

1 Symbiotic nitrogen fixation reduces belowground biomass carbon costs of nitrogen
2 acquisition under low, but not high, nitrogen availability

3 *Running title: Symbiotic N fixation reduces nitrogen acquisition costs under low soil N*

4

5 **Abstract**

6 Many plant species form symbiotic associations with nitrogen-fixing bacteria. Through this
7 symbiosis, plants allocate photosynthate belowground to the bacteria in exchange for nitrogen
8 fixed from the atmosphere. This symbiosis forms an important link between carbon and nitrogen
9 cycles in many ecosystems. However, the economics of this relationship under soil nitrogen
10 availability gradients is not well understood, as plant investment toward symbiotic nitrogen
11 fixation tends to decrease with increasing soil nitrogen availability. Here, we used a
12 manipulation experiment to examine how costs of nitrogen acquisition vary under a factorial
13 combination of soil nitrogen availability and inoculation with *Bradyrhizobium japonicum* in
14 *Glycine max* L. (Merr.). We found that inoculation decreased belowground biomass carbon costs
15 to acquire nitrogen and increased total leaf area and total biomass, but these patterns were only
16 observed under low fertilization. Treatment differences were the result of increased plant
17 nitrogen uptake and no change in belowground carbon allocation. These results suggest that
18 symbioses with nitrogen-fixing bacteria reduce carbon costs of nitrogen acquisition, but only
19 when soil nitrogen is low, allowing individuals to increase nitrogen allocation to structures that
20 support aboveground growth. This pattern may help explain the prevalence of plants capable of
21 forming these associations in less fertile areas and demonstrates responses that can help guide
22 models linking carbon and nitrogen cycles in terrestrial ecosystems.

23

24 **Keywords**

25 carbon-nitrogen interactions; nitrogen fixation; whole plant growth; greenhouse; crops; nutrient
26 acquisition strategy

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31 **Introduction**

32 Terrestrial ecosystems are regulated, in part, by interactions between carbon and nitrogen cycles.
33 As a result, terrestrial biosphere models are beginning to include coupled carbon and nitrogen
34 cycles to simulate past, present, and future atmosphere-biosphere fluxes more realistically
35 (Hungate *et al.* 2003; Prentice *et al.* 2015; Kou-Giesbrecht *et al.* 2023). Carbon and nutrient flux
36 simulations tend to converge across terrestrial biosphere model products using past and present
37 climate scenarios; however, these models often diverge under future environmental change
38 scenarios (Friedlingstein *et al.* 2014; Davies-Barnard *et al.* 2020). This divergence could be due
39 to an incomplete understanding of how changing environments modify processes that link
40 ecosystem carbon and nitrogen cycles (Wieder *et al.* 2015; Fay *et al.* 2015; Meyerholt *et al.*
41 2016).

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42 Plant nitrogen acquisition is a process in terrestrial ecosystems that links carbon and
43 nitrogen cycles. Plants allocate, photosynthetically derived carbon belowground in exchange for
44 nitrogen through different nitrogen acquisition strategies. These nitrogen acquisition strategies
45 can include direct uptake pathways such as mass flow or diffusion (Barber 1962; Fisher *et al.*
46 2010) or through symbioses with nitrogen-fixing bacteria (Vance and Heichel 1991; Vitousek *et*
47 *al.* 2002; Udvardi and Poole 2013). Plants cannot acquire nitrogen without first allocating carbon
48 belowground, which implies an inherent carbon cost to the plant for acquiring nitrogen (Chapin
49 *et al.* 1987). This carbon cost for acquiring nitrogen may vary in species with different nitrogen
50 acquisition strategies. For instance, carbon investment toward roots for direct nitrogen uptake
51 does not require costs beyond root development, as is the case for acquisition strategies that
52 involve other soil microbiota. However, the nitrogen acquired from a given belowground carbon
53 investment may be greater than direct uptake if carbon is allocated to bacterial symbionts in
54 exchange for nitrogen fixed from the atmosphere (Gutschick 1981; Vitousek and Field 1999;
55 Rastetter *et al.* 2001; Vitousek *et al.* 2002). Variations in the cost to acquire nitrogen may help
56 explain the prevalence of different nitrogen acquisition strategies in different environments, but
57 these costs have not been quantified outside of a few studies (Terrer *et al.* 2018; Perkowski *et al.*
58 2021; Lu *et al.* 2022) despite their inclusion in nitrogen uptake models (Fisher *et al.* 2010;
59 Brzostek *et al.* 2014; Allen *et al.* 2020) currently implemented in terrestrial biosphere models
60 (Shi *et al.* 2016; Lawrence *et al.* 2019; Braghieri *et al.* 2022). No studies to our knowledge have
61 directly investigated interactions between nitrogen fixation and nitrogen availability on carbon

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Deleted: if plants increase root exudation to supply soil microbial communities with substrate needed to decompose organic matter and increase inorganic soil nitrogen substrate available for root uptake . Alternatively, the nitrogen acquired from a given belowground carbon investment may be greater if carbon is allocated to fungal symbionts in exchange for nitrogen mined from the soil or converted to inorganic nitrogen from soil organic matter , or

80 costs to acquire nitrogen, which limits our ability to evaluate the ability of models to simulate
81 plant nitrogen uptake patterns across environmental gradients.

82 Carbon costs to acquire nitrogen are likely dependent on external environmental factors
83 such as atmospheric CO₂, light availability, and soil nutrient availability (Brzostek *et al.* 2014;
84 Terrer *et al.* 2018; Taylor and Menge 2018, 2021; Friel and Friesen 2019; Allen *et al.* 2020;
85 Perkowski *et al.* 2021; Lu *et al.* 2022). For instance, the amount of photosynthate allocated
86 belowground in exchange for nitrogen may increase with increased light and CO₂, as these
87 factors reduce the cost to produce photosynthate and increase plant demand for acquiring
88 nitrogen to build and maintain photosynthetic enzymes (Terrer *et al.* 2018; Taylor and Menge
89 2018; Friel and Friesen 2019; Perkowski *et al.* 2021; Waring *et al.* 2023). However, increasing
90 soil nitrogen availability may alternatively reduce costs for nitrogen acquisition due to increases
91 in plant nitrogen acquisition and reductions in belowground carbon allocation. This pattern may
92 be due to reduced soil resource mining (by roots or symbionts) needed to satisfy plant nitrogen
93 demand under greater nitrogen availability.

