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)

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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, availability, and acquisition strategy control plant responses to elevated CO2”, to *Plant, Cell & Environment*. Please find our revised manuscript attached. We have included a manuscript copy with changes noted through the “Track Changes” feature in Microsoft Word.

We thank the editor and two reviewers for their constructive feedback. The revised manuscript now includes organ mass fractions following a request from the first reviewer to include additional traits to contextualize the whole-plant responses to treatment combinations. We have also revised the Introduction to follow more closely with the two hypotheses tested by the manuscript, following recommendation from the second reviewer. Finally, we have included a justification for our decision to use 1000 ppm CO2 as the high CO2 treatment, following recommendation by both reviewers. Beyond the reviewer comments, we have also restructured the Discussion to follow more closely with the hypotheses tested and have made sentence-level revisions throughout to streamline content. We feel these changes have improved the manuscript and have improved its clarity and readability.

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

Please contact me using the e-mail listed above over any questions or concerns about our revised manuscript. We would prefer if the revised manuscript could be reviewed by the same referees as the previous manuscript version, although we certainly understand if other reviewers are needed.

Sincerely,

Evan A. Perkowski, Ph.D.

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

Associate Editor: 1

Comments to the Author:

(There are no comments.)

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.

**Response**: We appreciate the first reviewer’s positive feedback on the motivations of our manuscript. We acknowledge that Prof. Makino’s group has published several studies on the role of nitrogen availability on leaf and whole-plant responses to elevated CO2. As the reviewer points out, the plant materials are quite different, as the cited work from Prof. Makino’s group reports findings in rice, a species incapable of forming associations with symbiotic nitrogen-fixing bacteria. We directly manipulated the ability of soybean 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. The inoculation treatments used in our experiment provide additional context for understanding the role of symbiotic nitrogen fixation on plant responses to elevated CO2 across nitrogen fertilization gradients and provide novel insight into understanding the role of nutrient acquisition strategy on plant responses to elevated CO2. Understanding these dynamics is important because terrestrial biosphere models vary greatly in their formulation of nitrogen fixation, with downstream consequences for accurately simulating terrestrial carbon sink dynamics in response to increasing CO2 concentrations (Davies-Barnard et al., 2020). Data from experiments, such as the one done here, will refine the method in which these processes are formulated.

We agree that growth analyses would be a useful addition to the manuscript. However, detailed growth analyses were not practical due to growth chamber space limitation. We would have needed to conduct at least two additional experiment iterations to have enough replication in each of the 36 treatment combinations to be able to have multiple destructive harvest timepoints throughout the experiment. While we could calculate these growth parameters using final standing biomass, doing so would assume that individuals demonstrated a linear growth and nitrogen uptake pattern throughout the growth period, which is not the case for soybean and might therefore provide misleading results. Without additional harvest timepoints, these growth analyses would also likely not differ from the results reported for total biomass or whole-plant nitrogen biomass. Thus, we refrain from including these growth analyses in the manuscript, although the reviewer mentions a suite of traits that we will consider for future experiments.

While growth analyses were not practical, there are other traits at the whole-plant scale that we can include in the manuscript to contextualize the whole-plant responses to treatment combinations. The revised manuscript now includes a few relative allocation traits (root:shoot ratio and organ mass fractions) that help understand where plants allocated additional biomass across the different treatment combinations. In short, these analyses indicate that the root:shoot ratio decreased under elevated CO2, a response that stemmed from an increase in the leaf mass fraction and no change in the stem or root mass fractions. These results suggest that individuals were preferentially allocating biomass to aboveground tissues, specifically to leaves, contrasting previous work suggesting that elevated CO2 induces stronger belowground allocation responses. These allocation responses indicate that individuals responded to elevated CO2 by increasing the leaf surface area for light interception, possibly amplifying the positive effects of elevated CO2 on biomass accumulation by increasing total leaf area. These allocation results are now included in the main text (lines 470-486) and are referenced in the Discussion that explains whole-plant responses to treatment combinations (lines 587-618). Additionally, to avoid reader confusion, we have removed “growth” after “whole-plant” throughout the manuscript to clarify that we are assessing whole-plant responses to elevated CO2 and avoid misleading readers into thinking the manuscript reports the growth parameters mentioned by the reviewer.

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.

