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Dear Editorial Board at *Plant, Cell & Environment*,

Thank you for the opportunity to resubmit our manuscript (PCE-24-0855), titled “Nitrogen demand, supply, and acquisition strategy control plant responses to elevated CO2”, to *Plant, Cell & Environment*. Please find our revised manuscript attached. We have also included a copy of the revised manuscript with changes noted through the “Track Changes” feature in Microsoft Word.

We thank the two reviewers for their helpful and constructive feedback, as we find that the comments have helped clarify and improve the manuscript’s interpretation. The manuscript now includes additional growth analyses suggested by the first reviewer to contextualize whole-plant responses to treatment combinations. Specifically, we have added leaf area ratio to the main text, as well as organ mass fractions to the supplemental material. We have also attempted to clarify the Introduction as recommended by the second reviewer, and have included justification for our use of 1000 ppm CO2 as the high CO2 treatment as recommended by both reviewers.

Below, we provide a point-by-point response to reviewer comments. We first include the reviewer comment in black-colored font and include our response directly below each reviewer comment in red-colored font. Where possible, we reference line numbers and copy major text additions below each comment to facilitate review.

If you have any questions or concerns about our revised and resubmitted manuscript, please contact me using the e-mail listed above.

Sincerely,

Evan A. Perkowski, Ph.D.

*On behalf of coauthors Ezinwanne Ezekannagha and Nicholas G. Smith*

Referee: 1  
  
Comments to the Author  
In this manuscript, the authors examined effects of nitrogen fertilization and inoculation of N2 fixing bacteria on leaf photosynthesis and growth of soybean plants that were grown at two different CO2 concentrations. They set two interesting and different hypotheses on leaf photosynthesis or plant growth, respectively, and conducted enormous measurements of photosynthesis and growth. Some data are very interesting, and I think their data should be published. However, I cannot find any novelty of this study. Prof. Makino’s group already published similar results with rice plants more than 20 years ago (Nakano et al. 1997 Plant Physiol, 115: 191-198; Makino et al. 1997 Plant Physiol, 115: 199-203). The plant materials and presentations are quite different, but the essential aims are the same. What did the authors give us newly? Also, why did not the authors conduct growth analysis? Several parameters of growth analysis such as RGR, NAR, LAR, NNUR should tell us more direct evidence.

We thank the first reviewer for the kind words about our manuscript motivations. We acknowledge that work from Prof. Makino’s group has published a series of previous publications that suggest similar responses to nitrogen availability previous work. As the reviewer points out, the plant materials are quite different, as the cited work from Prof. Makino’s group report findings in rice, a species that is not capable of forming associations with symbiotic nitrogen-fixing bacteria. We directly manipulate ability to associate with nitrogen-fixing bacteria in our experiment, expecting that symbiotic nitrogen fixation would minimize any impact of nitrogen fertilization on leaf and whole-plant responses to elevated CO2. While the essential aims of our manuscript and Prof. Makino’s group are the same, inoculation treatments imposed in our experiment provide additional context for understanding the role of symbiotic nitrogen fixation on plant responses to elevated CO2. Studies that examine effects of symbiotic nitrogen fixation on leaf and whole plant responses to elevated CO2 across a nitrogen fertilization gradient are rare, but necessary as terrestrial biosphere models include explicit nitrogen cycle dynamics.

Growth analyses were not practical due to growth chamber space limitation and the large number of fertilization treatments that warranted destructive biomass harvest intervals throughout the experiment not feasible. We refrain from including RGR, NAR, and NNUR in the manuscript, as these variables would be calculated using final standing stock biomass and would therefore assume a linear growth pattern and nitrogen uptake rates throughout vegetative growth. Soybean does not exhibit a linear growth rate pattern throughout its vegetative growth phase, so the inclusion of these traits with the current dataset might be misleading. However, we have included LAR and a few relative allocation traits (e.g., root:shoot ratio and organ mass fractions) to contextualize growth responses to treatment combinations. In short, these analyses indicate… . These results are now included in the *Supplement* (Table SX; Fig. SX) and are referenced in the Discussion section (lines XX-XX).

1. I suggest that the authors firstly plot graphs of all photosynthetic parameters against leaf N per area, which should tell them whether the CO2 elevation or inoculation treatment independently affect relationships between photosynthetic parameters and leaf N.

The suggestion from the reviewer is useful if one is interested in understanding scenarios where leaf N-photosynthesis relationships become decoupled. Previous work from our lab has shown that changes in nitrogen availability and factors that influence demand to build and maintain photosynthetic enzymes (e.g., light) each modify relationships between leaf N and photosynthetic traits (Waring et al., 2023). However, understanding whether CO2 or inoculation treatment independently affected relationships between photosynthetic traits and leaf N is not a key component of our paper, especially because elevated CO2 increased root nodule biomass and the magnitude of nitrogen plants acquired through the symbiosis.

