

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 and were the result of increased plant nitrogen uptake and no
17 change in belowground carbon allocation. These results suggest that symbioses with nitrogen-
18 fixing bacteria reduce carbon costs of nitrogen acquisition by increasing plant nitrogen uptake,
19 but only when soil nitrogen is low, allowing individuals to increase nitrogen allocation to
20 structures that support aboveground growth. This pattern may help explain the prevalence of
21 plants capable of forming these associations in less fertile soils and provide useful insight into
22 understanding the role of nutrient acquisition strategy on plant nitrogen uptake across nitrogen
23 availability gradients.

24

25 **Keywords**

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

Deleted: and demonstrates responses that can help guide models linking carbon and nitrogen cycles in terrestrial ecosystems...

31 **Introduction**

32 Terrestrial ecosystems are regulated, in part, by interactions between carbon and nitrogen cycles
33 (Hungate *et al.* 2003; LeBauer and Treseder 2008; Wieder *et al.* 2015). One key process linking
34 these cycles is plant nitrogen acquisition, which involves the allocation of photosynthetically
35 derived carbon belowground in exchange for nitrogen. Plants can acquire nitrogen through
36 several strategies, including direct uptake from the soil (Barber 1962; Fisher *et al.* 2010) or by
37 forming symbiotic associations with soil microbial communities such as nitrogen-fixing bacteria
38 (Vance and Heichel 1991; Vitousek *et al.* 2002; Udvardi and Poole 2013). Carbon costs to
39 acquire nitrogen, or the amount of carbon plants allocate belowground per unit nitrogen
40 acquired, vary in species that have different acquisition strategies and are likely influenced by
41 abiotic factors that alter the supply of or demand for soil resources (Brzostek *et al.* 2014; Terrer
42 et al. 2018; Taylor and Menge 2018; Friel and Friesen 2019; Allen *et al.* 2020; Perkowski *et al.*
43 2021; Lu *et al.* 2022). Variations in the cost to acquire nitrogen across biotic and abiotic
44 thresholds may help explain the prevalence of different nitrogen acquisition strategies in
45 different environments. However, these costs have not been quantified outside of a few studies
46 (Terrer *et al.* 2018; Perkowski *et al.* 2021; Lu *et al.* 2022) even though they are included in
47 nitrogen uptake models (Fisher *et al.* 2010; Brzostek *et al.* 2014; Allen *et al.* 2020) used in
48 terrestrial biosphere models (Shi *et al.* 2016; Lawrence *et al.* 2019; Braghieri *et al.* 2022).

49 Carbon costs to acquire nitrogen vary in species with different nitrogen acquisition
50 strategies. For instance, species that acquire nitrogen through direct uptake pathways may have
51 reduced carbon costs to acquire nitrogen compared to plants that form symbiotic relationships
52 with soil microorganisms (Fisher *et al.* 2010; Brzostek *et al.* 2014; Perkowski *et al.* 2021). This is
53 likely because nitrogen uptake through direct uptake only requires carbon to develop and
54 Maintain root systems, while symbioses with soil microorganisms require additional carbon to
55 Maintain and exchange resources with microbial symbionts. Of the various symbioses plants
56 form with soil microbial communities, associations with nitrogen-fixing bacteria are particularly
57 notable due to their role in providing nitrogen inputs into ecosystems by fixing nitrogen from the
58 atmosphere (Vitousek *et al.* 2002). Plants form symbiotic relationships with nitrogen-fixing
59 bacteria by housing the bacteria in root nodules, supplying the bacteria with photosynthate in
60 exchange for nitrogen fixed from the atmosphere. In some cases, the costs to acquire nitrogen
61 through symbiotic nitrogen-fixing bacteria may be greater than costs to acquire nitrogen through

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Deleted: is one process in terrestrial ecosystems that links carbon and nitrogen cycles

Deleted: Plants can acquire nitrogen through direct uptake pathways such as mass flow or diffusion, or can form symbiotic relationships with soil microbial communities. Of the various microbial symbioses plants can form, associations with nitrogen-fixing bacteria are particularly notable, as they provide important nitrogen inputs through their ability to fix nitrogen from the atmosphere.¹

¹ For example, plants that acquire nitrogen through direct uptake pathways may have reduced carbon costs to acquire nitrogen compared to plants that associate with soil microbial communities, as individuals only need to allocate carbon belowground in order to build and maintain root systems, in exchange for nitrogen through different nitrogen acquisition strategies. Direct uptake pathways such as mass flow or diffusion implies an inherent carbon cost to the plant for acquiring nitrogen

Deleted: does not require costs beyond root development, as is the case for acquisition strategies that involve other soil microbiota.

85 direct uptake, as maintaining symbioses with nitrogen-fixing bacteria is both energetically
86 expensive and requires the allocation of carbon toward root nodule construction (Gutschick
87 1981; Vitousek and Howarth 1991). However, in certain environments (e.g., nitrogen-poor
88 environments), individuals who acquire nitrogen through associations with symbiotic nitrogen-
89 fixing bacteria may exhibit reduced carbon costs to acquire nitrogen compared to pathways that
90 rely on soil-derived nitrogen, as nitrogen fixation allows plants to tap into a greater nitrogen pool
91 (i.e., the atmosphere), which could allow plants to maximize the magnitude of nitrogen acquired
92 per unit carbon allocated belowground and therefore decrease the cost of acquiring nitrogen.

