

1 **SUPPLEMENTARY MATERIAL FOR:** “Nitrogen demand, supply, and acquisition strategy
2 control plant responses to elevated CO₂ at different scales”

3

4 **Methods (cont.)**

5 *Chlorophyll content*

6 Chlorophyll content was extracted from a second leaf in the same trifoliate leaf set as the leaf
7 used to generate A_{net}/C_i curves. A cork borer was used to punch 3-5 0.6 cm² disks from the leaf.
8 Images of each set of leaf disks were curated using a flat-bed scanner to determine wet leaf area,
9 again quantified using the 'LeafArea' R package (Katabuchi, 2015). Leaf disks were shuttled into
10 a test tube containing 10 mL dimethyl sulfoxide, vortexed, and incubated at 65°C for 120
11 minutes (Barnes *et al.*, 1992). Incubated test tubes were vortexed again before being loaded in
12 150 µL triplicate aliquots to a 96-well plate. Dimethyl sulfoxide was loaded in each plate as a
13 single 150 µL triplicate aliquot and used as a blank. Absorbance measurements at 649 nm (A_{649})
14 and 665 nm (A_{665}) were recorded using a plate reader (Bioteck Synergy H1; Bioteck Instruments,
15 Winooski, VT USA), with triplicate measurements averaged and corrected by the mean of the
16 blank absorbance value. Blank-corrected absorbance values were used to estimate Chl_a (µg mL⁻¹)
17 and Chl_b (µg mL⁻¹) following equations from (Wellburn, 1994):

18 $Chl_a = 12.47A_{665} - 3.62A_{649}$ (S1)

19 and

20 $Chl_b = 25.06A_{649} - 6.5A_{665}$ (S2)

21 Chl_a and Chl_b were converted to mmol mL⁻¹ using the molar masses of chlorophyll *a* (893.51 g
22 mol⁻¹) and chlorophyll *b* (907.47 g mol⁻¹), then added together to calculate the total chlorophyll
23 content in dimethyl sulfoxide extractant (mmol mL⁻¹). Total chlorophyll content (mmol) was
24 determined by multiplying the total chlorophyll content in dimethyl sulfoxide by the volume of
25 dimethyl sulfoxide extractant (10 mL). Area-based chlorophyll content (Chl_{area} ; mmol m⁻²) was
26 then calculated by dividing the total chlorophyll content by the total area of the leaf disks.

27

28 χ

29 Leaf δ¹³C was used to estimate the time-integrated ratio of leaf intercellular CO₂ concentration to
30 atmospheric CO₂ concentration (χ , unitless) using leaf δ¹³C and chamber air δ¹³C following
31 Farquhar *et al.* (1989):

$$32 \quad \chi = \frac{a^{13}c - a}{b - a} \quad (S1)$$

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

$$35 \quad \Delta^{13}C = \frac{\delta^{13}C_{air} - \delta^{13}C_{leaf}}{1 + \delta^{13}C_{leaf}} \quad (S2)$$

36 $\delta^{13}\text{C}_{\text{air}}$ is the chamber $\delta^{13}\text{C}$ air fractionation, a represents the fractionation between ^{12}C and ^{13}C
 37 due to diffusion in air, assumed to be 4.4‰, and b represents the fractionation caused by Rubisco
 38 carboxylation, assumed to be 27‰ (Farquhar *et al.*, 1989). $\delta^{13}\text{C}_{\text{air}}$ was quantified in each
 39 chamber by collecting air samples in triplicate for each CO_2 treatment using a 20 mL syringe
 40 (Air-Tite Products Co., Inc., Virginia Beach, VA, USA). Each air sample was plunged into a
 41 manually evacuated 10 mL Exetainer (Labco Ltd., Lampeter, UK) and sent to the University of
 42 California-Davis Stable Isotope Facility, where $\delta^{13}\text{C}_{\text{air}}$ was determined using a gas inlet system
 43 (GasBenchII; Thermo Fisher Scientific, Waltham, MA, USA) coupled to an isotope ratio mass
 44 spectrometer (Thermo Finnigan Delta Plus XL; Thermo Fisher Scientific, Waltham, MA, USA).
 45 $\delta^{13}\text{C}_{\text{air}}$ for each CO_2 treatment was estimated by calculating the mean of the triplicate $\delta^{13}\text{C}_{\text{air}}$
 46 samples within each chamber, then calculating the mean $\delta^{13}\text{C}_{\text{air}}$ across all chambers. Specifically,
 47 $\delta^{13}\text{C}_{\text{air}}$ was -8.81‰ for the ambient CO_2 treatment and -5.95‰ for the elevated CO_2 treatment.

