Evan A. Perkowski, Ph.D.

Postdoctoral Research Associate

Dept. of Biological Sciences

Texas Tech University

2901 Main St.

Lubbock, TX 79409

[Evan.a.perkowski@ttu.edu](mailto:Evan.a.perkowski@ttu.edu)

June 3, 2024

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, leaf mass fraction, stem mass fraction, and root mass fraction to the supplemental material of the manuscript. 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 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. Furthermore, we quantify leaf and whole-plant responses to elevated CO2 using the same set of plants, providing additional context to studies that quantify these responses using separate plants (e.g., Nakano et al., 1997; Makino et al., 1997), or rely on meta-analytic techniques to diagnose such responses ().

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 throughout vegetative growth. Soybean does not exhibit a linear growth rate pattern throughout its vegetative growth phase. However, we have included LAR and a few relative allocation traits (e.g., root, stem, and leaf 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 elevation or inoculation treatment independently affect 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 each treatment modifies 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 does not keep track of the cultivar. We have disclosed this in the manuscript on line XX.

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, 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.

We find that the Introduction is currently structured in a way that is based on the two hypotheses proposed in the experiment. The introduction starts by summarizing the large swath of previous literature that has investigated plant responses to elevated CO2. It then provides arguments for the nitrogen limitation hypothesis, followed by arguments for the eco-evolutionary optimality hypothesis. It then discusses that the two competing hypotheses may be a matter of scale and explains that experiments which measure both leaf and whole-plant responses to elevated CO2 concurrently would be a useful method for reconciling these competing hypotheses. We find that the information logic and flow through the introduction does exactly what the reviewer is requesting, though have gone through the subsection and refined language in efforts to minimize reader confusion.

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, we measured leaf photosynthetic traits on the most recent fully expanded leaf. 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. We have changed “leaf” to “leaflet” and have screened the rest of this section to make things clearer.

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. A final proofreading session prior to re-submission has been completed, which has corrected this point and any other discrepancies in the manuscript.

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.

These are common photosynthetic responses to elevated CO2. Reduced Anet,420 under elevated CO2 was likely the direct product of reduced apparent photosynthetic capacity and stomatal conductance under elevated CO2. This is because stronger downregulations in Vcmax than Jmax optimized investment toward photosynthetic capacity under elevated CO2, causing reduced net photosynthesis rates in plants grown under elevated CO2 compared to plants grown under ambient CO2. The increase in Anet,gc under elevated CO2 indicates that plants had greater net carbon assimilation rates under elevated CO2, which was likely due to optimized investment toward photosynthetic enzymes that maximized resource use efficiencies with greater substrate availability. These mechanisms are explained in the manuscript on lines 540-546. We have provided additional context by adding “under growth conditions” after “enhanced growth conditions”. These sentences are included below 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).”

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.

We disagree with the reviewer. Anet,gc does indeed reflect the effect of the CO2 treatment on net photosynthesis rates, as these are the carbon assimilation rates achieved under the growth conditions the plants were growing under. In other words, plants growing under elevated CO2 experienced greater net photosynthesis rates under their growth condition (1000 ppm CO2) than their ambient CO2 (420 ppm CO2) counterparts, for reasons explained in the previous comment. This partly explains why individuals growing under elevated CO2 had greater biomass, as greater photosynthesis and greater total leaf area fostered greater biomass accumulation compared to individuals growing under ambient CO2. The reviewer is correct that these rates do not tell us much about the effect of CO2 treatments on investment toward photosynthetic capacity and that Anet,420 is more reflective of this investment. This is why we included both measurements in the manuscript as well as why we conducted A/Ci curves to estimate apparent photosynthetic capacities. We have refined the language in the Discussion section to clarify these explanations.