93 Carbon costs to acquire nitrogen have been shown to decrease with increasing soil
94 nitrogen availability, a response that is typically the result of an increase in plant nitrogen uptake
95 and a decrease belowground carbon allocation (Perkowski et al. 2021; Lu et al. 2022). Negative
96 belowground carbon allocation responses to increasing nitrogen availability may be due to
97 reduced soil resource mining (by roots or symbionts) needed to satisfy plant nitrogen demand
98 under greater nitrogen availability and could be exacerbated by an increase in biomass allocation
99 to aboveground tissues (Li et al. 2020). Regardless, the effects of nitrogen availability on carbon
100 costs to acquire nitrogen likely vary across nutrient acquisition strategies. For example, plants
101 that form associations with symbiotic nitrogen-fixing bacteria often exhibit damped responses
102 to nitrogen availability despite reduced investment toward nitrogen fixation with increasing
103 nitrogen availability (Gutschick 1981; Taylor and Menge 2018; Friel and Friesen 2019;
104 McCulloch and Porder 2021; Schmidt et al. 2023). While previous work notes that plants can
105 still acquire nitrogen through symbiotic nitrogen fixation under high soil nitrogen availability
106 (Menge et al. 2023), resource optimization theory suggests that reduced sensitivity of plant
107 nitrogen uptake to changes in nitrogen availability in nitrogen-fixing plants may stem from
108 preferential investment toward the acquisition strategy that confers the lowest carbon cost and
109 greatest nitrogen gain (Bloom et al. 1985; Rastetter et al. 2001). If true, similar costs to acquire
110 nitrogen in nitrogen-fixing species may be achieved across nitrogen availability gradients due to
111 shifts away from nitrogen acquisition through nitrogen fixation to direct uptake as costs to
112 acquire nitrogen through direct uptake decrease (Fisher et al. 2010; Brzostek et al. 2014;
113 Perkowski et al. 2021).

114 Here, we sought to understand how nitrogen fixation and soil nitrogen fertilization
115 interact to influence belowground biomass carbon costs to acquire nitrogen in *Glycine max* L.

Deleted: Previous work suggests that the amount of photosynthate allocated belowground in exchange for nitrogen may increase with increased light and CO₂, as these factors reduce the cost to produce photosynthate and increase plant demand for acquiring nitrogen to build and maintain photosynthetic enzymes. However, increasing soil nitrogen availability tends to

Deleted: leading to a decrease in the carbon cost to acquire nitrogen

Deleted: decreased carbon costs to acquire nitrogen with increasing nitrogen availability may not be as robust in plant species with strong and specialized symbiotic relationships with nitrogen-acquiring partners that reduce the sensitivity of plant nitrogen uptake to changes in nitrogen availability (e.g., plant species that associate with symbiotic nitrogen-fixing bacteria). Indeed,

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

157 (Merr.) seedlings. To do this, we grew *Glycine max* L. (Merr.) seedlings under two soil nitrogen
158 fertilization treatments and manipulated whether seedlings were inoculated with symbiotic
159 nitrogen-fixing bacteria in a full factorial greenhouse experiment. We used this experiment to
160 test the following hypotheses:

- 161 (1) Soil nitrogen fertilization will decrease belowground biomass carbon costs of
162 nitrogen acquisition in uninoculated and inoculated individuals. This decrease will
163 manifest as a stronger increase in plant nitrogen uptake than belowground carbon
164 allocation.
- 165 (2) Inoculation with nitrogen-fixing bacteria will decrease belowground biomass carbon
166 costs to acquire nitrogen under low soil nitrogen availability. This is because
167 belowground biomass carbon costs to acquire nitrogen through symbiotic nitrogen
168 fixation will be less than the belowground biomass carbon cost to acquire nitrogen via
169 direct uptake. This pattern will be indexed as a stronger increase in plant nitrogen
170 uptake in inoculated plants under low nitrogen fertilization compared to uninoculated
171 plants. However, inoculation will not affect belowground biomass carbon costs to
172 acquire nitrogen under high soil nitrogen availability due to all plants shifting toward
173 a similar, direct uptake-dominated mode of nitrogen acquisition. This will be indexed
174 by similar belowground carbon allocation and nitrogen uptake patterns between
175 inoculation treatments under high nitrogen fertilization.
- 176 (3) Root nodulation and plant investment toward symbiotic nitrogen fixation will
177 decrease with increasing soil nitrogen availability. This pattern will be due to
178 increased plant nitrogen uptake through direct uptake with increasing nitrogen
179 fertilization as costs to acquire nitrogen through direct uptake pathways decrease.

180
181 **Materials and methods**

182 *Experimental Design*

183 *Glycine max* seeds were planted in 64, 6-liter pots (NS-600, Nursery Supplies, Orange, CA,
184 USA) containing unfertilized potting mix (Sungro Sunshine Mix #2, Agawam, MA, USA). The
185 experiment used *G. max* seedlings to compare observed responses from previous work that was
186 not able to disentangle species-specific effects on belowground biomass carbon costs to acquire
187 nitrogen from the explicit effects of nitrogen fixation (Perkowski *et al.* 2021). Pots and potting

188 mix were steam sterilized at 95°C for three hours to eliminate any bacterial or fungal growth.
189 Thirty-two randomly selected pots were planted with seeds inoculated with *Bradyrhizobium*
190 *japonicum* (Verdesian N-Dure™ Soybean, Cary, NC, USA) following a brief surface
191 sterilization in 20,000 ppm sodium hypochlorite for 5 minutes followed by three washes in
192 ultrapure water (Scouten and Beuchat 2002; Montville and Schaffner 2004). The remaining 32
193 pots were planted with seeds that did not receive any inoculation treatment. Uninoculated seeds
194 were also surface sterilized in 20,000 ppm sodium hypochlorite for 5 minutes followed by three
195 ultrapure water washes to ensure that the only difference between seed treatments was the
196 inoculation treatment.

197 Upon planting, all pots were immediately placed in one of four random blocks in a
198 greenhouse and received one of two nitrogen fertilization treatments as 150 mL of a modified
199 Hoagland's solution (Hoagland and Arnon 1950) equivalent to either 70 or 630 ppm N twice per
200 week for seven weeks. Nitrogen fertilization levels were chosen based on previous work using a
201 larger number of fertilization treatments (Perkowski *et al.*, 2021). Nitrogen fertilization doses
202 were received as topical agents to the soil surface and were modified to keep concentrations of
203 other macronutrients and micronutrients equivalent across the two treatments (Table S1).
204 Throughout the experiment, plants were routinely well-watered to minimize any chance of water
205 stress. Greenhouse maximum daytime temperatures averaged $42.4 \pm 3.9^\circ\text{C}$ (mean \pm standard
206 deviation) across blocks, while minimum nighttime temperature averaged $19.8 \pm 1.9^\circ\text{C}$ across
207 blocks. There was no evidence of growth limitation due to pot size at the time of biomass
208 harvest, indicated by total biomass: pot volume ratios less than 1 g L^{-1} within each treatment
209 combination (Table S2-3; Fig. S1; Poorter *et al.* 2012).