49 Results (cont.)

50 *Photosynthetic nitrogen-use efficiency*

51 eCO₂ increased *PNUE*_{growth} by 90% ($p<0.001$; Table S3; Fig. S2). Inoculation treatment did not
 52 modify the positive effect of eCO₂ on *PNUE*_{growth} (CO₂-by-inoculation interaction: $p>0.05$; Table
 53 2). An interaction between CO₂ and nitrogen fertilization ($p<0.05$; Table S3) indicated that the
 54 positive effect of eCO₂ on *PNUE*_{growth} decreased with increasing nitrogen fertilization (Fig. S2).
 55 This pattern resulted from a negative effect of increasing nitrogen fertilization on *PNUE*_{growth}
 56 ($p<0.001$; Table S3) that was stronger under eCO₂ than aCO₂ (Tukey test comparing the nitrogen
 57 fertilization-*PNUE*_{growth} slope between CO₂ treatments: $p<0.05$). An interaction between nitrogen
 58 fertilization and inoculation ($p<0.001$; Table S3; Fig. S3) indicated that the negative effect of
 59 increasing nitrogen fertilization on *PNUE*_{growth} was driven by inoculated plants (Tukey test of the
 60 nitrogen fertilization-*PNUE*_{growth} slope in inoculated plants: $p<0.001$), as there was no effect of

61 nitrogen fertilization on $PNUE_{growth}$ in uninoculated plants (Tukey test of the nitrogen
62 fertilization- $PNUE_{growth}$ slope in uninoculated plants: $p>0.05$).
63

64 χ

65 An interaction between CO₂ and nitrogen fertilization ($p<0.001$; Table S3) indicated that the
66 negative effect of increasing nitrogen fertilization on χ ($p<0.001$; Table S3) was stronger under
67 elevated CO₂ than ambient CO₂ (Tukey test comparing the nitrogen fertilization- χ slope between
68 CO₂ treatments: $p<0.05$; Fig. S3), resulting in a stronger downregulation of χ under elevated CO₂
69 with increasing fertilization. A three-way interaction ($p<0.001$; Table S3) indicated that
70 interactions between CO₂ and nitrogen fertilization were driven by inoculated plants (Tukey test
71 comparing the nitrogen fertilization- χ slope between inoculated plants grown under ambient CO₂
72 and inoculated plants grown under elevated CO₂: $p<0.001$), as there was no difference in the
73 effect of nitrogen fertilization on χ between CO₂ treatments in uninoculated plants (Tukey test
74 comparing the nitrogen fertilization- χ slope between uninoculated plants grown under ambient
75 CO₂ and uninoculated plants grown under elevated CO₂: $p>0.05$). An interaction between CO₂
76 and inoculation ($p<0.001$; Table S3) indicated that elevated CO₂ decreased χ in uninoculated
77 plants (Tukey test of the CO₂ effect in uninoculated plants: $p<0.001$) and increased χ in
78 inoculated plants (Tukey test of the CO₂ effect in inoculated plants: $p<0.001$).
79

80 *Components of carbon costs to acquire nitrogen*

81 Elevated CO₂ increased C_{bg} by 100% ($p<0.001$; Table S4). Nitrogen fertilization did not modify
82 this pattern (CO₂-by-nitrogen fertilization interaction: $p>0.05$; Table S4). An interaction between
83 CO₂ and inoculation ($p<0.05$; Table S4) indicated that the positive effect of inoculation on C_{bg}
84 ($p<0.001$; Table S4) was only apparent under ambient CO₂ (Tukey test of the inoculation effect
85 under ambient CO₂: $p<0.001$; Fig. S4), as there was no effect of inoculation on C_{bg} under
86 elevated CO₂ (Tukey test of the inoculation effect under elevated CO₂: $p>0.05$). An interaction
87 between nitrogen fertilization and inoculation ($p<0.001$; Table S3) indicated that the positive
88 effect of increasing nitrogen fertilization on C_{bg} ($p<0.001$; Table S3) was stronger in
89 uninoculated plants than inoculated plants (Tukey test comparing the nitrogen fertilization- C_{bg}
90 slope between inoculation treatments: $p<0.001$).

