



## Nitrogen demand, supply, and acquisition strategy control plant responses to elevated CO<sub>2</sub> at different scales

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Abstract:	Plants respond to elevated atmospheric CO <sub>2</sub> concentrations by reducing leaf nitrogen content and photosynthetic capacity – patterns that correspond with increased net photosynthesis rates, total leaf area, and total biomass. Nitrogen supply has been hypothesized to be the primary factor controlling these responses, as nitrogen availability limits net primary productivity globally. Recent work using evo-evolutionary optimality theory suggests that leaf photosynthetic responses to elevated CO <sub>2</sub> are independent of nitrogen supply and are instead driven by leaf nitrogen demand to build and maintain photosynthetic enzymes, which optimizes resource allocation to photosynthetic capacity and maximizes allocation to growth. Here, <i>Glycine max</i> L. (Merr) seedlings were grown under two CO <sub>2</sub> concentrations, with and without inoculation with <i>Bradyrhizobium japonicum</i> , and across nine soil nitrogen fertilization treatments in a full-factorial growth chamber experiment to reconcile the role of nitrogen supply and demand on leaf and whole-plant responses to elevated CO <sub>2</sub> . After seven weeks, elevated CO <sub>2</sub> increased net photosynthesis rates despite reduced leaf nitrogen content and maximum rates of Ribulose-1,5-bisphosphate (RuBP) carboxylase/oxygenase (Rubisco) carboxylation and electron transport for RuBP regeneration. Effects of elevated CO <sub>2</sub> on net photosynthesis and indices of photosynthetic capacity were independent of nitrogen fertilization and inoculation. However, increasing nitrogen fertilization enhanced positive effects of elevated CO <sub>2</sub> on total leaf area and total biomass due to increased nitrogen uptake and reduced carbon costs to acquire nitrogen. Whole-plant responses to elevated CO <sub>2</sub> were not modified by inoculation across the nitrogen fertilization gradient, as plant

investment toward symbiotic nitrogen fixation was similar between CO<sub>2</sub> treatments. These results indicate that leaf nitrogen demand to build and maintain photosynthetic enzymes drives leaf photosynthetic responses to elevated CO<sub>2</sub>, while nitrogen supply regulates whole-plant responses. Our findings build on previous work suggesting that terrestrial biosphere models may improve simulations of photosynthetic processes under future novel environments by adopting optimality principles.

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14

## 15 Abstract

16 Plants respond to elevated atmospheric CO<sub>2</sub> concentrations by reducing leaf nitrogen content and  
17 photosynthetic capacity – patterns that correspond with increased net photosynthesis rates, total  
18 leaf area, and total biomass. Nitrogen supply has been hypothesized to be the primary factor  
19 controlling these responses, as nitrogen availability limits net primary productivity globally.

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26 factorial growth chamber experiment to reconcile the role of nitrogen supply and demand on leaf  
27 and whole-plant responses to elevated CO<sub>2</sub>. After seven weeks, elevated CO<sub>2</sub> increased net  
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34 plant responses to elevated CO<sub>2</sub> were not modified by inoculation across the nitrogen  
35 fertilization gradient, as plant investment toward symbiotic nitrogen fixation was similar between  
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37 photosynthetic enzymes drives leaf photosynthetic responses to elevated CO<sub>2</sub>, while nitrogen  
38 supply regulates whole-plant responses. Our findings build on previous work suggesting that  
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41

## 42 **Keywords**

43 acclimation, eco-evolutionary optimality, growth chamber, least-cost theory, nitrogen acquisition  
44 strategy, photosynthesis, plant functional ecology, whole-plant growth

45

## 46 **Introduction**

47 Terrestrial ecosystems are regulated by complex carbon and nitrogen cycles. As a result,  
48 terrestrial biosphere models, which are beginning to include coupled carbon and nitrogen cycles  
49 (Shi *et al.*, 2016; Davies-Barnard *et al.*, 2020; Braghieri *et al.*, 2022), must accurately represent  
50 these cycles under different environmental scenarios to reliably simulate carbon and nitrogen  
51 fluxes (Oreskes *et al.*, 1994; Prentice *et al.*, 2015). While the inclusion of coupled carbon and  
52 nitrogen cycles in terrestrial biosphere models was intended to improve model reliability, large  
53 uncertainty in the role of nitrogen availability and nitrogen acquisition strategy on leaf and whole  
54 plant responses to increasing atmospheric CO<sub>2</sub> concentrations persists (Arora *et al.*, 2020;  
55 Davies-Barnard *et al.*, 2020; Kou-Giesbrecht *et al.*, 2023), contributing to widespread divergence  
56 in future carbon and nitrogen flux simulations across terrestrial biosphere models (Hungate *et al.*,  
57 2003; Friedlingstein *et al.*, 2014; Zaehle *et al.*, 2014; Wieder *et al.*, 2015; Meyerholt *et al.*,  
58 2020).

59 Over the past few decades, numerous studies have sought to elucidate plant responses to  
60 elevated CO<sub>2</sub>, revealing consistent leaf and whole-plant patterns. At the leaf level, C<sub>3</sub> plants  
61 grown under elevated CO<sub>2</sub> exhibit increased net photosynthesis rates compared to plants grown  
62 under ambient CO<sub>2</sub> (Medlyn *et al.*, 1999; Ainsworth & Long, 2005; Bernacchi *et al.*, 2005; Lee

63 *et al.*, 2011; Poorter *et al.*, 2022). These patterns correspond with reduced mass- and area-based  
64 leaf nitrogen content, increased leaf mass per area, reduced stomatal conductance, and reduced  
65 photosynthetic capacity, yielding increased photosynthetic nitrogen-use efficiency and water-use  
66 efficiency (Curtis, 1996; Drake *et al.*, 1997; Medlyn *et al.*, 1999; Ainsworth & Long, 2005;  
67 Ainsworth & Rogers, 2007; Lee *et al.*, 2011; Pastore *et al.*, 2019; Poorter *et al.*, 2022). At the  
68 whole-plant level, C<sub>3</sub> plants grown under elevated CO<sub>2</sub> exhibit increased total leaf area, which  
69 supports greater net primary productivity and total biomass compared to plants grown under  
70 ambient CO<sub>2</sub> (Coleman *et al.*, 1993; Ainsworth *et al.*, 2002; Ainsworth & Rogers, 2007; Finzi *et*  
71 *al.*, 2007; Poorter *et al.*, 2022). Some experiments suggest that elevated CO<sub>2</sub> increases  
72 belowground carbon allocation and the ratio of root biomass to shoot biomass compared to plants  
73 grown under ambient CO<sub>2</sub> (Nie *et al.*, 2013), though this allocation response is not consistently  
74 observed (Luo *et al.*, 1994; Poorter *et al.*, 2022).

75 Despite consistent plant responses to elevated CO<sub>2</sub> documented across experiments,  
76 mechanisms that drive these responses remain unresolved. Some have hypothesized that plant  
77 responses to elevated CO<sub>2</sub> are constrained by nitrogen availability, as net primary productivity is  
78 limited by nitrogen availability globally (Vitousek & Howarth, 1991; LeBauer & Treseder,  
79 2008). The progressive nitrogen limitation hypothesis predicts that elevated CO<sub>2</sub> will increase  
80 plant nitrogen uptake to support greater net primary productivity, which will cause nitrogen  
81 availability to decline over time (Luo *et al.*, 2004). The hypothesis predicts that this response  
82 should increase growth and net primary productivity under elevated CO<sub>2</sub> over short time scales  
83 that dampen with time as nitrogen becomes progressively more limiting and stored in longer-  
84 lived tissues. Growth responses to elevated CO<sub>2</sub> expected from the progressive nitrogen  
85 limitation hypothesis have received some support from free-air CO<sub>2</sub> enrichment experiments  
86 (Reich *et al.*, 2006; Norby *et al.*, 2010), though these patterns are not consistently observed  
87 (Finzi *et al.*, 2006, 2007; Moore *et al.*, 2006; Liang *et al.*, 2016).

88 Assuming positive relationships between soil nitrogen availability, leaf nitrogen content,  
89 and photosynthetic capacity (Field & Mooney, 1986; Evans, 1989; Evans & Seemann, 1989;  
90 Walker *et al.*, 2014; Firn *et al.*, 2019; Liang *et al.*, 2020), the progressive nitrogen limitation  
91 hypothesis implies that reductions in nitrogen availability over time might explain why C<sub>3</sub> plants  
92 exhibit decreased leaf nitrogen content and photosynthetic capacity under elevated CO<sub>2</sub>.  
93 However, results from free-air CO<sub>2</sub> enrichment experiments show that reductions in leaf nitrogen

94 content and photosynthetic capacity under elevated CO<sub>2</sub> are decoupled from changes in nitrogen  
95 availability (Crous *et al.*, 2010; Lee *et al.*, 2011; Pastore *et al.*, 2019). Additionally, variance in  
96 leaf nitrogen and photosynthetic capacity across environmental gradients tends to be more  
97 strongly determined through aboveground growth conditions that set demand to build and  
98 maintain photosynthetic enzymes than through changes in soil resource availability (Dong *et al.*,  
99 2017, 2020, 2022a; Smith *et al.*, 2019; Smith & Keenan, 2020; Paillassa *et al.*, 2020; Peng *et al.*,  
100 2021; Querejeta *et al.*, 2022; Westerband *et al.*, 2023; Waring *et al.*, 2023). These patterns  
101 indicate that leaf photosynthetic responses to elevated CO<sub>2</sub> may be a product of altered leaf  
102 nitrogen demand to build and maintain photosynthetic enzymes and may not be as strongly  
103 linked to changes in nitrogen availability.

104 Eco-evolutionary optimality theory provides a framework for understanding how leaf  
105 photosynthetic responses to elevated CO<sub>2</sub> may be determined through demand to build and  
106 maintain photosynthetic enzymes (Harrison *et al.*, 2021). Merging photosynthetic least-cost  
107 (Wright *et al.*, 2003; Prentice *et al.*, 2014) and optimal coordination (Chen *et al.*, 1993; Maire *et*  
108 *al.*, 2012) theories, eco-evolutionary optimality theory posits that reduced leaf nitrogen allocation  
109 under elevated CO<sub>2</sub> is the downstream result of a stronger downregulation in the maximum rate  
110 of Ribulose-1,5-bisphosphate (RuBP) carboxylase/oxygenase (Rubisco) carboxylation ( $V_{\text{cmax}}$ )  
111 than the maximum rate of electron transport for RuBP regeneration ( $J_{\text{max}}$ ), which reduces leaf  
112 nitrogen demand to build and maintain photosynthetic enzymes. Optimal leaf nitrogen allocation  
113 to photosynthetic capacity allows plants to make more efficient use of available light while  
114 avoiding overinvestment in Rubisco, which has high nitrogen and energetic costs of construction  
115 and maintenance (Evans, 1989; Sage, 1994; Evans & Clarke, 2019). Such optimal leaf nitrogen  
116 allocation responses to elevated CO<sub>2</sub> increases photosynthetic nitrogen-use efficiency and allows  
117 increased net photosynthesis rates to be achieved through increasingly equal co-limitation of  
118 Rubisco carboxylation and electron transport for RuBP regeneration (Chen *et al.*, 1993; Maire *et*  
119 *al.*, 2012; Wang *et al.*, 2017; Smith *et al.*, 2019). The expected optimal leaf response to elevated  
120 CO<sub>2</sub> has received some empirical support (Crous *et al.*, 2010; Lee *et al.*, 2011; Smith & Keenan,  
121 2020; Harrison *et al.*, 2021; Dong *et al.*, 2022b; Cui *et al.*, 2023), though no studies have  
122 connected these patterns with concurrently measured whole-plant responses.