210

211 *Plant trait measurements*

212 All individuals were harvested, and biomass was separated into major organ types (leaves, stems,
213 roots, and root nodules when present) approximately seven weeks after experiment initiation and
214 before the onset of reproduction. Leaf areas of all harvested leaves were measured using an LI-
215 3100C (Li-COR Biosciences, Lincoln, Nebraska, USA). Total leaf area (cm^2) was calculated as
216 the sum of all leaf areas. All harvested material was dried in an oven set to 65°C for at least 48
217 hours, weighed, and ground to homogeneity. Total dry biomass (g) was calculated as the sum of
218 dry leaf, stem, root, and root nodule biomass. Carbon and nitrogen content of each respective

219 organ was quantified through elemental combustion (Costech-4010, Costech, Inc., Valencia, CA,
220 USA) using subsamples of ground and homogenized organ tissue.

221 Belowground biomass carbon costs to acquire nitrogen were calculated as the ratio of
222 total belowground biomass carbon to whole plant nitrogen biomass ($\text{g C g}^{-1} \text{ N}$; Perkowski *et al.*,
223 2021). Belowground biomass carbon (g C) was calculated as the sum of total root carbon
224 biomass and total root nodule carbon biomass. Total root biomass carbon was calculated by
225 multiplying root carbon content by total root biomass, while total root nodule biomass carbon
226 was calculated by multiplying root nodule carbon content by total root nodule biomass. Whole-
227 plant nitrogen biomass (g N) was calculated by multiplying the nitrogen content of leaves, stems,
228 roots, and root nodules by biomass of each respective organ type, then calculating the sum of
229 nitrogen biomass of each organ type. This calculation only quantifies belowground biomass
230 carbon costs to acquire nitrogen and does not account for additional carbon costs of nitrogen
231 acquisition associated with root respiration, root exudation, or root turnover. An explicit
232 explanation of the limitations for interpreting this calculation can be found in Perkowski *et al.*
233 (2021) and Terrer *et al.* (2018).

234

235 *Statistical analyses*

236 A series of linear mixed-effects models were built to investigate the impacts of soil nitrogen
237 fertilization and inoculation on *G. max* belowground biomass carbon costs to acquire nitrogen
238 and plant growth. Any uninoculated individuals that formed nodules were removed prior to
239 model fitting. All models included soil nitrogen fertilization, inoculation, and interactions
240 between soil nitrogen fertilization and inoculation as categorical fixed effects. Block number was
241 included as a random intercept term to account for any environmental heterogeneity within the
242 greenhouse room. Models with this independent variable structure were constructed to quantify
243 relationships between soil nitrogen fertilization and inoculation on belowground biomass carbon
244 costs to acquire nitrogen, belowground biomass carbon, whole-plant nitrogen biomass, total leaf
245 area, total biomass, and root biomass.

Deleted: Individuals were categorized by inoculation treatment and not by whether they had formed nodules.

246 A second series of linear mixed-effects models were built to investigate the impacts of
247 soil nitrogen fertilization on *G. max* investment toward symbiotic nitrogen fixation. These
248 models included only measurements collected in inoculated individuals. Models included soil
249 nitrogen fertilization as the lone categorical fixed effect with block number included as a random

Deleted: , root nodule biomass: root biomass, root nodule biomass

254 effect. Two models with this independent variable structure were constructed to quantify
255 relationships between soil nitrogen fertilization and root nodule biomass and the ratio of root
256 nodule biomass to root biomass.

257 Shapiro-Wilk tests of normality were used to determine whether linear mixed-effects
258 models satisfied residual normality assumptions (Shapiro-Wilk: $p>0.05$). Models for whole-plant
259 nitrogen biomass, total leaf area, nodule biomass:root biomass, and root nodule biomass each
260 satisfied residual normality assumptions without data transformation. Models for belowground
261 biomass carbon costs to acquire nitrogen, belowground biomass carbon, total biomass, and root
262 biomass each satisfied residual normality assumptions after models were fit using dependent
263 variables that were natural log transformed (Shapiro-Wilk: $p>0.05$ in all cases).

264 We used the ‘lmer’ function in the ‘lme4’ R package (Bates et al. 2015) to fit each model
265 and the ‘Anova’ function in the ‘car’ R package (Fox and Weisberg 2019) to calculate Type II
266 Wald’s χ^2 and determine the significance ($\alpha=0.05$) of each fixed effect coefficient. We used the
267 ‘emmeans’ R package (Lenth 2019) to conduct post-hoc comparisons using Tukey’s tests, where
268 degrees of freedom were approximated using the Kenward-Roger approach (Kenward and Roger
269 1997). All analyses were conducted and plots were created using R version 4.2.0 (R Core Team
270 2021).

271 Results

272 Belowground biomass carbon costs to acquire nitrogen

273 Negative effects of inoculation ($p<0.001$; Table 1) on belowground biomass carbon costs to
274 acquire nitrogen were only apparent under low soil nitrogen fertilization (inoculation-by-nitrogen
275 fertilization interaction: $p<0.05$; Table 1; Fig. 1A). Increasing soil nitrogen fertilization
276 decreased belowground biomass carbon costs to acquire nitrogen ($p<0.001$; Table 1).

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

281 Positive effects of inoculation on whole-plant nitrogen biomass ($p<0.001$; Table 1) were
282 only apparent under low soil nitrogen fertilization (inoculation-by-nitrogen fertilization

Deleted: Specifically, models for belowground biomass carbon costs to acquire nitrogen, belowground biomass carbon, total biomass, and root biomass satisfied normality assumptions when response variables were fit using natural log transformed data, while models for nodule biomass: root biomass and root nodule biomass satisfied such assumptions when response variables were fit using square-root transformations.

