthetic enzymes and op-
1714 timize photosynthetic processes by maximizing resource use efficiency (Paillassa
1715 et al. 2020; Peng et al. 2021; Dong et al. 2022; Westerband et al. 2023).

1716 4.4.5 *Species identity traits modify effects of the environment on β , leaf $C_i:C_a$,*
1717 *and N_{area}*

1718 N-fixing species had greater N_{area} values on average compared to non-fixing species,
1719 a pattern driven by a stronger stimulation in N_{mass} in N-fixing species coupled with
1720 no change in M_{area} between species with different N-fixation ability. There was
1721 no evidence to suggest that N-fixing species had different β or leaf $C_i:C_a$ values
1722 compared to non-fixing species across the environmental gradient. These results
1723 follow patterns from previous environmental gradient experiments that investi-
1724 gate variance in leaf nitrogen allocation in N-fixing species (Adams et al. 2016;
1725 Dong et al. 2017; Dong et al. 2020), and that increases in N_{mass} and N_{area} in
1726 N-fixing species are not necessarily correlated to increases in water use efficiency
1727 or reductions in leaf $C_i:C_a$ (Adams et al. 2016). While results are consistent with
1728 results from previous environmental gradient experiments, they do not support
1729 hypotheses presented here or patterns expected from theory, which predicts that
1730 stimulations in N_{area} by N-fixing species should be driven by a reduction in β
1731 relative to non-fixing species, and that this response should decrease stomatal
1732 conductance and leaf $C_i:C_a$.

1733 C₄ species had reduced β , leaf $C_i:C_a$, and N_{area} than C₃ species. Reduced
1734 β and leaf $C_i:C_a$ values in C₄ species follow hypotheses listed above, a pattern

1735 that could be the result of either reduced costs of nitrogen acquisition and use,
1736 increased costs of water acquisition and use, or both (Wright et al. 2003; Prentice
1737 et al. 2014). Results also indicate that β in C₄ non-fixers was unresponsive to
1738 changes in soil nitrogen availability despite an apparent negative effect of increas-
1739 ing soil nitrogen availability on β in C₃ N-fixers and C₃ non-fixers. Combined
1740 with a general null response of β to soil moisture regardless of plant functional
1741 group, these patterns imply that reduced β values in C₄ species may be the re-
1742 sult of lower costs of nitrogen acquisition and use relative to C₃ species. While
1743 lower β values in C₄ species provides a possible explanation for why C₄ species
1744 often have lower leaf $C_i:C_a$ and greater water use efficiency, theory predicts that
1745 this response should cause C₄ species to have greater N_{area} values compared to
1746 C₃ species, though C₄ species commonly exhibit lower N_{area} and higher nitrogen
1747 use efficiency than C₃ species (Schmitt and Edwards 1981; Sage and Pearcy 1987;
1748 Ghannoum et al. 2011). We speculate that lowered costs of nitrogen acquisition
1749 and use in C₄ species could be driven by more efficient Rubisco carboxylation effi-
1750 ciency in C₄ species associated with CO₂ concentrating mechanisms that eliminate
1751 photorespiration (Ghannoum et al. 2011), which could reduce or eliminate the
1752 need to sacrifice inefficient nitrogen use for efficient water use to achieve optimal
1753 photosynthesis rates.

1754 4.4.6 *Next steps for optimality model development*

1755 Optimality models for both C₃ and C₄ species have been developed using principles
1756 from photosynthetic least-cost theory (Prentice et al. 2014; Wang et al. 2017;
1757 Smith et al. 2019; Stocker et al. 2020; Scott and Smith 2022). In both C₃ and C₄
1758 model variants, β values are held constant using global datasets of leaf $\delta^{13}\text{C}$ (Wang

1759 et al. 2017; Cornwell et al. 2018). Specifically, the C₃ optimality model initially
1760 assumed a constant β value of 240 (Wang et al. 2017), later corrected to 146
1761 (Stocker et al. 2020), while the C₄ optimality model assumes a constant β value of
1762 166 (Scott and Smith 2022). These results, which build on findings from Paillassa
1763 et al. (2020) and Lavergne et al. (2020), demonstrate high variability in calculated
1764 β values across the environmental gradient. Specifically, β values in C₃ species
1765 ranged from 1.7 to 188.0 (mean: 30.2; median: 23.1; standard deviation: 25.4),
1766 while ranged from 0.1 to 110.6 in C₄ species (mean: 7.2; median: 0.7; standard
1767 deviation: 18.6). Mean β values in both C₃ and C₄ species were consistently lower
1768 than values currently implemented in optimality models, though this was likely
1769 the result of increased water limitation across sites relative to global averages.
1770 Regardless, the high degree of β variability across this environmental gradient,
1771 together with findings from Lavergne et al. (2020) and Paillassa et al. (2020),
1772 suggests that the use of constant β values may contribute to erroneous errors when
1773 conducting optimality model simulations. Results from this experiment build
1774 on suggestions from Wang et al. (2017), suggesting that future photosynthetic
1775 least-cost optimality model developments should consider adopting frameworks
1776 for dynamically calculating β .

1777 4.4.7 *Conclusions*

1778 To summarize, variability in N_{area} across an environmental gradient in Texan
1779 grasslands was driven by indirect effects of climate and soil resource availability
1780 mediated by changes in β and leaf $C_i:C_a$. Results from this experiment provide
1781 strong and consistent support for patterns expected from photosynthetic least-

1782 cost theory, demonstrating that negative relationships between $C_i:C_a$ and N_{area}
1783 unify expected effects of climatic and edaphic characteristics on N_{area} across en-
1784 vironmental gradients. Results reported here also demonstrate a need to consider
1785 the dynamic nature of the relative cost of nitrogen versus water uptake (β) across
1786 environmental gradients in optimality models that leverage principles of photo-
1787 synthetic least-cost theory.

1788

Chapter 5

1789 Optimal resource investment to photosynthetic capacity maximizes
1790 nutrient allocation to whole plant growth under elevated CO₂

1791 5.1 Introduction

1792 Terrestrial ecosystems are regulated by complex carbon and nitrogen cycles. As
1793 a result, terrestrial biosphere models, which are beginning to include coupled
1794 carbon and nitrogen cycles (Shi et al. 2016; Davies-Barnard et al. 2020; Braghieri
1795 et al. 2022), must accurately represent these cycles under different environmental
1796 scenarios to reliably simulate carbon and nitrogen atmosphere-biosphere fluxes
1797 (Hungate et al. 2003; Prentice et al. 2015). While the inclusion of coupled carbon
1798 and nitrogen cycles tends to reduce model uncertainty (Arora et al. 2020), large
1799 uncertainty in role of soil nitrogen availability and nitrogen acquisition strategy
1800 on leaf and whole plant acclimation responses to CO₂ remains (Smith and Dukes
1801 2013; Terrer et al. 2018; Smith and Keenan 2020). This source of uncertainty
1802 likely contributes to the widespread divergence in future carbon and nitrogen flux
1803 simulations across terrestrial biosphere models (Friedlingstein et al. 2014; Zaehle
1804 et al. 2014; Meyerholt et al. 2020).

1805 Plants grown under elevated CO₂ generally have less leaf nitrogen content
1806 than those grown under ambient CO₂, a response that often corresponds with
1807 reductions in photosynthetic capacity and stomatal conductance at the leaf-level
1808 and biomass stimulation over time at the whole plant level (Curtis 1996; Drake
1809 et al. 1997; Ainsworth et al. 2002; Makino 2003; Morgan et al. 2004; Ainsworth
1810 and Long 2005; Ainsworth and Rogers 2007; Smith and Dukes 2013; Poorter et al.
1811 2022). As net primary productivity is generally limited by nitrogen availability

1812 (Vitousek and Howarth 1991; LeBauer and Treseder 2008; Fay et al. 2015), and
1813 soil nitrogen availability is often positively correlated with leaf nitrogen content
1814 and photosynthetic capacity (Field and Mooney 1986; Evans and Seemann 1989;
1815 Evans 1989; Walker et al. 2014; Firn et al. 2019; Liang et al. 2020), some
1816 have hypothesized that leaf and whole plant acclimation responses to CO₂ are
1817 constrained by soil nitrogen availability.

1818 The progressive nitrogen limitation hypothesis predicts that elevated CO₂
1819 will increase plant nitrogen demand, which will increase plant nitrogen uptake and
1820 progressively deplete soil nitrogen if soil nitrogen supply does not exceed plant
1821 nitrogen demand (Luo et al. 2004). The hypothesis predicts that this response
1822 should result in strong acute stimulations in whole plant growth and primary
1823 productivity that diminish over time as nitrogen becomes more limiting. Assuming
1824 a positive relationship between soil nitrogen availability, leaf nitrogen content, and
1825 photosynthetic capacity, this hypothesis also implies that progressive reductions in
1826 soil nitrogen availability should be the mechanism that drives the downregulation
1827 of leaf nitrogen content and photosynthetic capacity under elevated CO₂. This
1828 hypothesis has received some support from free air CO₂ enrichment experiments
1829 (Reich et al. 2006; Norby et al. 2010), although is not consistently observed across
1830 experiments (Finzi et al. 2006; Moore et al. 2006; Liang et al. 2016).

1831 While possible that progressive nitrogen limitation may determine leaf and
1832 whole plant acclimation responses to CO₂, growing evidence indicates that leaf ni-
1833 trogen and photosynthetic capacity are more strongly determined through above-
1834 ground growing conditions than by soil resource availability (Dong et al. 2017;
1835 Dong et al. 2020; Dong et al. 2022; Smith et al. 2019; Smith and Keenan 2020;

1836 Paillassa et al. 2020; Peng et al. 2021; Querejeta et al. 2022; Westerband et al.
1837 2023), and satellite-derived chlorophyll fluorescence data indicate that increasing
1838 atmospheric CO₂ may decrease leaf and canopy demand for nitrogen (Dong et al.
1839 2022). Together, results from these studies suggest that the downregulation in
1840 leaf nitrogen content and photosynthetic capacity due to increasing CO₂ may not
1841 be as tightly linked to progressive nitrogen limitation as previously hypothesized.

1842 A unification of optimal coordination and least-cost theories predicts that
1843 leaves acclimate to elevated CO₂ by downregulating nitrogen allocation to Ribulose-
1844 1,5-bisphosphate (RuBP) carboxylase/oxygenase (Rubisco) to optimize resource
1845 use efficiencies at the leaf level, which allows for greater resource allocation to
1846 whole plant growth (Drake et al. 1997; Wright et al. 2003; Prentice et al. 2014;
1847 Smith et al. 2019). The theory predicts that the downregulation in nitrogen
1848 allocation to Rubisco results in a stronger downregulation in the maximum rate
1849 of Rubisco carboxylation (V_{cmax}) than the maximum rate of RuBP regeneration
1850 (J_{max}), which maximizes photosynthetic efficiency by allowing net photosynthesis
1851 rates to be equally co-limited by Rubisco carboxylation and RuBP regeneration
1852 (Chen et al. 1993; Maire et al. 2012). This acclimation response allows plants to
1853 make more efficient use of available light while avoiding overinvestment in Rubisco,
1854 which has high nitrogen and energetic costs of building and maintaining (Evans
1855 1989; Evans and Clarke 2019). Instead, additional acquired resources not needed
1856 to optimize leaf photosynthesis are allocated to the maintenance of structures that
1857 support whole plant growth (e.g., total leaf area, whole plant biomass, etc.) or
1858 to allocation processes not related to leaf photosynthesis or growth, such as plant
1859 defense mechanisms. Regardless, optimized resource allocation at the leaf level

1860 should allow for greater resource allocation to whole plant growth. The theory
1861 indicates that leaf acclimation responses to CO₂ should be independent of changes
1862 in soil nitrogen availability. While this leaf acclimation response maximizes nitro-
1863 gen allocation to structures that support whole plant growth, the theory suggests
1864 that the positive effect of elevated CO₂ on whole plant growth may be further
1865 stimulated by soil nitrogen availability through reductions in the cost of acquiring
1866 nitrogen (Bae et al. 2015; Perkowski et al. 2021; Lu et al. 2022).

1867 Plants acquire nitrogen by allocating photosynthetically derived carbon be-
1868 lowground in exchange for nitrogen through different nitrogen acquisition strate-
1869 gies. These nitrogen acquisition strategies can include direct uptake pathways
1870 such as mass flow or diffusion (Barber 1962), symbioses with mycorrhizal fungi or
1871 symbiotic nitrogen-fixing bacteria (Vance and Heichel 1991; Marschner and Dell
1872 1994; Smith and Read 2008; Udvardi and Poole 2013), or through the release
1873 of root exudates that prime free-living soil microbial communities (Phillips et al.
1874 2011; Wen et al. 2022). Plants cannot acquire nitrogen without first allocating
1875 carbon belowground, which implies an inherent carbon cost to the plant for acquir-
1876 ing nitrogen regardless of nitrogen acquisition strategy. Carbon costs to acquire
1877 nitrogen often vary in species with different nitrogen acquisition strategies and
1878 are dependent on external environmental factors such as atmospheric CO₂, light
1879 availability, and soil nitrogen availability (Brzostek et al. 2014; Terrer et al. 2016;
1880 Terrer et al. 2018; Allen et al. 2020; Perkowski et al. 2021; Lu et al. 2022). These
1881 patterns suggest that acquisition strategy may at least partially determine the net
1882 effect of soil nitrogen availability on leaf and whole plant acclimation responses to
1883 elevated CO₂.

1884 A recent meta-analysis using data across 20 grassland and forest CO₂ en-
1885 richment experiments suggested that species which acquire nitrogen from sym-
1886 biotic nitrogen-fixing bacteria had reduced costs of nitrogen acquisition under
1887 elevated CO₂ (Terrer et al. 2018). Though these analyses only included data
1888 from two experimental sites, findings from this meta-analysis indicated that re-
1889 ductions in costs of nitrogen acquisition in species that form associations with
1890 symbiotic nitrogen-fixing bacteria under elevated CO₂ may drive stronger stim-
1891 ulations in whole plant growth and downregulations in V_{cmax} than species that
1892 associate with arbuscular mycorrhizal fungi (Smith and Keenan 2020), which gen-
1893 erally have greater costs of nitrogen acquisition under elevated CO₂ (Terrer et al.
1894 2018). However, plant investments in symbiotic nitrogen fixation generally de-
1895 cline with increasing nitrogen availability (Dovrat et al. 2018; Perkowski et al.
1896 2021), a response that has been previously inferred to be the result of a shift in
1897 the dominant mode of nitrogen acquisition to direct uptake pathways as costs of
1898 direct uptake decrease with increasing soil nitrogen availability (Rastetter et al.
1899 2001; Perkowski et al. 2021). Thus, effects of symbiotic nitrogen fixation on plant
1900 acclimation responses to CO₂ should decline with increasing soil nitrogen avail-
1901 ability, although manipulative experiments that directly test these patterns are
1902 rare.