91 Elevated CO₂ increased N_{wp} by 27% ($p<0.001$; Table S4). This pattern was enhanced
92 with increasing nitrogen fertilization (CO₂-by-nitrogen fertilization interaction: $p<0.05$; Table
93 S4) but was not modified by inoculation (CO₂-by-inoculation interaction: $p>0.05$; Table S4). An
94 interaction between nitrogen fertilization and inoculation ($p<0.001$; Table S4) indicated that the
95 positive effect of increasing nitrogen fertilization on N_{wp} ($p<0.001$; Table S4) was stronger in
96 uninoculated plants than inoculated plants (Tukey test comparing the nitrogen fertilization- N_{wp}
97 slope between inoculation treatments: $p<0.001$).
98

99 *Nitrogen fixation*

100 There was no effect of CO₂ treatment on root nodule: root biomass ($p>0.05$; Table S5). This null
101 response was not modified by nitrogen fertilization (CO₂-by-nitrogen fertilization interaction:
102 $p>0.05$; Table S5). However, an interaction between CO₂ and inoculation ($p<0.001$; Table S5)
103 indicated that the positive effect of inoculation on root nodule: root biomass ($p<0.001$; Table S5)
104 was stronger under ambient CO₂ (3129% increase; Tukey test comparing the inoculation effect
105 under ambient CO₂: $p<0.001$) than elevated CO₂ (379% increase; Tukey test comparing the
106 inoculation effect under elevated CO₂: $p<0.001$). An interaction between nitrogen fertilization
107 and inoculation ($p<0.001$; Table S5) indicated that the negative effect of increasing nitrogen
108 fertilization on root nodule: root biomass ($p<0.001$; Table S5) was stronger in inoculated pots
109 than uninoculated plants (Tukey test comparing the nitrogen fertilization-root nodule: root
110 biomass slope between inoculation treatments: $p<0.001$; Fig. S5).

111 Root nodule biomass increased by 30% under elevated CO₂ ($p<0.001$; Table S5). This
112 pattern was not modified by nitrogen fertilization (CO₂-by-nitrogen fertilization interaction:
113 $p>0.05$; Table S5) or inoculation (CO₂-by-inoculation interaction: $p>0.05$; Table S5; Fig. S5).
114 An interaction between nitrogen fertilization and inoculation ($p<0.001$; Table S5) indicated that
115 the negative effect of increasing nitrogen fertilization on root nodule biomass ($p<0.001$; Table
116 S5) was driven by inoculated plants (Tukey test comparing the nitrogen fertilization-root nodule
117 biomass slope in inoculated plants: $p<0.001$), as there was no effect of nitrogen fertilization on
118 root nodule biomass in uninoculated plants (Tukey test comparing the nitrogen fertilization-root
119 nodule biomass slope in uninoculated plants: $p>0.05$; Fig. S5).

120 Root biomass increased by 96% under elevated CO₂ ($p<0.001$; Table S5). An interaction
121 between CO₂ concentration and fertilization ($p<0.001$; Table S5) indicated that the positive

122 effect of increasing nitrogen fertilization on root biomass ($p<0.001$; Table S5) was stronger
123 under ambient CO₂ (Tukey test comparing the nitrogen fertilization-root biomass slope between
124 CO₂ treatments: $p=0.001$). An interaction between CO₂ and inoculation ($p<0.001$; Table S5)
125 indicated that the positive effect of inoculation on root biomass ($p<0.001$; Table S5) was driven
126 by the ambient CO₂ treatment (Tukey test comparing inoculation effect under ambient CO₂:
127 $p<0.001$), as there was no inoculation effect on root biomass under elevated CO₂ (Tukey test
128 comparing inoculation effect under elevated CO₂: $p>0.05$). An interaction between nitrogen
129 fertilization and inoculation ($p<0.001$; Table S5) indicated that the positive effect of increasing
130 nitrogen fertilization on root biomass ($p<0.001$; Table S5) was stronger in uninoculated plants
131 (Tukey test comparing the fertilization-root biomass slope between inoculation treatments:
132 $p=0.001$).