292 interaction: $p<0.001$; Fig. 1C). Increasing soil nitrogen fertilization increased whole-plant
293 nitrogen biomass ($p<0.001$; Table 1).

294

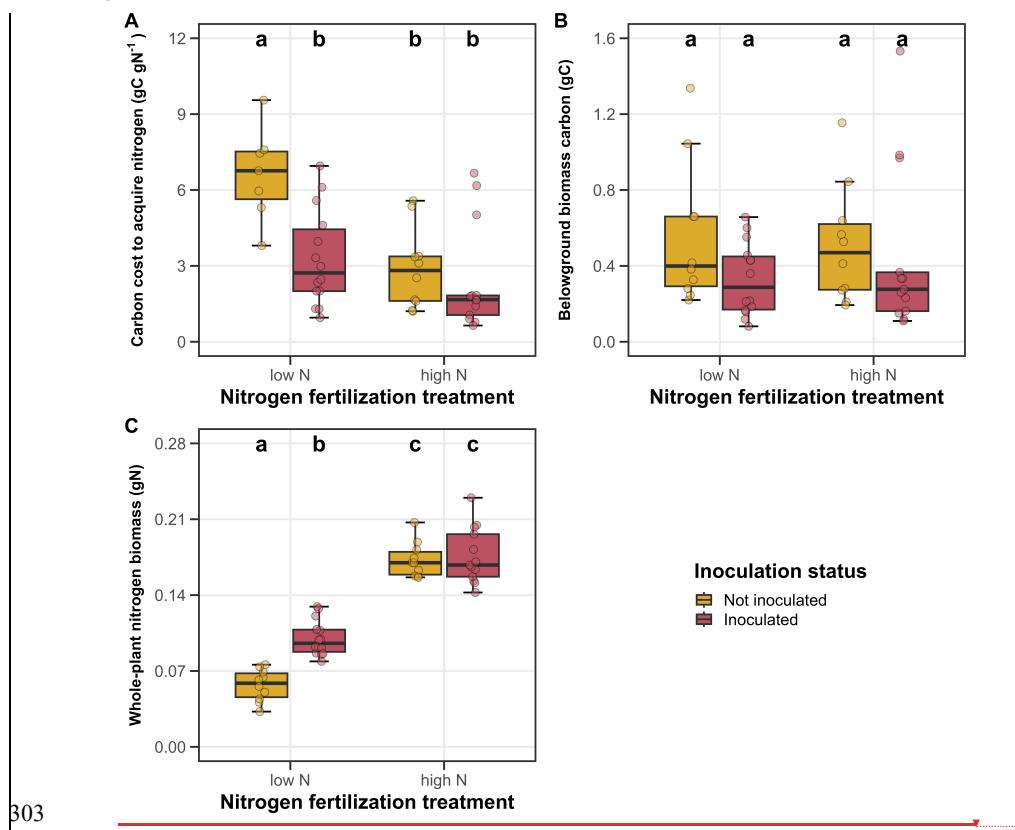
295 **Table 1** Analysis of variance results exploring effects of soil nitrogen fertilization, inoculation with *B. japonicum*, and interactions
 296 between soil nitrogen fertilization and inoculation on belowground biomass carbon costs to acquire nitrogen, whole-plant growth, and
 297 investment toward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

	Carbon cost to acquire nitrogen			Belowground biomass carbon		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
N fertilization (N)	1	17.101	<0.001	0.062	0.804	281.094	<0.001	200.584	<0.001	35.592	<0.001
Inoculation (I)	1	15.443	<0.001	4.318	0.038	17.991	<0.001	23.242	<0.001	0.695	0.404
N*I	1	4.460	0.035	0.264	0.607	12.771	<0.001	16.783	<0.001	0.759	0.384

	Nodule biomass: root biomass			Nodule biomass		Root biomass	
	df	χ^2	p	χ^2	p	χ^2	p
N fertilization (N)	1	4.663	0.031	6.391	0.011	0.016	0.900
Inoculation (I)	1	—	—	—	—	3.884	0.049
N*I	1	—	—	—	—	0.223	0.637

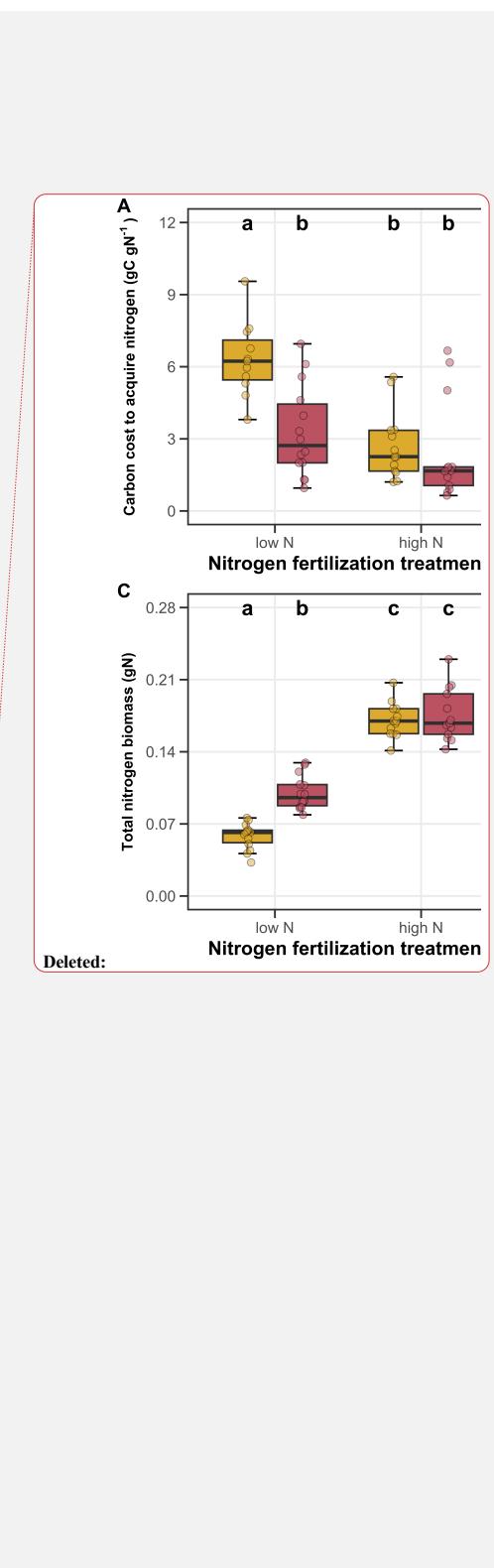
298 *Significance determined using Type II Wald χ^2 tests ($\alpha=0.05$). P-values less than 0.05 are in bold and P-values between 0.05 and 0.1
 299 are italicized. Models for nodule biomass:root biomass and root nodule biomass were fit using nitrogen fertilization as the lone fixed
 300 effect.