94 Reductions in the cost of nitrogen acquisition due to increasing soil nitrogen availability
95 may not be as robust in plant species with strong and specialized symbiotic relationships with
96 nitrogen-acquiring partners that reduce the sensitivity of plant nitrogen uptake to changes in
97 nitrogen availability (e.g., plant species that associate with symbiotic nitrogen-fixing bacteria)
98 (Perkowski *et al.* 2021). Indeed, plants that form associations with symbiotic nitrogen-fixing
99 bacteria often exhibit reduced responses to changes in nitrogen availability despite reduced
100 investment toward nitrogen fixation with increasing nitrogen availability (Gutschick 1981;
101 Taylor and Menge 2018; Friel and Friesen 2019; McCulloch and Porder 2021; Menge *et al.*
102 2023; Schmidt *et al.* 2023). While previous work notes that plants can still acquire nitrogen
103 through symbiotic nitrogen fixation under high soil nitrogen availability (Menge *et al.* 2023),
104 resource optimization theory suggests that reduced sensitivity of plant nitrogen uptake to changes
105 in nitrogen availability in nitrogen-fixing plants may stem from preferential investment toward
106 the acquisition strategy that confers the lowest carbon cost and greatest nitrogen gain (Bloom *et*
107 *al.* 1985; Rastetter *et al.* 2001). If true, similar costs to acquire nitrogen in nitrogen-fixing species
108 may be achieved across nitrogen availability gradients due to shifts away from nitrogen
109 acquisition through nitrogen fixation to direct uptake as costs to acquire nitrogen through direct
110 uptake decrease (Fisher *et al.* 2010; Brzostek *et al.* 2014; Perkowski *et al.* 2021).

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122 In a recent study, Perkowski *et al.* (2021) showed that increasing soil nitrogen
123 fertilization decreased belowground biomass carbon costs to acquire nitrogen in *Gossypium*
124 *hirsutum* (L.) and *Glycine max* L. (Merr.). *Gossypium hirsutum* can acquire nutrients via direct
125 uptake pathways, while *G. max* can acquire nutrients via direct uptake pathways or through
126 symbioses with nitrogen-fixing bacteria. In the experiment, belowground biomass carbon costs
127 to acquire nitrogen in *G. max* were less responsive to increasing soil nitrogen fertilization than *G.*
128 *hirsutum*. This pattern coincided with reduced *G. max* root nodulation with increasing
129 fertilization. The authors speculated that this response may have been driven by resource
130 optimization, where *G. max* shifted their dominant mode of nitrogen acquisition from nitrogen
131 fixation to direct uptake with increasing nitrogen fertilization once the cost to acquire nitrogen
132 via direct uptake became less than the cost to acquire nitrogen via nitrogen fixation (Bloom *et al.*
133 1985; Rastetter *et al.* 2001). However, the authors were not able to make robust conclusions
134 about whether the carbon cost to acquire nitrogen responses to soil nitrogen fertilization differed
135 between *G. hirsutum* and *G. max* due to differences in the ability to associate with symbiotic
136 nitrogen-fixing bacteria. This was because the two species are not phylogenetically related and
137 adopt different growth forms and growth durations.

138 To understand how nitrogen fixation and soil nitrogen fertilization interact to influence
139 belowground biomass carbon costs to acquire nitrogen, *Glycine max* L. (Merr.) seedlings were
140 grown under two soil nitrogen fertilization treatments and were either inoculated or not
141 inoculated with symbiotic nitrogen-fixing bacteria, in a full factorial greenhouse experiment. We
142 used this experiment to test the following hypotheses:

- 143 (1) Soil nitrogen fertilization will decrease belowground biomass carbon costs of
144 nitrogen acquisition in both uninoculated and inoculated individuals. This decrease
145 will manifest as an increase in the amount of nitrogen acquired per belowground
146 carbon investment, indexed by a stronger increase in plant nitrogen uptake than
147 belowground carbon allocation.
- 148 (2) Inoculation with nitrogen-fixing bacteria will decrease belowground biomass carbon
149 costs to acquire nitrogen under low soil nitrogen availability. This is because
150 belowground biomass carbon costs to acquire nitrogen through symbiotic nitrogen
151 fixation will be less than the belowground biomass carbon cost to acquire nitrogen via
152 direct uptake. However, inoculation will not affect belowground biomass carbon costs

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162 to acquire nitrogen under high soil nitrogen availability due to all plants shifting
163 toward a similar, direct uptake-dominated mode of nitrogen acquisition.

- 164 (3) Root nodulation and plant investment toward symbiotic nitrogen fixation will
165 decrease with increasing soil nitrogen availability. This pattern will be due to reduced
166 belowground biomass carbon costs to obtain nitrogen from direct uptake with
167 increasing soil nitrogen fertilization that will increase relative plant investment
168 toward nutrient uptake through direct uptake pathways.

169

170 **Materials and methods**

171 *Experimental Design*

172 *Glycine max* seeds were planted in 64, 6-liter pots (NS-600, Nursery Supplies, Orange, CA,
173 USA) containing unfertilized potting mix (Sungro Sunshine Mix #2, Agawam, MA, USA). The
174 experiment used *G. max* seedlings to compare observed responses from previous work that was
175 not able to disentangle species-specific effects on belowground biomass carbon costs to acquire
176 nitrogen from the explicit effects of nitrogen fixation (Perkowski et al. 2021). Pots and potting
177 mix were steam sterilized at 95°C for three hours to eliminate any bacterial or fungal growth.
178 Thirty-two randomly selected pots were planted with seeds inoculated with *Bradyrhizobium*
179 *japonicum* (Verdesian N-Dure™ Soybean, Cary, NC, USA) following a brief surface
180 sterilization in 20,000 ppm sodium hypochlorite for 5 minutes followed by three washes in
181 ultrapure water (Scouten and Beuchat 2002; Montville and Schaffner 2004). The remaining 32
182 pots were planted with seeds that did not receive any inoculation treatment. Uninoculated seeds
183 were also surface sterilized in 20,000 ppm sodium hypochlorite for 5 minutes followed by three
184 ultrapure water washes to ensure that the only difference between seed treatments was the
185 inoculation treatment.