123 The eco-evolutionary optimality hypothesis deviates from the progressive nitrogen  
124 limitation hypothesis by indicating that photosynthetic responses to elevated CO<sub>2</sub> are driven by

leaf nitrogen demand to build and maintain photosynthetic enzymes and are independent of changes in soil nitrogen supply. However, the eco-evolutionary optimality hypothesis does not discount the role of soil nitrogen availability on whole-plant responses to elevated CO<sub>2</sub>, where the expected optimal strategy in response to elevated CO<sub>2</sub> is to allocate surplus nitrogen not needed to satisfy leaf nitrogen demand toward the construction of a greater quantity of optimally coordinated leaves and other plant organs. Thus, whether the supply-driven progressive nitrogen limitation hypothesis or demand-driven eco-evolutionary optimality hypothesis controls plant responses to elevated CO<sub>2</sub> may be a matter of scale, where leaf photosynthetic responses to elevated CO<sub>2</sub> are determined through demand to build and maintain photosynthetic enzymes and whole-plant responses to elevated CO<sub>2</sub> are regulated by changes in nitrogen supply.

Plants allocate carbon belowground in exchange for nutrients through different nutrient acquisition strategies, including direct uptake pathways or symbioses with mycorrhizal fungi and symbiotic nitrogen-fixing bacteria (Gutschick, 1981; Smith & Read, 2008). Carbon costs to acquire nitrogen, or the amount of carbon allocated belowground per unit nitrogen acquired, vary in species with different nitrogen acquisition strategies and are dependent on environmental factors such as atmospheric CO<sub>2</sub>, temperature, light availability, and nutrient availability (Brzostek *et al.*, 2014; Terrer *et al.*, 2018; Allen *et al.*, 2020; Eastman *et al.*, 2021; Perkowski *et al.*, 2021; Lu *et al.*, 2022; Peng *et al.*, 2023). Therefore, nitrogen acquisition strategy cannot be ignored when considering effects of nitrogen availability on plant responses to elevated CO<sub>2</sub>. To date, few studies account for acquisition strategy when considering the role of nitrogen availability on leaf and whole-plant responses to elevated CO<sub>2</sub> (e.g., Terrer *et al.*, 2016, 2018; Smith & Keenan, 2020). Such studies found that nitrogen acquisition strategies with reduced carbon costs to acquire nitrogen may buffer the effect of nitrogen limitation at the whole-plant level (Terrer *et al.*, 2018), but leaf-level responses remain inconsistent (Terrer *et al.*, 2018; Smith & Keenan, 2020).

Here, we conducted a growth chamber experiment using *Glycine max* L. (Merr.) seedlings grown under full factorial combinations of two CO<sub>2</sub> concentrations, two inoculation treatments, and nine soil nitrogen fertilization treatments to reconcile the role of nitrogen supply and demand on plant responses to elevated CO<sub>2</sub>. We used this experimental setup to test the following hypotheses:

- 155 (1) Following the demand-driven eco-evolutionary optimality hypothesis, elevated CO<sub>2</sub> will  
156 downregulate  $V_{cmax}$  more strongly than  $J_{max}$ , increasing  $J_{max}:V_{cmax}$  and allowing increased  
157 net photosynthesis rates to approach equal co-limitation of Rubisco carboxylation and  
158 electron transport for RuBP regeneration. Leaf photosynthetic responses to elevated CO<sub>2</sub>  
159 will be independent of nitrogen fertilization and inoculation treatment and will  
160 correspond with increased photosynthetic nitrogen-use efficiency.
- 161 (2) Following the supply-driven nitrogen limitation hypothesis, positive effects of elevated  
162 CO<sub>2</sub> on total leaf area and total biomass will be enhanced with increasing nitrogen  
163 fertilization due to increased plant nitrogen uptake and reduced carbon costs to acquire  
164 nitrogen. Inoculation with symbiotic nitrogen-fixing bacteria will enhance positive  
165 growth responses to elevated CO<sub>2</sub>, though these responses will only be apparent under  
166 low nitrogen fertilization levels where individuals will have increased investment in  
167 nitrogen acquisition through symbiotic nitrogen fixation.

168

169 **Methods**170 *Seed treatments and experimental design*

171 *Glycine max* seeds were planted in 144 6-liter surface sterilized pots (NS-600, Nursery Supplies,  
172 Orange, CA, USA) containing a steam-sterilized 70:30 volume: volume mix of *Sphagnum* peat  
173 moss (Premier Horticulture, Quakertown, PA, USA) to sand (Pavestone, Atlanta, GA, USA).  
174 Before planting, all *G. max* seeds were surface sterilized in 2% sodium hypochlorite for 3  
175 minutes, followed by three separate 3-minute washes with ultrapure water (MilliQ 7000;  
176 MilliporeSigma, Burlington, MA USA). Subsets of surface-sterilized seeds were inoculated with  
177 *Bradyrhizobium japonicum* (Verdesian N-Dure™ Soybean, Cary, NC, USA) in a slurry  
178 following manufacturer recommendations (3.12 g inoculant and 241 g ultrapure water per 1 kg  
179 seed).

180 Seventy-two pots were randomly planted with surface-sterilized seeds inoculated with *B.*  
181 *japonicum*, while the remaining 72 pots were planted with surface-sterilized uninoculated seeds.  
182 Thirty-six pots in each inoculation treatment were randomly placed in one of two atmospheric  
183 CO<sub>2</sub> treatments (420 and 1000  $\mu\text{mol mol}^{-1}$  CO<sub>2</sub>). Plants in each unique inoculation-by-CO<sub>2</sub>  
184 treatment combination randomly received one of nine nitrogen fertilization treatments equivalent  
185 to 0 (0 mM), 35 (2.5 mM), 70 (5 mM), 105 (7.5 mM), 140 (10 mM), 210 (15 mM), 280 (20

186 mM), 350 (25 mM), or 630 ppm (45 mM) N. Nitrogen fertilization treatments were created using  
187 a modified Hoagland's solution (Hoagland & Arnon, 1950) designed to keep concentrations of  
188 all other macronutrients and micronutrients equivalent across treatments (Table S1). Plants  
189 received the same nitrogen fertilization treatment twice per week in 150 mL doses as topical  
190 agents to the soil surface.

191

#### 192 *Growth chamber conditions*

193 Plants were randomly placed in one of six Percival LED-41L2 growth chambers (Percival  
194 Scientific Inc., Perry, IA, USA) over two experimental iterations due to chamber space  
195 limitation. Two iterations were conducted such that one iteration included all plants grown under  
196 elevated CO<sub>2</sub> plants, and the second iteration included all plants grown under ambient CO<sub>2</sub>.  
197 Average ( $\pm$  SD) CO<sub>2</sub> concentrations across chambers throughout the experiment were 439 $\pm$ 5  
198  $\mu\text{mol mol}^{-1}$  CO<sub>2</sub> for the ambient treatment and 989 $\pm$ 4  $\mu\text{mol mol}^{-1}$  CO<sub>2</sub> for the elevated treatment.

199 Daytime growth conditions were simulated using a 16-hour photoperiod, with incoming  
200 light radiation set to chamber maximum (mean $\pm$ SD: 1230 $\pm$ 12  $\mu\text{mol m}^{-2} \text{s}^{-1}$  across chambers), air  
201 temperature set to 25°C, and relative humidity set to 50%. The remaining 8-hour period  
202 simulated nighttime growing conditions, with incoming light radiation set to 0  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ,  
203 chamber temperature set to 17°C, and relative humidity set to 50%. Transitions between daytime  
204 and nighttime growing conditions were simulated by ramping incoming light radiation in 45-  
205 minute increments and temperature in 90-minute increments over a 3-hour period (Table S2).

206 Plants grew under average ( $\pm$  SD) daytime light intensity of 1049 $\pm$ 27  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ,  
207 including ramping periods. In the elevated CO<sub>2</sub> iteration, plants grew under 24.0 $\pm$ 0.2°C during  
208 the day, 16.4 $\pm$ 0.8°C during the night, and 51.6 $\pm$ 0.4% relative humidity. In the ambient CO<sub>2</sub>  
209 iteration, plants grew under 23.9 $\pm$ 0.2°C during the day, 16.0 $\pm$ 1.4°C during the night, and  
210 50.3 $\pm$ 0.2% relative humidity. Within each experiment iteration, any differences in climate  
211 conditions across the six chambers were accounted for by shuffling the same group of plants  
212 throughout the growth chambers. This process was done by iteratively moving the group of  
213 plants on the top rack of a chamber to the bottom rack of the same chamber, while  
214 simultaneously moving the group of plants on the bottom rack of a chamber to the top rack of the  
215 adjacent chamber. Plants were moved within and across chambers daily during each experiment  
216 iteration.

217

218 *Leaf gas exchange measurements*

219 Leaf gas exchange measurements were collected on the seventh week of development, before the  
220 onset of reproduction. All gas exchange measurements were collected on the center leaf of the  
221 most recent fully expanded trifoliate leaf set using LI-6800 portable photosynthesis machines  
222 configured with a 6800-01A fluorometer head and 6 cm<sup>2</sup> aperture (LI-COR Biosciences,  
223 Lincoln, NE, USA). Specifically, net photosynthesis ( $A_{\text{net}}$ ;  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ), stomatal conductance  
224 ( $g_{\text{sw}}$ ;  $\text{mol m}^{-2} \text{s}^{-1}$ ), and intercellular CO<sub>2</sub> ( $C_i$ ;  $\mu\text{mol mol}^{-1}$ ) concentrations were measured across a  
225 range of atmospheric CO<sub>2</sub> concentrations (i.e., an  $A_{\text{net}}/C_i$  curve) using the Dynamic  
226 Assimilation™ Technique. The Dynamic Assimilation™ Technique corresponds well with  
227 traditional steady-state  $A_{\text{net}}/C_i$  curves in *G. max* (Saathoff & Welles, 2021).  $A_{\text{net}}/C_i$  curves were  
228 generated along a reference CO<sub>2</sub> ramp down from 420  $\mu\text{mol mol}^{-1}$  CO<sub>2</sub> to 20  $\mu\text{mol mol}^{-1}$  CO<sub>2</sub>,  
229 followed by a ramp up from 420  $\mu\text{mol mol}^{-1}$  CO<sub>2</sub> to 1620  $\mu\text{mol mol}^{-1}$  CO<sub>2</sub> after a 90-second wait  
230 period at 420  $\mu\text{mol mol}^{-1}$  CO<sub>2</sub>. The ramp rate for each curve was set to 200  $\mu\text{mol mol}^{-1} \text{min}^{-1}$ ,  
231 logging every five seconds, which generated 96 data points per response curve. All  $A_{\text{net}}/C_i$  curves  
232 were generated after  $A_{\text{net}}$  and  $g_{\text{sw}}$  stabilized in a LI-6800 cuvette set to a 500 mol s<sup>-1</sup> flow rate,  
233 10000 rpm mixing fan speed, 1.5 kPa vapor pressure deficit, 25°C leaf temperature, 2000  $\mu\text{mol}$   
234 m<sup>-2</sup> s<sup>-1</sup> incoming light radiation, and initial reference CO<sub>2</sub> set to 420  $\mu\text{mol mol}^{-1}$ .

235 Snapshot  $A_{\text{net}}$  measurements were extracted from each  $A_{\text{net}}/C_i$  curve, both at a common  
236 CO<sub>2</sub> concentration, 420  $\mu\text{mol mol}^{-1}$  CO<sub>2</sub> ( $A_{\text{net},420}$ ;  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ), and under each individual's  
237 growth CO<sub>2</sub> concentration, 420 and 1000  $\mu\text{mol mol}^{-1}$  CO<sub>2</sub> ( $A_{\text{net,growth}}$ ;  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ). Dark  
238 respiration ( $R_d$ ;  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) measurements were collected with the same leaf used to generate  
239  $A_{\text{net}}/C_i$  curves following at least 30 minutes of darkness. Measurements were collected on a 5-  
240 second log interval for 60 seconds after the leaf stabilized in a LI-6800 cuvette set to a 500 mol  
241 s<sup>-1</sup> flow rate, 10000 rpm mixing fan speed, 1.5 kPa vapor pressure deficit, 25°C leaf temperature,  
242 and 420  $\mu\text{mol mol}^{-1}$  reference CO<sub>2</sub> concentration (regardless of CO<sub>2</sub> treatment), with incoming  
243 light radiation set to 0  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . A single dark respiration value was determined for each leaf  
244 by calculating the mean dark respiration value across the logging interval.