301

302 **Figure 1**

303

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



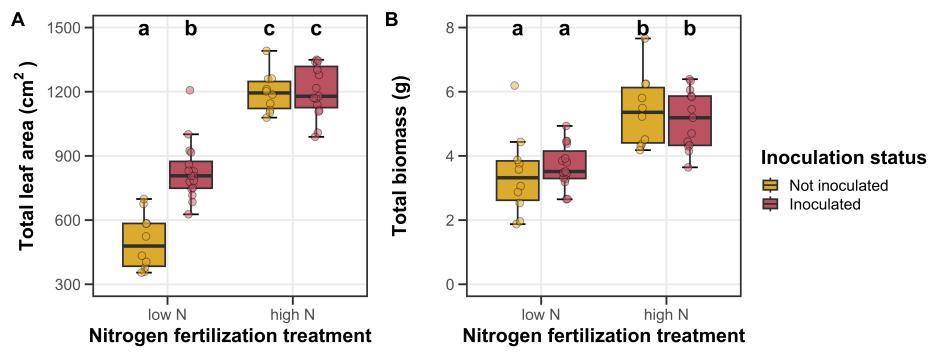
315 *Whole-plant growth*

316 Positive effects of inoculation on total leaf area ($p<0.001$; Table 1) were only apparent under low
317 soil nitrogen fertilization (inoculation-by-nitrogen fertilization interaction: $p<0.001$; Table 1; Fig.
318 2A). Increasing soil nitrogen fertilization increased total leaf area ($p<0.001$; Table 1; Fig. 2A).

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

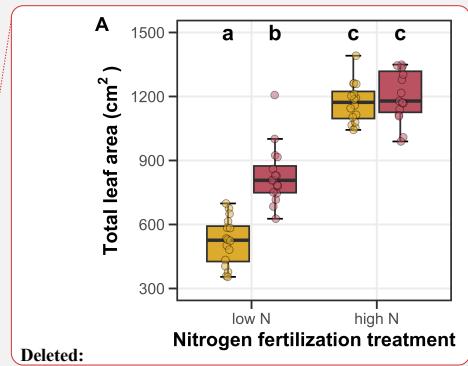
320 This pattern was not modified by inoculation (inoculation-by-nitrogen fertilization interaction:
321 $p>0.05$; Table 1). Inoculation had no effect on total biomass ($p>0.05$; Table 1; Fig. 2B).