186 Upon planting, all pots were immediately placed in one of four random blocks in a
187 greenhouse and received one of two nitrogen fertilization treatments as 150 mL of a modified
188 Hoagland's solution (Hoagland and Arnon 1950) equivalent to either 70 or 630 ppm N twice per
189 week for seven weeks. Nitrogen fertilization levels were chosen based on previous work using a
190 larger number of fertilization treatments (Perkowski et al., 2021). Nitrogen fertilization doses
191 were received as topical agents to the soil surface and were modified to keep concentrations of
192 other macronutrients and micronutrients equivalent across the two treatments (Table S1).

193 Throughout the experiment, plants were routinely well-watered to minimize any chance of water
194 stress. Greenhouse maximum daytime temperatures averaged $42.4 \pm 3.9^\circ\text{C}$ (mean \pm standard
195 deviation) across blocks, while minimum nighttime temperature averaged $19.8 \pm 1.9^\circ\text{C}$ across
196 blocks. There was no evidence of growth limitation due to pot size at the time of biomass
197 harvest, indicated by total biomass: pot volume ratios less than 1 g L^{-1} within each treatment
198 combination (Table S2-3; Fig. S1; Poorter et al., 2012).

199

200 *Plant trait measurements*

201 All individuals were harvested, and biomass was separated into major organ types (leaves, stems,
202 roots, and root nodules when present) approximately seven weeks after experiment initiation and
203 before the onset of reproduction. Leaf areas of all harvested leaves were measured using an LI-
204 3100C (LI-COR Biosciences, Lincoln, Nebraska, USA). Total leaf area (cm^2) was calculated as
205 the sum of all leaf areas. All harvested material was dried in an oven set to 65°C for at least 48
206 hours, weighed, and ground to homogeneity. Total dry biomass (g) was calculated as the sum of
207 dry leaf, stem, root, and root nodule biomass. Carbon and nitrogen content of each respective
208 organ was quantified through elemental combustion (Costech-4010, Costech, Inc., Valencia, CA,
209 USA) using subsamples of ground and homogenized organ tissue.

210 Belowground biomass carbon costs to acquire nitrogen were calculated as the ratio of
211 total belowground biomass carbon to whole plant nitrogen biomass ($\text{g C g}^{-1} \text{ N}$; Perkowski *et al.*,
212 2021). Belowground biomass carbon (g C) was calculated as the sum of total root carbon
213 biomass and total root nodule carbon biomass. Total root biomass carbon was calculated by
214 multiplying root carbon content by total root biomass, while total root nodule biomass carbon
215 was calculated by multiplying root nodule carbon content by total root nodule biomass. Whole-
216 plant nitrogen biomass (g N) was calculated by multiplying the nitrogen content of leaves, stems,
217 roots, and root nodules by biomass of each respective organ type, then calculating the sum of
218 nitrogen biomass of each organ type. This calculation only quantifies belowground biomass
219 carbon costs to acquire nitrogen and does not account for additional carbon costs of nitrogen
220 acquisition associated with root respiration, root exudation, or root turnover. An explicit
221 explanation of the limitations for interpreting this calculation can be found in Perkowski *et al.*
222 (2021) and Terrer *et al.* (2018).

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231 *Statistical analyses*
232 A series of linear mixed-effects models were built to investigate the impacts of soil nitrogen
233 fertilization and inoculation on *G. max* belowground biomass carbon costs to acquire nitrogen
234 and investment toward symbiotic nitrogen fixation. All models included soil nitrogen
235 fertilization, inoculation, and interactions between soil nitrogen fertilization and inoculation as
236 categorical fixed effects. Block number was included as a random intercept term to account for
237 any environmental heterogeneity within the greenhouse room. Individuals were categorized by
238 inoculation treatment and not by whether they had formed nodules. Models with this independent
239 variable structure were constructed to quantify relationships between soil nitrogen fertilization
240 and inoculation on belowground biomass carbon costs to acquire nitrogen, belowground biomass
241 carbon, whole-plant nitrogen biomass, total leaf area, total biomass, root nodule biomass: root
242 biomass, root nodule biomass, and root biomass.

243 Shapiro-Wilk tests of normality were used to determine whether linear mixed-effects
244 models satisfied residual normality assumptions (Shapiro-Wilk: $p>0.05$). Models for whole-plant
245 nitrogen biomass and total leaf area satisfied residual normality assumptions without data
246 transformation. We attempted to satisfy residual normality assumptions by fitting the other
247 models using dependent variables that were natural log transformed. If residual normality
248 assumptions were still not met after a natural-log transformation (Shapiro-Wilk: $p<0.05$), then
249 models were fit using dependent variables that were square root transformed. All residual
250 normality assumptions were met with either a natural log or square root data transformation
251 (Shapiro-Wilk: $p>0.05$ in all cases). Specifically, models for belowground biomass carbon costs
252 to acquire nitrogen, belowground biomass carbon, total biomass, root biomass, and biomass: pot
253 volume satisfied normality assumptions when response variables were fit using natural log
254 transformed data, while models for nodule biomass: root biomass and root nodule biomass
255 satisfied such assumptions when response variables were fit using square-root transformations.

256 We used the ‘lmer’ function in the ‘lme4’ R package (Bates *et al.* 2015) to fit each model
257 and the ‘Anova’ function in the ‘car’ R package (Fox and Weisberg 2019) to calculate Type II
258 Wald’s χ^2 and determine the significance ($\alpha=0.05$) of each fixed effect coefficient. We then used
259 the ‘emmeans’ R package (Lenth, 2019) to conduct post-hoc comparisons using Tukey’s tests,
260 where degrees of freedom were approximated using the Kenward-Roger approach (Kenward and

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266 Roger 1997). All analyses were conducted and plots were created using R version 4.2.0 (R Core
267 Team, 2021).