245

246 *A/C<sub>i</sub> curve-fitting and parameter estimation*

247  $A_{\text{net}}/C_i$  curves were fit using the 'fitaci' function in the 'plantecophys' R package (Duursma,  
248 2015). This function estimates the maximum rate of Rubisco carboxylation ( $V_{\text{cmax}}$ ;  $\mu\text{mol m}^{-2} \text{s}^{-1}$ )  
249 and maximum rate of electron transport for RuBP regeneration ( $J_{\text{max}}$ ;  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) based on the  
250 Farquhar *et al.* (1980) biochemical model of C<sub>3</sub> photosynthesis. Triose phosphate utilization  
251 (TPU) limitation was included as an additional rate-limiting step in all curve fits after visually  
252 observing clear TPU limitation for most curves. All curve fits included measured dark respiration  
253 values. As  $A_{\text{net}}/C_i$  curves were generated using a common leaf temperature (25°C), curves were  
254 fit using Michaelis-Menten coefficients for Rubisco affinity to CO<sub>2</sub> ( $K_c$ ;  $\mu\text{mol mol}^{-1}$ ) and O<sub>2</sub> ( $K_o$ ;  
255  $\mu\text{mol mol}^{-1}$ ), and the CO<sub>2</sub> compensation point ( $T^*$ ;  $\mu\text{mol mol}^{-1}$ ) reported in Bernacchi *et al.*  
256 (2001). Specifically,  $K_c$  was set to 404.9  $\mu\text{mol mol}^{-1}$ ,  $K_o$  was set to 278.4  $\mu\text{mol mol}^{-1}$ , and  $T^*$  was  
257 set to 42.75  $\mu\text{mol mol}^{-1}$ . For clarity,  $V_{\text{cmax}}$ ,  $J_{\text{max}}$ , and  $R_d$  estimates are referenced throughout the  
258 rest of the paper as  $V_{\text{cmax}25}$ ,  $J_{\text{max}25}$ , and  $R_{d25}$ .

259

#### 260 *Leaf trait measurements*

261 The leaf used to generate  $A_{\text{net}}/C_i$  curves and dark respiration measurements was harvested  
262 immediately following gas exchange measurements. Images of each focal leaf were curated  
263 using a flat-bed scanner to determine fresh leaf area using the 'LeafArea' R package (Katabuchi,  
264 2015), which automates leaf area calculations using ImageJ software (Schneider *et al.*, 2012).  
265 Post-processed images were visually assessed to check against errors in the automation process.  
266 Each leaf was dried at 65°C for at least 48 hours and subsequently weighed and ground until  
267 homogenized. Leaf mass per area ( $M_{\text{area}}$ ; g m<sup>-2</sup>) was calculated as the ratio of dry leaf biomass to  
268 fresh leaf area. Leaf nitrogen content ( $N_{\text{mass}}$ ; gN g<sup>-1</sup>) was quantified using a subsample of ground  
269 and homogenized leaf tissue through elemental combustion analysis (Costech-4010, Costech,  
270 Inc., Valencia, CA, USA). Leaf nitrogen content per unit leaf area ( $N_{\text{area}}$ ; gN m<sup>-2</sup>) was calculated  
271 by multiplying  $N_{\text{mass}}$  and  $M_{\text{area}}$ . Photosynthetic nitrogen-use efficiency ( $PNUE_{\text{growth}}$ ;  $\mu\text{mol CO}_2$   
272 g<sup>-1</sup> N s<sup>-1</sup>) was estimated as the ratio of  $A_{\text{net,growth}}$  to  $N_{\text{area}}$ .

273 Chlorophyll content was extracted from a second leaf in the same trifoliate leaf set as the  
274 leaf used to generate  $A_{\text{net}}/C_i$  curves. A cork borer was used to punch between 3-5 0.6 cm<sup>2</sup> disks  
275 from the leaf. Images of each set of leaf disks were curated using a flat-bed scanner to determine  
276 wet leaf area, again quantified using the 'LeafArea' R package (Katabuchi, 2015). Leaf disks  
277 were shuttled into a test tube containing 10 mL dimethyl sulfoxide, vortexed, and incubated at

278 65°C for 120 minutes (Barnes *et al.*, 1992). Incubated test tubes were vortexed again before  
 279 being loaded in 150 µL triplicate aliquots to a 96-well plate. Dimethyl sulfoxide was loaded in  
 280 each plate as a single 150 µL triplicate aliquot and used as a blank. Absorbance measurements at  
 281 649 nm ( $A_{649}$ ) and 665 nm ( $A_{665}$ ) were recorded in each well using a plate reader (Biotek Synergy  
 282 H1; Biotek Instruments, Winooski, VT USA), with triplicates averaged and corrected by the  
 283 mean of the blank absorbance value. Blank-corrected absorbance values were used to estimate  
 284  $Chl_a$  (µg mL<sup>-1</sup>) and  $Chl_b$  (µg mL<sup>-1</sup>) following equations from Wellburn (1994):

$$285 \quad Chl_a = 12.47A_{665} - 3.62A_{649} \quad (1)$$

286 and

$$287 \quad Chl_b = 25.06A_{649} - 6.5A_{665} \quad (2)$$

288  $Chl_a$  and  $Chl_b$  were converted to mmol mL<sup>-1</sup> using the molar masses of chlorophyll *a* (893.51 g  
 289 mol<sup>-1</sup>) and chlorophyll *b* (907.47 g mol<sup>-1</sup>), then added together to calculate the total chlorophyll  
 290 content in dimethyl sulfoxide extractant (mmol mL<sup>-1</sup>). Total chlorophyll content (mmol) was  
 291 determined by multiplying the total chlorophyll content in dimethyl sulfoxide by the volume of  
 292 dimethyl sulfoxide extractant (10 mL). Area-based chlorophyll content ( $Chl_{area}$ ; mmol m<sup>-2</sup>) was  
 293 then calculated by dividing the total chlorophyll content by the total area of the leaf disks.

294 Subsamples of ground and homogenized leaf tissue were sent to the University of  
 295 California-Davis Stable Isotope Facility to determine leaf  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  using an elemental  
 296 analyzer (Elementar vario MICRO cube elemental analyzer; Elementar Analysensysteme GmbH,  
 297 Langenselbold, Germany) interfaced to an isotope ratio mass spectrometer (PDZ Europa 20-20  
 298 Isotope Ratio Mass Spectrometer, Sercon Ltd., Cheshire, UK). Leaf  $\delta^{13}\text{C}$  was used to estimate  
 299 the time-integrated ratio of leaf intercellular CO<sub>2</sub> concentration to atmospheric CO<sub>2</sub>  
 300 concentration ( $\chi$ , unitless) using leaf  $\delta^{13}\text{C}$  and chamber air  $\delta^{13}\text{C}$  following Farquhar *et al.* (1989):

$$301 \quad \chi = \frac{\Delta^{13}\text{C} - a}{b - a} \quad (3)$$

302 where  $\Delta^{13}\text{C}$  represents the relative difference between leaf  $\delta^{13}\text{C}$  (‰) and air  $\delta^{13}\text{C}$  (‰), and is  
 303 calculated as:

$$304 \quad \Delta^{13}\text{C} = \frac{\delta^{13}\text{C}_{air} - \delta^{13}\text{C}_{leaf}}{1 + \delta^{13}\text{C}_{leaf}} \quad (4)$$

305  $\delta^{13}\text{C}_{air}$  is the chamber  $\delta^{13}\text{C}$  air fractionation, *a* represents the fractionation between <sup>12</sup>C and <sup>13</sup>C  
 306 due to diffusion in air, assumed to be 4.4‰, and *b* represents the fractionation caused by Rubisco  
 307 carboxylation, assumed to be 27‰ (Farquhar *et al.*, 1989).  $\delta^{13}\text{C}_{air}$  was quantified in each

308 chamber by collecting air samples in triplicate for each CO<sub>2</sub> treatment using a 20 mL syringe  
309 (Air-Tite Products Co., Inc., Virginia Beach, VA, USA). Each air sample was plunged into a  
310 manually evacuated 10 mL Exetainer (Labco Ltd., Lampeter, UK) and sent to the University of  
311 California-Davis Stable Isotope Facility, where δ<sup>13</sup>C<sub>air</sub> was determined using a gas inlet system  
312 (GasBenchII; Thermo Fisher Scientific, Waltham, MA, USA) coupled to an isotope ratio mass  
313 spectrometer (Thermo Finnigan Delta Plus XL; Thermo Fisher Scientific, Waltham, MA, USA).  
314 δ<sup>13</sup>C<sub>air</sub> for each CO<sub>2</sub> treatment was estimated by calculating the mean of the triplicate δ<sup>13</sup>C<sub>air</sub>  
315 samples within each chamber, then calculating the mean δ<sup>13</sup>C<sub>air</sub> across all chambers. Specifically,  
316 δ<sup>13</sup>C<sub>air</sub> was -8.81‰ for the ambient CO<sub>2</sub> treatment and -5.95‰ for the elevated CO<sub>2</sub> treatment.

317 Finally, the percent of leaf nitrogen acquired from the atmosphere (%N<sub>dfa</sub>; %) was  
318 estimated using leaf δ<sup>15</sup>N and the following equation adapted from Andrews *et al.* (2011):

$$319 \%N_{dfa} = \frac{\delta^{15}N_{direct} - \delta^{15}N_{sample}}{\delta^{15}N_{direct} - \delta^{15}N_{fixation}} \quad (5)$$

320 where δ<sup>15</sup>N<sub>direct</sub> refers to the δ<sup>15</sup>N value from plants that exclusively acquired nitrogen via direct  
321 uptake, δ<sup>15</sup>N<sub>sample</sub> refers to an individual's leaf δ<sup>15</sup>N, and δ<sup>15</sup>N<sub>fixation</sub> refers to the δ<sup>15</sup>N value from  
322 individuals that were entirely reliant on nitrogen fixation. δ<sup>15</sup>N<sub>direct</sub> was calculated as the mean  
323 leaf δ<sup>15</sup>N of uninoculated individuals within each unique nitrogen fertilization-by-CO<sub>2</sub> treatment  
324 combination. Any individual with visual evidence of root nodule formation or nodule initiation  
325 was omitted from the calculation of δ<sup>15</sup>N<sub>direct</sub>. δ<sup>15</sup>N<sub>fixation</sub> was calculated within each CO<sub>2</sub>  
326 treatment using the mean leaf δ<sup>15</sup>N of inoculated individuals that received 0 ppm N. δ<sup>15</sup>N<sub>fixation</sub>  
327 was not calculated within each unique nitrogen fertilization-by-CO<sub>2</sub> treatment combination, as  
328 previous studies suggest decreased reliance on nitrogen fixation with increasing nitrogen  
329 fertilization (e.g., Perkowski *et al.*, 2021).

330

### 331 *Whole-plant measurements*

332 Seven weeks after experiment initiation and immediately following gas exchange measurements,  
333 all individuals were harvested, and biomass of major organ types (leaves, stems, roots, and  
334 nodules when present) were separated. Fresh leaf area of all harvested leaves was measured  
335 using a LI-3100C (LI-COR Biosciences, Lincoln, Nebraska, USA). Total fresh leaf area (cm<sup>2</sup>)  
336 was calculated as the sum of all leaf areas, including the leaf used to collect gas exchange data  
337 and the leaf used to extract chlorophyll content. All harvested material was dried in an oven set

338 to 65°C for at least 48 hours to a constant mass, weighed, and ground to homogeneity. Leaves  
339 and root nodules were ground using a mortar and pestle, while stems and roots were ground  
340 using an E3300 Single Speed Mini Cutting Mill (Eberbach Corp., MI, USA). Total biomass (g)  
341 was calculated as the sum of dry leaf, stem, root, and root nodule biomass. Carbon and nitrogen  
342 content was measured for each organ type through elemental combustion (Costech-4010,  
343 Costech, Inc., Valencia, CA, USA) using subsamples of ground and homogenized organ tissue.  
344 The ratio of root nodule biomass to root biomass was calculated as an additional indicator of  
345 investment toward symbiotic nitrogen fixation.