133

134 *The ratio of total biomass to pot volume*

135 Total biomass: pot volume increased with elevated CO₂, inoculation, and nitrogen fertilization
136 ($p<0.001$ in all cases; Table S6). The positive effect of increasing nitrogen fertilization on
137 biomass: pot volume was stronger in uninoculated plants than inoculated plants (Tukey test
138 comparing the nitrogen fertilization-biomass: pot volume slope between inoculation treatments:
139 $p<0.05$; Fig. S6), and when plants were grown under elevated CO₂ compared to ambient CO₂
140 (Tukey test comparing the nitrogen fertilization-biomass: pot volume slope between CO₂
141 treatments: $p<0.001$; Fig. S6).

Table S1 Summary table containing volumes of compounds used to create modified Hoagland's solutions for each soil nitrogen fertilization treatment. All volumes are expressed as milliliters per liter (mL/L)

Compound	0 ppm N (0 mM N)	35 ppm N (2.5 mM N)	70 ppm N (5 mM N)	105 ppm N (7.5 mM N)	140 ppm N (10 mM N)
1 M NH₄H₂PO₄	0	0.165	0.33	0.5	0.67
2 M KNO₃	0	0.335	0.67	1	1.33
2 M Ca(NO₃)₂	0	0.335	0.67	1	1.33
1 M NH₄NO₃	0	0.165	0.33	0.5	0.67
8 M NH₄NO₃	0	0	0	0	0
1 M KH₂PO₄	1	0.85	0.67	0.5	0.33
1 M KCl	3	2.45	2	1.5	1
1 M CaCO₃	4	3.33	2.67	2	1.33
2 M MgSO₄	1	1	1	1	1
10% Fe-EDTA	1	1	1	1	1
Trace elements	1	1	1	1	1

Compound	210 ppm N (15 mM N)	280 ppm N (20 mM N)	350 ppm N (25 mM N)	630 ppm N (45 mM N)
1 M NH₄H₂PO₄	1	1	1	1
2 M KNO₃	2	2	2	2
2 M Ca(NO₃)₂	2	2	2	2
1 M NH₄NO₃	1	3.5	0	0
8 M NH₄NO₃	0	0	0.75	2
1 M KH₂PO₄	0	0	0	0
1 M KCl	0	0	0	0
1 M CaCO₃	0	0	0	0
2 M MgSO₄	1	1	1	1
10% Fe-EDTA	1	1	1	1
Trace elements	1	1	1	1

Table S2 Summary of the daily growth chamber growing condition program

Time	Air temperature (°C)	PAR ± SD ($\mu\text{mol m}^{-2} \text{s}^{-1}$)
09:00	21	278±2
09:45		557±4
10:30	25	797±4
11:15		1230±12
22:45	21	797±4
23:30		557±4
00:15	17	278±2
01:00		0±0

Table S3 Effects of CO₂ concentration, inoculation, and nitrogen fertilization on photosynthetic nitrogen-use efficiency and χ^*

		<i>PNUE</i> _{growth}		χ	
	df	χ^2	p	χ^2	p
CO ₂	1	300.197	<0.001	6.809	0.009
Inoculation (I)	1	9.897	0.002	5.827	0.016
N fertilization (N)	1	29.695	<0.001	109.544	<0.001
CO ₂ *I	1	0.944	0.331	20.644	<0.001
CO ₂ *N	1	5.359	0.021	11.839	<0.001
I*N	1	10.883	<0.001	0.013	0.909
CO ₂ *I*N	1	0.369	0.544	16.901	<0.001