268

269 Results

270 *Belowground biomass* carbon costs to acquire nitrogen

271 The interaction between soil nitrogen fertilization and inoculation ($p<0.05$; Table 1) indicated
272 that negative effects of inoculation ($p<0.001$; Table 1) on *belowground biomass* carbon costs to
273 acquire nitrogen were only apparent under low soil nitrogen fertilization (Tukey test comparing
274 the inoculation effect under low soil nitrogen fertilization: $p<0.001$; Fig. 1A). There was no
275 inoculation effect on *belowground biomass* carbon costs to acquire nitrogen under high soil
276 nitrogen fertilization (Tukey test comparing the inoculation effect under high soil nitrogen
277 fertilization: $p>0.05$; Fig. 1A). Increasing soil nitrogen fertilization decreased structural carbon
278 costs to acquire nitrogen ($p<0.001$; Table 1).

279 Inoculation decreased belowground biomass *carbon* ($p<0.05$; Table 1). This response was
280 not modified by soil nitrogen fertilization (inoculation-by-fertilization interaction: $p>0.05$; Table
281 1; Fig. 1B). Soil nitrogen fertilization had no effect on belowground biomass *carbon* ($p>0.05$;
282 Table 1).

283 The interaction between soil nitrogen fertilization and inoculation ($p<0.001$; Table 1)
284 indicated that positive effects of inoculation on whole-plant nitrogen biomass ($p<0.001$; Table 1)
285 were only apparent under low soil nitrogen fertilization (Tukey test comparing the inoculation
286 effect under low soil nitrogen fertilization: $p<0.001$; Fig. 1C). There was no effect of inoculation
287 on whole-plant nitrogen biomass under high soil nitrogen fertilization (Tukey test comparing the
288 inoculation effect under high soil nitrogen fertilization: $p>0.05$; Fig. 1C). Increasing soil nitrogen
289 fertilization generally increased whole-plant nitrogen biomass ($p<0.001$; Table 1).

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300 **Table 1** Analysis of variance results exploring effects of soil nitrogen fertilization, inoculation with *B. japonicum*, and interactions
 301 between soil nitrogen fertilization and inoculation on belowground biomass carbon costs to acquire nitrogen, whole-plant growth, and
 302 investment toward symbiotic nitrogen fixation*

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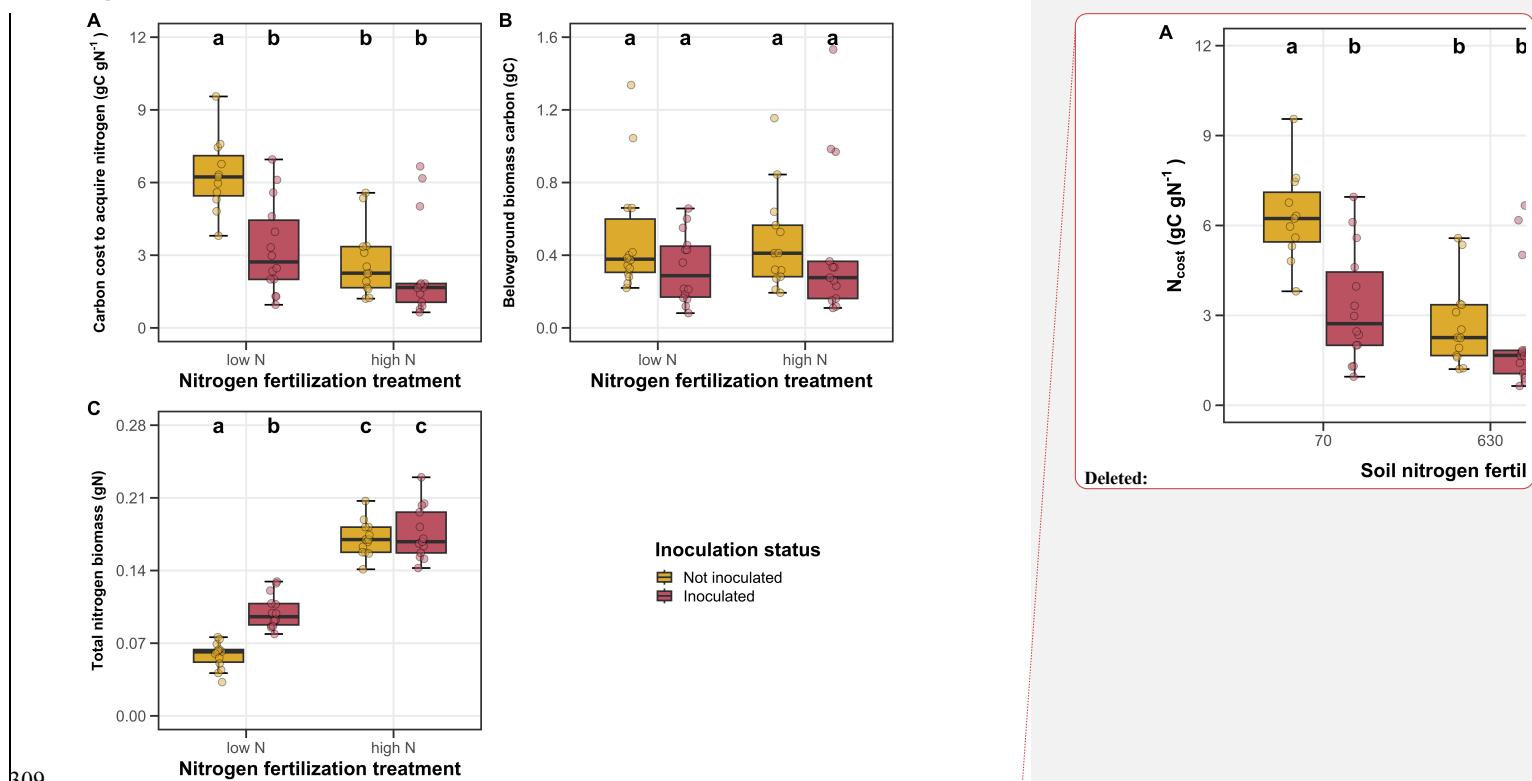
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	Carbon cost to acquire nitrogen			Belowground biomass			Whole-plant nitrogen biomass			Total leaf area		Whole plant biomass	
	df	χ^2	p	χ^2	p	χ^2	p	χ^2	p	χ^2	p	χ^2	p
N fertilization (N)	1	23.340	<0.001	0.076	0.782	358.695	<0.001	292.458	<0.001	52.427	<0.001		
Inoculation (I)	1	16.749	<0.001	4.166	0.041	24.113	<0.001	35.095	<0.001	2.042	0.153		
N*I	1	4.833	0.028	0.265	0.607	13.515	<0.001	17.898	<0.001	1.230	0.267		
Nodule biomass:													
root biomass				Nodule biomass			Root biomass						
df		χ^2	p	χ^2	p	χ^2	p	χ^2	p				
N fertilization (N)	1	1.291	0.256	1.364	0.243	0.011	0.918						
Inoculation (I)	1	27.375	<0.001	30.788	<0.001	3.268	0.071						
N*I	1	0.493	0.483	1.005	0.316	0.254	0.614						

303 *Significance determined using Type II Wald χ^2 tests ($\alpha=0.05$). P-values less than 0.05 are in bold and P-values between 0.05 and 0.1
 304 are italicized.