346 Following Perkowski *et al.* (2021), carbon costs to acquire nitrogen were quantified as  
347 the ratio of belowground carbon biomass to total nitrogen biomass ( $N_{\text{cost}}$ ; gC gN<sup>-1</sup>). Belowground  
348 carbon biomass ( $C_{\text{bg}}$ ; gC) was calculated as the sum of root carbon biomass and root nodule  
349 carbon biomass. Root carbon biomass and root nodule carbon biomass were calculated as the  
350 product of the organ biomass and respective organ carbon content. Total nitrogen biomass ( $N_{\text{wp}}$ ;  
351 gN) was calculated as the sum of total leaf, stem, root, and root nodule nitrogen biomass. Leaf,  
352 stem, root, and root nodule nitrogen biomass was calculated as the product of the organ biomass  
353 and respective organ nitrogen content. This calculation does not account for additional carbon  
354 costs associated with respiration, root exudation, or root turnover, and therefore may  
355 underestimate carbon costs to acquire nitrogen (Perkowski *et al.*, 2021).

356

### 357 *Statistical analyses*

358 Uninoculated plants that had substantial root nodule formation (root nodule biomass: root  
359 biomass values greater than 0.05 g g<sup>-1</sup>) were removed from analyses under the assumption that  
360 plants were either incompletely sterilized or were colonized by symbiotic nitrogen-fixing  
361 bacteria from neighboring plants in the chamber. This decision resulted in the removal of sixteen  
362 plants from the analysis: two plants in the elevated CO<sub>2</sub> treatment that received 35 ppm N, three  
363 plants in the elevated CO<sub>2</sub> treatment that received 70 ppm N, one plant in the elevated CO<sub>2</sub>  
364 treatment that received 210 ppm N, two plants in the elevated CO<sub>2</sub> treatment that received 280  
365 ppm N, two plants in the ambient CO<sub>2</sub> treatment that received 0 ppm N, three plants in the  
366 ambient CO<sub>2</sub> treatment that received 70 ppm N, two plants in the ambient CO<sub>2</sub> treatment that  
367 received 105 ppm N, and one plant in the ambient CO<sub>2</sub> treatment that received 280 ppm N.

368 A series of linear mixed-effects models were built to investigate the impacts of CO<sub>2</sub>  
369 concentration, nitrogen fertilization, and inoculation on *G. max* leaf nitrogen allocation, gas  
370 exchange, whole-plant growth, and investment in nitrogen fixation. All models included CO<sub>2</sub>  
371 treatment as a categorical fixed effect, inoculation treatment as a categorical fixed effect, and  
372 nitrogen fertilization as a continuous fixed effect, with all possible interaction terms between all  
373 three fixed effects also included. Models accounted for climatic differences between chambers  
374 across experiment iterations by including a random intercept term that nested the starting  
375 chamber rack by CO<sub>2</sub> treatment. Models with this independent variable structure were created for  
376 each of the following dependent variables:  $N_{\text{area}}$ ,  $M_{\text{area}}$ ,  $N_{\text{mass}}$ ,  $Chl_{\text{area}}$ ,  $A_{\text{net},420}$ ,  $A_{\text{net,growth}}$ ,  $V_{\text{cmax}25}$ ,  
377  $J_{\text{max}25}$ ,  $J_{\text{max}25}:V_{\text{cmax}25}$ ,  $R_{\text{d}25}$ ,  $PNUE_{\text{growth}}$ ,  $\chi$ , total leaf area, total biomass,  $N_{\text{cost}}$ ,  $C_{\text{bg}}$ ,  $N_{\text{wp}}$ , % $N_{\text{dfa}}$ ,  
378 root nodule biomass: root biomass, root nodule biomass, and root biomass.

379 Shapiro-Wilk tests of normality were used to assess whether linear mixed-effects models  
380 satisfied residual normality assumptions. All models that did not satisfy residual normality  
381 assumptions satisfied such assumptions when response variables were fit using either a natural  
382 log or square root data transformation (Shapiro-Wilk:  $p>0.05$  in all cases). Specifically, models  
383 for  $N_{\text{area}}$ ,  $N_{\text{mass}}$ ,  $Chl_{\text{area}}$ ,  $A_{\text{net},420}$ ,  $A_{\text{net,growth}}$ ,  $V_{\text{cmax}25}$ ,  $J_{\text{max}25}$ ,  $J_{\text{max}25}:V_{\text{cmax}25}$ ,  $R_{\text{d}25}$ ,  $PNUE_{\text{growth}}$ ,  $\chi$ , total  
384 leaf area, and  $N_{\text{cost}}$  each satisfied residual normality assumptions without data transformation.  
385 Models for  $M_{\text{area}}$ , total biomass, and  $C_{\text{bg}}$  satisfied residual normality assumptions with a natural  
386 log data transformation, while models for  $N_{\text{wp}}$ , root nodule biomass: root biomass, root nodule  
387 biomass, root biomass, and % $N_{\text{dfa}}$  satisfied residual normality assumptions with a square root  
388 data transformation.

389 In all models, we used the ‘lmer’ function in the ‘lme4’ R package (Bates *et al.*, 2015) to  
390 fit each model and the ‘Anova’ function in the ‘car’ R package (Fox & Weisberg, 2019) to  
391 calculate Type II Wald's  $\chi^2$  and determine the significance ( $\alpha=0.05$ ) of each fixed effect  
392 coefficient. We used the ‘emmeans’ R package (Lenth, 2019) to conduct post-hoc comparisons  
393 using Tukey's tests, where degrees of freedom were approximated using the Kenward-Roger  
394 approach (Kenward & Roger, 1997). Trendlines and error ribbons representing the 95%  
395 confidence intervals were drawn in all figures using ‘emmeans’ outputs across the range in  
396 nitrogen fertilization values. All analyses and plots were conducted in R version 4.1.0 (R Core  
397 Team, 2021). Model results for  $\chi$ ,  $C_{\text{bg}}$ ,  $N_{\text{wp}}$ , root nodule biomass: root biomass, root nodule

398 biomass, and root biomass are reported in the *Supplemental Material* (Tables S3-S6; Figs. S3-  
399 S6).

400

## 401 Results

### 402 Leaf nitrogen content

403 Elevated CO<sub>2</sub> reduced  $N_{\text{area}}$ ,  $N_{\text{mass}}$ , and  $Chl_{\text{area}}$  by 29%, 50%, and 31%, respectively, and  
404 increased  $M_{\text{area}}$  by 44% ( $p < 0.001$  in all cases; Table 1). Interactions between nitrogen  
405 fertilization and CO<sub>2</sub> ( $p < 0.05$  in all cases; Table 1) indicated that positive effects of increasing  
406 nitrogen fertilization on  $N_{\text{area}}$ ,  $N_{\text{mass}}$ , and  $M_{\text{area}}$  ( $p < 0.001$  in all cases; Table 1) were stronger under  
407 ambient CO<sub>2</sub> than elevated CO<sub>2</sub> (Tukey test of the nitrogen fertilization-trait slope between CO<sub>2</sub>:  
408  $p < 0.05$  in all cases). These responses resulted in a stronger reduction in  $N_{\text{area}}$  and  $N_{\text{mass}}$  and a  
409 stronger increase in  $M_{\text{area}}$  under elevated CO<sub>2</sub> with increasing nitrogen fertilization than ambient  
410 CO<sub>2</sub> (Fig. S1). Nitrogen fertilization did not modify reductions in  $Chl_{\text{area}}$  due to elevated CO<sub>2</sub>  
411 (Tukey test of the nitrogen fertilization- $Chl_{\text{area}}$  slope between CO<sub>2</sub> treatments:  $p > 0.05$ ).

412 An interaction between inoculation and CO<sub>2</sub> ( $p < 0.05$ ; Table 1) indicated that reductions  
413 in  $N_{\text{area}}$  due to elevated CO<sub>2</sub> were stronger in uninoculated plants (36% reduction; Tukey test of  
414 the CO<sub>2</sub> effect in uninoculated plants:  $p < 0.001$ ) than inoculated plants (22% reduction; Tukey  
415 test of the CO<sub>2</sub> effect in inoculated plants:  $p < 0.001$ ). Inoculation did not modify  $N_{\text{mass}}$ ,  $M_{\text{area}}$ , or  
416  $Chl_{\text{area}}$  responses to elevated CO<sub>2</sub> (CO<sub>2</sub>-by-inoculation interaction:  $p > 0.05$  in all cases; Table 1).  
417 However, an interaction between nitrogen fertilization and inoculation ( $p < 0.05$  in all cases; Table  
418 1; Figs. 1a-d) indicated that positive effects of increasing nitrogen fertilization on  $N_{\text{area}}$ ,  $N_{\text{mass}}$ ,  
419  $M_{\text{area}}$ , and  $Chl_{\text{area}}$  ( $p < 0.001$  in all cases; Table 1) were stronger in uninoculated plants compared  
420 to inoculated plants (Tukey test of the nitrogen fertilization-trait slope between inoculation  
421 treatments:  $p < 0.05$  in all cases).

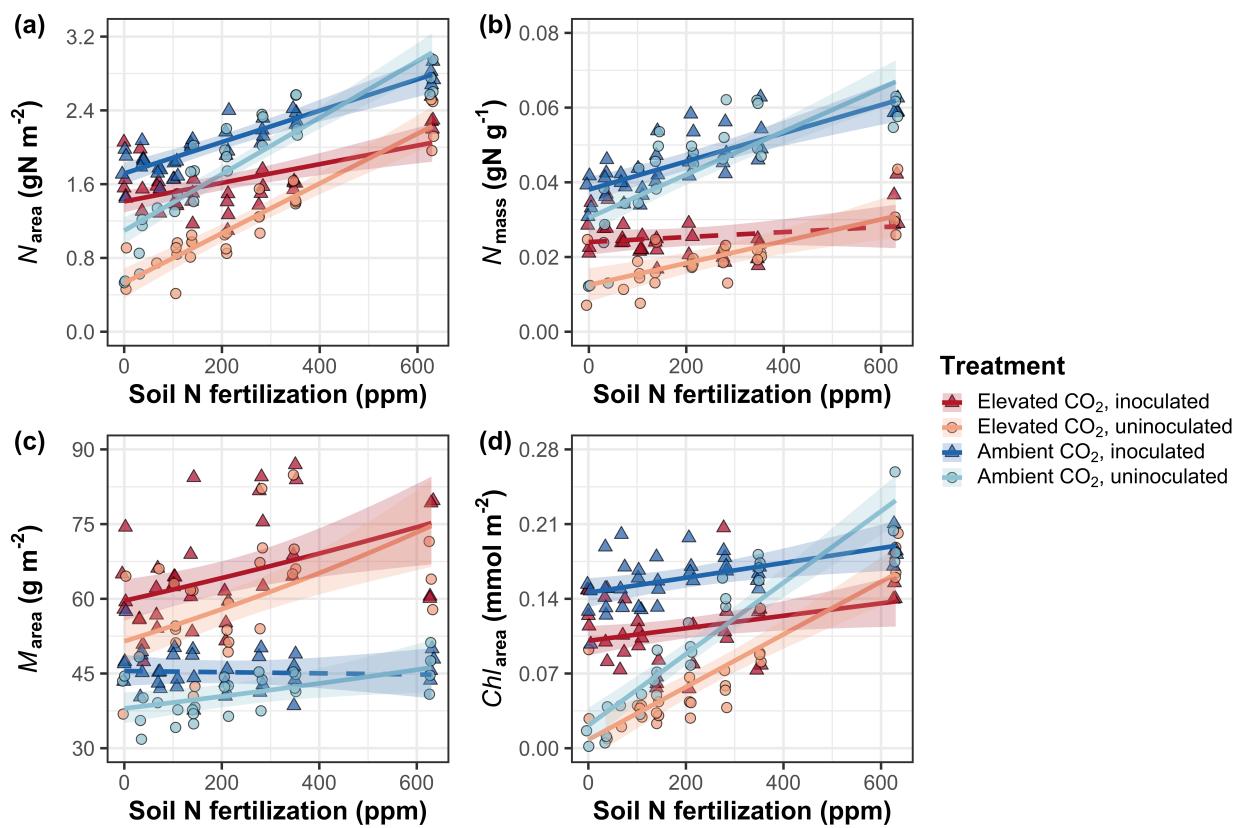
422

423 **Table 1** Effects of CO<sub>2</sub> concentration, inoculation, and nitrogen fertilization on leaf nitrogen allocation\*