1903 Here, I conducted a 7-week growth chamber experiment using *Glycine max*
1904 L. (Merr.) to examine the effects of soil nitrogen fertilization and inoculation with
1905 symbiotic nitrogen-fixing bacteria on leaf and whole plant acclimation responses
1906 to elevated CO₂. Following patterns expected from theory, I hypothesized that in-
1907 dividual leaves should acclimate to elevated CO₂ by more strongly downregulating

1908 V_{cmax} relative to J_{max} , allowing leaf photosynthesis to approach optimal coordi-
1909 nation. I expected this response to correspond with a stronger downregulation in
1910 leaf nitrogen content than V_{cmax} and J_{max} , which would increase the fraction of
1911 leaf nitrogen content allocated to photosynthesis and photosynthetic nitrogen use
1912 efficiency. At the whole-plant level, I hypothesized that plants would acclimate
1913 to elevated CO₂ by stimulating whole plant growth and productivity, a response
1914 that would be driven by a strong positive response of total leaf area and above-
1915 ground biomass to elevated CO₂. I predicted that leaf acclimation responses to
1916 elevated CO₂ would be independent of soil nitrogen fertilization and inoculation
1917 with symbiotic nitrogen-fixing bacteria; however, I expected that increasing soil
1918 nitrogen fertilization would increase the positive effect of elevated CO₂ on mea-
1919 sures of whole plant growth due to a stronger reduction in the cost of acquiring
1920 nitrogen under elevated CO₂ with increasing fertilization. I also expected stronger
1921 stimulations in whole plant growth due to inoculation, but that this effect would
1922 only be apparent under low fertilization due to a reduction in root nodulation
1923 with increasing fertilization.

1924 5.2 Methods

1925 5.2.1 *Seed treatments and experimental design*

1926 *Glycine max* L. (Merr) seeds were planted in 144 6-liter surface sterilized pots (NS-
1927 600, Nursery Supplies, Orange, CA, USA) containing a steam-sterilized 70:30 v:v
1928 mix of Sphagnum peat moss (Premier Horticulture, Quakertown, PA, USA) to
1929 sand (Pavestone, subsidiary of Quikrete Companies, Atlanta, GA, USA). Before
1930 planting, all *G. max* seeds were surface sterilized in 2% sodium hypochlorite for 3

1931 minutes, followed by three separate 3-minute washes with ultrapure water (MilliQ
1932 7000; MilliporeSigma, Burlington, MA USA). A subset of surface sterilized seeds
1933 were inoculated with *Bradyrhizobium japonicum* (Verdesian N-DureTM Soybean,
1934 Cary, NC, USA) in a slurry following manufacturer recommendations (3.12 g
1935 inoculant and 241 g deionized water per 1 kg seed).

1936 Seventy-two pots were randomly planted with surface-sterilized seeds inoc-
1937 ulated with *B. japonicum*, while the remaining 72 pots were planted with surface-
1938 sterilized uninoculated seeds. Thirty-six pots within each inoculation treatment
1939 were randomly placed in one of two atmospheric CO₂ treatments (ambient and
1940 1000 $\mu\text{mol mol}^{-1}$ CO₂). Pots within each unique inoculation-by-CO₂ treatment
1941 combination randomly received one of nine soil nitrogen fertilization treatments
1942 equivalent to 0, 35, 70, 105, 140, 210, 280, 350, or 630 ppm N. Nitrogen fertil-
1943 ization treatments were created using a modified Hoagland solution (Hoagland
1944 and Arnon 1950) designed to keep concentrations of other macronutrients and
1945 micronutrients equivalent across treatments (Table D1). Pots received the same
1946 fertilization treatment throughout the entire duration experiment, which were ap-
1947 plied twice per week in 150 mL doses as topical agents to the soil surface. This
1948 experimental design yielded a fully factorial experiment with four replicates per
1949 unique fertilization-by-inoculation-by-CO₂ combination.

1950 5.2.2 *Growth chamber conditions*

1951 Upon experiment initiation, pots were randomly placed in one of six Percival
1952 LED-41L2 growth chambers (Percival Scientific Inc., Perry, IA, USA) over two
1953 experimental iterations due to chamber space limitation. Two iterations were

1954 conducted such that one iteration included all elevated CO₂ pots and the second
1955 iteration included all ambient CO₂ pots. Mean (\pm SD) CO₂ concentrations across
1956 chambers throughout the experiment were $439 \pm 5 \mu\text{mol mol}^{-1}$ CO₂ for the ambient
1957 CO₂ treatment and $989 \pm 4 \mu\text{mol mol}^{-1}$ CO₂ for the elevated CO₂ treatment.

1958 Daytime growing conditions were simulated using a 16-hour photoperiod,
1959 with incoming light radiation set to chamber maximum (mean \pm SD: 1240 ± 32
1960 $\mu\text{mol m}^{-2} \text{s}^{-1}$ across chambers), air temperature set to 25°C, and relative humid-
1961 ity set to 50%. The remaining 8 hours simulated nighttime growing conditions,
1962 with incoming light radiation set to 0 $\mu\text{mol m}^{-2} \text{s}^{-1}$, chamber temperature set
1963 to 17°C, and relative humidity set to 50%. Transitions between daytime and
1964 nighttime growing conditions were simulated by ramping incoming light radiation
1965 in 45-minute increments and temperature in 90-minute increments over a 3-hour
1966 period (Table D2).

1967 Including the two, 3-hour ramping periods, pots grew under average (\pm SD)
1968 daytime light intensity of $1049 \pm 27 \mu\text{mol m}^{-2} \text{s}^{-1}$. In the elevated CO₂ iteration,
1969 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
1970 51.6 $\pm 0.4\%$ relative humidity. In the ambient CO₂ iteration, pots grew under
1971 $23.9 \pm 0.2^\circ\text{C}$ during the day, $16.0 \pm 1.4^\circ\text{C}$ during the night, and 50.3 $\pm 0.2\%$ relative
1972 humidity. I accounted for any climatic differences across the six chambers by
1973 shuffling the same group of pots daily throughout the growth chambers. This
1974 process was done by iteratively moving the group of pots on the top rack of a
1975 chamber to the bottom rack of the same chamber, while simultaneously moving
1976 the group of pots on the bottom rack of a chamber to the top rack of the adjacent
1977 chamber. I moved pots within and across chambers every day throughout the

1978 course of each experiment iteration.

1979 5.2.3 *Leaf gas exchange measurements*

1980 Gas exchange measurements were collected for all individuals on the seventh week
1981 of development. All gas exchange measurements were collected on the center leaf
1982 of the most recent fully expanded trifoliate leaf set. Specifically, I measured net
1983 photosynthesis (A_{net} ; $\mu\text{mol m}^{-2} \text{s}^{-1}$), stomatal conductance (g_{sw} ; $\text{mol m}^{-2} \text{s}^{-1}$),
1984 and intercellular CO_2 (C_i ; $\mu\text{mol mol}^{-1}$) concentrations across a range of atmo-
1985 spheric CO_2 concentrations (i.e., an A_{net}/C_i curve) using the Dynamic Assimila-
1986 tion Technique™. The Dynamic Assimilation Technique™ has been shown to
1987 correspond well with traditional steady-state CO_2 response curves in *G. max*
1988 (Saathoff and Welles 2021). A_{net}/C_i curves were generated along a reference CO_2
1989 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
1990 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
1991 period at $420 \mu\text{mol mol}^{-1} \text{CO}_2$. The ramp rate for each curve was set to 200
1992 $\mu\text{mol mol}^{-1} \text{min}^{-1}$, logging every five seconds, which generated 96 data points per
1993 response curve. All A_{net}/C_i curves were generated after A_{net} and g_{sw} stabilized
1994 in a LI-6800 cuvette set to a 500 mol s^{-1} , 10,000 rpm mixing fan speed, 1.5 kPa
1995 vapor pressure deficit, 25°C leaf temperature, $2000 \mu\text{mol m}^{-2} \text{s}^{-1}$ incoming light
1996 radiation, and initial reference CO_2 set to $420 \mu\text{mol mol}^{-1}$.

1997 With the same focal leaf used to generate A_{net}/C_i curves, I measured dark
1998 respiration (R_{d25} ; $\mu\text{mol m}^{-2} \text{s}^{-1}$) following at least a 30-minute period of darkness.
1999 Measurements were collected on a 5-second log interval for 60 seconds after stabi-
2000 lizing in a LI-6800 cuvette set to a 500 mol s^{-1} , 10,000 rpm mixing fan speed, 1.5

2001 kPa vapor pressure deficit, 25°C leaf temperature, and 420 $\mu\text{mol mol}^{-1}$ reference
2002 CO₂ concentration (for both CO₂ concentrations), with incoming light radiation
2003 set to 0 $\mu\text{mol m}^{-2} \text{s}^{-1}$. A single dark respiration value was determined for each
2004 focal leaf by calculating the mean dark respiration value (i.e. the absolute value
2005 of A_{net} during the logging period) across the logging interval.

2006 5.2.4 *Leaf trait measurements*

2007 The focal leaf used to generate A_{net}/C_i curves and dark respiration was harvested
2008 immediately following gas exchange measurements. Images of each focal leaf were
2009 curated using a flat-bed scanner to determine wet leaf area using the ‘LeafArea’ R
2010 package (Katabuchi 2015), which automates leaf area calculations using ImageJ
2011 software (Schneider et al. 2012). Each leaf was dried at 65°C for at least 48
2012 hours, and subsequently weighed and ground until homogenized. Leaf mass per
2013 area (M_{area} ; g m^{-2}) was calculated as the ratio of dry leaf biomass to fresh leaf
2014 area. Using subsamples of ground and homogenized leaf tissue, I measured leaf
2015 nitrogen content (N_{mass} ; gN g^{-1}) through elemental combustion analysis (Costech-
2016 4010, Costech, Inc., Valencia, CA, USA). Leaf nitrogen content per unit leaf area
2017 (N_{area} ; gN m^{-2}) was calculated by multiplying N_{mass} and M_{area} . Subsamples of
2018 ground and homogenized leaf tissue were also sent to the UC-Davis Stable Isotope
2019 Facility to quantify leaf $\delta^{15}\text{N}$, later used to estimate the fraction of leaf nitrogen
2020 derived from the atmosphere.

2021 I extracted chlorophyll content from a second leaf in the same trifoliolate
2022 leaf set as the focal leaf used to generate A_{net}/C_i curves. Prior to chlorophyll
2023 extraction, I used a cork borer to punch between 3 and 5 0.6 cm² disks from the

2024 leaf. Separate images of each punched leaf and set of leaf disks were curated using
2025 a flat-bed scanner to determine wet leaf area, again quantified using the ‘LeafArea’
2026 R package (Katabuchi 2015). The punched leaf was dried and weighed after at
2027 least 65°C in the drying oven to determine M_{area} of the chlorophyll leaf.

2028 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):

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

2038 and

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

2039 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

2044 content by dividing by the total area of the leaf disks (Chl_{area} ; mmol m⁻²). Mass-
2045 based chlorophyll content (Chl_{mass} ; mmol g⁻¹) was calculated by dividing Chl_{area}
2046 by the leaf mass per area of the punched leaf.

2047 5.2.5 *A/C_i curve fitting and parameter estimation*

2048 I fit A_{net}/C_i curves of each individual using the ‘fitaci’ function in the ‘plante-
2049 cophys’ R package (Duursma 2015). This function estimates the maximum rate
2050 of Rubisco carboxylation (V_{cmax} ; $\mu\text{mol m}^{-2} \text{s}^{-1}$) and maximum rate of electron
2051 transport for RuBP regeneration (J_{max} ; $\mu\text{mol m}^{-2} \text{s}^{-1}$) based on the Farquhar
2052 biochemical model of C₃ photosynthesis (Farquhar et al. 1980). Triose phosphate
2053 utilization (TPU) limitation was included in all curve fits, and all curve fits in-
2054 cluded measured dark respiration values. As A_{net}/C_i curves were generated using
2055 a common leaf temperature, curves were fit using Michaelis-Menten coefficients
2056 for Rubisco affinity to CO₂ (K_c ; $\mu\text{mol mol}^{-1}$) and O₂ (K_o ; $\mu\text{mol mol}^{-1}$), and the
2057 CO₂ compensation point (Γ^* ; $\mu\text{mol mol}^{-1}$) reported in Bernacchi et al. (2001).
2058 Specifically, K_c was set to 404.9 $\mu\text{mol mol}^{-1}$, K_o was set to 278.4 $\mu\text{mol mol}^{-1}$,
2059 and Γ^* was set to 42.75 $\mu\text{mol mol}^{-1}$. All curve fits were visually examined for
2060 goodness-of-fit. The use of a common leaf temperature across curves and dark
2061 respiration measurements eliminated the need to temperature standardize rate
2062 estimates. For clarity, I reference V_{cmax} , J_{max} , and R_d estimates throughout the
2063 rest of the chapter as V_{cmax25} , J_{max25} , and R_{d25} .

2064 5.2.6 *Stomatal limitation*

2065 I quantified the extent by which stomatal conductance limited photosynthesis (l ;

2066 unitless) following equations originally described in Farquhar and Sharkey (1982).

2067 Stomatal limitation was calculated as:

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

2068 where A_{mod} represents the photosynthetic rate where $C_i = C_a$. A_{mod} was calculated

2069 as:

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

2070 K_m is the Michaelis-Menten coefficient for Rubisco-limited photosynthesis, calcu-

2071 lated as:

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

2072 where O_i refers to leaf intercellular O_2 concentrations, set to $210 \mu\text{mol mol}^{-1}$.

2073 5.2.7 *Proportion of leaf nitrogen allocated to photosynthesis and structure*

2074 I used equations from Niinemets and Tenhunen (1997) to estimate the proportion

2075 of leaf nitrogen content allocated to Rubisco, bioenergetics, and light harvesting

2076 proteins. The proportion of leaf nitrogen allocated to Rubisco ($\rho_{rubisco}$; gN g^{-1})

2077 was calculated as a function of V_{cmax25} and N_{area} :

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

2078 where N_r is the amount of nitrogen in Rubisco, set to 0.16 gN (gN in Rubisco) $^{-1}$
2079 and V_{cr} is the maximum rate of RuBP carboxylation per unit Rubisco protein,
2080 set to 20.5 $\mu\text{mol CO}_2$ (g Rubisco) $^{-1}$. The proportion of leaf nitrogen allocated to
2081 bioenergetics (ρ_{bioe} ; gN gN $^{-1}$) was similarly calculated as a function of J_{max25} and
2082 N_{area} :

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

2083 where N_b is the amount of nitrogen in cytochrome f, set to 0.12407 gN (μmol
2084 cytochrome f) $^{-1}$ assuming a constant 1: 1: 1.2 cytochrome f: ferredoxin NADP
2085 reductase: coupling factor molar ratio (Evans and Seemann 1989; Niinemets and
2086 Tenhunen 1997), and J_{mc} is the capacity of electron transport per cytochrome f,
2087 set to 156 $\mu\text{mol electron}$ ($\mu\text{mol cytochrome f}$) $^{-1}\text{s}^{-1}$.