*Significance determined using Type II Wald χ^2 tests ($\alpha=0.05$). P-values less than 0.05 are in bold. Key: df=degrees of freedom, χ^2 =Wald chi-square test statistic, *PNUE*_{growth}=photosynthetic nitrogen-use efficiency ($\mu\text{mol CO}_2 \text{ gN}^{-1} \text{ s}^{-1}$), χ =isotope-based ratio of intercellular CO₂ to extracellular CO₂, inversely related to water-use efficiency (unitless)

Table S4 Effects of CO₂ concentration, inoculation, and nitrogen fertilization on components of the carbon cost to acquire nitrogen*

			<i>C_{bg}</i> ^a		<i>N_{wp}</i> ^b	
	df	χ^2	<i>p</i>	χ^2	<i>p</i>	
CO ₂	1	84.134	<0.001	23.890	<0.001	
Inoculation (I)	1	41.030	<0.001	134.460	<0.001	
N fertilization (N)	1	152.248	<0.001	529.021	<0.001	
CO ₂ *I	1	8.965	0.003	1.190	0.275	
CO ₂ *N	1	1.188	0.276	5.915	0.015	
I*N	1	22.648	<0.001	55.562	<0.001	
CO ₂ *I*N	1	1.109	0.292	0.620	0.431	

*Significance determined using Type II Wald χ^2 tests ($\alpha=0.05$). A superscript “^a” is included after trait labels to indicate if models were fit with natural-log transformed response variables, while a superscript “^b” is included if models were fit with square-root transformed response variables. *P*-values less than 0.05 are in bold. Key: df=degrees of freedom, *C_{bg}*=belowground carbon biomass (gC, numerator of *N_{cost}*), *N_{wp}*=total nitrogen biomass (gN, denominator of *N_{cost}*).

Table S5 Effects of CO₂ concentration, inoculation, and nitrogen fertilization on investment toward symbiotic nitrogen fixation*

	Root nodule biomass ^b			Root biomass ^b			Root nodule: root biomass ^b	
	df	χ^2	p	χ^2	p	χ^2		p
CO ₂	1	19.258	<0.001	93.249	<0.001	0.010	0.921	
Inoculation (I)	1	755.02	<0.001	6.983	0.008	902.063	<0.001	
N fertilization (N)	1	84.376	<0.001	195.843	<0.001	254.741	<0.001	
CO ₂ *I	1	0.950	0.330	3.873	0.049	21.632	<0.001	
CO ₂ *N	1	2.106	0.147	11.456	<0.001	1.590	0.207	
I*N	1	44.622	<0.001	7.435	0.006	132.463	<0.001	
CO ₂ *I*N	1	0.196	0.658	0.065	0.799	2.481	0.115	

*Significance determined using Type II Wald χ^2 tests ($\alpha=0.05$). A superscript “^a” is included after trait labels to indicate if models were fit with natural log-transformed response variables, while a superscript “^b” is included if models were fit with square-root transformed response variables. P-values less than 0.05 are in bold. Key: df=degrees of freedom, root nodule biomass (g), root biomass (g), root nodule: root biomass (unitless).

Table S6 Effects of CO₂ concentration, inoculation, and nitrogen fertilization on the ratio of total biomass to pot volume (g L⁻¹)^{*}

	df	χ^2	p
CO ₂	1	146.004	<0.001
Inoculation (I)	1	19.320	<0.001
N fertilization (N)	1	279.388	<0.001
CO ₂ *I	1	0.007	0.934
CO ₂ *N	1	49.725	<0.001
I*N	1	9.007	0.003
CO ₂ *I*N	1	0.640	0.434