		<i>N<sub>area</sub></i>		<i>N<sub>mass</sub></i>		<i>M<sub>area</sub><sup>a</sup></i>		<i>Chl<sub>area</sub></i>	
	df	$\chi^2$	<i>p</i>	$\chi^2$	<i>p</i>	$\chi^2$	<i>p</i>	$\chi^2$	<i>p</i>
CO <sub>2</sub>	1	155.908	<0.001	272.362	<0.001	151.319	<0.001	69.233	<0.001
Inoculation (I)	1	86.029	<0.001	15.576	<0.001	19.158	<0.001	136.341	<0.001
N fertilization (N)	1	316.408	<0.001	106.659	<0.001	21.440	<0.001	163.111	<0.001
CO <sub>2</sub> *I	1	4.729	<b>0.030</b>	2.025	0.155	0.029	0.866	2.102	0.147
CO <sub>2</sub> *N	1	5.723	<b>0.017</b>	22.542	<0.001	7.619	<b>0.006</b>	2.999	0.083
I*N	1	43.381	<0.001	11.137	<b>0.001</b>	5.022	<b>0.025</b>	75.769	<0.001
CO <sub>2</sub> *I*N	1	0.489	0.484	0.041	0.839	0.208	0.649	2.144	0.143

424 \*Significance determined using Type II Wald  $\chi^2$  tests ( $\alpha=0.05$ ). *P*-values less than 0.05 are in bold. A superscript “a” is included after  
 425 trait labels to indicate if models were fit with natural log-transformed response variables. Key: df=degrees of freedom,  $\chi^2$ =Wald chi-  
 426 square test statistic, *N<sub>area</sub>*=leaf nitrogen content per unit leaf area (g N m<sup>-2</sup>), *N<sub>mass</sub>*=leaf nitrogen content (g N g<sup>-1</sup>), *M<sub>area</sub>*=leaf mass per  
 427 unit leaf area (g m<sup>-2</sup>).

428

429 **Figure 1**

430

431 **Figure 1** Effects of CO<sub>2</sub> concentration, nitrogen fertilization, and inoculation on leaf nitrogen per  
 432 unit leaf area (a), leaf nitrogen per unit leaf mass (b), leaf mass per unit leaf area (c), and  
 433 chlorophyll content per unit leaf area (d). Nitrogen fertilization is represented on the x-axis in all  
 434 panels. Red shaded points and trendlines indicate plants grown under elevated CO<sub>2</sub>, while blue  
 435 shaded points and trendlines indicate plants grown under ambient CO<sub>2</sub>. Light blue and red  
 436 circular points and trendlines indicate measurements collected from uninoculated plants, while  
 437 dark blue and red triangular points indicate measurements collected from inoculated plants. Solid  
 438 trendlines indicate regression slopes that are different from zero ( $p < 0.05$ ), while dashed  
 439 trendlines indicate slopes that are not distinguishable from zero ( $p > 0.05$ ).

440

441 *Gas exchange*

442 Elevated CO<sub>2</sub> decreased  $A_{\text{net},420}$  by 17% ( $p<0.001$ ; Table 2) and increased  $A_{\text{net,growth}}$  by 33%  
443 ( $p<0.001$ ; Table 2). Nitrogen fertilization did not modify effects of elevated CO<sub>2</sub> on  $A_{\text{net},420}$  or  
444  $A_{\text{net,growth}}$  (CO<sub>2</sub>-by-nitrogen fertilization interaction:  $p>0.05$  in both cases; Table 2; Fig. 2a-b).  
445 Inoculation did not modify  $A_{\text{net},420}$  responses to elevated CO<sub>2</sub> (CO<sub>2</sub>-by-inoculation interaction:  
446  $p>0.05$ ). However, an interaction between CO<sub>2</sub> and inoculation ( $p<0.05$ ; Table 2) indicated that  
447 inoculated plants experienced a stronger increase in  $A_{\text{net,growth}}$  due to elevated CO<sub>2</sub> (38% increase;  
448 Tukey test of the CO<sub>2</sub> effect in inoculated plants:  $p<0.001$ ) compared to uninoculated plants  
449 (26% increase; Tukey test of the CO<sub>2</sub> effect in uninoculated plants:  $p<0.05$ ). An interaction  
450 between nitrogen fertilization and inoculation ( $p<0.001$  in both cases; Table 2) indicated that  
451 positive effects of increasing nitrogen fertilization on  $A_{\text{net},420}$  and  $A_{\text{net,growth}}$  ( $p<0.001$  in both  
452 cases; Table 2; Fig. 2a-b) were stronger in uninoculated plants than inoculated plants (Tukey test  
453 comparing the nitrogen fertilization-trait slope between inoculation treatments:  $p<0.001$  in both  
454 cases).

455 Elevated CO<sub>2</sub> decreased  $V_{\text{cmax}25}$  and  $J_{\text{max}25}$  by 16% and 10%, respectively, increasing  
456  $J_{\text{max}25}:V_{\text{cmax}25}$  by 8% ( $p<0.05$  in all cases; Table 2; Fig. 2c-e).  $V_{\text{cmax}25}$ ,  $J_{\text{max}25}$ , and  $J_{\text{max}25}:V_{\text{cmax}25}$   
457 responses to elevated CO<sub>2</sub> were not modified by nitrogen fertilization (CO<sub>2</sub>-by-nitrogen  
458 fertilization interaction:  $p>0.05$  in all cases; Table 2; Fig. 2c-e) or inoculation (CO<sub>2</sub>-by-  
459 inoculation interaction:  $p>0.05$  in all cases; Table 2). An interaction between nitrogen  
460 fertilization and inoculation ( $p<0.05$  in both cases; Table 2) indicated that positive effects of  
461 increasing nitrogen fertilization on  $V_{\text{cmax}25}$  and  $J_{\text{max}25}$  ( $p<0.001$  in both cases; Table 2) and  
462 negative effects of increasing nitrogen fertilization on  $J_{\text{max}25}:V_{\text{cmax}25}$  ( $p<0.001$ ; Table 2) were  
463 driven by uninoculated plants (Tukey test of the nitrogen fertilization-trait slope in uninoculated  
464 plants:  $p<0.001$  in all cases), as there was no effect of nitrogen fertilization on  $V_{\text{cmax}25}$ ,  $J_{\text{max}25}$ , or  
465  $J_{\text{max}25}:V_{\text{cmax}25}$  in inoculated plants (Tukey test of the nitrogen fertilization-trait slope in inoculated  
466 plants:  $p>0.05$  in all cases).

467 There was no effect of CO<sub>2</sub> concentration on  $R_{\text{d}25}$  ( $p>0.05$ ; Table 2). An interaction  
468 between nitrogen fertilization and inoculation ( $p<0.001$ ; Table 2) indicated that the positive  
469 effect of increasing nitrogen fertilization on  $R_{\text{d}25}$  ( $p<0.05$ ; Table 2) was driven by uninoculated  
470 plants (Tukey test of the nitrogen fertilization- $R_{\text{d}25}$  slope in uninoculated plants:  $p<0.001$ ), as

471 there was no effect of nitrogen fertilization on  $R_{d25}$  in inoculated plants (Tukey test of the  
472 nitrogen fertilization- $R_{d25}$  slope in inoculated plants:  $p>0.05$ ).

473

474 *Photosynthetic nitrogen-use efficiency*

475 Elevated CO<sub>2</sub> increased  $PNUE_{growth}$  by 90% ( $p<0.001$ ; Table 2; Fig. 3), a pattern that was not  
476 modified by inoculation treatment (CO<sub>2</sub>-by-inoculation interaction:  $p>0.05$ ; Table 2). An  
477 interaction between CO<sub>2</sub> and nitrogen fertilization ( $p<0.05$ ; Table 2) indicated that the positive  
478 effect of elevated CO<sub>2</sub> on  $PNUE_{growth}$  decreased with increasing nitrogen fertilization (Fig. S2).  
479 This pattern was driven by a negative effect of increasing nitrogen fertilization on  $PNUE_{growth}$   
480 ( $p<0.001$ ; Table 2) that was stronger under elevated CO<sub>2</sub> than ambient CO<sub>2</sub> (Tukey test  
481 comparing the nitrogen fertilization- $PNUE_{growth}$  slope between CO<sub>2</sub> treatments:  $p<0.05$ ). An  
482 interaction between nitrogen fertilization and inoculation ( $p<0.001$ ; Table 2; Fig. 3) indicated  
483 that the negative effect of increasing nitrogen fertilization on  $PNUE_{growth}$  was driven by  
484 inoculated plants (Tukey test of the nitrogen fertilization- $PNUE_{growth}$  slope in inoculated plants:  
485  $p<0.001$ ), as there was no effect of nitrogen fertilization on  $PNUE_{growth}$  in uninoculated plants  
486 (Tukey test of the nitrogen fertilization- $PNUE_{growth}$  slope in uninoculated plants:  $p>0.05$ ).

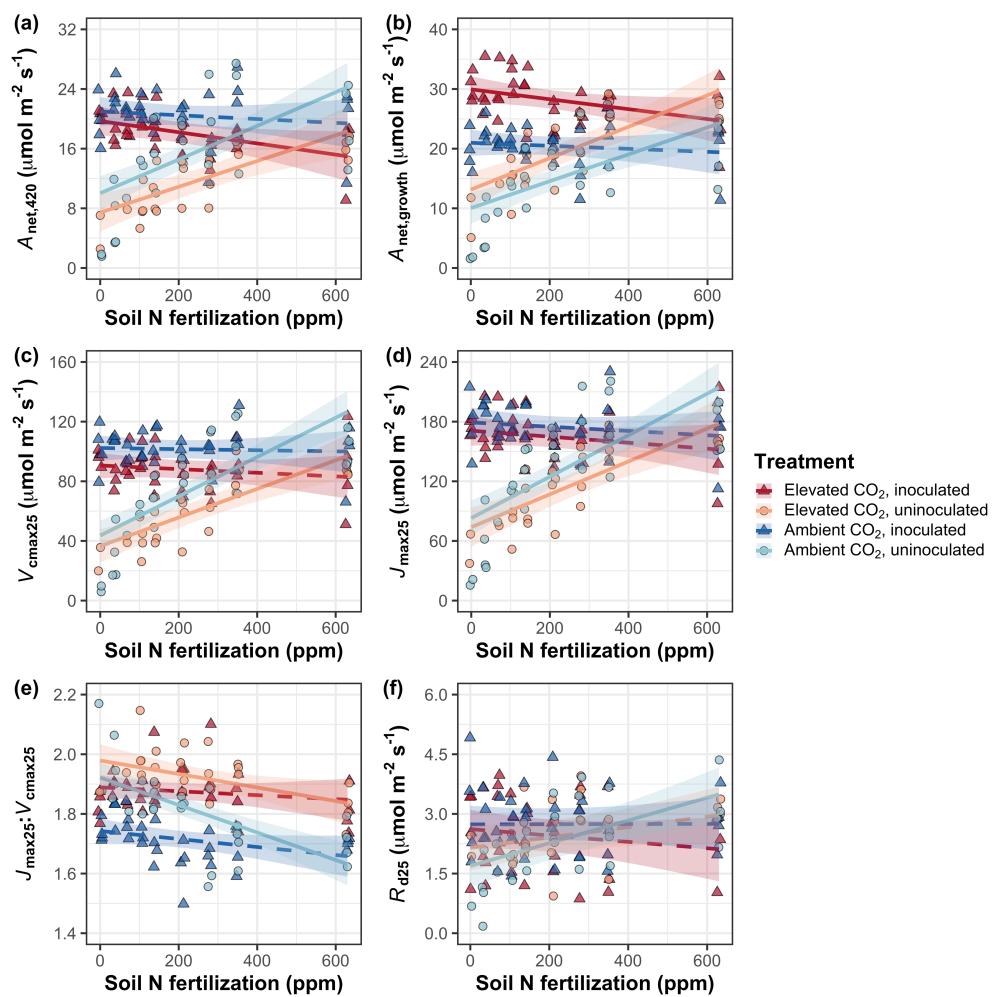
487

488 **Table 2** Effects of CO<sub>2</sub> concentration, inoculation, and nitrogen fertilization on leaf gas exchange\*