2088 The proportion of leaf nitrogen allocated to light harvesting proteins (ρ_{light} ;
2089 gN gN $^{-1}$) was calculated as a function of Chl_{mass} and N_{mass} :

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

2090 where c_b is the stoichiometry of the light-harvesting chlorophyll complexes of
2091 photosystem II, set to 2.75 mmol chlorophyll (gN in chlorophyll) $^{-1}$. I used the
2092 N_{mass} value of the focal leaf used to generate A_{net}/C_i curves instead of the leaf
2093 used to extract chlorophyll content, as the two leaves are from the same trifoliolate
2094 leaf set and are highly correlated (Figure D1).

2095 The proportion of leaf nitrogen content allocated to photosynthetic tissue
2096 (ρ_{photo} ; gN gN $^{-1}$) was estimated as the sum of $\rho_{rubisco}$, ρ_{bioe} , and ρ_{light} . Finally,
2097 the proportion of leaf nitrogen content allocated to structural tissue ($\rho_{structure}$; gN

2098 gN^{-1}) was estimated as:

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

2099 where N_{cw} is the leaf nitrogen content allocated to cell walls (gN m^{-2}), calculated

2100 as a function of M_{area} using an empirical equation from Onoda et al. (2017):

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

2101 5.2.8 *Whole plant traits*

2102 Seven weeks after experiment initiation and immediately following gas exchange

2103 measurements, I harvested all experimental individuals and separated biomass of

2104 each experimental individual into major organ types (leaves, stems, roots, and

2105 nodules when present). Fresh leaf area of all harvested leaves was measured using

2106 an LI-3100C (Li-COR Biosciences, Lincoln, Nebraska, USA). Total fresh leaf area

2107 (cm^2) was calculated as the sum of all leaf areas, including the focal leaf used to

2108 collect gas exchange data and the focal leaf used to extract chlorophyll content. All

2109 harvested material was dried in an oven set to 65°C for at least 48 hours, weighed,

2110 and ground to homogeneity. Leaves and nodules were manually ground with a

2111 mortar and pestle, while stems and roots were ground using a Wiley mill (E3300

2112 Mini Mill; Eberbach Corp., MI, USA). Total dry biomass (g) was calculated as

2113 the sum of dry leaf (including focal leaf for both the A_{net}/C_i curve and leaf used

2114 to extract chlorophyll content), stem, root, and root nodule biomass. I quantified

2115 carbon and nitrogen content of each respective organ type through elemental

2116 combustion (Costech-4010, Costech, Inc., Valencia, CA, USA) using subsamples

2117 of ground and homogenized organ tissue.

2118 Following the approach explained in the first experimental chapter, I calcu-
 2119 lated structural carbon costs to acquire nitrogen as the ratio of total belowground
 2120 carbon biomass to whole plant nitrogen biomass (N_{cost} ; gC gN⁻¹). Belowground
 2121 carbon biomass (C_{bg} ; gC) was calculated as the sum of root carbon biomass
 2122 and root nodule carbon biomass. Root carbon biomass and root nodule carbon
 2123 biomass was calculated as the product of the organ biomass and the respective
 2124 organ carbon content. Whole plant nitrogen biomass (N_{wp} ; gN) was similarly
 2125 calculated as the sum of total leaf, stem, root, and root nodule nitrogen biomass,
 2126 including the focal leaf used for A_{net}/C_i curve and chlorophyll extractions. Leaf,
 2127 stem, root, and root nodule nitrogen biomass was calculated as the product of
 2128 the organ biomass and the respective organ nitrogen content. This calculation
 2129 only quantifies plant structural carbon costs to acquire nitrogen and does not
 2130 include any additional costs of nitrogen acquisition associated with respiration,
 2131 root exudation, or root turnover. An explicit explanation of the limitations for
 2132 interpreting this calculation can be found in Perkowski et al. (2021) and Terrer
 2133 et al. (2018).

2134 Finally, plant investments in nitrogen fixation were calculated as the ratio
 2135 of root nodule biomass to root biomass, where increasing values indicate an in-
 2136 crease in plant investments to nitrogen fixation (Dovrat et al. 2018; Dovrat et al.
 2137 2020; Perkowski et al. 2021). I also calculated the percent of leaf nitrogen ac-
 2138 quired from the atmosphere (% N_{dfa} ; %) using leaf $\delta^{15}\text{N}$ and the following equation
 2139 from Andrews et al. (2011):

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

2140 where $\delta^{15}\text{N}_{\text{reference}}$ refers to a reference plant that exclusively acquires nitrogen via
2141 direct uptake, $\delta^{15}\text{N}_{\text{sample}}$ refers to an individual's leaf $\delta^{15}\text{N}$, and B refers to individuals
2142 that are entirely reliant on nitrogen fixation. Within each unique nitrogen
2143 fertilization treatment-by-CO₂ treatment combination, I calculated the mean leaf
2144 $\delta^{15}\text{N}$ for individuals growing in the non-inoculated treatment for $\delta^{15}\text{N}_{\text{reference}}$. Any
2145 individuals with visual confirmation of root nodule formation were omitted from
2146 the calculation of $\delta^{15}\text{N}_{\text{reference}}$. Following recommendations from Andrews et al.
2147 (2011) I calculated B within each CO₂ treatment using the mean leaf $\delta^{15}\text{N}$ of
2148 inoculated individuals that received 0 ppm N. I did not calculate B within each
2149 unique soil nitrogen-by-CO₂ treatment combination, as previous studies suggest
2150 decreased reliance on nitrogen fixation with increasing soil nitrogen availability
2151 (Perkowski et al. 2021).

2152 5.2.9 *Statistical analyses*

2153 Uninoculated pots that had substantial root nodule formation (nodule biomass:
2154 root biomass values greater than 0.05 g g⁻¹) were removed from all analyses, as
2155 pots were assumed to have been colonized by symbiotic nitrogen-fixing bacteria
2156 from outside sources. This decision resulted in the removal of sixteen pots from
2157 analyses: two pots in the elevated CO₂ treatment that received 35 ppm N, three
2158 pots in the elevated CO₂ treatment that received 70 ppm N, one pot in the elevated
2159 CO₂ treatment that received 210 ppm N, two pots in the elevated CO₂ treatment
2160 that received 280 ppm N, two pots in the ambient CO₂ treatment that received
2161 0 ppm N, three pots in the ambient CO₂ treatment that received 70 ppm N, two
2162 pots in the ambient CO₂ treatment that received 105 ppm N, and one pot in the

2163 ambient CO₂ treatment that received 280 ppm N.

2164 I built a series of linear mixed effects models to investigate the impacts of
2165 CO₂ concentration, soil nitrogen fertilization, and inoculation with *B. japonicum*
2166 on *G. max* gas exchange, tradeoffs between nitrogen and water use, whole plant
2167 growth, and investment in nitrogen fixation. All models included CO₂ treatment
2168 as a categorical fixed effect, inoculation treatment as a categorical fixed effect,
2169 soil nitrogen fertilization as a continuous fixed effect, with interaction terms be-
2170 tween all three fixed effects. All models also accounted for climatic difference
2171 between chambers across experiment iterations by including a random intercept
2172 term that nested starting chamber rack by CO₂ treatment. Models with this
2173 independent variable structure were created for each of the following dependent
2174 variables: N_{area} , M_{area} , N_{mass} , Chl_{area} , V_{cmax25} , J_{max25} , $J_{\text{max25}}:V_{\text{cmax25}}$, R_{d25} , g_{sw} ,
2175 stomatal limitation, ρ_{rubisco} , ρ_{bioe} , ρ_{light} , ρ_{photo} , $\rho_{\text{structure}}$, total biomass, total leaf
2176 area, N_{cost} , C_{bg} , N_{wp} , nodule biomass, the ratio of nodule biomass to root biomass,
2177 and % N_{dfa} .

2178 I used Shapiro-Wilk tests of normality to determine whether linear mixed
2179 effects models satisfied residual normality assumptions. If residual normality as-
2180 sumptions were not met (Shapiro-Wilk: $p < 0.05$), then models were fit using de-
2181 pendent variables that were natural log transformed. If residual normality as-
2182 sumptions were still not met (Shapiro-Wilk: $p < 0.05$), then models were fit using
2183 dependent variables that were square root transformed. All residual normality
2184 assumptions that did not originally satisfy residual normality assumptions were
2185 met with either a natural log or square root data transformation (Shapiro-Wilk:
2186 $p > 0.05$ in all cases). Specifically, models for N_{area} , N_{mass} , Chl_{area} , V_{cmax25} , J_{max25} ,

2187 $J_{\max25}$: $V_{\text{cmax}25}$, R_{d25} , g_{sw} , stomatal limitation, ρ_{rubisco} , ρ_{bioe} , ρ_{light} , ρ_{photo} , total leaf
2188 area, N_{cost} satisfied residual normality assumptions without data transformation.
2189 Models for M_{area} , $\rho_{\text{structure}}$, C_{bg} , and total biomass satisfied residual normality as-
2190 sumptions with a natural log data transformation, while models for N_{wp} , nodule
2191 biomass, nodule biomass: root biomass, and $\%N_{dfa}$ satisfied residual normality
2192 assumptions with a square root data transformation.

2193 In all statistical models, I used the ‘lmer’ function in the ‘lme4’ R package
2194 (Bates et al. 2015) to fit each model and the ‘Anova’ function in the ‘car’ R
2195 package (Fox and Weisberg 2019) to calculate Type II Wald’s χ^2 and determine
2196 the significance ($\alpha=0.05$) of each fixed effect coefficient. I used the ‘emmeans’
2197 R package (Lenth 2019) to conduct post-hoc comparisons using Tukey’s tests,
2198 where degrees of freedom were approximated using the Kenward-Roger approach
2199 (Kenward and Roger 1997). All analyses and plots were conducted in R version
2200 4.2.0 (R Core Team 2021).

2201 5.3 Results

2202 5.3.1 Leaf nitrogen and chlorophyll content

2203 Elevated CO₂ reduced N_{area} , N_{mass} , and Chl_{area} by 29%, 50%, and 31%, respec-
2204 tively, and stimulated M_{area} by 44% ($p<0.001$ in all cases; Table 5.1). An inter-
2205 action between fertilization and CO₂ (CO₂-by-fertilization interaction: $p_{N_{\text{area}}}=$
2206 0.017, $p_{N_{\text{mass}}}<0.001$, $p_{Chl_{\text{area}}}=0.083$; Table 5.1) indicated that the positive effect
2207 of increasing fertilization on N_{area} , N_{mass} , and Chl_{area} ($p<0.001$ in all cases; Table
2208 5.1) was stronger under ambient CO₂ (Tukey _{N_{area}} : $p=0.026$; Tukey _{N_{mass}} : $p<0.001$;
2209 Tukey _{Chl_{area}} : $p=0.065$; Table 5.1; Figs. 5.1a, 5.1b, 5.1d). An interaction between

2210 fertilization and CO₂ on M_{area} (CO₂-by-fertilization interaction: $p=0.006$; Ta-
2211 ble 5.1) indicated that the positive effect of increasing fertilization on M_{area} was
2212 stronger under elevated CO₂ (Tukey: $p=0.009$; Fig. 5.1c). Overall, interactions
2213 between fertilization and CO₂ resulted in stronger reductions in N_{area} , N_{mass} , and
2214 Chl_{area} , and a stronger stimulation in M_{area} under elevated CO₂ with increasing
2215 fertilization.

2216 An interaction between inoculation and CO₂ on N_{area} (CO₂-by-inoculation
2217 interaction: $p=0.030$; Table 5.1) indicated that the positive effect of inoculation
2218 on N_{area} ($p<0.001$; Table 5.1) was stronger under elevated CO₂ (45% increase;
2219 Tukey: $p<0.001$) than under ambient CO₂ (18% increase; Tukey: $p<0.001$), a
2220 result that increased the reduction in N_{area} in inoculated pots under elevated
2221 CO₂. Inoculation treatment did not modify the downregulation in N_{mass} (CO₂-
2222 by-inoculation interaction: $p=0.148$; Table 5.1) and Chl_{area} ($p = 0.147$; Table
2223 5.1) or the stimulation in M_{area} ($p=0.866$; Table 5.1) under elevated CO₂. How-
2224 ever, interactions between fertilization and inoculation on N_{area} , N_{mass} , M_{area} ,
2225 and Chl_{area} (fertilization-by-inoculation interaction: $p_{N_{\text{area}}}<0.001$, $p_{N_{\text{mass}}}=0.001$,
2226 $p_{M_{\text{area}}}=0.025$, $p_{Chl_{\text{area}}}=0.083$; Table 5.1) indicated that the positive effect of in-
2227 creasing fertilization on each trait was stronger in uninoculated pots (Tukey _{N_{area}} :
2228 $p<0.001$; Tukey _{N_{mass}} : $p=0.001$; Tukey _{M_{area}} : $p=0.031$; Tukey _{Chl_{area}} : $p<0.001$;
2229 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						

131

2230 *Significance determined using Type II Wald χ^2 tests ($\alpha=0.05$). P-values less than 0.05 are in bold, while p-values
 2231 between 0.05 and 0.1 are italicized. A superscript “a” is included after trait labels to indicate if models were fit with
 2232 natural log transformed response variables. Key: df=degrees of freedom, χ^2 =Wald Type II chi-square test statistic.

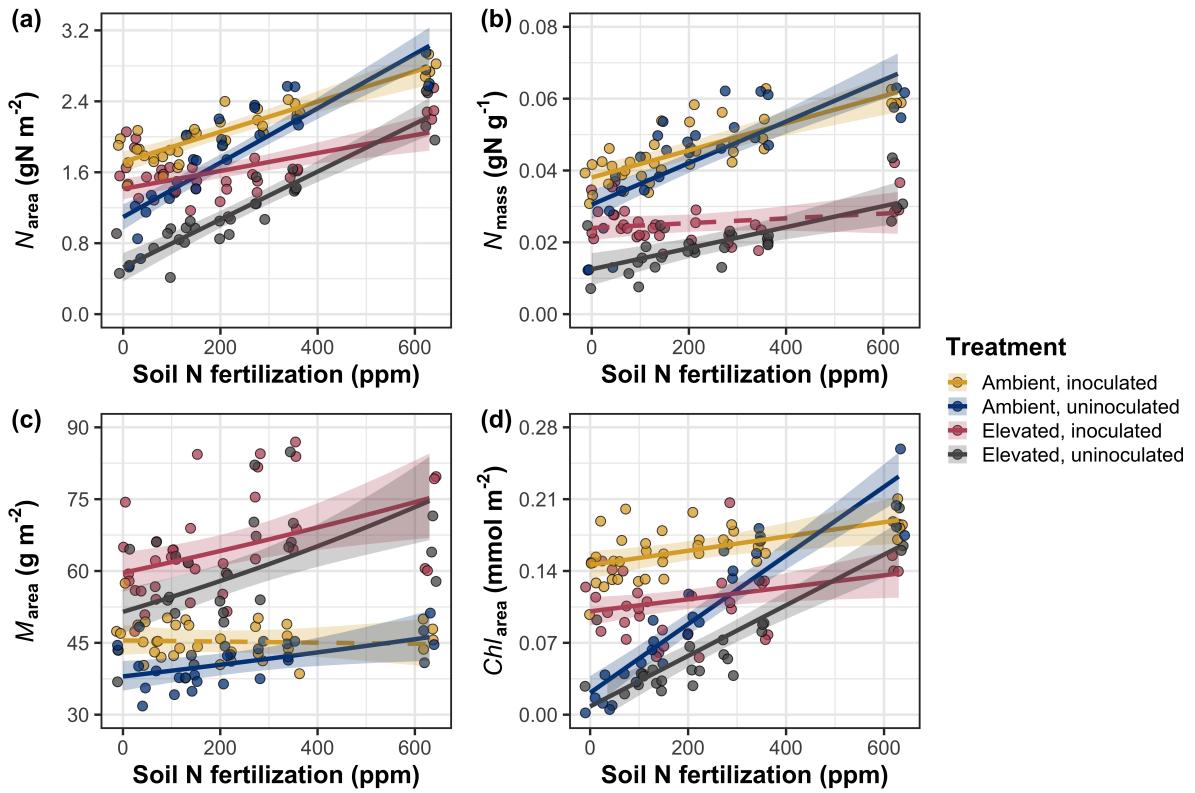


Figure 5.1. Effects of CO_2 , fertilization, and inoculation on leaf nitrogen per unit leaf area (a), leaf nitrogen content (b), leaf mass per unit leaf area (c), and chlorophyll content per unit leaf area (d). Soil nitrogen fertilization is represented on the x-axis in all panels. Yellow points and 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 ($p > 0.05$).