Our present analyses allow us to make inferences about the effects of our treatment combinations about leaf N-photosynthesis relationships without altering the fundamental structure of the paper. Specifically, elevated CO2 decreased area-based leaf nitrogen content more strongly than it decreased Vcmax25 and Jmax25, while inoculation increased Vcmax25 and Jmax25 more strongly than it increased area-based leaf nitrogen content. Following equations in Niinemets et al. (1997), these patterns indicate that elevated CO2 increased the fractional pool of leaf nitrogen content allocated to Rubisco and bioenergetics, consistent with the idea that elevated CO2 increased photosynthetic nitrogen use efficiency, while inoculation decreased the fractional pool of leaf nitrogen content allocated to Rubisco and bioenergetics. These patterns indicate that all treatments modified leaf N-photosynthesis relationships. This content is currently included in the *Modeling Implications* subsection of the Discussion, starting on line 614. We have made efforts to make this subsection more explicit by adding the following sentence starting on line 619:

“Specifically, elevated CO2 reduced leaf nitrogen content more strongly than it increased *A*net,gc and decreased *V*cmax25 and *J*max25, while inoculation increased *V*cmax25 and *J*max25 more strongly than it increased *N*area. These patterns indicate that elevated CO2 increased the fractional pool of leaf nitrogen content allocated to Rubisco and bioenergetics, while inoculation decreased the fraction of leaf nitrogen content allocated to Rubisco and bioenergetics (Niinemets & Tenhunen, 1997).”

1. What cultivar of soybean did the authors use? Responses to elevated CO2 or nitrogen should largely change depending on cultivar differences.

This information is not available to us. We purchased seeds from Territorial Seed Company, who do not keep track of the cultivar. We have disclosed this in the manuscript on line XX and have included where seeds were purcahsed.

1. Why did the authors use 1000 ppm CO2 as the elevated CO2 condition? This concentration seems a bit too high.

While 1000 ppm CO2 is indeed high compared to previous elevated CO2 studies, the Intergovernmental Panel on Climate Change predicts that atmospheric CO2 concentrations will likely exceed 1000 ppm CO2 by 2100 under the Shared Socioeconomic Pathway 5-8.5 (Figure TS.4; Figure 4.3; IPCC 2021). We have included the following sentence to the Methods to justify this decision, starting on line 193:

“These treatments were based on current ambient CO2 concentrations and projections from the Intergovernmental Panel on Climate Change indicating that CO2 concentrations could surpass 1000 ppm by 2100 under the Shared socioeconomic Pathway 5-8.5 (IPCC 2021).”

Referee: 2

Comments to the Author

The manuscript entitled “Nitrogen demand, availability, and acquisition strategy control plant responses to elevated CO2” by Perkowski et al. concerns an interesting topic. The authors conducted experiments with 2 CO2, 2 inoculation, and 9 nitrogen fertilization treatments to explore the responses at the leaf and whole plant scales. This research result supports the eco-evolutionary optimality hypothesis at the leaf scale, where elevated CO2 increased photosynthetic rate by optimizing leaf nitrogen allocation. In addition, this study also supports the nitrogen limitation hypothesis at the whole plant scale, nitrogen availability enhanced whole-plant responses to elevated CO2 due to increased plant nitrogen uptake and reduced costs of nitrogen acquisition. These results are beneficial for further optimizing the model and better understanding the carbon and nitrogen cycling of ecosystems in the context of global change. However, the manuscript had some issues here reported. I recommend the manuscript to be accepted after major revision.

We thank the reviewer for their accurate summary of our manuscript and have made efforts to address these issues mentioned below.

General comments:  
1.      The introduction section is confusing and redundant. The author needs to reorganize the introduction section based on the two hypotheses proposed in this experiment. The current version mostly only lists previous research results.

Thank you for this comment. We have streamlined the introduction to reduce its redundancy and lessen confusion. This was primarily accomplished by removing two paragraphs: the second paragraph that summarized consistent leaf- and whole-plant responses to elevated CO2, and the fourth paragraph that speculated on the role of nitrogen limitation on leaf responses to elevated CO2. The

This was primarily accomplished by removing the second paragraph that summarized consistent leaf and whole-plant responses to elevated CO2 that have been documented over the past few decades, and a paragraph dis

, as these citations and general patterns are already explained and cited in the paragraphs that discuss the nitrogen limitation and eco-evolutionary optimality hypotheses. We have also clarified sentences where necessary and have revised topic sentences of a few paragraphs to lessen confusion and clarify the information logic.

2.      The concentration of elevated CO2 treatment is 1000 μmol mol-1, which is very high relative to the current CO2 level. What is the basis for setting this concentration? Please further explain in the manuscript the reasons why the author set this concentration.