		<i>A</i> <sub>net,420</sub>		<i>A</i> <sub>net,growth</sub>		<i>V</i> <sub>cmax25</sub>		<i>J</i> <sub>max25</sub>	
	df	$\chi^2$	p	$\chi^2$	p	$\chi^2$	p	$\chi^2$	p
CO <sub>2</sub>	1	15.747	<0.001	52.716	<0.001	18.039	<0.001	6.042	0.014
Inoculation (I)	1	77.137	<0.001	83.008	<0.001	98.579	<0.001	85.064	<0.001
N fertilization (N)	1	11.986	<0.001	14.658	<0.001	37.053	<0.001	25.356	<0.001
CO <sub>2</sub> *I	1	1.032	0.310	5.634	0.018	0.065	0.799	0.667	0.414
CO <sub>2</sub> *N	1	1.998	0.158	0.135	0.713	1.758	0.185	0.742	0.389
I*N	1	46.800	<0.001	50.774	<0.001	60.394	<0.001	57.41	<0.001
CO <sub>2</sub> *I*N	1	0.002	0.964	1.332	0.248	0.748	0.387	0.377	0.539

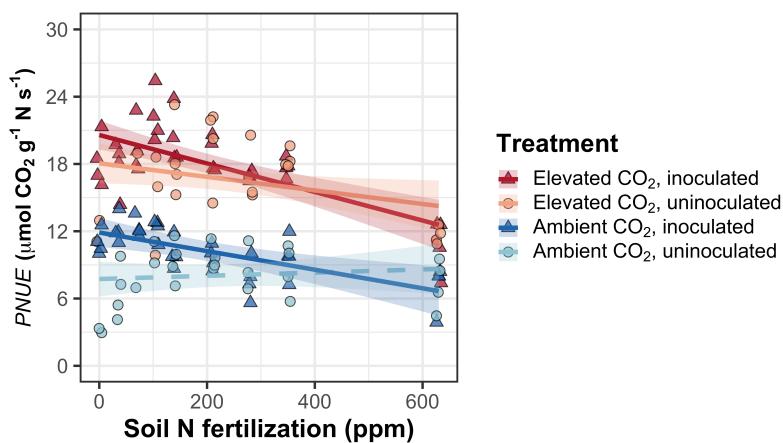
	<i>J</i> <sub>max25:<i>V</i><sub>cmax25</sub></sub>		<i>R</i> <sub>d25</sub>		<i>PNUE</i> <sub>growth</sub>		
	$\chi^2$	p	$\chi^2$	p	$\chi^2$	p	
CO <sub>2</sub>	1	92.010	<0.001	0.256	0.613	300.197	<0.001
Inoculation (I)	1	27.768	<0.001	3.094	0.079	9.897	0.002
N fertilization (N)	1	28.147	<0.001	5.965	0.015	29.695	<0.001
CO <sub>2</sub> *I	1	2.916	0.088	2.563	0.109	0.944	0.331
CO <sub>2</sub> *N	1	3.210	0.073	2.675	0.102	5.359	0.021
I*N	1	9.607	0.002	12.083	0.001	10.883	<0.001
CO <sub>2</sub> *I*N	1	1.102	0.294	0.244	0.622	0.369	0.544

489 \*Significance determined using Type II Wald  $\chi^2$  tests ( $\alpha=0.05$ ). P-values less than 0.05 are in bold. Key: df=degrees of freedom,490  $\chi^2$ =Wald chi-square test statistic, *A*<sub>net</sub>=net photosynthesis rate ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ ), *V*<sub>cmax25</sub>=maximum rate of Rubisco carboxylation at 25°C  
491 ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ ), *J*<sub>max25</sub>=maximum rate of electron transport for RuBP regeneration at 25°C ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ ), *J*<sub>max25:*V*<sub>cmax25</sub>=ratio of *J*<sub>max25</sub>  
492 to *V*<sub>cmax25</sub> (unitless), *R*<sub>d25</sub>=dark respiration at 25°C ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ ), *PNUE*<sub>growth</sub>=photosynthetic nitrogen-use efficiency ( $\mu\text{mol CO}_2 \text{ gN}^{-1}$   
493  $\text{s}^{-1}$ )</sub>

494 **Figure 2**

495

496 **Figure 2** Effects of CO<sub>2</sub>, nitrogen fertilization, and inoculation on net photosynthesis measured  
 497 at 420  $\mu\text{mol mol}^{-1}$  CO<sub>2</sub> (a), net photosynthesis measured under growth CO<sub>2</sub> concentration (b), the  
 498 maximum rate of Rubisco carboxylation at 25°C (c), the maximum rate of electron transport for  
 499 RuBP regeneration at 25°C (d), the ratio of the maximum rate of electron transport for RuBP  
 500 regeneration to the maximum rate of Rubisco carboxylation (e), and dark respiration at 25°C (f).  
 501 Nitrogen fertilization is represented on the x-axis. Red shaded points and trendlines indicate  
 502 plants grown under elevated CO<sub>2</sub>, while blue shaded points and trendlines indicate plants grown  
 503 under ambient CO<sub>2</sub>. Light blue and red circular points and trendlines indicate measurements  
 504 collected from uninoculated plants, while dark blue and red triangular points indicate  
 505 measurements collected from inoculated plants. Solid trendlines indicate regression slopes that  
 506 are different from zero ( $p < 0.05$ ), while dashed trendlines indicate slopes that are not  
 507 distinguishable from zero ( $p > 0.05$ ).

508 **Figure 3**

509

510 **Figure 3** Effects of CO<sub>2</sub>, nitrogen fertilization, and inoculation on photosynthetic nitrogen-use  
511 efficiency. Nitrogen fertilization is represented on the x-axis. Red shaded points and trendlines  
512 indicate plants grown under elevated CO<sub>2</sub>, while blue shaded points and trendlines indicate  
513 plants grown under ambient CO<sub>2</sub>. Light blue and red circular points and trendlines indicate  
514 measurements collected from uninoculated plants, while dark blue and red triangular points  
515 indicate measurements collected from inoculated plants. Solid trendlines indicate regression  
516 slopes that are different from zero ( $p < 0.05$ ), while dashed trendlines indicate slopes that are not  
517 distinguishable from zero ( $p > 0.05$ ).

518

519 *Whole-plant traits*

520 Elevated CO<sub>2</sub> increased total leaf area and total biomass by 51% and 102%, respectively  
521 ( $p<0.001$  in both cases; Table 3). Positive effects of elevated CO<sub>2</sub> on total leaf area and total  
522 biomass were enhanced with increasing nitrogen fertilization (CO<sub>2</sub>-by-nitrogen fertilization  
523 interaction:  $p<0.001$  in both cases; Table 3; Fig. 4a-b) but not inoculation (CO<sub>2</sub>-by-inoculation  
524 interaction:  $p>0.05$  in both cases; Table 3). An interaction between nitrogen fertilization and  
525 inoculation ( $p<0.001$  in both cases; Table 3) indicated that positive effects of increasing nitrogen  
526 fertilization on total leaf area and total biomass ( $p<0.001$  in both cases; Table 3) were stronger in  
527 uninoculated plants than inoculated plants (Tukey tests comparing the nitrogen fertilization-trait  
528 slopes between inoculation treatments:  $p<0.05$  for both traits).

529 Elevated CO<sub>2</sub> increased  $N_{cost}$  by 62% ( $p<0.001$ ; Table 3), a pattern that was not modified  
530 by nitrogen fertilization (CO<sub>2</sub>-by-nitrogen fertilization interaction:  $p>0.05$ ; Table 3). An  
531 interaction between CO<sub>2</sub> and inoculation ( $p<0.05$ ; Table 3) indicated that the positive effect of  
532 elevated CO<sub>2</sub> on  $N_{cost}$  was stronger in uninoculated plants (99% increase; Tukey test evaluating  
533 the CO<sub>2</sub> effect on  $N_{cost}$  in uninoculated plants:  $p<0.001$ ) than inoculated plants (21% increase  
534 Tukey test evaluating the CO<sub>2</sub> effect on  $N_{cost}$  in inoculated plants:  $p<0.05$ ). An interaction  
535 between nitrogen fertilization and inoculation ( $p<0.001$ ; Table 3) indicated that the negative  
536 effect of increasing nitrogen fertilization on  $N_{cost}$  ( $p<0.001$ ; Table 3) was stronger in  
537 uninoculated plants (Tukey test comparing the nitrogen fertilization- $N_{cost}$  slope between  
538 inoculation treatments:  $p<0.001$ ). A three-way interaction ( $p<0.001$ ; Table 3) indicated that  
539 interactions between nitrogen fertilization and inoculation were stronger under elevated CO<sub>2</sub> than  
540 ambient CO<sub>2</sub>. This pattern was driven by greater  $N_{cost}$  in uninoculated plants grown under  
541 elevated CO<sub>2</sub> and low nitrogen fertilization than any other CO<sub>2</sub>-by-inoculation treatment  
542 combination under low nitrogen fertilization (Tukey test comparing  $N_{cost}$  in uninoculated  
543 individuals grown under elevated CO<sub>2</sub> and 0 ppm N to all other CO<sub>2</sub>-inoculation treatment  
544 combinations grown under 0 ppm N:  $p<0.001$  in all cases; Fig. 4c).  $N_{cost}$  was generally reduced  
545 in inoculated plants ( $p<0.001$ ; Table 3). Negative effects of increasing nitrogen fertilization and  
546 inoculation on  $N_{cost}$  were driven by stronger positive effects of each treatment on  $N_{wp}$  than  $C_{bg}$ ,  
547 while positive effects of elevated CO<sub>2</sub> on  $N_{cost}$  were driven by stronger positive effects on  $C_{bg}$   
548 than  $N_{wp}$  (Table S4; Fig. S4).

549

550 *Nitrogen fixation*

551 Elevated CO<sub>2</sub> had no effect on %N<sub>dfa</sub> ( $p=0.472$ ; Table 3; Fig. 4d). An interaction between  
552 nitrogen fertilization and inoculation ( $p<0.001$ ; Table 3) indicated that the negative effect of  
553 increasing nitrogen fertilization on %N<sub>dfa</sub> ( $p<0.001$ ; Table 3) was driven by inoculated plants  
554 (Tukey test of the nitrogen fertilization-%N<sub>dfa</sub> slope in inoculated plants:  $p<0.001$ ), as there was  
555 no effect of nitrogen fertilization on %N<sub>dfa</sub> in uninoculated plants (Tukey test of the nitrogen  
556 fertilization-%N<sub>dfa</sub> slope in uninoculated plants:  $p>0.05$ ; Fig. 4d).