2233 5.3.2 *Leaf biochemistry and stomatal conductance*

2234 Elevated CO₂ resulted in plants with 16% lower V_{cmax25} ($p<0.001$; Table 5.2) and
2235 10% lower J_{max25} ($p=0.014$; Table 5.2) compared to those grown under ambient
2236 CO₂. However, CO₂ concentration did not influence R_{d25} ($p=0.613$; Table 5.2;
2237 Fig. 5.2d). A relatively stronger downregulation in V_{cmax25} than J_{max25} resulted
2238 in an 8% stimulation in $J_{max25}:V_{cmax25}$ under elevated CO₂ ($p<0.001$; Table 5.2).
2239 The downregulatory effect of CO₂ on V_{cmax25} and J_{max25} was not modified across
2240 the fertilization gradient (CO₂-by-fertilization interaction: $p=0.185$ and $p=0.389$
2241 for V_{cmax25} and J_{max25} , respectively; Table 5.2; Figs. 5.2a, 5.2b) or between in-
2242 oculation treatments (CO₂-by-inoculation interaction: $p=0.799$ and $p=0.714$ for
2243 V_{cmax25} and J_{max25} , respectively; Table 5.2). However, a strong interaction between
2244 fertilization and inoculation (fertilization-by-inoculation interaction: $p\leq0.001$ in
2245 all cases; Table 5.2) indicated that the positive effect of increasing fertilization
2246 on V_{cmax25} ($p<0.001$; Table 5.2), J_{max25} ($p<0.001$; Table 5.2), and R_{d25} ($p=0.015$;
2247 Table 5.2) was only observed in uninoculated pots (Tukey: $p\leq0.001$ in all cases;
2248 Figs. 5.2a, 5.2b). A stronger positive effect of increasing fertilization on V_{cmax25}
2249 than J_{max25} resulted in a reduction in $J_{max25}:V_{cmax25}$ with increasing fertilization
2250 ($p<0.001$; Table 5.2), though this pattern was only observed in uninoculated pots
2251 (fertilization-by-inoculation interaction: $p=0.002$; Table 5.2; Fig. 5.2c).

2252 Elevated CO₂ reduced stomatal conductance by 20% ($p<0.001$; Table 5.2;
2253 Fig. 5.2e), but this downregulation did not influence stomatal limitation of pho-
2254 tosynthesis ($p=0.355$; Table 5.2; Fig. 5.2f). As with V_{cmax25} and J_{max25} , the down-
2255 regulation of stomatal conductance due to elevated CO₂ was not modified across
2256 the fertilization gradient (CO₂-by-fertilization interaction: $p=0.141$; Table 5.2) or

2257 between inoculation treatments (CO_2 -by-inoculation interaction: $p=0.179$; Table
2258 5.2). Fertilization also did not modify the null effect of CO_2 on stomatal limitation
2259 (CO_2 -by-fertilization interaction: $p=0.554$; Table 5.2), although an interaction
2260 between CO_2 and inoculation (CO_2 -by-inoculation interaction: $p=0.043$; Table
2261 5.2) indicated that inoculation increased stomatal limitation under ambient CO_2
2262 (Tukey: $p=0.021$), but not under elevated CO_2 (Tukey: $p>0.999$). An interaction
2263 between inoculation and fertilization on stomatal conductance (fertilization-by-
2264 inoculation interaction: $p<0.001$; Table 5.2) indicated that increasing fertilization
2265 increased stomatal conductance in uninoculated pots (Tukey: $p=0.003$) but de-
2266 creased stomatal conductance in inoculated pots (Tukey: $p=0.021$). The similar
2267 in magnitude, but opposite direction, trend in the effect of increasing fertiliza-
2268 tion on stomatal conductance between inoculation treatments likely drove a null
2269 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

	$J_{\text{max}25}:V_{\text{cmax}25}$			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

2270 *Significance determined using Type II Wald χ^2 tests ($\alpha=0.05$). P-values less than 0.05 are in bold, while p-values
 2271 between 0.05 and 0.1 are italicized. Key: df=degrees of freedom; χ^2 =Wald Type II chi-square test statistic.

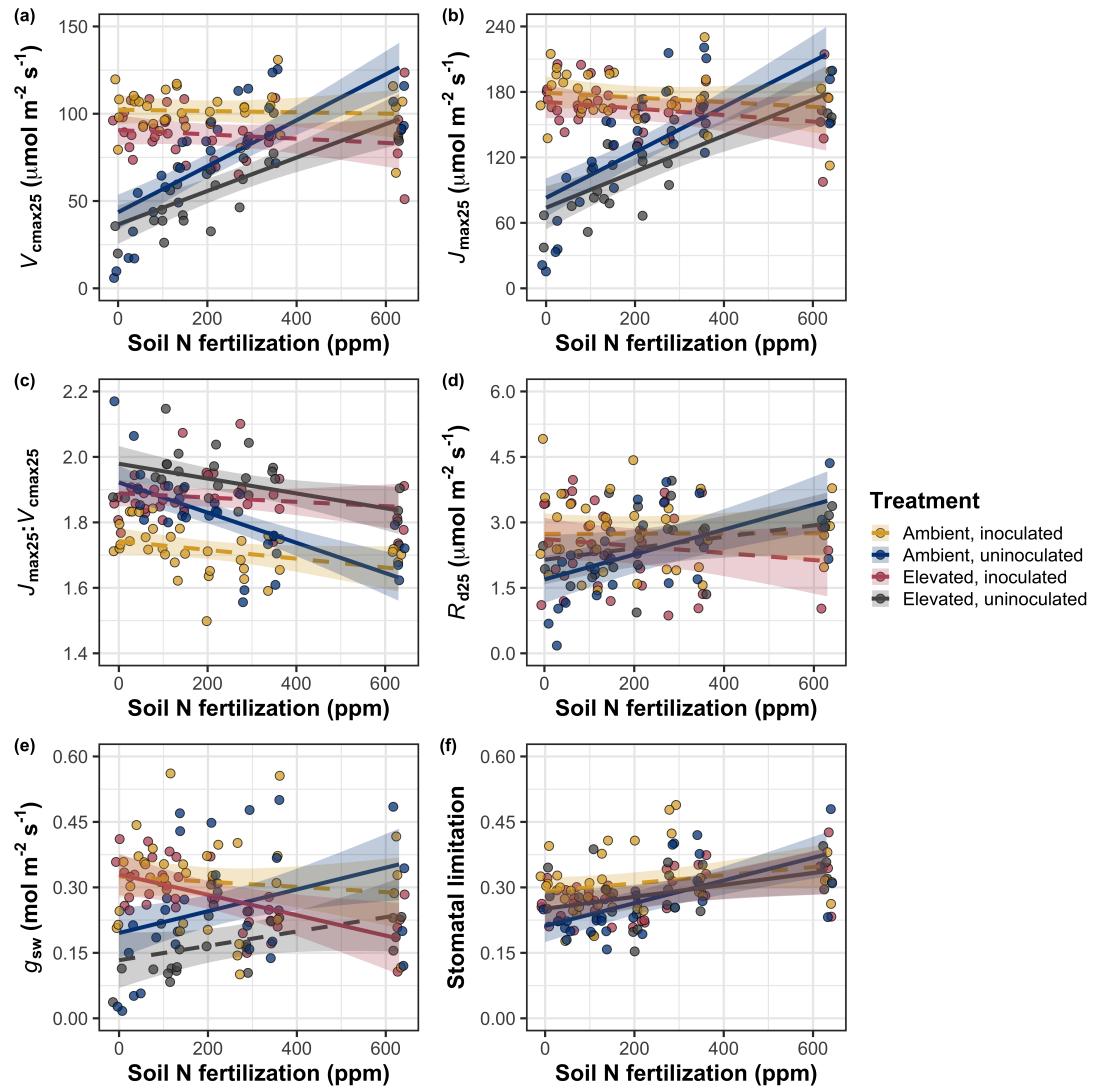


Figure 5.2. Effects of CO₂, fertilization, and inoculation on maximum rate of Rubisco carboxylation (a), the maximum rate of RuBP regeneration (b), and the ratio of the maximum rate of RuBP regeneration to the maximum rate of Rubisco carboxylation leaf mass per unit leaf area (c), dark respiration (d), 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.

2272 5.3.3 *Leaf nitrogen allocation*

2273 A relatively stronger downregulation in N_{area} than $V_{\text{cmax}25}$ and $J_{\text{max}25}$ resulted in
2274 an 20% and 29% respective stimulation in ρ_{rubisco} and ρ_{bioe} under elevated CO₂
2275 ($p<0.001$ in both cases; Table 5.3). There was no effect of CO₂ on ρ_{light} ($p=0.700$;
2276 Table 5.3), but the stimulation in ρ_{rubisco} and ρ_{bioe} resulted in a 21% stimulation
2277 of ρ_{photo} under elevated CO₂ ($p<0.001$; Table 5.3; Fig. 5.3a). The stimulation
2278 of ρ_{rubisco} , ρ_{bioe} , and ρ_{photo} under elevated CO₂ was not modified across the fer-
2279 tilization gradient (CO₂-by-fertilization interaction: $p_{\text{rubisco}}=0.269$, $p_{\text{bioe}}=0.298$,
2280 $p_{\text{photo}}=0.281$; Table 5.3). A marginal interaction between inoculation and CO₂ on
2281 ρ_{rubisco} and ρ_{photo} (CO₂-by-inoculation interaction: $p_{\text{rubisco}}=0.057$, $p_{\text{photo}}=0.055$;
2282 Table 5.3) indicated that the positive effect of inoculation on ρ_{rubisco} and ρ_{photo}
2283 ($p<0.001$ in both cases; Table 5.3) was only apparent under ambient CO₂ (Tukey:
2284 $p<0.001$ in both cases). Inoculation did not modify the stimulation of ρ_{bioe} un-
2285 der elevated CO₂ (CO₂-by-inoculation interaction: $p=0.122$; Table 5.3) or the
2286 null effect of CO₂ on ρ_{bioe} (CO₂-by-inoculation interaction: $p=0.298$; Table 5.3).
2287 An interaction between fertilization and inoculation on ρ_{rubisco} , ρ_{bioe} , and ρ_{photo}
2288 (fertilization-by-inoculation interaction: $p<0.001$ in all cases; Table 5.3) indicated
2289 that the negative effect of increasing fertilization on each trait ($p<0.001$ in all
2290 cases; Table 5.3) was only observed in inoculated pots (Tukey: $p<0.001$ in all
2291 cases). An additional interaction between fertilization and inoculation on ρ_{light}
2292 (fertilization-by-inoculation interaction: $p<0.001$; Table 5.3) indicated a negative
2293 effect of increasing fertilization on ρ_{light} in inoculated pots (Tukey: $p=0.041$), but
2294 a positive effect of increasing fertilization in uninoculated pots (Tukey: $p<0.001$).
2295 The stimulation in M_{area} under elevated CO₂ resulted in an 133% stimu-

2296 lation of $\rho_{\text{structure}}$ ($p<0.001$; Table 5.3; Fig 5.3b). An interaction between fertil-
2297 ization and CO₂ (CO₂-by-fertilization interaction: $p=0.039$; Table 5.3) indicated
2298 that the negative effect of increasing fertilization ($p<0.001$; Table 5.3) on $\rho_{\text{structure}}$
2299 was marginally stronger under ambient CO₂ (Tukey: $p=0.055$). A marginal inter-
2300 action between inoculation and CO₂ (CO₂-by-inoculation interaction: $p=0.057$;
2301 Table 5.3) indicated that the positive effect of inoculation on $\rho_{\text{structure}}$ ($p<0.001$;
2302 Table 5.3) was only observed under elevated CO₂ (Tukey: $p<0.001$), with no ap-
2303 parent inoculation effect observed under ambient CO₂ (Tukey: $p=0.513$). Finally,
2304 an interaction between fertilization and inoculation (fertilization-by-inoculation
2305 interaction: $p<0.001$; Table 5.3) indicated that, while increasing fertilization in-
2306 creased $\rho_{\text{structure}}$ ($p<0.001$; Table 5.3), this response was stronger in uninoculated
2307 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

2308 *Significance determined using Type II Wald χ^2 tests ($\alpha=0.05$). P-values less than 0.05 are in bold, while p-values
 2309 between 0.05 and 0.1 are italicized. A superscript “a” is included after trait labels to indicate if models were fit with
 2310 natural log transformed response variable. Key: df=degrees of freedom; χ^2 =Wald Type II chi-square test statistic.