This CO2 treatment was assigned based on the idea that atmospheric CO2 concentrations under the Shared Socioeconomic Pathway 5-8.5 are predicted to exceed 1000 ppm CO2 (Fig. 4.3, IPCC 2021). We have included the following sentence to the Methods, starting on line 193:

“These treatments were based on current ambient CO2 concentrations and projections from the Intergovernmental Panel on Climate Change indicating that CO2 concentrations could surpass 1000 ppm by 2100 under the Shared socioeconomic Pathway 5-8.5 (IPCC 2021).”

3.      The method description in the study is not detailed enough. For example, in line 229, “the center leaf of the most recent fully expanded trifoliate leaf” was selected for measuring leaf photosynthesis. How many leaves do plants have in total, and why did they choose the leaf? Is there a difference in the observation indicators of different leaf positions?

Soybean forms a pair of opposite unifoliate leaves after the cotyledons open. After unifoliate leaves form, soybean grows a series of alternating trifoliate leaf sets, which are compound leaves that are made up of three leaflets at the end of a single petiole. Following standard plant ecophysiology practice (e.g., as explained in Busch et al., 2024), we measured leaf photosynthetic traits on the most recent fully expanded leaflet set. We chose to measure photosynthetic traits on the center leaflet of the most recent fully expanded trifoliate leaf set to standardize across individuals of different treatment combinations. This was also done to minimize any difference between investment toward photosynthetic tissues between leaflets, though one might expect photosynthetic processes in leaflets to be non-independent of each other given their compound leaf structure. We have changed “leaf” to “leaflet” and have screened the rest of this section to avoid reader confusion.

4.      There are still some errors in the manuscript, such as in line 419, which should refer to Table S3 instead of Table S4. The authors need to further check the details of the manuscript.

We thank the reviewer for their careful eye, although note that the manuscript correctly identified that dark respiration results were reported in Table S4. Regardless, a final proofreading session prior to re-submission has been completed to ensure no other major notational or grammatical errors are present.

5.      Line 403, “Elevated CO2 decreased Anet,420 by 17% and increased Anet,gc”. Please explain the reason in the discussion. Line 411-416, elevated CO2 reduced Vcmax more than Jmax, please explain the reason in the discussion.

We have added an explanation for the Anet,420 response starting on line 564:

“Individuals grown under elevated CO2 experienced a reduction in *A*net,420 that stemmed from a reduction in apparent photosynthetic capacity and stomatal conductance compared to individuals grown under ambient CO2”

An explanation for the increase in *A*net,gc under elevated CO2 is already included in the manuscript starting on line 540. However, we have added “under growth CO2 conditions” to clarify that optimal resource investment to photosynthetic capacity likely drove increased operational net photosynthesis rates. These sentences are included below, with the addition underlined, for ease of review:

“In further support of the hypothesis, increased *J*max25:*V*cmax25 and *PNUE*gc provide strong support for the idea that leaves were downregulating *V*cmax25 in response to elevated CO2 such that enhanced net photosynthesis rates under growth conditions approached optimal coordination of Rubisco carboxylation and electron transport for RuBP regeneration (Chen *et al.*, 1993; Maire *et al.*, 2012; Smith & Keenan, 2020), decreasing leaf-level demand for building and maintaining photosynthetic enzymes (Dong et al., 2022b) while maximizing net carbon assimilation.”

6.      In the first paragraph of the discussion section, the author compared Anet,gc under different treatments. When measuring Anet,gc, the CO2 concentration set by the photosynthetic instrument is different under different CO2 treatments, which cannot reflect the effect of CO2 treatment. The difference in Anet,gc may be mainly due to different CO2 concentrations set by the photosynthetic instrument during the measurement process. Anet,420 can better reflect the photosynthetic capacity of different treatments because the photosynthetic instrument is set under the same conditions.

The reviewer is correct that Anet,gc were measured under different CO2 conditions; however, these CO2 conditions were reflective of the growth CO2 conditions each plant was grown under. Anet,gc was quantified at 420 μmol mol-1 for individuals that received the ambient CO2 treatment, while Anet,gc was quantified at 1000 μmol mol-1 for individuals that received the elevated CO2 treatment. We disagree with the reviewer’s suggestion that Anet,gc does not reflect the effect of CO2 treatment, as greater net carbon assimilation rates and total leaf area under elevated CO2 fostered greater biomass accumulation compared to individuals grown under ambient CO2. However, we agree that Anet,gc does not give us much information about how CO2 treatments altered investment toward photosynthetic enzymes, as implied by the reviewer, which is why we include both Anet,gc and Anet,420 in the manuscript to allow us to scale leaf photosynthetic responses to elevated CO2 to whole-plant responses (i.e. through Anet,gc) and also investigate how CO2 treatments altered biochemical investment toward photosynthesis.

**References**

**Busch FA, Ainsworth EA, Amtmann A, Cavanagh AP, Driever SM, Ferguson JN, Kromdijk J, Lawson T, Leakey ADB, Matthews JSA, *et al.*** **2024**. A guide to photosynthetic gas exchange measurements: Fundamental principles, best practice and potential pitfalls. *Plant Cell and Environment*.