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

Figure S1

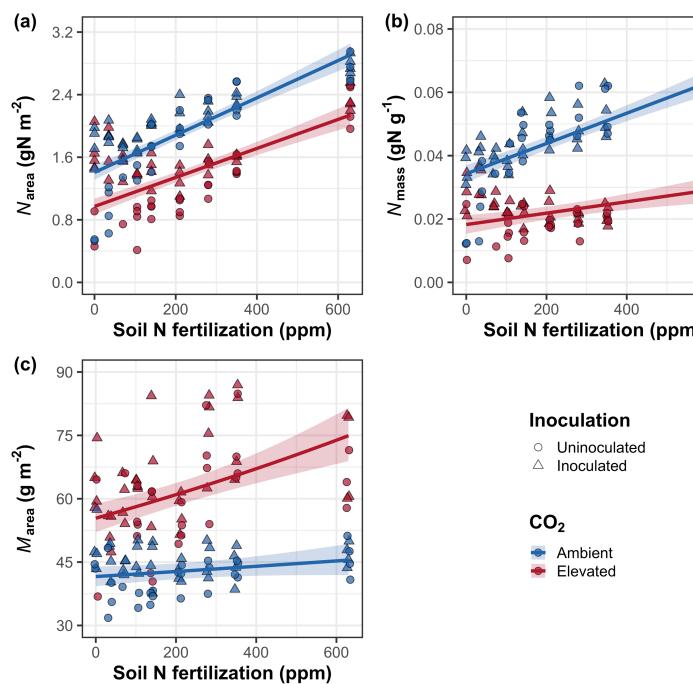


Figure S1 Effects of CO₂ and fertilization inoculation on area-based leaf nitrogen content (a), mass-based leaf nitrogen content (b), and leaf biomass per unit leaf area (c). Fertilization is represented on the x-axis. Red shaded points and trendlines indicate plants grown under elevated CO₂, while blue shaded points and trendlines indicate plants grown under ambient CO₂. Light blue and red circular points and trendlines indicate measurements collected from uninoculated plants, while dark blue and red triangular points indicate measurements collected from inoculated plants. Solid trendlines indicate regression slopes that are different from zero ($p < 0.05$), while dashed trendlines indicate slopes that are not distinguishable from zero ($p > 0.05$).

Figure S2

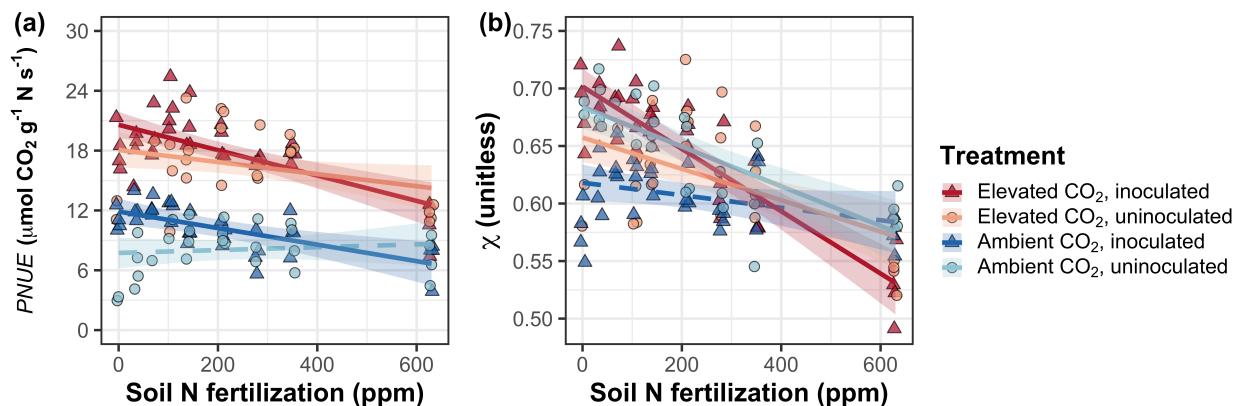


Figure S2 Effects of nitrogen fertilization, inoculation treatment, and CO₂ treatment on photosynthetic nitrogen-use efficiency (a) and χ (b). Fertilization is represented on the x-axis. Red shaded points and trendlines indicate plants grown under eCO₂, while blue shaded points and trendlines indicate plants grown under aCO₂. Light blue and red circular points and trendlines indicate measurements collected from uninoculated plants, while dark blue and red triangular points indicate measurements collected from inoculated plants. Solid trendlines indicate regression slopes that are different from zero ($p < 0.05$), while dashed trendlines indicate slopes that are not distinguishable from zero ($p > 0.05$).