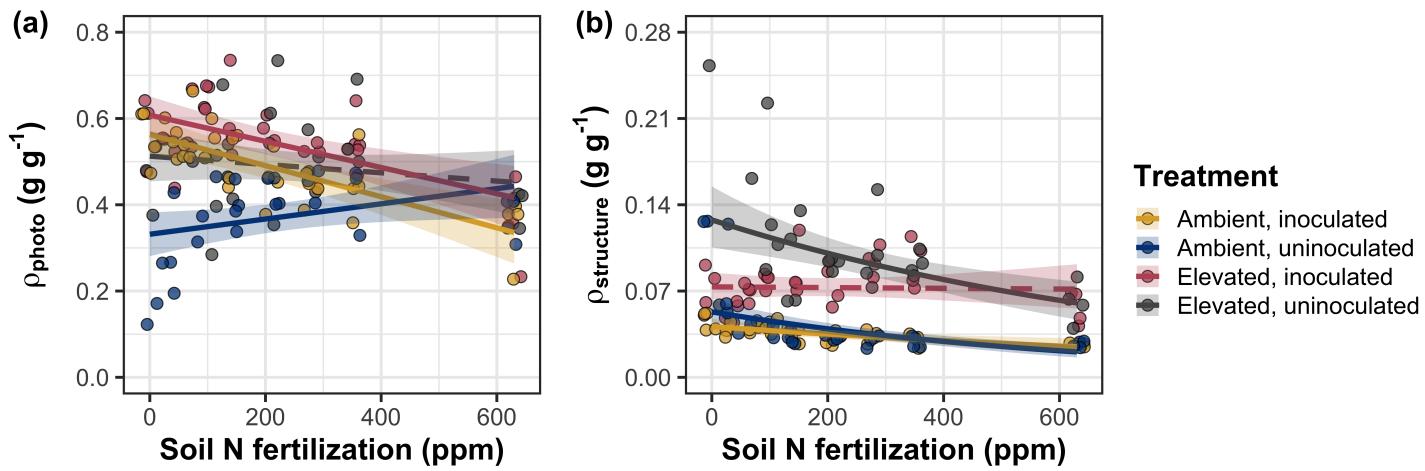


Figure 5.3. Effects of CO_2 , fertilization, and inoculation on the relative fraction of leaf nitrogen allocated to photosynthesis (a) and the fraction of leaf nitrogen allocated to structure (b). Soil nitrogen fertilization is represented on the x-axis in both panels. Colored points and trendlines are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

2311 5.3.4 *Whole plant traits*

2312 Total leaf area and total biomass were 51% and 102% greater under elevated CO₂,
2313 respectively ($p<0.001$ in both cases; Table 5.4). The stimulation in total leaf area
2314 and total biomass under elevated CO₂ was enhanced by increasing fertilization
2315 (CO₂-by-fertilization interaction: $p<0.001$ in both cases; Table 5.4; Figs. 5.4a,
2316 5.4b) but was not modified across inoculation treatments (CO₂-by-inoculation
2317 interaction: $p_{total_leaf_area}=0.151$, $p_{total_biomass}=0.472$; Table 5.4). The positive
2318 effect of increasing fertilization on total leaf area and total biomass was modified by
2319 inoculation treatment (fertilization-by-inoculation interaction: $p<0.001$ in both
2320 cases; Table 5.4), indicating a stronger positive effect of increasing fertilization in
2321 uninoculated pots (Tukey: $p_{total_leaf_area}=0.002$, $p_{total_biomass}=0.001$, Figs. 5.4a,
2322 5.4b).

2323 A 62% stimulation in N_{cost} under elevated CO₂ was modified through a
2324 strong three-way interaction between CO₂, fertilization, and inoculation (CO₂-
2325 by-inoculation-by-fertilization interaction: $p<0.001$; Table 5.4; Fig. 5.4). This
2326 interaction revealed a general negative effect of increasing fertilization on N_{cost}
2327 ($p<0.001$; Table 5.4) that was observed in all treatment combinations (Tukey:
2328 $p<0.001$ in all cases) except for inoculated pots grown under elevated CO₂ (Tukey:
2329 $p=0.779$; Fig. 5.4c). This response also resulted in stronger negative effects of in-
2330 creasing fertilization on N_{cost} in uninoculated pots grown under elevated CO₂ than
2331 uninoculated pots grown under ambient CO₂ (Tukey: $p=0.001$) and inoculated
2332 pots grown under either ambient CO₂ (Tukey: $p<0.001$) or elevated CO₂ (Tukey:
2333 $p<0.001$), while uninoculated pots grown under ambient CO₂ had stronger nega-
2334 tive effects of increasing fertilization on N_{cost} than inoculated pots grown under

2335 elevated CO₂ (Tukey: $p=0.002$), but not inoculated pots grown under ambient
2336 CO₂ (Tukey: $p=0.216$; Fig. 5.4). The reduction in N_{cost} with increasing fertiliza-
2337 tion and in uninoculated pots were driven by a stronger positive effect of increasing
2338 fertilization on N_{wp} (denominator of N_{cost}) than C_{bg} (numerator of N_{cost}), while
2339 the stimulation in N_{cost} under elevated CO₂ was driven by a stronger positive
2340 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

2341 *Significance determined using Type II Wald χ^2 tests ($\alpha=0.05$). *P*-values less than 0.05 are in bold. Superscripts
2342 included after trait labels indicate if models were fit with natural log (^a) or square root (^b) transformed response
2343 variables. Key: df=degrees of freedom; χ^2 =Wald Type II chi-square test statistic.

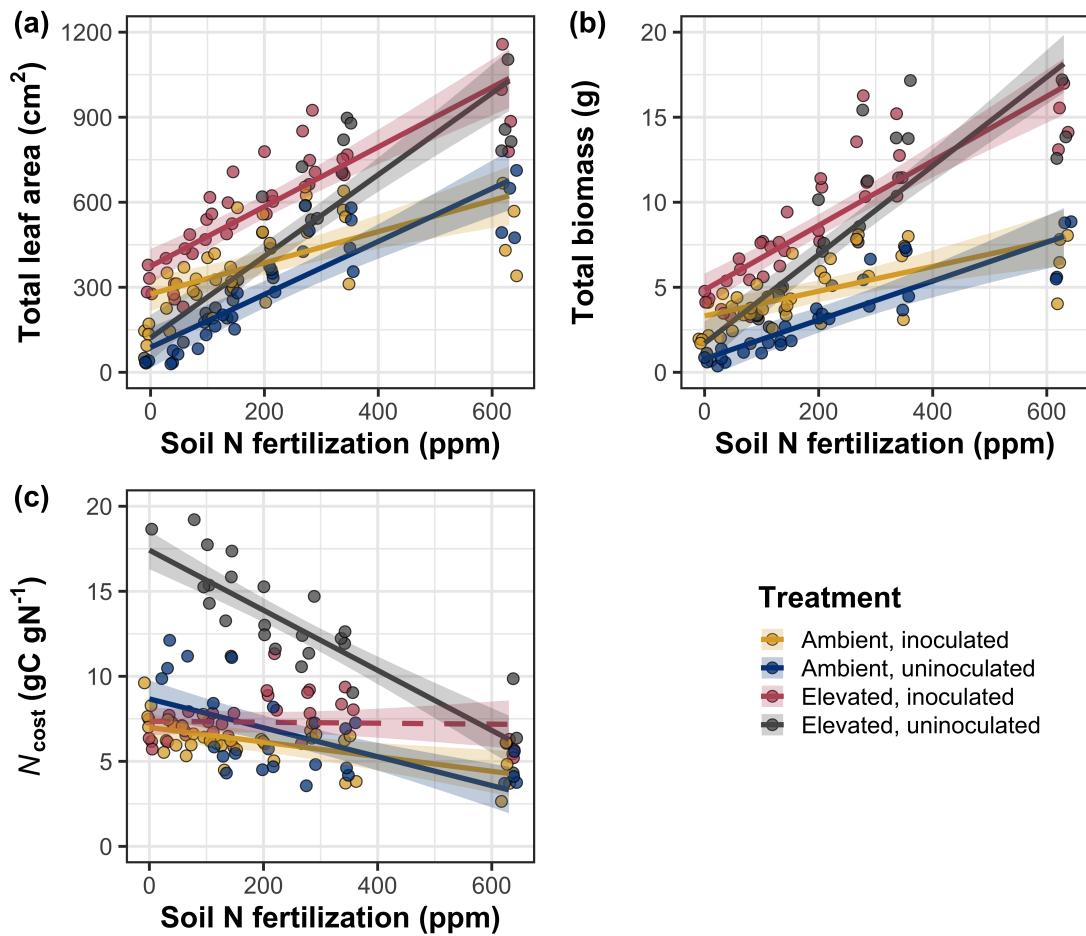


Figure 5.4. Effects of CO_2 , fertilization, and inoculation on total leaf area (a), total biomass (b), and structural carbon costs to acquire nitrogen (c). Soil nitrogen fertilization is represented on the x-axis in all panels. Colored points and trendlines are as explained in Figure 5.1.

2344 5.3.5 *Nitrogen fixation*

2345 Nodule biomass was stimulated by 30% under elevated CO₂ ($p<0.001$; Table 5.5),
2346 a pattern that was modified across the fertilization gradient (CO₂-by-fertilization
2347 interaction: $p=0.479$; Table 5.5), but not between inoculation treatments (CO₂-
2348 by-inoculation interaction: $p=0.404$; Table 5.5). Specifically, the negative effect
2349 of increasing fertilization on nodule biomass ($p<0.001$; Table 5.5) was stronger
2350 under elevated CO₂ (Tukey: $p<0.001$; Fig. 5.5a). An interaction between fertil-
2351 ization and inoculation (fertilization-by-inoculation interaction: $p<0.001$; Table
2352 5.5) indicated a stronger negative effect of increasing fertilization in inoculated
2353 pots (Tukey: $p<0.001$; Fig. 5.5a).

2354 There was no effect of CO₂ on nodule: root biomass ($p=0.767$; Table 5.5),
2355 although an interaction between CO₂ and inoculation (CO₂-by-inoculation in-
2356 teraction: $p<0.001$; Table 5.5) indicated that the positive effect of inoculation
2357 on nodule: root biomass ($p<0.001$; Table 5.5) was stronger under ambient CO₂
2358 (3129% increase; Tukey: $p<0.001$) than elevated CO₂ (379% increase; Tukey:
2359 $p<0.001$; Fig. 5.5b). The null effect of CO₂ on nodule: root biomass was consis-
2360 tently observed across the fertilization gradient (CO₂-by-fertilization interaction:
2361 $p=0.183$; Table 5.5; Fig. 5.5b). An interaction between fertilization and inocula-
2362 tion (fertilization-by-inoculation interaction: $p<0.001$; Table 5.5) indicated that
2363 the negative effect of increasing fertilization on nodule: root biomass ($p<0.001$;
2364 Table 5.5) was stronger in inoculated pots (Tukey: $p<0.001$; Fig. 5.5b).

2365 There was no effect of CO₂ on %N_{dfa} ($p=0.472$; Table 5.5), a pattern
2366 that was not modified by inoculation (CO₂-by-inoculation interaction: $p=0.156$;
2367 Table 5.5) or fertilization (CO₂-by-fertilization interaction: $p=0.099$; Table 5.5).

- 2368** An interaction between fertilization and inoculation (fertilization-by-inoculation
2369 interaction: $p<0.001$; Table 5.5) indicated that the negative effect of increasing
2370 fertilization on $\%N_{dfa}$ ($p<0.001$; Table 5.5) was only observed in inoculated pots
2371 (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

2372 *Significance determined using Type II Wald χ^2 tests ($\alpha=0.05$). P-values less than 0.05 are in bold, while p-values
 2373 between 0.05 and 0.1 are italicized. Superscript letters indicate model coefficients fit to square-root (^b) transformed
 2374 data. Key: df=degrees of freedom; χ^2 =Wald Type II chi-square test statistic.

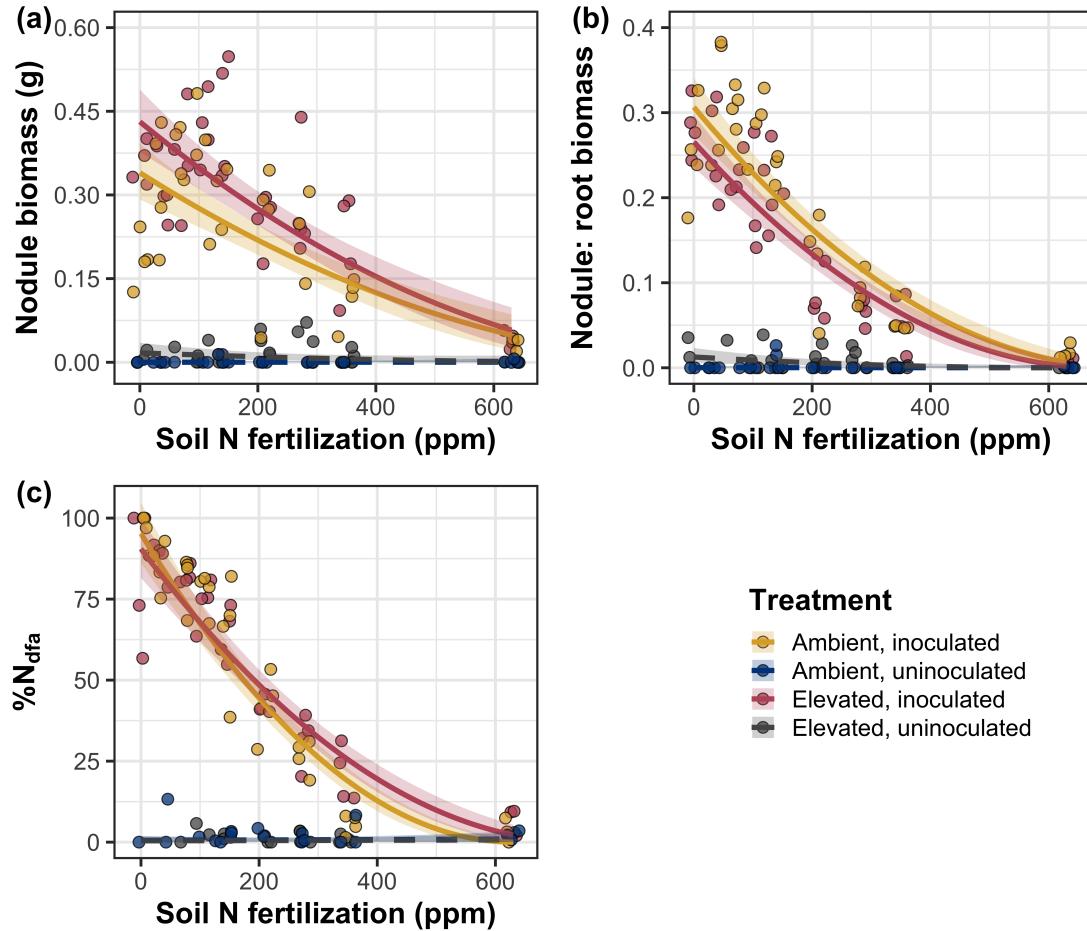


Figure 5.5. Effects of CO₂, fertilization, and inoculation on nodule biomass (a), nodule biomass: root biomass (b), and percent nitrogen fixed from the atmosphere (c). Soil nitrogen fertilization is represented on the x-axis. Colored points and trendlines are as explained in Figure 5.1. Curvilinear trendlines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

2375 5.4 Discussion

2376 In this study, I determined leaf and whole plant acclimation responses of 7-week *G.*
2377 *max* seedlings grown under two CO₂ concentrations, two inoculation treatments,
2378 and nine soil nitrogen fertilization treatments in a full-factorial growth chamber
2379 experiment. In support of hypotheses and patterns expected from theory, elevated
2380 CO₂ reduced N_{area} , V_{cmax25} , and J_{max25} . The relatively stronger downregulation in
2381 V_{cmax25} than J_{max25} under elevated CO₂ resulted in a stimulation in $J_{\text{max25}}:V_{\text{cmax25}}$
2382 under elevated CO₂. The downregulation of V_{cmax25} and J_{max25} under elevated
2383 CO₂ was similar across fertilization and inoculation treatments, indicating that
2384 the CO₂ responses were not associated with nitrogen limitation. Interestingly,
2385 results indicate that elevated CO₂ increased the fraction of leaf nitrogen allocated
2386 to photosynthesis and structure, leading to a stimulation in nitrogen use efficiency
2387 under elevated CO₂ despite the apparent downregulation in N_{area} , V_{cmax25} , and
2388 J_{max25} .