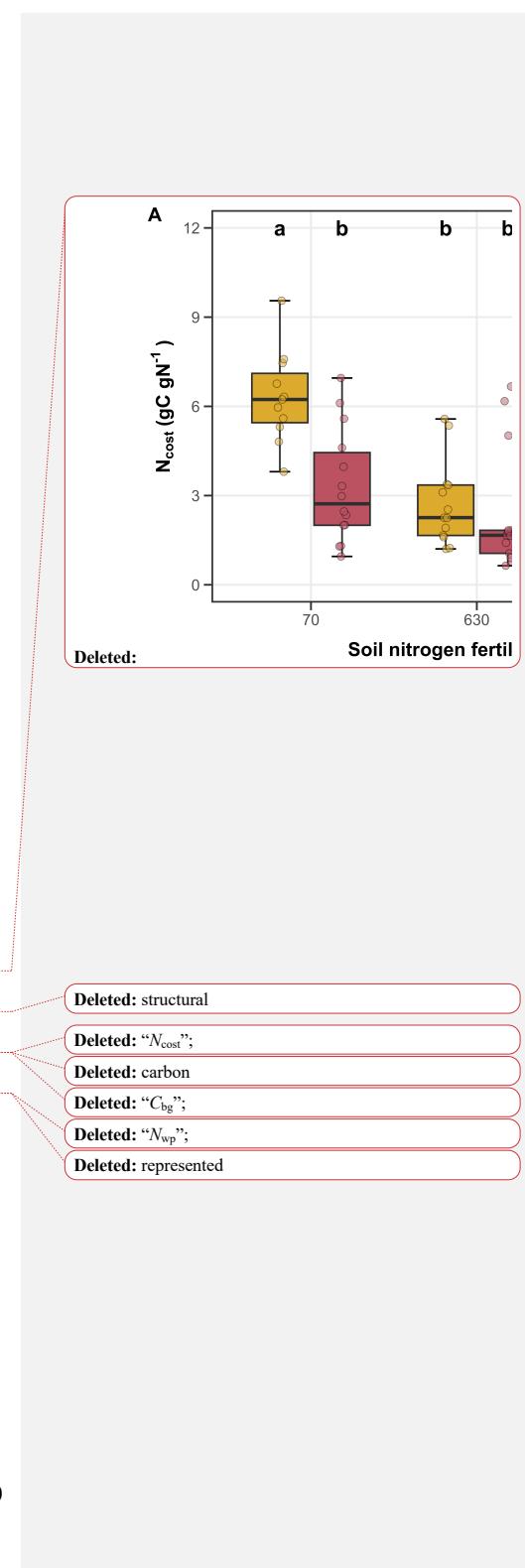
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Figure 1

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310 **Figure 1** Effects of soil nitrogen fertilization and inoculation on *G. max* belowground biomass
 311 carbon costs to acquire nitrogen (panel A), belowground biomass carbon (panel B), and whole-
 312 plant nitrogen biomass (panel C). Soil nitrogen fertilization treatment is on the x-axis, while
 313 inoculation treatment is represented by colored boxplots. Yellow shaded boxplots indicate
 314 individuals that were not inoculated with *B. japonicum*, while red shaded boxplots indicate
 315 individuals that were inoculated with *B. japonicum*. Boxes are the upper (75% percentile) and
 316 lower (25% percentile) quartile. The whiskers are the minimum and maximum value, calculated
 317 as 1.5 times the upper and lower quartile value. Colored dots are individual data points, jittered
 318 for visibility. The lettering above each box indicates the results from post-hoc Tukey's tests with
 319 different lettering indicating statistically different groups (Tukey: $p < 0.05$).

320



328 *Whole-plant growth*

329 The interaction between soil nitrogen fertilization and inoculation ($p<0.001$; Table 1) indicated
330 that positive effects of inoculation on total leaf area ($p<0.001$; Table 1) were only apparent under
331 low soil nitrogen fertilization (Tukey test comparing the inoculation effect under low soil
332 nitrogen fertilization: $p<0.001$). There was no **effect of inoculation treatment** on total leaf area
333 under high soil nitrogen fertilization (Tukey test comparing the inoculation effect under high soil
334 nitrogen fertilization: $p>0.05$; Fig. 2A). Increasing soil nitrogen fertilization generally increased
335 total leaf area ($p<0.001$; Table 1; Fig. 2A).

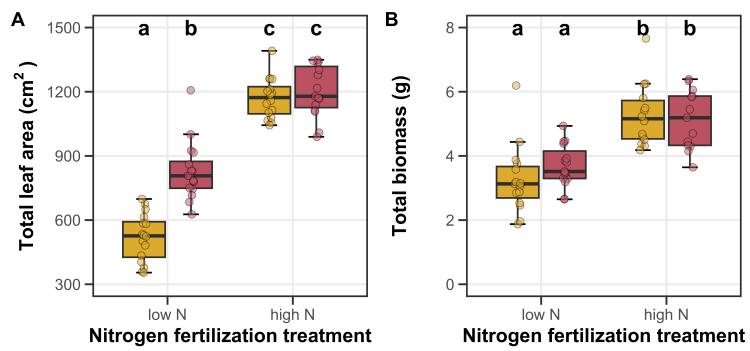
336 Increasing soil nitrogen fertilization increased total biomass ($p<0.001$; Table 1; Fig. 2B).