557

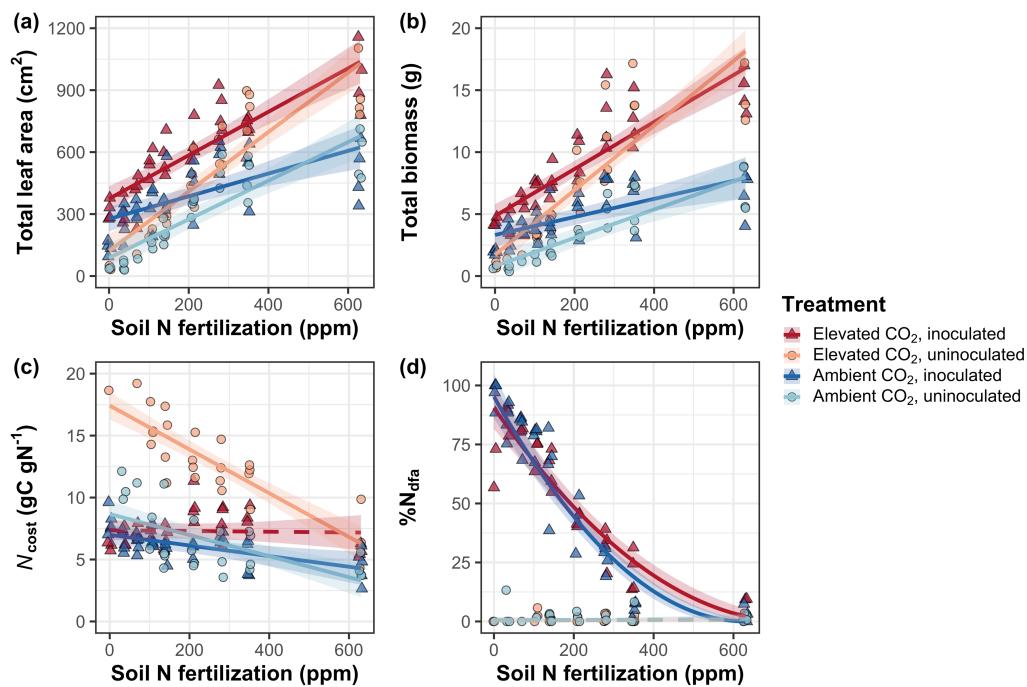
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558 **Table 3** Effects of CO<sub>2</sub> concentration, inoculation, and nitrogen fertilization on whole-plant growth, carbon costs to acquire nitrogen,  
 559 and investment toward symbiotic nitrogen fixation\*

		Total leaf area		Total biomass <sup>b</sup>		Carbon cost to acquire nitrogen		%N <sub>dfa</sub> <sup>b</sup>	
	df	$\chi^2$	p	$\chi^2$	p	$\chi^2$	p	$\chi^2$	p
CO <sub>2</sub>	1	69.291	<0.001	131.477	<0.001	88.189	<0.001	0.518	0.472
Inoculation (I)	1	35.715	<0.001	34.264	<0.001	136.343	<0.001	955.57	<0.001
N fertilization (N)	1	274.199	<0.001	269.046	<0.001	80.501	<0.001	292.938	<0.001
CO <sub>2</sub> *I	1	2.064	0.151	0.518	0.472	85.237	<0.001	2.010	0.156
CO <sub>2</sub> *N	1	18.655	<0.001	16.877	<0.001	1.050	0.306	2.716	0.099
I*N	1	10.804	0.001	15.779	<0.001	46.489	<0.001	231.29	<0.001
CO <sub>2</sub> *I*N	1	<0.001	0.990	0.023	0.880	18.125	<0.001	2.119	0.145

560 \*Significance determined using Type II Wald  $\chi^2$  tests ( $\alpha=0.05$ ). P-values less than 0.05 are in bold and p-values between 0.05 and 0.10  
 561 are italicized. A superscript “<sup>b</sup>” after trait labels indicates if models were fit using square root transformed variables. Key: df=degrees  
 562 of freedom,  $\chi^2$ =Wald chi-square test statistic, total leaf area (cm<sup>2</sup>), total biomass (g), carbon cost to acquire nitrogen (gC gN<sup>-1</sup>),  
 563 %N<sub>dfa</sub>=percent leaf nitrogen content fixed from the atmosphere (%).

564

565 **Figure 4**

566

567 **Figure 4.** Effects of CO<sub>2</sub>, nitrogen fertilization, and inoculation on total leaf area (a), total  
 568 biomass (b), structural carbon costs to acquire nitrogen (c), and percent of leaf nitrogen content  
 569 derived from the atmosphere (d). Nitrogen fertilization is represented on the x-axis. Red shaded  
 570 points and trendlines indicate plants grown under elevated CO<sub>2</sub>, while blue shaded points and  
 571 trendlines indicate plants grown under ambient CO<sub>2</sub>. Light blue and red circular points and  
 572 trendlines indicate measurements collected from uninoculated plants, while dark blue and red  
 573 triangular points indicate measurements collected from inoculated plants. Solid trendlines  
 574 indicate regression slopes that are different from zero ( $p < 0.05$ ), while dashed trendlines indicate  
 575 slopes that are not distinguishable from zero ( $p > 0.05$ ).  
 576

577 **Discussion**

578 *Glycine max* seedlings were grown under two CO<sub>2</sub> concentrations, two inoculation treatments,  
579 and nine soil nitrogen fertilization treatments in a full-factorial growth chamber experiment to  
580 reconcile the role of nitrogen supply, demand, and acquisition strategy on leaf and whole-plant  
581 responses to elevated CO<sub>2</sub>.

582 Results revealed that elevated CO<sub>2</sub> increased  $A_{\text{net,growth}}$  despite reduced  $N_{\text{area}}$ ,  $V_{\text{cmax25}}$ , and  
583  $J_{\text{max25}}$ . Larger reductions in  $V_{\text{cmax25}}$  than  $J_{\text{max25}}$  increased  $J_{\text{max25}}:V_{\text{cmax25}}$ , while respective increases  
584 and decreases in  $A_{\text{net,growth}}$  and  $N_{\text{area}}$  increased photosynthetic nitrogen-use efficiency. Effects of  
585 elevated CO<sub>2</sub> on  $A_{\text{net,growth}}$ ,  $V_{\text{cmax25}}$ ,  $J_{\text{max25}}$ , and  $J_{\text{max25}}:V_{\text{cmax25}}$  were similar across the nitrogen  
586 fertilization gradient, suggesting that leaf photosynthetic responses to elevated CO<sub>2</sub> were  
587 decoupled from changes in nitrogen supply. Instead, increased  $J_{\text{max25}}:V_{\text{cmax25}}$  under elevated CO<sub>2</sub>  
588 indicated that plants responded to increasing atmospheric CO<sub>2</sub> concentrations by allowing  
589 enhanced net photosynthesis rates to be achieved by approaching equal co-limitation of Rubisco  
590 carboxylation rate-limited photosynthesis and electron transport for RuBP regeneration rate-  
591 limited photosynthesis (Chen *et al.*, 1993; Maire *et al.*, 2012). These responses supported our  
592 hypothesis that leaf photosynthetic responses to elevated CO<sub>2</sub> would be driven by leaf nitrogen  
593 demand to build and maintain photosynthetic enzymes and would be independent of nitrogen  
594 supply. Leaf photosynthetic responses to elevated CO<sub>2</sub> corresponded with increased total leaf  
595 area and total biomass, patterns that were enhanced with increasing nitrogen fertilization and  
596 associated with increased nitrogen uptake efficiency. These results supported our hypothesis that  
597 whole-plant responses to elevated CO<sub>2</sub> would be constrained by nitrogen supply. However,  
598 contrasting our hypothesis, inoculation did not modify whole-plant responses to elevated CO<sub>2</sub>  
599 due to similar plant investment in symbiotic nitrogen fixation between CO<sub>2</sub> treatments.

600 Combined, results indicate that nitrogen supply and demand were each important factors  
601 that determined plant responses to elevated CO<sub>2</sub> – leaf nitrogen demand to build and maintain  
602 photosynthetic enzymes drove leaf photosynthetic responses to elevated CO<sub>2</sub>, while nitrogen  
603 supply constrained whole-plant growth responses to elevated CO<sub>2</sub>. These findings support leaf-  
604 level patterns expected from eco-evolutionary optimality theory, suggesting that terrestrial  
605 biosphere models may improve simulations of leaf photosynthetic processes under future novel  
606 environments by considering frameworks that adopt optimality principles (Smith & Keenan,

607 2020; Harrison *et al.*, 2021; Luo *et al.*, 2021). Below, we expand and contextualize these  
608 conclusions and suggest their implications for terrestrial biosphere model development.  
609

610 *Nitrogen supply and demand regulate leaf and whole-plant responses to elevated CO<sub>2</sub> at*  
611 *different scales*

612 Leaf photosynthetic responses to elevated CO<sub>2</sub> were consistent with previous studies that have  
613 investigated or reviewed leaf responses to elevated CO<sub>2</sub> (Drake *et al.*, 1997; Makino *et al.*, 1997;  
614 Ainsworth *et al.*, 2002; Ainsworth & Long, 2005; Ainsworth & Rogers, 2007; Crous *et al.*, 2010;  
615 Lee *et al.*, 2011; Smith & Dukes, 2013; Poorter *et al.*, 2022), and follow patterns expected from  
616 eco-evolutionary optimality theory (Chen *et al.*, 1993; Wright *et al.*, 2003; Maire *et al.*, 2012;  
617 Prentice *et al.*, 2014; Wang *et al.*, 2017; Smith *et al.*, 2019; Smith & Keenan, 2020; Harrison *et*  
618 *al.*, 2021). Positive effects of elevated CO<sub>2</sub> on  $A_{\text{net,growth}}$  and  $J_{\text{max25}}:V_{\text{cmax25}}$  and negative effects of  
619 elevated CO<sub>2</sub> on  $V_{\text{cmax25}}$  and  $J_{\text{max25}}$  were similar across the nitrogen fertilization gradient,  
620 indicating that leaf photosynthetic responses to elevated CO<sub>2</sub> were decoupled from changes in  
621 nitrogen supply. Increased  $J_{\text{max25}}:V_{\text{cmax25}}$  and photosynthetic nitrogen-use efficiency under  
622 elevated CO<sub>2</sub> provide strong support for the idea that leaves were downregulating  $V_{\text{cmax25}}$  in  
623 response to elevated CO<sub>2</sub> such that enhanced net photosynthesis rates approached becoming  
624 equally co-limited by Rubisco carboxylation and RuBP regeneration (Chen *et al.*, 1993; Maire *et*  
625 *al.*, 2012; Smith & Keenan, 2020). These patterns suggest that leaf photosynthetic responses to  
626 elevated CO<sub>2</sub> were likely the result of reduced demand to build and maintain photosynthetic  
627 enzymes, following patterns expected from eco-evolutionary optimality theory (Harrison *et al.*,  
628 2021; Dong *et al.*, 2022b).

629 Whole-plant responses were also consistent with previous studies that have investigated  
630 or reviewed whole-plant responses to elevated CO<sub>2</sub> (Makino *et al.*, 1997; Ainsworth *et al.*, 2002;  
631 Hungate *et al.*, 2003; Ainsworth & Long, 2005; Norby *et al.*, 2010; Smith & Dukes, 2013;  
632 Poorter *et al.*, 2022). Greater whole-plant growth under elevated CO<sub>2</sub> was associated with greater  
633 carbon costs to acquire nitrogen through stronger increases in belowground carbon allocation  
634 than whole-plant nitrogen uptake. These patterns indicate that plants grown under elevated CO<sub>2</sub>  
635 supported greater total leaf area and total biomass through increased plant nitrogen uptake,  
636 though at reduced nitrogen uptake efficiency. Unlike leaf photosynthetic responses to elevated  
637 CO<sub>2</sub>, positive whole-plant responses to elevated CO<sub>2</sub> were enhanced with increasing nitrogen

638 fertilization, supporting our hypothesis that nitrogen supply would constrain whole-plant  
639 responses to elevated CO<sub>2</sub> (Hungate *et al.*, 2003; Luo *et al.*, 2004; Finzi *et al.*, 2007). Positive  
640 effects of increasing nitrogen fertilization on total leaf area and total biomass were associated  
641 with reductions in carbon costs to acquire nitrogen, a pattern that was driven by stronger  
642 increases in whole-plant nitrogen uptake than belowground carbon allocation (Perkowski *et al.*,  
643 2021). While reductions in carbon costs to acquire nitrogen due to increasing nitrogen  
644 fertilization were similar between CO<sub>2</sub> treatments, increasing nitrogen fertilization increased  
645 whole-plant nitrogen uptake more strongly under elevated CO<sub>2</sub>. This pattern, coupled with  
646 similar effects of nitrogen fertilization on belowground carbon allocation responses to elevated  
647 CO<sub>2</sub>, indicated that stronger growth responses to elevated CO<sub>2</sub> with increasing nitrogen  
648 fertilization were likely driven by enhanced nitrogen uptake efficiency. These findings suggest  
649 that positive short-term effects of nitrogen supply on whole-plant responses to elevated CO<sub>2</sub> are  
650 linked to reduced costs of acquiring nitrogen and increased nitrogen uptake efficiency,  
651 supporting conclusions from Terrer *et al.* (2018).