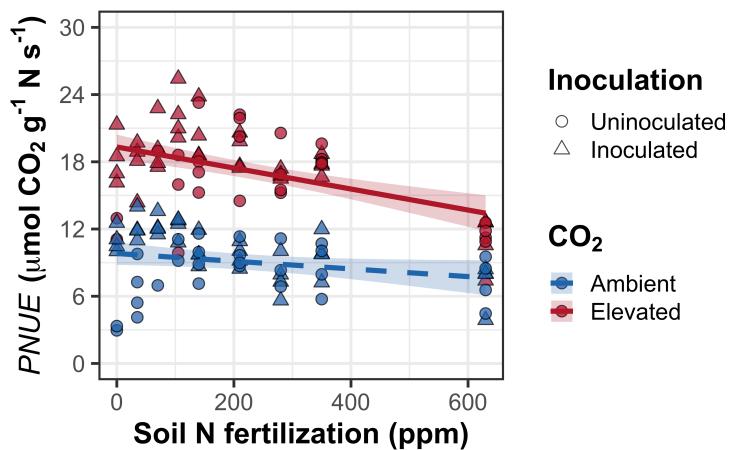
Figure S3

Figure S3 Effects of CO₂ and fertilization inoculation on photosynthetic nitrogen-use efficiency. Fertilization is represented on the x-axis. Red shaded points and trendlines indicate plants grown under eCO₂, while blue shaded points and trendlines indicate plants grown under aCO₂. Light blue and red circular points and trendlines indicate measurements collected from uninoculated plants, while dark blue and red triangular points indicate measurements collected from inoculated plants. Solid trendlines indicate regression slopes that are different from zero ($p < 0.05$), while dashed trendlines indicate slopes that are not distinguishable from zero ($p > 0.05$).

Figure S4

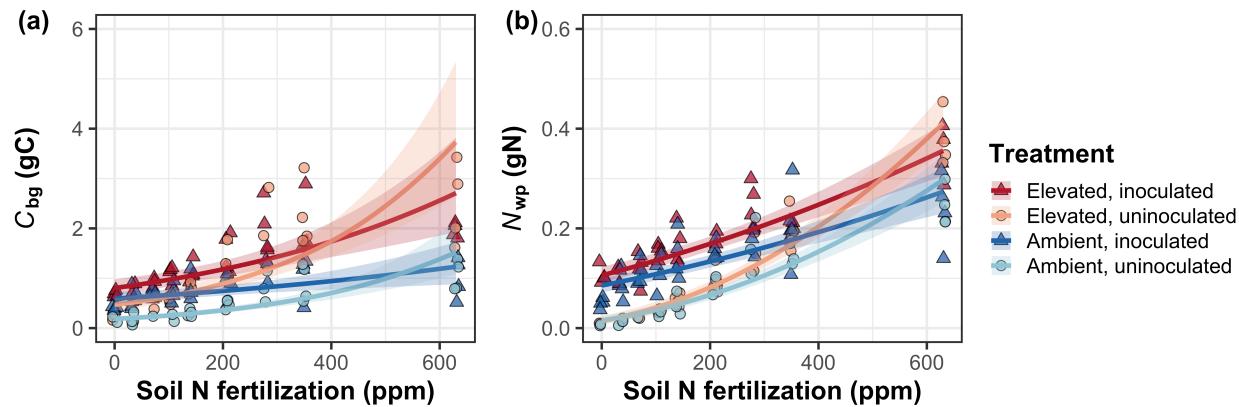


Figure S4 Effects of CO₂ and fertilization inoculation on belowground carbon biomass (a) and total nitrogen biomass (b). Belowground carbon biomass is the numerator of N_{cost} , while total nitrogen biomass is the denominator of N_{cost} . Fertilization is represented on the x-axis in all panels. Red shaded points and trendlines indicate plants grown under eCO₂, while blue shaded points and trendlines indicate plants grown under aCO₂. Light blue and red circular points and trendlines indicate measurements collected from uninoculated plants, while dark blue and red triangular points indicate measurements collected from inoculated plants. Solid trendlines indicate regression slopes that are different from zero ($p < 0.05$), while dashed trendlines indicate slopes that are not distinguishable from zero ($p > 0.05$).