322

323 **Figure 2**

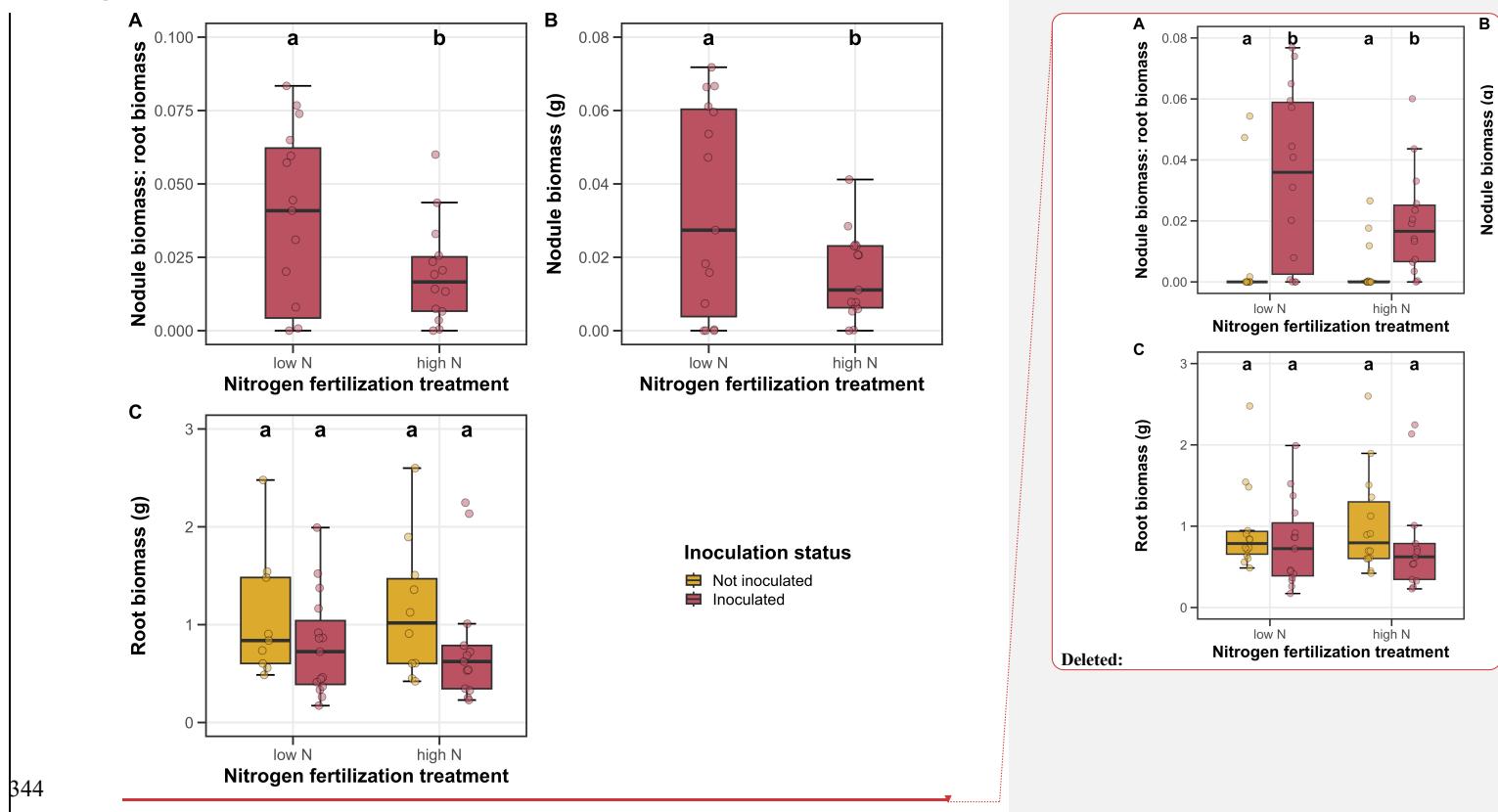
324

325 **Figure 2** Effects of soil nitrogen fertilization and inoculation on *G. max* total leaf area (panel A)
 326 and total biomass (panel B). Soil nitrogen fertilization treatment is on the x-axis, while
 327 inoculation treatment is represented by colored boxplots. Yellow shaded boxplots indicate
 328 individuals that were not inoculated with *B. japonicum*, while red shaded boxplots indicate
 329 individuals that were inoculated with *B. japonicum*. Boxes are the upper (75% percentile) and
 330 lower (25% percentile) quartile. The whiskers are the minimum and maximum value, calculated
 331 as 1.5 times the upper and lower quartile value. Colored dots are individual data points, jittered
 332 for visibility. The lettering above each box indicates the results from post-hoc Tukey's tests with
 333 different lettering indicating statistically different groups (Tukey: $p < 0.05$).
 334



336 *Plant investment toward symbiotic nitrogen fixation*
337 Increasing soil nitrogen fertilization decreased root nodule biomass:root biomass ($p \leq 0.05$; Table
338 1; Fig 3A) through a reduction in root nodule biomass ($p < 0.05$; Table 1; Fig. 3B) and no change
339 in root biomass ($p > 0.05$; Table 1; Fig. 3c). Inoculation decreased root biomass ($p < 0.05$; Table 1;
340 Fig. 3C), a pattern was not modified by soil nitrogen fertilization treatment (inoculation-by-
341 fertilization interaction: $p > 0.05$; Table 1).

342

343 **Figure 3**

344

345 **Figure 3** Effects of soil nitrogen fertilization and inoculation on *G. max* nodule biomass: root
 346 biomass (panel A), nodule biomass (panel B), and root biomass (panel C). Soil nitrogen
 347 fertilization treatment is on the x-axis. Inoculation treatment is represented by colored boxplots.
 348 Yellow shaded boxplots in panel C indicate individuals that were not inoculated with *B.*
 349 *japonicum*, while red shaded boxplots in all panels indicate individuals that were inoculated with
 350 *B. japonicum*. Boxes are the upper (75% percentile) and lower (25% percentile) quartile range.
 351 The whiskers are the minimum and maximum value, calculated as 1.5 times the upper and lower
 352 quartile value. Colored dots are individual data points, jittered for visibility. The lettering above
 353 each box indicates the results from post-hoc Tukey's tests with different lettering indicating
 354 statistically different groups (Tukey: $p < 0.05$).

356 **Discussion**

357 Here, we quantified the interactive effect of soil nitrogen fertilization and inoculation with
358 symbiotic nitrogen-fixing bacteria on relationships between *G. max* belowground biomass
359 carbon and whole-plant nitrogen biomass. We did this to understand the effects of nitrogen
360 fertilization and nitrogen acquisition strategy on plant carbon costs to acquire nitrogen.
361 Inoculation with symbiotic nitrogen-fixing bacteria increased whole-plant nitrogen biomass, but
362 this pattern was only observed under low nitrogen fertilization and was not associated with a
363 change in belowground biomass carbon. The positive effects of inoculation on whole-plant
364 nitrogen biomass diminished with increasing nitrogen fertilization, as there was no effect of
365 inoculation treatment on whole-plant nitrogen biomass under high nitrogen fertilization. These
366 patterns indicate that, under low soil nitrogen fertilization, inoculation with symbiotic nitrogen-
367 fixing bacteria increased plant nitrogen uptake and the magnitude of nitrogen acquired per unit
368 carbon allocated belowground compared to their uninoculated counterparts, supporting our
369 hypothesis. However, positive effects of inoculation on plant nitrogen uptake diminished with
370 increasing nitrogen fertilization, as plants may have invested less toward symbiotic nitrogen
371 fixation and instead invested more strongly in direct uptake pathways as costs to acquire nitrogen
372 between direct uptake and symbiotic nitrogen fixation became similar (Perkowski *et al.* 2021).
373 Increasing nitrogen fertilization increased whole-plant nitrogen biomass, again while maintaining
374 the same belowground biomass carbon, which increased the magnitude of nitrogen acquired per
375 unit carbon allocated belowground in plants grown under the high nitrogen fertilization
376 treatment. These findings indicate that symbiotic nitrogen fixation increased plant nitrogen
377 uptake under low nitrogen fertilization, which decreased the cost of acquiring nitrogen.
378 ▼

379 *The impact of inoculation on belowground biomass carbon costs to acquire nitrogen depends on*
380 *soil nitrogen availability*