2389 The downregulation in leaf photosynthetic processes under elevated CO₂
2390 corresponded with a strong stimulation in total leaf area and total biomass. Strong
2391 stimulations in whole plant growth due to elevated CO₂ were generally enhanced
2392 with increasing fertilization and were negatively related to structural carbon costs
2393 to acquire nitrogen. Inoculation generally did not modify whole plant responses
2394 to elevated CO₂ across the fertilization gradient, likely due to a strong reduc-
2395 tion in root nodulation with increasing fertilization. However, strong positive
2396 effects of inoculation on whole plant growth were observed under low fertilization,
2397 consistent with hypotheses. Overall, observed leaf and whole plant acclimation
2398 responses to CO₂ support hypotheses and patterns expected from photosynthetic

2399 least-cost theory, showing that leaf acclimation responses to CO₂ were decoupled
2400 from soil nitrogen availability and ability to acquire nitrogen via symbiotic nitro-
2401 gen fixation. Instead, leaf and whole plant acclimation responses to CO₂ were
2402 driven by optimal resource investment to photosynthetic capacity, where optimal
2403 resource investment at the leaf level maximized nitrogen allocation to structures
2404 that support whole plant growth.

2405 5.4.1 *Soil nitrogen fertilization has divergent effects on leaf and whole plant*
2406 *acclimation responses to CO₂*

2407 Elevated CO₂ reduced N_{area} , V_{cmax25} , J_{max25} , and stomatal conductance by 29%,
2408 16%, 10%, and 20%, respectively. The larger downregulation of V_{cmax25} than
2409 J_{max25} led to an 8% stimulation in $J_{\text{max25}}:V_{\text{cmax25}}$, while the larger downregulation
2410 of N_{area} than V_{cmax25} resulted in a 21% stimulation in the fraction of leaf nitro-
2411 gen allocated to photosynthesis under elevated CO₂. These acclimation responses
2412 are directionally consistent with previous studies that have investigated or re-
2413 viewed leaf acclimation responses to CO₂ (Drake et al. 1997; Makino et al. 1997;
2414 Ainsworth et al. 2002; Ainsworth and Long 2005; Ainsworth and Rogers 2007;
2415 Smith and Dukes 2013; Smith and Keenan 2020; Poorter et al. 2022), and fol-
2416 low patterns expected from photosynthetic least-cost theory (Wright et al. 2003;
2417 Prentice et al. 2014; Smith et al. 2019; Smith and Keenan 2020). Together, the
2418 stimulation in $J_{\text{max25}}:V_{\text{cmax25}}$ and the fraction of leaf nitrogen allocated to pho-
2419 tosynthesis under elevated CO₂ provide strong support for the idea that leaves
2420 were downregulating V_{cmax25} in response to elevated CO₂ in order to optimally co-
2421 ordinate photosynthesis such that net photosynthesis rates approached becoming

2422 equally co-limited by Rubisco carboxylation and RuBP regeneration (Chen et al.
2423 1993; Maire et al. 2012) while optimizing resource use efficiency.

2424 Increasing fertilization and inoculation induced strong positive effects on
2425 N_{area} , V_{cmax25} , J_{max25} . The general positive response of N_{area} to increasing fertiliza-
2426 tion and in inoculated pots was enhanced under ambient CO₂, which, paired with
2427 the general downregulation of N_{area} under elevated CO₂, resulted in a stronger
2428 downregulation of N_{area} under elevated CO₂ with increasing fertilization and in
2429 inoculated pots. These patterns suggest that N_{area} responses to CO₂ were at least
2430 partially dependent on soil nitrogen fertilization and nitrogen acquisition strat-
2431 egy. However, the general stimulation in the fraction of leaf nitrogen allocated to
2432 Rubisco, bioenergetics, or photosynthesis under elevated CO₂ was not modified
2433 across the fertilization gradient and was only marginally enhanced in inoculated
2434 pots. These patterns suggest that the increased downregulation of N_{area} under
2435 elevated CO₂ with increasing fertilization was not necessarily associated with a
2436 change in relative investment to photosynthetic tissue, providing another line of
2437 evidence suggesting that leaf acclimation responses to CO₂ are decoupled from
2438 changes in soil nitrogen availability.

2439 Leaf acclimation responses to elevated CO₂ corresponded with a 62% and
2440 100% stimulation in total leaf area and total biomass, respectively. The stimula-
2441 tion in total leaf area and total biomass under elevated CO₂ corresponded with
2442 generally larger structural carbon costs to acquire nitrogen, a pattern driven by
2443 a stimulation in belowground carbon biomass and reduction in whole plant ni-
2444 trogen biomass. This result suggests that elevated CO₂ reduces plant nitrogen
2445 uptake efficiency, which does not explain why plants grown under elevated CO₂

2446 generally had higher biomass and total leaf area, unless growth stimulations un-
2447 der elevated CO₂ were driven by reductions in per-tissue nitrogen demand (Dong
2448 et al. 2022). Interestingly, strong negative effects of increasing fertilization on
2449 structural carbon costs to acquire nitrogen, which were generally similar between
2450 CO₂ concentrations, were driven by stronger increases in whole plant nitrogen
2451 biomass than belowground carbon biomass. This response allowed plants to in-
2452 crease nitrogen uptake efficiency with increasing fertilization, which could be the
2453 mechanism that drove the enhanced growth stimulation under elevated CO₂ with
2454 increasing fertilization.

2455 Interestingly, results indicate that the stimulation in total leaf area and
2456 whole plant growth under elevated CO₂ was not modified by inoculation despite
2457 an apparent general negative effect of inoculation on N_{cost} . This response could
2458 have been due to strong negative effect of increasing fertilization on nodulation,
2459 which may have caused the strong increase in the positive effect of elevated CO₂ on
2460 whole plant growth with increasing fertilization to mask any increase in the posi-
2461 tive effect of elevated CO₂ on whole plant growth due to inoculation. Reductions
2462 in nodulation with increasing fertilization are commonly observed patterns that
2463 have been inferred to be a response that allows species optimize nitrogen uptake
2464 efficiency as costs to acquire nitrogen via direct uptake become more similar (Gib-
2465 son and Harper 1985; Rastetter et al. 2001). In this study, pairwise comparisons
2466 indicated strong positive effects of inoculation on total leaf area and total biomass
2467 (158% increase in total leaf area, 119% increase in total biomass) under elevated
2468 CO₂ at 0 ppm N ($p < 0.05$ in both cases), but no observable inoculation effect on
2469 total leaf area or total biomass under elevated CO₂ at 350 ppm N or 630 ppm N

2470 ($p>0.05$ in both cases). While these responses did not generally differ from those
2471 observed under ambient CO₂, they do confirm the hypothesis that positive effects
2472 of inoculation on whole plant growth responses to elevated CO₂ would decrease
2473 with increasing fertilization.

2474 Combined, results reported here suggest that soil nitrogen availability plays
2475 divergent roles in shaping leaf and whole plant acclimation responses to CO₂. Leaf
2476 acclimation responses were generally decoupled from fertilization, while whole
2477 plant acclimation responses relied heavily on an increase in nitrogen uptake ef-
2478 ficiency and consequent reduction in costs of acquiring nitrogen associated with
2479 increasing fertilization. Whole plant responses to CO₂ indicated that fertilization
2480 may play a more important role in determining whole plant acclimation responses
2481 to CO₂ than nitrogen acquisition strategy, although any inoculation effect was
2482 likely masked by the strong reduction in root nodulation with increasing fertil-
2483 ization. These results suggest that plants acclimate to CO₂ in nitrogen-limited
2484 systems by minimizing the number of optimally coordinated leaves, and that
2485 downregulations in leaf nitrogen content under elevated CO₂ are not driven by
2486 changes in soil nitrogen availability as has been previously implied.

2487 5.4.2 *Implications for future model development*

2488 Many terrestrial biosphere models predict photosynthetic capacity through plant
2489 functional group-specific linear regressions between N_{area} and V_{cmax} (Rogers 2014;
2490 Rogers et al. 2017), which assumes that leaf nitrogen-photosynthesis relation-
2491 ships are constant across growing environments. These results build on previ-
2492 ous work suggesting that leaf nitrogen-photosynthesis relationships dynamically
2493 change across growing environments (Luo et al. 2021; Dong et al. 2022), showing

2494 that CO₂ concentration increases the fraction of leaf nitrogen content allocated to
2495 photosynthesis independent of fertilization or acquisition strategy. Additionally,
2496 increasing fertilization strongly decreased the fraction of leaf nitrogen allocated
2497 to photosynthesis, a response that was largely determined by acquisition strategy.
2498 Specifically, reductions in the fraction of leaf nitrogen allocated to photosynthesis
2499 with increasing fertilization were only observed in inoculated pots that had less
2500 finite access to nitrogen, suggesting that constant leaf nitrogen-photosynthesis
2501 relationships may only be apparent in environments where nitrogen is limiting.
2502 Terrestrial biosphere models that parameterize photosynthetic capacity through
2503 linear relationships between N_{area} and V_{cmax} (Rogers 2014; Rogers et al. 2017) may
2504 therefore be overestimating photosynthetic capacity in systems where nitrogen is
2505 not as limiting. Such models are also not capable of detecting stimulations in the
2506 fraction of leaf nitrogen allocated to photosynthesis with increasing CO₂ concen-
2507 tration. The inability of models to predict these responses likely contributes to the
2508 widespread divergence of model simulations under future environmental scenarios
2509 (Friedlingstein et al. 2014; Davies-Barnard et al. 2020), and should therefore be
2510 a target for resolving in future generations of terrestrial biosphere models.

2511 These results demonstrate that optimal resource investment to photosyn-
2512 thetic capacity defines leaf acclimation responses to elevated CO₂, and that these
2513 responses were independent of fertilization or inoculation treatment. Current
2514 model approaches for simulating photosynthetic responses to CO₂ generally invoke
2515 patterns expected from progressive nitrogen limitation, where the downregulation
2516 in N_{area} , and therefore photosynthetic capacity, due to elevated CO₂ is formu-
2517 lated as a function of progressive reductions in soil nitrogen availability. Results

2518 reported here contradict this formulation, suggesting that the leaf acclimation re-
2519 sponse is driven by optimal resource investment to photosynthetic capacity and
2520 is independent of soil resource supply. Optimality models that leverage prin-
2521 ciples from optimal coordination and photosynthetic least-cost theories (Wang
2522 et al. 2017; Stocker et al. 2020; Scott and Smith 2022) are capable of capturing
2523 such acclimation responses to CO₂ (Smith and Keenan 2020), suggesting that the
2524 implementation of these models may improve the simulation of photosynthetic
2525 processes in terrestrial biosphere models under increasing CO₂ concentrations.

2526 5.4.3 *Study limitations and future directions*

2527 There are two study limitations that must be addressed to contextualize patterns
2528 observed in this study. First, restricting the volume of belowground substrate
2529 via a potted experiment does not adequately replicate belowground environments
2530 of natural systems, and therefore may modify effects of soil resource availability
2531 and inoculation on plant nitrogen uptake. This limitation may be particularly
2532 relevant if pot size limits whole plant growth (Poorter et al. 2012). I attempted
2533 to minimize the extent of pot size limitation experienced in the first experimen-
2534 tal chapter while accounting for the expected stimulation in whole plant growth
2535 under elevated CO₂ by using 6-liter pots. Despite attempts to minimize growth
2536 limitation imposed by pot volume, fertilization and CO₂ treatments increased the
2537 biomass: pot volume ratio such that all treatment combinations to exceed 1 g L⁻¹
2538 biomass: pot volume under high fertilization (Table D3; Fig. D2). The 1 g L⁻¹
2539 biomass: pot volume recommendation from Poorter et al. (2012) was designated
2540 to avoid growth limitation imposed by pot volume. However, if pot size limita-

2541 tion indeed limited whole plant growth, then structural carbon costs to acquire
2542 nitrogen, belowground carbon biomass, whole plant nitrogen biomass, and whole
2543 plant biomass should each exhibit strong saturation points with increasing fertil-
2544 ization, which was not observed here. Importantly, leaf acclimation responses to
2545 CO₂ observed in this study are consistent with findings reported in (Smith and
2546 Keenan 2020), who used data from field manipulation experiments that did not
2547 have any belowground space limitation.

2548 Second, this study evaluated leaf and whole plant responses to CO₂ in 7-
2549 week seedlings. Given the long-term scale of the progressive nitrogen limitation
2550 hypothesis, patterns observed here should be validated in longer-term nitrogen
2551 manipulation experiments. Previous work in free air CO₂ enrichment experiments
2552 show some support for patterns expected from the progressive nitrogen limitation
2553 hypothesis (Reich et al. 2006; Norby et al. 2010), although results are not consis-
2554 tent across experimental sites (Finzi et al. 2006; Moore et al. 2006; Liang et al.
2555 2016). I found some support for patterns expected by the progressive nitrogen
2556 limitation hypothesis, namely the increase in plant nitrogen uptake under elevated
2557 CO₂ (Luo et al. 2004), though leaf acclimation responses to CO₂ were strongly
2558 indicative of optimal resource investment to photosynthetic capacity as expected
2559 from photosynthetic least-cost theory (Prentice et al. 2014; Smith et al. 2019;
2560 Smith and Keenan 2020).

2561 5.4.4 *Conclusions*

2562 This study provides strong evidence suggesting that leaf acclimation responses
2563 to elevated CO₂ did not vary with soil nitrogen fertilization or ability to acquire
2564 nitrogen through symbiotic nitrogen fixation. However, whole plant acclimation

2565 responses to CO₂ were dependent on fertilization, where increasing fertilization
2566 increased the positive effect of whole plant growth under elevated CO₂. Results
2567 also indicate that fertilization played a relatively more important role in modify-
2568 ing whole plant responses to CO₂ than inoculation with symbiotic nitrogen-fixing
2569 bacteria, perhaps due to a reduction in nodulation across the fertilization gra-
2570 dient. These patterns strongly support the hypothesis that leaf and whole plant
2571 acclimation responses are driven by optimal resource investment to photosynthetic
2572 capacity, and that leaf acclimation responses to CO₂ were not modified by changes
2573 in soil nitrogen availability. These results build on previous work suggesting that
2574 constant leaf nitrogen-photosynthesis relationships are dynamic and change across
2575 growing environments, calling the current formulation of photosynthetic processes
2576 used in many terrestrial biosphere models into question.