337 This pattern was not modified by inoculation (inoculation-by-fertilization interaction: $p>0.05$;
338 Table 1). Inoculation had no effect on total biomass ($p>0.05$; Table 1; Fig. 2B).

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344 **Figure 2**

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346 **Figure 2** Effects of soil nitrogen fertilization and inoculation on *G. max* total leaf area (panel A)

347 and total biomass (panel B). Soil nitrogen fertilization treatment is on the x-axis, while

348 inoculation treatment is represented by colored boxplots. Yellow shaded boxplots indicate

349 individuals that were not inoculated with *B. japonicum*, while red shaded boxplots indicate350 individuals that were inoculated with *B. japonicum*. Boxes are the upper (75% percentile) and

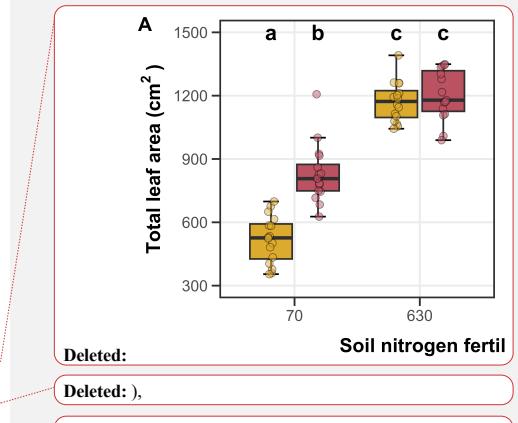
351 lower (25% percentile) quartile. The whiskers are the minimum and maximum value, calculated

352 as 1.5 times the upper and lower quartile value. Colored dots are individual data points, jittered

353 for visibility. The lettering above each box indicates the results from post-hoc Tukey's tests with

354 different lettering indicating statistically different groups (Tukey: $p < 0.05$).

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Inoculation status
 Not inoculated
 Inoculated


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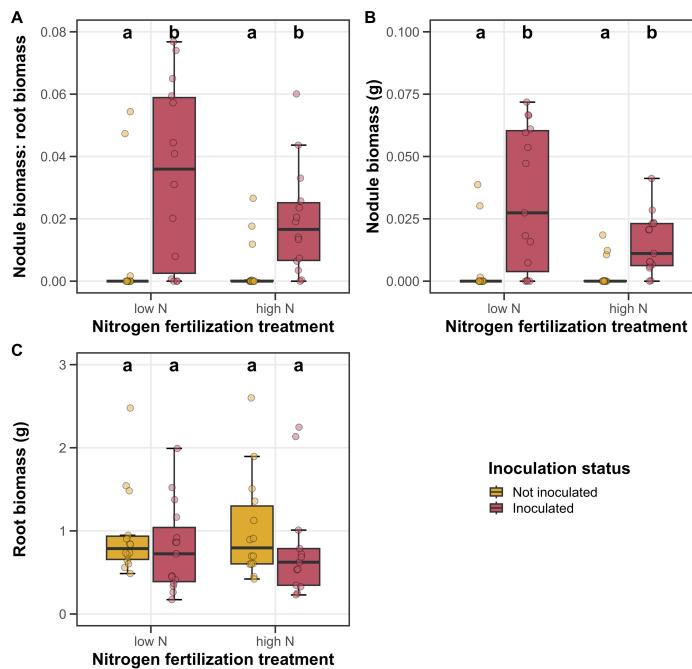
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359 *Plant investment toward symbiotic nitrogen fixation*
360 Inoculation increased root nodule biomass: root biomass ($p<0.001$; Table 1; Fig 3A). This
361 pattern was not modified by soil nitrogen fertilization (inoculation-by-fertilization interaction:
362 $p>0.05$; Table 1). Soil nitrogen fertilization had no effect on root nodule biomass: root biomass
363 ($p>0.05$; Table 1; Fig 3A).

364 Inoculation increased root nodule biomass ($p<0.001$; Table 1; Fig 3B). This pattern was
365 not modified by soil nitrogen fertilization (inoculation-by-fertilization interaction: $p>0.05$; Table
366 1). Soil nitrogen fertilization had no effect on root nodule biomass ($p>0.05$; Table 1; Fig. 3B).

367 Inoculation had a marginal negative effect on root biomass ($p<0.1$; Table 1; Fig. 3C).
368 This pattern was not modified by soil nitrogen fertilization (inoculation-by-fertilization
369 interaction: $p>0.05$; Table 1). Soil nitrogen fertilization had no effect on root biomass ($p>0.05$;
370 Table 1; Fig. 3C).

371

372 **Figure 3**

Inoculation status
■ Not inoculated
■ Inoculated

373

374 **Figure 3** Effects of soil nitrogen fertilization and inoculation on *G. max* nodule biomass: root

375 biomass (panel A), nodule biomass (panel B), and root biomass (panel C). Soil nitrogen

376 fertilization treatment is on the x-axis, while inoculation treatment is represented by colored

377 boxplots. Yellow shaded boxplots indicate individuals that were not inoculated with *B.*378 *japonicum*, while red shaded boxplots indicate individuals that were inoculated with *B.*379 *japonicum*. Boxes are the upper (75% percentile) and lower (25% percentile) quartile range. The

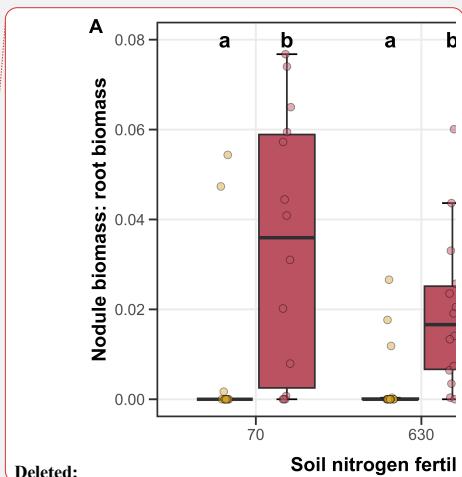
380 whiskers are the minimum and maximum value, calculated as 1.5 times the upper and lower

381 quartile value. Colored dots are individual data points, jittered for visibility. The lettering above

382 each box indicates the results from post-hoc Tukey's tests with different lettering indicating

383 statistically different groups (Tukey: $p < 0.05$).