381 Our results provide direct evidence that, under low soil nitrogen availability, increased nitrogen
382 uptake through symbioses with nitrogen-fixing bacteria reduces belowground biomass carbon
383 costs to acquire nitrogen compared to nitrogen uptake through direct uptake pathways. This
384 result corroborates results from past theory (Vitousek *et al.* 2002), modeling exercises (Brzostek
385 *et al.* 2014), and cross-species experimental studies (Perkowski *et al.* 2021). Here, we used
386 individuals of the same species to confirm that the ability to form symbioses with nitrogen-fixing

Deleted: reduced belowground biomass carbon costs to acquire nitrogen under the low soil nitrogen fertilization treatment. This pattern was due to similar belowground carbon allocation between inoculation treatments, but significantly greater whole-plant nitrogen uptake in inoculated individuals compared to their uninoculated counterparts. Inoculation effects on belowground biomass carbon costs to acquire nitrogen diminished with increasing nitrogen fertilization, as there was no effect of inoculation treatment on belowground biomass carbon costs to acquire nitrogen under the high soil nitrogen fertilization treatment. That said, belowground biomass carbon costs to acquire nitrogen were the lowest under high soil nitrogen fertilization irrespective of inoculation treatment, a pattern that was also driven by enhanced plant nitrogen uptake coupled with no change in belowground carbon allocation. Overall, results indicate that increased nitrogen supply, either through symbiotic nitrogen under low soil nitrogen fertilization or direct uptake under high soil nitrogen fertilization, reduces the costs of nitrogen acquisition.¶

407 bacteria are the primary drivers of this response. Despite a strong inoculation effect on nitrogen
408 uptake in the low soil nitrogen fertilization treatment, there was no impact (positive or negative)
409 of inoculation on nitrogen uptake in the high soil nitrogen fertilization treatment, yielding similar
410 carbon costs to acquire nitrogen between inoculation treatments. Similar results were shown in a
411 previous cross-species study that observed similar belowground biomass carbon costs to acquire
412 nitrogen under high nitrogen fertilization between a nitrogen-fixing and non-fixing species and
413 reduced belowground biomass carbon costs to acquire nitrogen in the nitrogen-fixing species
414 under low nitrogen fertilization (Perkowski et al. 2021). The differential role of symbiotic
415 nitrogen fixation on plant nitrogen uptake under the two nitrogen fertilization treatments may
416 help to explain the greater prevalence of plants capable of symbiotic nitrogen fixation where soil
417 nitrogen availability is low (Monks et al. 2012), as expected from theory (Vitousek and Field
418 1999; Vitousek et al. 2002; Menge et al. 2008) and simulated in plant nitrogen uptake models
419 (Brzostek et al. 2014).

420 Our results indicate that symbiotic nitrogen fixation may provide a competitive advantage
421 in nitrogen-poor soils by increasing plant nitrogen uptake relative to direct uptake pathways.
422 However, the longer-term outcomes of this advantage are difficult to predict because nitrogen
423 fixation brings in nitrogen to the ecosystem that may alleviate nitrogen limitation in non-fixing
424 plant species. Additionally, long-term consequences of these dynamics are difficult to predict
425 because nitrogen-fixing species may inhibit nitrogen fixation to minimize resource facilitation to
426 neighboring non-fixing species (Nasto et al. 2017; Taylor and Menge 2021). Other bottom-up
427 (e.g., soil resources) and top-down (e.g., herbivory) factors may also limit the competitive ability
428 of species that associate with symbiotic nitrogen-fixing bacteria in terrestrial ecosystems (Eisele
429 et al. 1989; Ritchie et al. 1998; Vitousek and Field 1999; Rastetter et al. 2001; Vitousek et al.
430 2002, 2013). Longer term field and mesocosm experiments (e.g., Finzi and Rodgers, 2009;
431 Taylor et al., 2017; Lai et al., 2018) coupled with targeted model experiments (e.g., Brzostek et
432 al. 2014; Allen et al. 2020; Braghieri et al. 2022) could help to clarify the role of these different
433 drivers.

434
435 *Soil nitrogen availability and inoculation modify whole-plant nitrogen, but not belowground*
436 *carbon allocation*

Deleted: belowground biomass carbon costs to acquire nitrogen

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445 Plant nitrogen uptake increased with increasing soil nitrogen fertilization and in inoculated plants
446 grown under low soil nitrogen fertilization. Belowground carbon allocation was not impacted by
447 any of our treatments. The increase in nitrogen uptake was predominantly used to support
448 aboveground tissue, which demonstrated a strong increase under increasing soil nitrogen
449 fertilization and with inoculation when soil nitrogen was low. Specifically, increases in plant
450 nitrogen uptake were associated with increased total leaf area, which likely increased total
451 biomass due to greater surface area for light interception and whole-plant primary productivity.
452 Theory suggests that increasing nitrogen availability (from soil or symbionts) should increase
453 relative plant investment in aboveground tissues (Ågren and Franklin 2003), as was observed
454 here. Meta-analyses also find consistent positive increases in aboveground biomass with
455 increasing soil nitrogen availability but inconsistent impacts on belowground biomass (Li et al.
456 2020).

457 Our findings provide an empirical benchmark for models that use carbon costs of
458 nitrogen acquisition to simulate terrestrial carbon-nitrogen dynamics (e.g., Brzostek et al. 2014;
459 Shi et al. 2016; Braghieri et al. 2022). Integrating our results with findings presented in
460 Perkowski et al. (2021), changes in the belowground cost of nitrogen acquisition due to
461 increasing soil nitrogen availability or ability to associate with symbiotic nitrogen-fixing bacteria
462 should be the result of stronger differences in plant nitrogen uptake than belowground carbon
463 allocation. Thus, models that omit variability in costs to acquire nitrogen are likely to bias
464 estimates of plant carbon-nitrogen economics across environmental gradients. However, it must
465 be noted that, in both studies, additional carbon costs that resulted from differences in root
466 exudation, turnover, or respiration were not quantified. It is unclear whether these unaccounted
467 allocation patterns are proportional to belowground biomass carbon costs and future studies
468 should be performed to validate this assumption.
469

470 Soil nitrogen fertilization reduced plant investment toward symbiotic nitrogen fixation
471 Consistent with our hypothesis, root nodulation and plant investment toward symbiotic nitrogen
472 fixation decreased with increasing nitrogen fertilization in inoculated plants. These patterns
473 corresponded with diminished effects of inoculation treatment on belowground biomass carbon,
474 whole-plant nitrogen biomass, and total leaf area with increasing nitrogen fertilization. These
475 results are consistent with previous results showing that plants decrease reliance on nitrogen-

Deleted: Reduced belowground biomass carbon costs to acquire nitrogen with both increasing soil nitrogen fertilization and inoculation under low soil nitrogen were the result of increased plant nitrogen uptake.