2577

Chapter 6

2578

Conclusions

2579 The experiments included in this dissertation test mechanisms that drive patterns
2580 expected from photosynthetic least-cost theory across various edaphic and climatic
2581 gradients. Specifically, I investigate environmental drivers of carbon costs to ac-
2582 quire nitrogen, tradeoffs between nitrogen and water use, and plant acclimation
2583 responses to CO₂. These experiments provide important empirical data needed to
2584 test assumptions made in optimality models that leverage photosynthetic least-
2585 cost frameworks, and are among the first manipulative experiments to show sup-
2586 port for patterns expected from theory. Below, I summarize main findings of each
2587 chapter, synthesize common patterns observed across experiments, and conclude
2588 with a few study ideas that I think will help refine our understanding of plant
2589 nutrient acquisition and allocation responses to environmental change leveraging
2590 patterns predicted by photosynthetic least-cost theory.

2591 In the first experimental chapter, I quantified carbon costs to acquire ni-
2592 trogen in a species capable of forming associations with symbiotic nitrogen-fixing
2593 bacteria (*Glycine max*) and a species not capable of forming such associations
2594 (*Gossypium hirsutum*) grown under four soil nitrogen fertilization treatments and
2595 four light availability treatments in a full factorial greenhouse experiment. Sup-
2596 porting hypotheses, increasing light availability increased carbon costs to acquire
2597 nitrogen in both species due to a larger increase in belowground carbon biomass
2598 than whole plant nitrogen biomass. In further support of hypotheses, increasing
2599 fertilization decreased carbon costs to acquire nitrogen due to a larger increase in

2600 whole plant nitrogen biomass than belowground carbon biomass. Root nodulation
2601 data indicated that *G. max* shifted relative carbon allocation from nitrogen fixa-
2602 tion to direct uptake with increasing fertilization, which may explain the reduced
2603 responsiveness of *G. max* carbon costs to acquire nitrogen across the fertilization
2604 gradient.

2605 Despite evidence that reductions in the response of *G. max* carbon costs
2606 to acquire nitrogen to increasing fertilization may have been driven by shifts away
2607 from nitrogen fixation with increasing fertilization, I urge caution in assigning
2608 causality to the differential response of carbon costs to acquire nitrogen between
2609 species. This is because *G. max* and *G. hirsutum* are not phylogenetically related
2610 and have different life histories. Differences in life history between the two species
2611 limit my ability to assess whether reductions in the negative effect of increasing
2612 fertilization on carbon costs to acquire nitrogen in *G. max* were driven by shifts
2613 to direct uptake with increasing fertilization. However, these patterns were later
2614 confirmed in the fourth experimental chapter, where similar weaker negative ef-
2615 fects of increasing fertilization on carbon costs to acquire nitrogen were observed
2616 in *G. max* that were inoculated with symbiotic nitrogen-fixing bacteria compared
2617 to *G. max* that were left uninoculated across a similar soil nitrogen fertilization
2618 gradient.

2619 In the second experimental chapter, I assessed whether changes in soil
2620 nitrogen availability or soil pH drove changes in nitrogen-water use tradeoffs pre-
2621 dicted by photosynthetic least-cost theory. I measured leaf traits of mature upper
2622 canopy deciduous trees growing in a nine-year nitrogen-by-sulfur field manipula-
2623 tion experiment, where experimental sulfur additions were added with intent to

2624 acidify plots. Following patterns expected from the theory, increasing soil nitrogen
2625 availability was associated with increased leaf nitrogen content, but not net photo-
2626 tosynthesis, resulting in an increase in photosynthetic nitrogen use efficiency. In
2627 further support of theory, increasing soil nitrogen availability exhibited slight, but
2628 nonsignificant, decreases in leaf $C_i:C_a$ and increases in measures of photosynthetic
2629 capacity. Perhaps the strongest evidence for the theory was a strong negative
2630 relationship between leaf nitrogen content and leaf $C_i:C_a$, of which increased with
2631 increasing soil nitrogen availability through a stronger increase in leaf nitrogen
2632 content than leaf $C_i:C_a$.

2633 I found no effect of soil pH on nitrogen-water use tradeoffs aside from a
2634 marginal reduction in net photosynthesis rates that marginally reduced photosyn-
2635 thetic nitrogen use efficiency with increasing soil pH. Directionally, reductions in
2636 photosynthetic nitrogen use efficiency with increasing soil pH were expected per
2637 theory; however, this response was driven by no change in leaf nitrogen content
2638 and a reduction in net photosynthesis. Theory predicts that these tradeoffs should
2639 be driven by no change in net photosynthesis and an increase in leaf nitrogen con-
2640 tent. The general null leaf response to changing soil pH may have been due to
2641 experimental treatments directly increased soil nitrogen availability and affected
2642 soil pH in opposite patterns, suggesting that soil nitrogen availability may be more
2643 important in dictating nitrogen-water use tradeoffs than soil pH per se.

2644 In the third experimental chapter, I quantified variance in leaf nitrogen
2645 content across a precipitation and soil resource availability gradient in Texan
2646 grasslands. Specifically, I measured area-based leaf nitrogen content, components
2647 of area-based leaf nitrogen content (leaf mass per unit leaf area, leaf nitrogen per

2648 unit dry biomass), leaf $C_i:C_a$, and the unit cost of acquiring nitrogen relative to
2649 water in 520 individuals comprising 57 species. I found that variance in area-
2650 based leaf nitrogen content was positively associated with increasing soil nitrogen
2651 availability, soil moisture, vapor pressure deficit, and was negatively related to
2652 increasing leaf $C_i:C_a$. Following patterns expected from theory, a path analysis
2653 revealed that the positive soil nitrogen-leaf nitrogen relationship was driven by a
2654 positive relationship between soil nitrogen availability and the unit cost of acquir-
2655 ing and using nitrogen relative to water, a positive relationship between the unit
2656 cost of acquiring and using nitrogen relative to water, and negative relationship
2657 between leaf $C_i:C_a$ and leaf mass per unit leaf area. Interestingly, there was no
2658 effect of $C_i:C_a$ on leaf nitrogen content per unit dry biomass, indicating that vari-
2659 ance in area-based leaf nitrogen content across the environmental gradient was
2660 driven by a change in leaf morphology and not leaf chemistry.

2661 In the fourth experimental chapter, I quantified leaf and whole plant accli-
2662 mation responses in *G. max* grown under two atmospheric CO₂ levels, with and
2663 without inoculation with *Bradyrhizobium japonicum*, and across nine nitrogen fer-
2664 tilization treatments in a full factorial growth chamber experiment. I found strong
2665 evidence that leaf nitrogen content, V_{cmax} , and J_{max} were each downregulated un-
2666 der elevated CO₂. A stronger downregulation in V_{cmax} than J_{max} and stronger
2667 downregulation in leaf nitrogen content than V_{cmax} or J_{max} provided strong sup-
2668 port suggesting that leaves were acclimating to elevated CO₂ by optimizing leaf
2669 photosynthetic resource use efficiency to achieve optimal coordination. In striking
2670 support of my hypotheses, I find strong evidence suggesting that leaf acclimation
2671 responses to elevated CO₂ were decoupled from soil nitrogen fertilization and in-

2672 oculation treatment, despite apparent strong increases in leaf nitrogen content,
2673 V_{cmax} , and J_{max} with increasing fertilization and in inoculated pots. These find-
2674 ings contrast the current formulation of photosynthetic processes in terrestrial
2675 biosphere models, where many models simulate downregulations in leaf nitrogen
2676 content under elevated CO₂ as a function of progressive nitrogen limitation.

2677 There are currently two iterations of optimality models that employ the
2678 use of patterns expected from photosynthetic least-cost theory, one for C₃ species
2679 (Wang et al. 2017; Smith et al. 2019; Stocker et al. 2020) and one more recently
2680 developed for C₄ species (Scott and Smith 2022). In both model variants, costs
2681 to acquire and use nitrogen relative to water are held constant using a global
2682 dataset of δ¹³C (Cornwell et al. 2018). Throughout experiments, I show strong
2683 evidence suggesting that costs to acquire and use nitrogen are dynamic and vary
2684 predictably across environmental gradients, and that changes in these costs scale
2685 to alter leaf nitrogen-water use tradeoffs and acclimation responses to changing
2686 environments in ways predicted through photosynthetic least-cost theory. Thus,
2687 while optimality model simulations show good agreement with measured data
2688 (Smith et al. 2019; Stocker et al. 2020), such models may not be capturing an
2689 important source of variability in leaf nitrogen-water use tradeoffs by holding costs
2690 of resource use constant across environmental gradients.

2691 First principles of photosynthetic least-cost theory suggest that, in a given
2692 environment, plants optimize photosynthesis rates by sacrificing inefficient use of
2693 a relatively more abundant (and less costly to acquire) resource for more efficient
2694 use of a relatively less abundant (and more costly to acquire) resource. Through-
2695 out experimental chapters, I show strong support for these patterns across ex-

2696 periments, where increasing soil nitrogen fertilization generally decreased the cost
2697 of acquiring nitrogen relative to water, a pattern that scaled to influence leaf
2698 nitrogen-water use tradeoffs. I did not find evidence to suggest that soil moisture
2699 influenced nitrogen-water use tradeoffs, though this was due to strong covariation
2700 between soil moisture and soil nitrogen availability. Overall, findings across exper-
2701 iments provide empirical validation of photosynthetic least-cost theory needed to
2702 further develop optimality models and eventually implement such models in ter-
2703 restrial biosphere model products. Many terrestrial biosphere model products do
2704 not include robust frameworks for simulating acclimation responses to changing
2705 environmental conditions, and empirical findings shown here provide some support
2706 that optimality models that leverage photosynthetic least-cost theory predictions
2707 may improve the ability of terrestrial biosphere models to accurately simulate
2708 photosynthetic processes.

2709 Many terrestrial biosphere models predict photosynthetic capacity through
2710 plant functional group-specific linear regressions between area-based leaf nitrogen
2711 content and V_{cmax} (Rogers 2014; Rogers et al. 2017), which assumes that leaf
2712 nitrogen-photosynthesis relationships are constant across growing environments.
2713 I found constant leaf nitrogen-photosynthesis relationships with increasing soil ni-
2714 trogen availability in the nitrogen-by-sulfur field manipulation experiment. How-
2715 ever, results from the CO₂-by-nitrogen-by-inoculation manipulation experiment
2716 indicated that leaf nitrogen-photosynthesis responses to soil nitrogen availability
2717 were dependent on whether nitrogen was limiting. Further investigation regard-
2718 ing the effect of soil nitrogen availability in modifying leaf nitrogen-photosynthesis
2719 relationships is warranted to better understand the generality of leaf nitrogen pho-

2720 tosynthesis relationships across environmental gradients. However, findings from
2721 these experiments suggest that representing photosynthetic processes through pos-
2722 itive relationships between soil nitrogen availability, leaf nitrogen, and photosyn-
2723 thetic capacity are likely contributing to erroneous errors in model simulations and
2724 may explain the high degree of divergence in simulated processes across terrestrial
2725 biosphere models (Friedlingstein et al. 2014; Davies-Barnard et al. 2020).

2726 The experiments included in this dissertation have provided a strong foun-
2727 dation for me to continue growing as a plant physiological ecologist. I envision
2728 five primary avenues for future research that build on the work presented here,
2729 which are briefly summarized below:

- 2730** 1. Manipulative and environmental gradient experiments included here were
2731 designed to provide empirical data needed to test photosynthetic least-cost
2732 theory assumptions. While these results show promising patterns for pat-
2733 terns expected from photosynthetic least-cost theory, they do not necessarily
2734 address whether these patterns follow those simulated by optimality models
2735 that leverage photosynthetic least-cost principles. Thus, a clear future di-
2736 rection of these experiments would be to conduct model-data comparisons
2737 using data collected here (or similar experiments) to compare against opti-
2738 mality model simulations.

- 2739** 2. Experiments included here explicitly quantify effects of symbiotic nitrogen
2740 fixation on carbon costs to acquire nitrogen, nitrogen-water use tradeoffs,
2741 and leaf nitrogen-photosynthesis relationships. However, carbon costs to ac-
2742 quire nitrogen also vary in species that associate with different mycorrhizal
2743 types (Brzostek et al. 2014; Terrer et al. 2018), and dominant mycorrhizal

2744 type in an ecosystem has been shown to determine net biogeochemical cycle
2745 dynamics in deciduous forests of the northeastern United States (Phillips
2746 et al. 2013). Thus, future work should consider conducting similar experi-
2747 ments while manipulating mycorrhizal association to better understand how
2748 microbial symbioses modify leaf and whole plant acclimation responses to
2749 changing environments.

2750 3. Recent work indicates a high degree of variance in symbiotic nitrogen fixa-
2751 tion rates across terrestrial biosphere models (Meyerholt et al. 2016; Davies-
2752 Barnard et al. 2020), perhaps due to nitrogen fixation rates that are im-
2753 plemented across terrestrial biosphere models as a function of temperature
2754 (Houlton et al. 2008). While energetic costs of nitrogen fixation are de-
2755 pendent on temperature, I show that structural carbon costs to acquire
2756 nitrogen via symbiotic nitrogen fixation are driven by factors that influence
2757 demand to acquire nitrogen (i.e. CO₂, light) and are modified by soil ni-
2758 trogen supply. The light-by-nitrogen greenhouse experiment was published
2759 in *Journal of Experimental Botany*, and a reviewer encouraged future work
2760 to include a model-data comparison comparing structural carbon costs to
2761 acquire nitrogen measured in the experiment to carbon costs to acquire ni-
2762 trogen simulated by the FUN biogeochemical model (Fisher et al. 2010;
2763 Brzostek et al. 2014; Allen et al. 2020). Conveniently, FUN calculates car-
2764 bon costs to acquire nitrogen following the same calculation used in the first
2765 and fourth experimental chapter. Conducting such a model-data comparison
2766 would be a useful step toward identifying biases in the FUN biogeochemi-
2767 cal model, which is currently coupled to several terrestrial biosphere models

2768 (Clark et al. 2011; Shi et al. 2016; Lawrence et al. 2019; Davies-Barnard
2769 et al. 2020).

2770 4. Carbon costs to acquire nitrogen relative to water were quantified at the
2771 leaf level as a function of $\delta^{13}\text{C}$ and vapor pressure deficit, while structural
2772 carbon costs to acquire nitrogen were quantified at the whole plant level
2773 as the ratio of belowground carbon allocation per unit whole plant nitro-
2774 gen biomass. As increasing soil nitrogen availability decreases both leaf and
2775 whole plant estimates of costs to acquire and use nitrogen, one might expect
2776 leaf and whole plant carbon cost to acquire nitrogen estimates to covary. Fu-
2777 ture work should consider investigating if leaf and whole plant estimates of
2778 carbon costs to acquire nitrogen covary and evaluate whether environmental
2779 conditions (or species acquisition strategy) modifies any of this possible co-
2780 variance. Strong covariance between leaf and whole plant costs of nitrogen
2781 acquisition could be a possible avenue to implement frameworks for allowing
2782 costs of nitrogen acquisition to vary in optimality models, as the FUN model
2783 calculates carbon costs of nitrogen acquisition at the whole plant level.