652 Our findings indicate that nitrogen supply and demand could each explain plant responses  
653 to elevated CO<sub>2</sub>, though these factors operated at different scales. Specifically, photosynthetic  
654 responses to elevated CO<sub>2</sub> were determined through reduced leaf nitrogen demand to build and  
655 maintain photosynthetic enzymes. Reduced leaf nitrogen demand resulted in a shift in nitrogen  
656 allocation to photosynthetic enzymes independent of soil nitrogen supply that increased  
657 photosynthetic nitrogen use efficiency and allowed net photosynthesis rates to occur by  
658 approaching optimal coordination of Rubisco carboxylation-limited and RuBP regeneration-  
659 limited photosynthesis. Whole-plant responses to elevated CO<sub>2</sub> were enhanced with increasing  
660 soil nitrogen supply. Interestingly, optimized nitrogen allocation to photosynthetic capacity may  
661 have resulted in nitrogen savings at the leaf level that could have maximized nitrogen allocation  
662 to growth. These results suggest that plants grown under elevated CO<sub>2</sub> responded to increased  
663 nitrogen supply by increasing the number of optimally coordinated leaves and that the  
664 downregulation in photosynthetic capacity under elevated CO<sub>2</sub> was not a direct response to  
665 changes in nitrogen supply.

666

667 *Inoculation with symbiotic nitrogen-fixing bacteria does not modify leaf or whole-plant*  
668 *responses to elevated CO<sub>2</sub>*

669 Inoculation increased  $N_{\text{area}}$ ,  $A_{\text{net},420}$ ,  $A_{\text{net,growth}}$ ,  $V_{\text{cmax}25}$ ,  $J_{\text{max}25}$ , photosynthetic nitrogen-use  
670 efficiency, total leaf area, and total biomass, and decreased  $J_{\text{max}25}:V_{\text{cmax}25}$  and  $R_{\text{d}25}$ . These patterns  
671 support previous literature suggesting that species that form associations with symbiotic  
672 nitrogen-fixing bacteria often have increased leaf nitrogen content, photosynthetic capacity, and  
673 growth compared to species that do not form such associations (Adams *et al.*, 2016; Bytnerowicz  
674 *et al.*, 2023). Positive effects of inoculation on leaf and whole-plant traits were strongest under  
675 low nitrogen fertilization and rapidly diminished with increasing nitrogen fertilization as  
676 investment in symbiotic nitrogen fixation decreased (Andrews *et al.*, 2011; Friel & Friesen,  
677 2019; McCulloch & Porder, 2021; Perkowski *et al.*, 2021), supporting the idea that nitrogen  
678 fixation is a nutrient acquisition strategy that may confer competitive benefits for nitrogen-fixing  
679 species growing in low soil nitrogen environments (Rastetter *et al.*, 2001; Vitousek *et al.*, 2002).

680 Interestingly, inoculation did not modify effects of elevated CO<sub>2</sub> on  $V_{\text{cmax}25}$ ,  $J_{\text{max}25}$ ,  
681  $J_{\text{max}25}:V_{\text{cmax}25}$ , photosynthetic nitrogen-use efficiency, total leaf area, or total biomass. These  
682 patterns corresponded with null effects of elevated CO<sub>2</sub> on % $N_{\text{dfa}}$  and the ratio of root nodule  
683 biomass to root biomass, suggesting that null inoculation effects on plant responses to elevated  
684 CO<sub>2</sub> were primarily due to similar plant investments toward symbiotic nitrogen fixation between  
685 CO<sub>2</sub> treatments. We observed these patterns regardless of nitrogen fertilization level, contrasting  
686 our hypothesis that inoculation would enhance whole-plant responses to elevated CO<sub>2</sub> under low  
687 nitrogen fertilization where individuals were expected to be invested more strongly in symbiotic  
688 nitrogen fixation. These patterns also contrast previous work showing that inoculated *G. max* is  
689 generally more responsive to increasing atmospheric CO<sub>2</sub> concentrations (Ainsworth *et al.*,  
690 2002) and that plant investment toward symbiotic nitrogen fixation tends to be greater under  
691 scenarios that increase whole-plant demand to acquire nitrogen (Taylor & Menge, 2018; Friel &  
692 Friesen, 2019; McCulloch & Porder, 2021).

693

#### 694 *Implications for future model development*

695 Many terrestrial biosphere models predict photosynthetic capacity through parameterized  
696 relationships between  $N_{\text{area}}$  and  $V_{\text{cmax}}$  (Rogers, 2014; Rogers *et al.*, 2017), which assumes that  
697 leaf nitrogen-photosynthesis relationships are constant across growing environments. Our results  
698 build on previous work suggesting that leaf nitrogen-photosynthesis relationships dynamically  
699 change across growing environments (Smith & Keenan, 2020; Luo *et al.*, 2021; Dong *et al.*,

700 2022b; Waring *et al.*, 2023), as elevated CO<sub>2</sub> reduced leaf nitrogen content more strongly than it  
701 increased  $A_{\text{net,growth}}$  and decreased  $V_{\text{cmax25}}$  and  $J_{\text{max25}}$ . Additionally, positive effects of increasing  
702 nitrogen fertilization on indices of photosynthetic capacity were only apparent in uninoculated  
703 plants, as there was no effect of nitrogen fertilization on  $V_{\text{cmax25}}$  or  $J_{\text{max25}}$  in inoculated plants.  
704 Positive effects of increasing nitrogen fertilization on  $N_{\text{area}}$  and  $\text{Chl}_{\text{area}}$  were also markedly  
705 weaker in inoculated plants compared to uninoculated plants. These patterns indicate that leaf  
706 nitrogen-photosynthesis relationships are context-dependent on nitrogen acquisition strategy,  
707 may only be constant in environments where nitrogen supply limits leaf physiology, and will  
708 likely shift in response to increasing atmospheric CO<sub>2</sub> concentrations. Terrestrial biosphere  
709 models that predict photosynthetic capacity through parameterized relationships between  $N_{\text{area}}$   
710 and  $V_{\text{cmax}}$  (e.g., Kattge *et al.*, 2009; Walker *et al.*, 2014) may risk overestimating photosynthetic  
711 capacity, therefore net primary productivity and the magnitude of the land carbon sink, under  
712 future novel growth environments.

713 Our results demonstrate that optimal resource allocation to photosynthetic capacity  
714 defines leaf photosynthetic responses to elevated CO<sub>2</sub> and that these responses are independent  
715 of nitrogen supply. Current approaches for simulating photosynthetic responses to CO<sub>2</sub> in  
716 terrestrial biosphere models with coupled carbon and nitrogen cycles often invoke patterns  
717 expected from progressive nitrogen limitation, where photosynthetic responses to elevated CO<sub>2</sub>  
718 are modeled as a function of positive relationships between nitrogen availability and leaf  
719 nitrogen content. Our results contradict this framework, suggesting that photosynthetic responses  
720 to elevated CO<sub>2</sub> are driven by optimal nitrogen investment to satisfy leaf nitrogen demand to  
721 build and maintain photosynthetic enzymes. Optimality models that use principles from optimal  
722 coordination and photosynthetic least-cost theories (Wang *et al.*, 2017; Stocker *et al.*, 2020; Scott  
723 & Smith, 2022) are capable of capturing responses to CO<sub>2</sub> independent of nitrogen supply (Smith  
724 & Keenan, 2020; Harrison *et al.*, 2021), suggesting that including optimality frameworks in  
725 terrestrial biosphere models may improve the accuracy by which photosynthetic processes are  
726 simulated in response to increasing atmospheric CO<sub>2</sub> concentrations.

727 Previous work has highlighted the fact that pot experiments restrict belowground rooting  
728 volume and may alter plant allocation responses to environmental change (Ainsworth *et al.*,  
729 2002; Poorter *et al.*, 2012). In this study, the ratio of pot volume to total biomass was greater  
730 under elevated CO<sub>2</sub> and increased with increasing nitrogen fertilization such that several

731 treatment combinations exceeded values recommended by Poorter *et al.* (2012) to avoid growth  
732 limitation imposed by restricted pot volume ( $<1\text{ g L}^{-1}$ ; Table S6; Fig. S6). While pot size may  
733 have limited plant responses to elevated CO<sub>2</sub>, similar responses to elevated CO<sub>2</sub> have been  
734 observed using field measurements that do not restrict belowground rooting volume (Bernacchi  
735 *et al.*, 2005; Crous *et al.*, 2010; Lee *et al.*, 2011; Pastore *et al.*, 2019; Smith & Keenan, 2020).  
736 Additionally, there was no apparent saturating effect of increasing fertilization on total biomass,  
737 belowground carbon biomass, or root biomass under conditions where biomass: pot volume  
738 ratios exceeded 1 g L<sup>-1</sup> (e.g., individuals of either inoculation status grown under high  
739 fertilization and elevated CO<sub>2</sub>), which might be expected if pot volume had limited plant growth.  
740 The lack of such responses indicate that the pot volume used in this study (6 L) was sufficient to  
741 avoid growth limitation.

742

743 *Conclusions*

744 Our results indicate that nitrogen supply and demand each helped explain *G. max* responses to  
745 elevated CO<sub>2</sub>, though operated at different scales. Supporting eco-evolutionary optimality theory,  
746 leaf photosynthetic responses to elevated CO<sub>2</sub> were independent of soil nitrogen supply and  
747 ability to associate with symbiotic nitrogen-fixing bacteria and were instead driven by leaf  
748 nitrogen demand to build and maintain photosynthetic enzymes such that net photosynthesis  
749 rates approached optimal coordination. Supporting the progressive nitrogen limitation  
750 hypothesis, whole-plant responses to elevated CO<sub>2</sub> were enhanced with increasing nitrogen  
751 fertilization due to increased plant nitrogen uptake efficiency coupled with possible cascading  
752 effects of nitrogen savings at the leaf level that may have maximized nitrogen allocation to  
753 whole-plant growth. However, inoculation did not modify whole-plant responses to elevated  
754 CO<sub>2</sub>, as plants invested similarly in symbiotic nitrogen fixation between CO<sub>2</sub> treatments. Results  
755 suggest that plants grown under elevated CO<sub>2</sub> responded to increased nitrogen supply by  
756 increasing the number of optimally coordinated leaves and that the downregulation in  
757 photosynthetic capacity under elevated CO<sub>2</sub> was not modified by changes in nitrogen supply.  
758 The differential role of nitrogen supply on leaf and whole-plant responses to elevated CO<sub>2</sub>  
759 coupled with dynamic leaf nitrogen-photosynthesis relationships across CO<sub>2</sub> and nitrogen  
760 fertilization treatments suggests that terrestrial biosphere models may improve simulations of

761 photosynthetic responses to increasing atmospheric CO<sub>2</sub> concentrations by adopting frameworks  
762 that include optimality principles.

763

#### 764 **Conflicts of Interest**

765 The authors declare no conflicts of interest.

766

#### 767 **Acknowledgements**

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774

#### 775 **Data Availability**

776 All R scripts, data, and metadata are available at <https://doi.org/10.5281/zenodo.10177575> (or on  
777 GitHub at: [https://github.com/eaperkowski/NxCO2xI\\_ms\\_data](https://github.com/eaperkowski/NxCO2xI_ms_data))

778

#### 779 **Author contributions**

780 EAP conceptualized the study objectives and designed the experiment in collaboration with  
781 NGS, collected data, conducted data analysis, and wrote the first manuscript draft. EE assisted  
782 with data collection and experiment maintenance. NGS conceptualized study objectives and  
783 experimental design with EAP and oversaw experiment progress. All authors provided  
784 manuscript feedback and approved the manuscript in its current form for submission to *Global  
785 Change Biology*.

786

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