Deleted: Plant investment toward symbiotic nitrogen fixation decreased with increasing nitrogen fertilization in inoculated plants. Root nodule biomass and the ratio of root nodule biomass to root biomass were each reduced in the high nitrogen fertilization treatment compared to the low nitrogen fertilization treatment, which corresponded with reduced effects of inoculation treatments. The level of nitrogen availability did not impact the strength of the symbiosis between *G. max* and *B. japonicum*. This result followed our expectations and previous

490 fixing symbionts as soil nitrogen availability increases (Vitousek et al. 2002; Perkowski et al.
491 2021). Though recent work suggests that plants can still acquire nitrogen through symbiotic
492 nitrogen fixation under high nitrogen availability (Menge et al. 2023), these patterns indicate that
493 inoculated individuals likely shifted their relative mode of nitrogen acquisition away from
494 nitrogen fixation and toward direct uptake pathways with increasing nitrogen fertilization.
495

496 Study limitations

497 This study has a few limitations that deserve recognition and limit the generality of the observed
498 responses. First, effects of soil nitrogen fertilization on root nodulation may be nonlinear, and a
499 two-level fertilization experiment is not equipped to address possible nonlinearities that might
500 explain the interaction between soil nitrogen fertilization and root nodulation. Future work
501 should consider conducting similar experiments using a larger number of nitrogen fertilization
502 treatments than presented here. Additionally, this study used a single plant species and an
503 inoculant comprising a single bacterial species. While this allowed us to isolate mechanisms that
504 drove *G. max* responses to nitrogen fertilization and inoculation independent of phylogeny or
505 genetic diversity, a key factor that limited inferences in Perkowski et al. (2021), future work
506 should consider conducting similar experiments using a larger number of leguminous species, as
507 well as multi-species mixes of different *Rhizobium* or *Actinobacteria* species. Doing so would
508 better allow us to generalize patterns observed here and would more accurately replicate soil
509 microbial communities that are observed in nature. Finally, the belowground biomass carbon
510 cost to acquire nitrogen metric used in this study does not account for changes in belowground
511 carbon allocation due to root turnover, respiration, or root exudation. It is possible that nitrogen
512 fertilization and inoculation with symbiotic nitrogen-fixing bacteria may modify metabolic
513 pathways that alter carbon investment (e.g., bacterial respiration). Future studies should carefully
514 assess whether these carbon pools should be measured as failure to measure these pools could
515 risk underestimating the belowground biomass carbon cost of nitrogen acquisition.

516

517 Conclusions

518 Here, we used a single-pair symbiosis to quantify the impact of symbiotic nitrogen fixation on
519 belowground biomass carbon and whole-plant nitrogen biomass under varying soil nitrogen
520 environments. Regardless of nitrogen fertilization level, individuals that were inoculated with

Deleted: was counter to the expectation that increasing soil nitrogen availability would reduce plant reliance on nitrogen fixing symbionts

Deleted: even if investment in the nitrogen fixation pathway trends in a negative direction

Deleted: Indeed, we observed a negative, albeit nonsignificant, trend in the effect of increasing fertilization on plant investment toward symbiotic nitrogen fixation, where inoculated individuals grown under high soil nitrogen availability had mean root nodule biomass and root nodule biomass: root biomass values that were 46% and 40% less than individuals grown under low soil nitrogen availability. Regardless, null effects of soil nitrogen availability on plant investment toward symbiotic nitrogen fixation may imply stronger bacterial control over the symbiosis than previously thought. If true, greater carbon costs for nitrogen acquisition may have been observed in inoculated plants grown under high soil nitrogen if increased amounts of unquantified plant carbon were allocated toward bacterial respiration. Carbon and nitrogen tracing experiments would be useful for examining this hypothesis.¶

Deleted: It should be noted that uninoculated individuals that formed nodules were kept in the analysis.

Deleted: belowground biomass carbon costs to acquire nitrogen

546 symbiotic nitrogen-fixing bacteria exhibited no change in belowground carbon allocation
547 compared to their uninoculated counterparts. Under low nitrogen fertilization, inoculated
548 individuals increased plant nitrogen uptake, decreasing the cost of acquiring nitrogen compared
549 to uninoculated individuals. However, inoculation treatment had no effect on plant nitrogen
550 uptake under high nitrogen fertilization. Increasing nitrogen fertilization decreased costs of
551 acquiring nitrogen by increasing plant nitrogen uptake despite no change in belowground carbon
552 allocation. These results indicate that symbiotic nitrogen fixation may provide a competitive
553 advantage to plants growing in nitrogen-poor soils, though these advantages diminish with
554 increasing nitrogen availability as investment in nitrogen uptake through direct uptake pathways
555 increase.

556 **Supplementary data**

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

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

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

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

566

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exhibited reduced belowground biomass carbon costs to acquire nitrogen under the low soil nitrogen fertilization treatment compared to uninoculated counterparts. In contrast, there was no effect of inoculation treatment on belowground biomass carbon costs of nitrogen acquisition under the high soil nitrogen fertilization treatment. Belowground biomass carbon cost to acquire nitrogen differences between treatment combinations were entirely due to changes in plant nitrogen uptake rather than belowground carbon allocation. Treatments that increased plant nitrogen uptake corresponded with enhanced total leaf area and total biomass, suggesting that additional plant nitrogen acquired was being allocated to support aboveground growth.

Deleted: These findings can be used to help improve simulations of carbon-nitrogen economics in terrestrial biosphere models.

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