2784 5. While experiments included here target effects of soil nitrogen availability
2785 on carbon costs to acquire nitrogen and associated leaf nitrogen-water use
2786 tradeoffs, photosynthetic least-cost theory predicts that plants acclimate
2787 their photosynthetic processes by minimizing the summed cost of nutrient
2788 (not just nitrogen) and water use. Therefore, the theory would predict
2789 similar leaf acclimation responses across soil phosphorus or other nutrient
2790 availability gradients. Recent iterations of the FUN biogeochemical cycle
2791 includes a framework for determining the carbon and nitrogen cost of ac-

2792 quiring and using phosphorus, which similarly varies in species with different
2793 nutrient acquisition strategies (Allen et al. 2020). The implementation of
2794 this model in a terrestrial biosphere model (E3SM) was also recently shown
2795 to improve model performance of ecosystem nutrient limitation (Braghieri
2796 et al. 2022). As nitrogen and phosphorus commonly co-limit leaf photo-
2797 synthesis and primary productivity, extending experiments reported here to
2798 investigate carbon and nitrogen costs of phosphorus use, and whether these
2799 patterns scale to leaf nutrient-water use tradeoffs would be a useful next
2800 step in understanding extensions and limitations of photosynthetic least-
2801 cost theory.

2802 The experiments included in this dissertation and the proposed experiments sum-
2803 marized above provide a snapshot view of the things that I have learned through-
2804 out my time as a graduate student. I am excited to continue learning and growing
2805 as a plant ecophysiologicalist, ecologist, and scientist, and look forward to continuing
2806 along my journey of investigating nutrient acquisition and allocation responses to
2807 global change.

2808

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3668 Appendix A: Supplemental material for "Structural carbon costs to
3669 acquire nitrogen are determined by nitrogen and light availability in
3670 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

3671 *Significance determined using Wald's χ^2 tests ($p=0.05$). P-values less than 0.05
3672 are in bold and p-values between 0.05 and 0.1 are italicized. Negative coefficients
3673 for light treatments indicate a positive effect of increasing light availability on
3674 all response variables, as light availability is treated as percent shade cover in all
3675 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>

3676 *Slopes represent estimated marginal mean slopes from linear mixed-effects models described in the Methods. Slopes
3677 were calculated using the ‘emmeans’ R package (Lenth 2019). Superscripts indicate slopes fit to natural-log (^a) or
3678 square root (^b) transformed data. Slopes statistically different from zero (Tukey: $p < 0.05$) are indicated in bold.
3679 Marginally significant slopes (Tukey: $0.05 < p < 0.1$) are italicized.

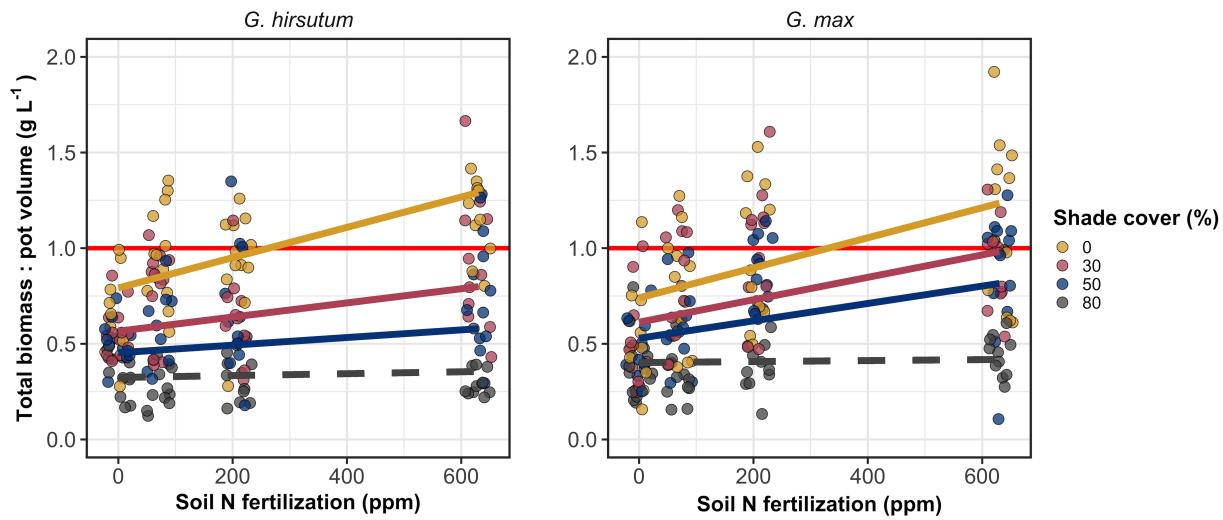


Figure A1. Effects of shade cover and nitrogen fertilization on the ratio of plant biomass to rooting volume in *G. hirsutum* (left panel) and *G. max* (right panel). The red horizontal line indicates the recommended 1 g L^{-1} threshold for BVR recommended by Poorter et al. (2012) to avoid pot size-induced 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.

3680 Appendix B: Supplemental material for "Soil nitrogen availability
3681 modifies leaf nitrogen economies in mature temperate deciduous
3682 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

3683 *Plots within each site are represented based on nitrogen and sulfur addition
3684 status. The final column on the right depicts total sample size per plot in each
3685 site (N_{plot}) and the final row on the bottom represents cumulative species sample
3686 size across all plots and all sites (N_{spp}). Key: ACRU=*A. rubrum*; ACSA=*A.*
3687 *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

3688 *Results detail linear mixed effects model where temperature was regressed against
3689 net photosynthesis or stomatal conductance, with site and species designated as
3690 random intercept terms. Significance was determined using Type II Wald χ^2 tests
3691 ($\alpha=0.05$).

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

3692 *Net photosynthesis and stomatal conductance values were fit to the log-polynomial
3693 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

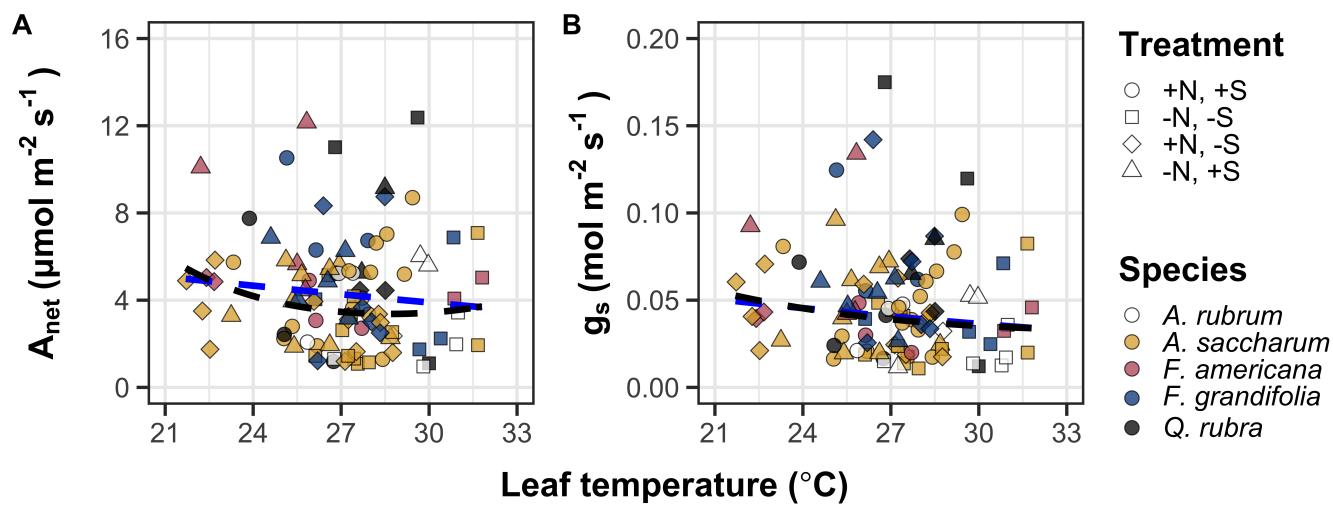


Figure B1. Effects of leaf temperature on net photosynthesis rate (A) and stomatal conductance (B) values when measured at $400 \mu\text{mol mol}^{-1} \text{CO}_2$. Leaf temperature is represented on the x-axis, while species are represented as colored points. Colored points and shapes are as explained 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.

**3694 Appendix C: Supplemental material for "The relative cost of resource
3695 use for photosynthesis drives variance in leaf nitrogen content across a
3696 climate and soil resource availability gradient"**

3697 C.1 Calculations for soil water holding capacity

3698 Water holding capacity (θ_{WHC} ; mm) was calculated as a function of the volumetric
3699 soil water storage at field capacity (W_{FC} ; m³ m⁻³), and the volumetric soil water
3700 storage at wilting point (W_{PWP} ; m³ m⁻³):

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

3701 where f_{gravel} (%) is the fraction of gravel content in soil, $z_{bedrock}$ (mm) is the
3702 distance to bedrock, and z_{max} (mm) is the maximum allowable distance to bedrock,
3703 set to 2000mm. W_{FC} is calculated as:

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

3704 where

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

3705 W_{PWP} is calculated as:

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

3706 where

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

3707 In Equations C4.4 and C4.5, f_{sand} (%) is the fraction of sand content in soil

3708 (%), f_{clay} (%) is the fraction of clay content in soil (%), and f_{OM} is the fraction of

3709 organic matter in soil (%). Organic matter in the soil was calculated by converting

3710 soil organic carbon data extracted from SoilGrids 2.0 to soil organic matter using

3711 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 gooddingii</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

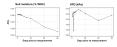


Figure C1. Model selection results exploring relevant timescales for soil moisture (left panel) and vapor pressure deficit (right panel). The x-axis indicates the number of days before each site visit and the y-axis notes the corrected Akaike Information Criterion value. The timescale with the lowest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

**3712 Appendix D: Supplemental material for "Optimal resource investment
 3713 to photosynthetic capacity maximizes nutrient allocation to whole
 3714 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

3715 *Significance determined using Type II Wald χ^2 tests ($\alpha=0.05$). P-values less
3716 than 0.05 are in bold. Key: df=degrees of freedom; χ^2 =Wald Type II chi-square
3717 test statistic.

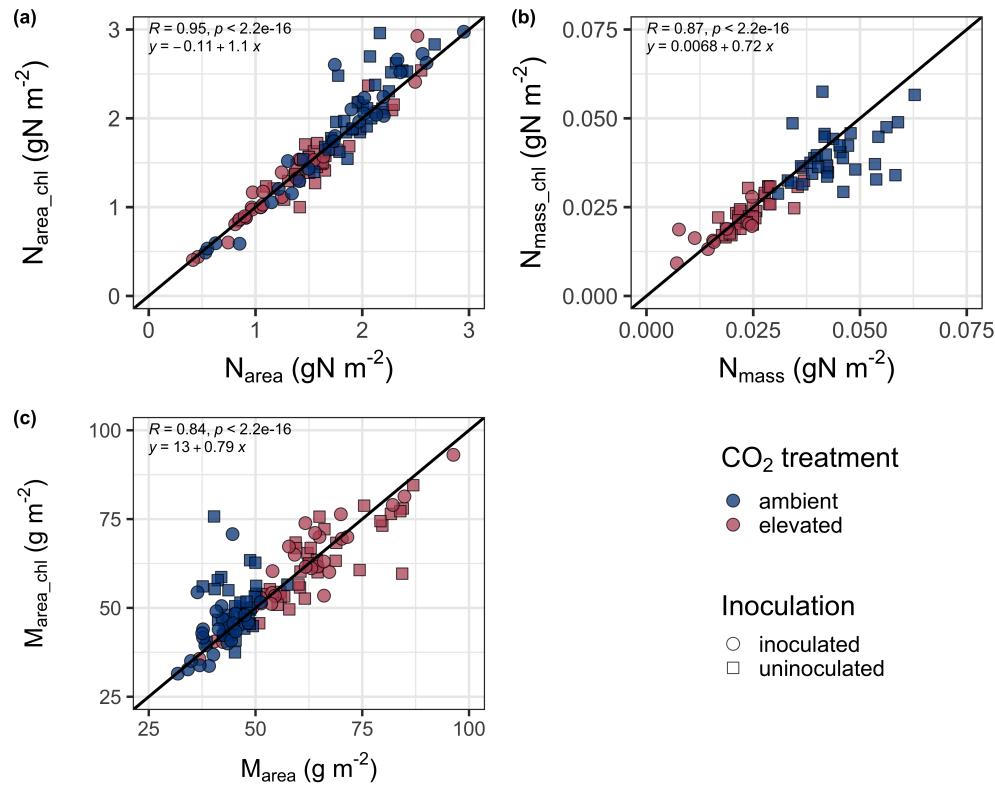


Figure D1. Relationships between area-based leaf nitrogen content (a), mass-based leaf nitrogen content (b), and leaf mass per unit leaf area (c) measured on the focal leaf used to generate A_{net}/C_i curves (x-axis) and leaf nitrogen content measured on the leaf used for chlorophyll extractions (y-axis). 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.

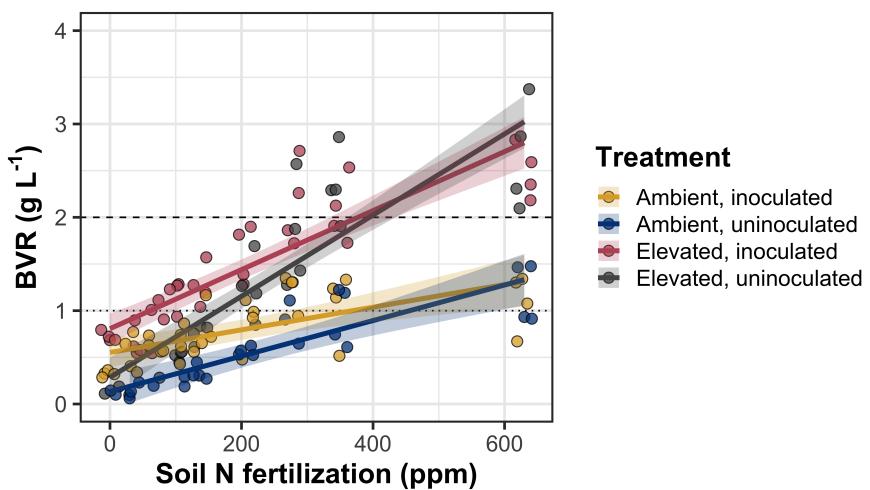


Figure D2. Effects of CO₂, fertilization, and inoculation on the ratio of whole plant biomass to pot volume. Soil nitrogen fertilization is represented on the x-axis in all panels. Yellow points and trendlines indicate inoculated individuals grown under ambient CO₂, blue points and trendlines indicate 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.