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387 **Discussion**

388 Here, we quantified the interactive effect of soil nitrogen fertilization and inoculation with
389 symbiotic nitrogen-fixing bacteria on *G. max* belowground biomass carbon costs to acquire
390 nitrogen using a full-factorial greenhouse manipulation experiment. We found that inoculation
391 reduced belowground biomass carbon costs to acquire nitrogen under the low soil nitrogen
392 fertilization treatment. This pattern was due to similar belowground carbon allocation between
393 inoculation treatments, but significantly greater whole-plant nitrogen uptake in inoculated
394 individuals compared to their uninoculated counterparts. Inoculation effects on belowground
395 biomass carbon costs to acquire nitrogen diminished with increasing nitrogen fertilization, as
396 there was no effect of inoculation treatment on belowground biomass carbon costs to acquire
397 nitrogen under the high soil nitrogen fertilization treatment. That said, belowground biomass,
398 carbon costs to acquire nitrogen were the lowest under high soil nitrogen fertilization irrespective
399 of inoculation treatment, a pattern that was also driven by enhanced plant nitrogen uptake
400 coupled with no change in belowground carbon allocation. Overall, results indicate that
401 increased nitrogen supply, either through symbiotic nitrogen under low soil nitrogen fertilization
402 or direct uptake under high soil nitrogen fertilization, reduces the costs of nitrogen acquisition.

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404 *The impact of inoculation on belowground biomass* carbon costs to acquire nitrogen depend on
405 soil nitrogen availability

Deleted: This pattern was observed despite no significant differences in belowground carbon allocation across the treatments. Instead, inoculated individuals grown under the low soil nitrogen fertilization treatment exhibited greater whole-plant nitrogen uptake than uninoculated individuals. These results suggest that symbioses with nitrogen-fixing bacteria exhibit reduced costs of nitrogen acquisition under low nitrogen availability by enhancing nitrogen uptake efficiency compared to individuals restricted to nitrogen acquisition through direct uptake pathways.

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406 Our results provide direct evidence that, under low soil nitrogen availability, nitrogen uptake
407 through symbioses with nitrogen-fixing bacteria reduces belowground biomass carbon costs to
408 acquire nitrogen compared to nitrogen uptake through direct uptake pathways. This result
409 corroborates results from past theory (Vitousek *et al.* 2002), modeling exercises (Brzostek *et al.*
410 2014), and cross-species experimental studies (Perkowski *et al.* 2021). Here, we used individuals
411 of the same species to confirm that the ability to form symbioses with nitrogen-fixing bacteria
412 are the primary drivers of this response. Despite a strong inoculation effect on belowground
413 biomass carbon costs to acquire nitrogen in the low soil nitrogen fertilization treatment, there
414 was no impact (positive or negative) of inoculation on belowground biomass carbon costs to
415 acquire nitrogen in the high soil nitrogen fertilization treatment. Similar results were shown in a
416 previous cross-species study that observed similar belowground biomass carbon costs to acquire
417 nitrogen under high nitrogen fertilization between a nitrogen-fixing and non-fixing species and

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435 reduced belowground biomass carbon costs to acquire nitrogen in the nitrogen-fixing species
436 under low nitrogen fertilization (Perkowski *et al.* 2021). The differential role of symbiotic
437 nitrogen fixation on belowground biomass carbon costs to acquire nitrogen under the two
438 nitrogen fertilization treatments may help to explain the greater prevalence of plants capable of
439 symbiotic nitrogen fixation where soil nitrogen availability is low (Monks *et al.* 2012), as
440 expected from theory (Vitousek and Field 1999; Vitousek *et al.* 2002; Menge *et al.* 2008) and
441 simulated in plant nitrogen uptake models (Brzostek *et al.* 2014).

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442 Our results indicate that symbiotic nitrogen fixation may provide a competitive advantage
443 in nitrogen-poor soils by reducing belowground biomass carbon costs for acquiring nitrogen
444 relative to direct uptake pathways. However, the longer-term outcomes of this advantage are
445 difficult to predict because nitrogen fixation brings in nitrogen to the ecosystem that may
446 alleviate nitrogen limitation in non-fixing plant species. Additionally, long-term consequences of
447 these dynamics are difficult to predict because nitrogen-fixing species may inhibit nitrogen
448 fixation to minimize resource facilitation to neighboring non-fixing species (Nasto *et al.* 2017;
449 Taylor and Menge 2021). Other bottom-up (e.g., soil resources) and top-down (e.g., herbivory)
450 factors may also limit the competitive ability of species that associate with symbiotic nitrogen-
451 fixing bacteria in terrestrial ecosystems (Eisele *et al.* 1989; Ritchie *et al.* 1998; Vitousek and
452 Field 1999; Rastetter *et al.* 2001; Vitousek *et al.* 2002, 2013). Longer term field and mesocosm
453 experiments (e.g., Finzi and Rodgers, 2009; Taylor *et al.*, 2017; Lai *et al.*, 2018) coupled with
454 targeted model experiments (e.g., Brzostek *et al.*, 2014; Allen *et al.*, 2020; Braghieri *et al.*, 2022)
455 could help to clarify the role of these different drivers.
456

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457 *Soil nitrogen availability and inoculation modify whole-plant nitrogen, but not belowground*
458 *carbon allocation*

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459 Reduced belowground biomass carbon costs to acquire nitrogen with both increasing soil
460 nitrogen fertilization and inoculation under low soil nitrogen were the result of increased plant
461 nitrogen uptake. Belowground carbon allocation was not impacted by any of our treatments. The
462 increase in nitrogen uptake was predominantly used to support aboveground tissue, which
463 demonstrated a strong increase under increasing soil nitrogen fertilization and with inoculation
464 when soil nitrogen was low. Specifically, increases in plant nitrogen uptake were associated with
465 increased total leaf area, which likely increased total biomass due to greater surface area for light

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475 interception and whole-plant primary productivity. Theory suggests that increasing nitrogen
476 availability (from soil or symbionts) should increase relative plant investment in aboveground
477 tissues (Ågren and Franklin 2003), as was observed here. Meta-analyses also find consistent
478 positive increases in aboveground biomass with increasing soil nitrogen availability but
479 inconsistent impacts on belowground biomass (Li *et al.* 2020).