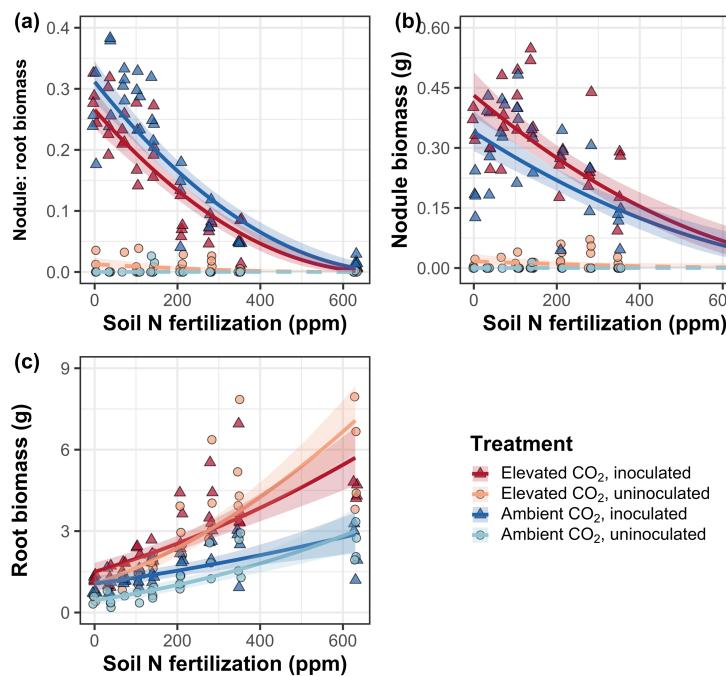
Figure S5

Figure S5 Effects of nitrogen fertilization, inoculation treatment, and CO₂ treatment on root nodule biomass: root biomass (a), root nodule biomass (b), and root biomass (c). Nitrogen fertilization is represented on the x-axis. Red shaded points and trendlines indicate plants grown under eCO₂, while blue shaded points and trendlines indicate plants grown under aCO₂. Light blue and red circular points and trendlines indicate measurements collected from uninoculated plants, while dark blue and red triangular points indicate measurements collected from inoculated plants. Solid trendlines indicate regression slopes that are different from zero ($p < 0.05$), while dashed trendlines indicate slopes that are not distinguishable from zero ($p > 0.05$).

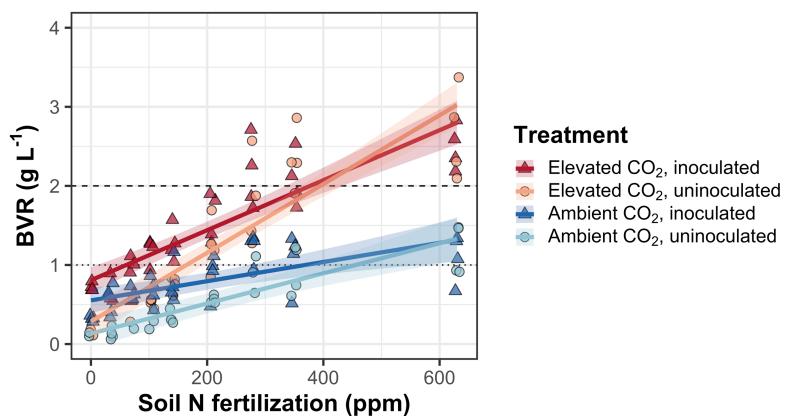
Figure S6

Figure S6 Effects of CO₂, fertilization, and inoculation on the ratio of whole plant biomass to pot volume. Fertilization is represented on the x-axis. Red shaded points and trendlines indicate plants grown under eCO₂, while blue shaded points and trendlines indicate plants grown under aCO₂. Light blue and red circular points and trendlines indicate measurements collected from uninoculated plants, while dark blue and red triangular points indicate measurements collected from inoculated plants. Solid trendlines indicate regression slopes that are different from zero ($p<0.05$). The dotted horizontal line indicates the point where biomass: pot volume exceeds 1 g L⁻¹, and the dashed line indicates the point where biomass: pot volume exceeds 2 g L⁻¹.