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480 Our findings provide an empirical benchmark for models that use carbon costs of
481 nitrogen acquisition to simulate terrestrial carbon-nitrogen dynamics (e.g., Brzostek *et al.*, 2014;
482 Shi *et al.*, 2016; Braghieri *et al.*, 2022). Integrating our results with findings presented in
483 Perkowski *et al.* (2021), changes in the belowground cost of nitrogen acquisition due to
484 increasing soil nitrogen availability or ability to associate with symbiotic nitrogen-fixing bacteria
485 should be the result of stronger differences in plant nitrogen uptake than belowground carbon
486 allocation. Thus, models that omit variability in costs to acquire nitrogen are likely to bias
487 estimates of plant carbon-nitrogen economics across environmental gradients. However, it must
488 be noted that, in both studies, additional carbon costs that resulted from differences in root
489 exudation, turnover, or respiration were not quantified. It is unclear whether these unaccounted
490 allocation patterns are proportional to belowground biomass carbon costs and future studies
491 should be performed to validate this assumption.

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492
493 *Soil nitrogen fertilization does not significantly reduce plant investment toward symbioses with*
494 *nitrogen fixing bacteria.*

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495 Inoculated plants exhibited similar levels of nodulation under both soil nitrogen fertilization
496 treatments, indicating that the level of nitrogen availability did not impact the strength of the
497 symbiosis between *G. max* and *B. japonicum*. This result was counter to the expectation that
498 increasing soil nitrogen availability would reduce plant reliance on nitrogen fixing symbionts
499 (Vitousek *et al.* 2002; Perkowski *et al.* 2021), though recent work suggests that plants do still
500 acquire nitrogen through symbiotic nitrogen fixation under high nitrogen availability even if
501 investment in the nitrogen fixation pathway trends in a negative direction (Menge *et al.* 2023).
502 Indeed, we observed a negative, albeit nonsignificant, trend in the effect of increasing
503 fertilization on plant investment toward symbiotic nitrogen fixation, where inoculated
504 individuals grown under high soil nitrogen availability had mean root nodule biomass and root
505 nodule biomass: root biomass values that were 46% and 40% less than individuals grown under

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513 low soil nitrogen availability. Regardless, null effects of soil nitrogen availability on plant
514 investment toward symbiotic nitrogen fixation may imply stronger bacterial control over the
515 symbiosis than previously thought. If true, greater carbon costs for nitrogen acquisition may have
516 been observed in inoculated plants grown under high soil nitrogen if increased amounts of
517 unquantified plant carbon were allocated toward bacterial respiration. Carbon and nitrogen
518 tracing experiments would be useful for examining this hypothesis.

519

520 *Study limitations*

521 This study has a few limitations that deserve recognition and limit the generality of the observed Deleted: our
522 responses. First, effects of soil nitrogen fertilization on root nodulation may be nonlinear, and a
523 two-level fertilization experiment is not equipped to address possible nonlinearities that might
524 explain the interaction between soil nitrogen fertilization and root nodulation. Future work
525 should consider conducting similar experiments using a larger number of nitrogen fertilization
526 treatments than presented here. Additionally, this study used a single plant species and an
527 inoculant comprising a single bacterial species. While this allowed us to isolate mechanisms that
528 drove *G. max* responses to nitrogen fertilization and inoculation independent of phylogeny or
529 genetic diversity, a key factor that limited inferences in Perkowski *et al.* (2021), future work
530 should consider conducting similar experiments using a larger number of leguminous species, as
531 well as multi-species mixes of different *Rhizobium* or *Actinobacteria* species. Doing so would
532 better allow us to generalize patterns observed here and would more accurately replicate soil
533 microbial communities that are observed in nature. It should be noted that uninoculated
534 individuals that formed nodules were kept in the analysis. Finally, the belowground biomass
535 carbon cost to acquire nitrogen metric used in this study does not account for changes in
536 belowground carbon allocation due to root turnover, respiration, or root exudation. It is possible
537 that nitrogen fertilization and inoculation with symbiotic nitrogen-fixing bacteria may modify
538 metabolic pathways that alter carbon investment (e.g., bacterial respiration). Future studies
539 should carefully assess whether these carbon pools should be measured as failure to measure
540 these pools could risk underestimating the belowground biomass carbon cost of nitrogen
541 acquisition.

542

543 *Conclusions*

545 Here, we used a single-pair symbiosis to quantify the impact of symbiotic nitrogen fixation on
546 belowground biomass carbon costs to acquire nitrogen under varying soil nitrogen environments.
547 Individuals that were inoculated with symbiotic nitrogen-fixing bacteria exhibited reduced
548 belowground biomass carbon costs to acquire nitrogen under the low soil nitrogen fertilization
549 treatment compared to uninoculated counterparts. In contrast, there was no effect of inoculation
550 treatment on belowground biomass carbon costs of nitrogen acquisition under the high soil
551 nitrogen fertilization treatment. Belowground biomass carbon cost to acquire nitrogen
552 differences between treatment combinations were entirely due to changes in plant nitrogen
553 uptake rather than belowground carbon allocation. Treatments that increased plant nitrogen
554 uptake corresponded with enhanced total leaf area and total biomass, suggesting that additional
555 plant nitrogen acquired was being allocated to support aboveground growth. These results
556 indicate that symbiotic nitrogen fixation may provide a competitive advantage to plants growing
557 in nitrogen-poor soils, though these advantages diminish with increasing nitrogen availability as
558 investment in nitrogen uptake through direct uptake pathways increase. These findings can be
559 used to help improve simulations of carbon-nitrogen economics in terrestrial biosphere models.
560

561 Supplementary data

562 **Table S1** Summary table containing volumes of compounds used to create modified Hoagland's
563 solutions for each soil nitrogen fertilization treatment.

564 **Table S2** Analysis of variance results exploring effect of nitrogen fertilization, inoculation with
565 *B. japonicum*, and interactions between soil nitrogen fertilization and inoculation status on whole
566 plant biomass: pot volume

567 **Table S3** Marginal mean, degrees of freedom, and 95% confidence intervals of whole plant
568 biomass: pot volume values across nitrogen fertilization and inoculation treatment combinations

569 **Figure S1** Effects of soil nitrogen fertilization and inoculation status on whole plant biomass: pot
570 volume

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