**Response**: Thank you for this comment. This 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) modify relationships between leaf N and photosynthetic traits (Waring et al., 2023). However, upon some reflection, understanding whether CO2 or inoculation treatment independently affected relationships between photosynthetic traits and leaf nitrogen content is not necessary to understand whether photosynthetic responses to elevated CO2 followed patterns expected from the nitrogen limitation or eco-evolutionary optimality hypothesis. Additionally, all treatments (CO2, inoculation, and nitrogen fertilization) influenced root nodulation and the magnitude of nitrogen that inoculated plants acquired through the symbiosis, so it would be a challenge to make inferences about the independence of treatment combinations through this approach. Instead, we refer to leaf N-photosynthesis relationships in the Discussion section (lines 657-672) as an implication of the work and do not seek to evaluate these relationships explicitly. This would, however, be an interesting follow up manuscript.

Our present analyses allow us to make inferences about the effects of our treatment combinations on 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 *V*cmax25 and *J*max25, while inoculation increased *V*cmax25 and *J*max25 more strongly than it increased area-based leaf nitrogen content. Following equations from Niinemets & Tenhunen (1997), these patterns indicate that elevated CO2 increased the fractional pool of leaf nitrogen content allocated to Rubisco and bioenergetics, consistent with our finding 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 CO2 and inoculation treatments each modified leaf N-photosynthesis relationships. This content is currently included in the *Modeling Implications* subsection of the Discussion, starting on line 654. We have made efforts to make this subsection more explicit by adding the following sentence starting on line 660:

“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 may have 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.

**Response**: This information unfortunately is not available to us. We purchased seeds from Territorial Seed Company, who do not keep track of the cultivar used. However, personal communication with the company implies that all seeds from a given lot number are sourced from the same cultivar. We have disclosed this in the manuscript on line 177 and have included the company where seeds were purchased. However, it is important to note that, while the magnitude of change in the response of plants to elevated CO2 or nitrogen might change depending on cultivar choice, we do not expect the directionality of these responses to change based on previous meta-analyses reporting soybean responses to elevated CO2 using studies that have different cultivars (Ainsworth et al., 2002)

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

**Response**: The reviewer makes a great point here. While 1000 ppm CO2 is indeed high compared to previous elevated CO2 studies, this treatment was based on projections from the Intergovernmental Panel on Climate Change that indicated that CO2 concentrations will likely surpass 1000 ppm by 2100 under the Shared Socioeconomic Pathway 5-8.5 (IPCC 2021). We have included this justification in the main text starting on line 189:

“CO2 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.

**Response**: 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.

**Response**: Thank you for this comment. We have revised the Introduction section in efforts to reduce its redundancy and lessen confusion. We have reduced redundancy by removing the paragraph that discussed the lack of evidence supporting the nitrogen limitation hypothesis at the leaf level, as the sentences and citations included in this paragraph were interspersed throughout the second part of the Introduction that discussed patterns expected from eco-evolutionary optimality. We have also reduced redundancy by shortening the second paragraph that summarizes consistent leaf and whole-plant responses to elevated CO2. Finally, we have attempted to reduce confusion through small sentence-level changes that aim to clarify and increase sentence and paragraph flow throughout the section. These are indicated through ‘Tracked Changes’ in the Introduction section of the revised manuscript.

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.

**Response**: The reviewer makes a great point, and the fact that this was brought up by both reviewers indicates that we need to include this justification in the manuscript. 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 189:

“CO2 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?

**Response**: Soybean forms a pair of opposite unifoliate leaves after the cotyledons open. After the pair of opposite 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 leaflet set to standardize measurements 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 that photosynthetic processes in leaflets would 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 clarify and avoid reader confusion. The total leaf area of the plants is indicated in the manuscript, which could be used as a proxy for the total number of leaves.

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.

**Response**: We thank the reviewer for their careful eye; however, 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, paying particular attention to the table and figure reference details throughout.

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.

**Response**: We have added an explanation for the *A*net,420 and *A*net,gc responses, starting on line 567:

“Individuals grown under elevated CO2 experienced a reduction in *A*net,420, leaf nitrogen content, *V*cmax25, and *J*max25 compared to plants grown under ambient CO2. These patterns are consistent with a downregulation of leaf-level investment toward photosynthetic enzymes, and were likely driven by increased Rubisco affinity for carboxylation relative to oxygenation that decreased leaf-level demand to build and maintain photosynthetic enzymes. Despite this apparent reduction in leaf-level investment toward photosynthetic enzymes, elevated CO2 increased *A*net,gc. This response was associated with a larger reduction in *V*cmax25 than *J*max25, which increased *J*max25: *V*cmax25 and allowed enhanced *A*net,gc to be achieved by approaching optimal coordination (Chen *et al.*, 1993; Maire *et al.*, 2012; Smith & Keenan, 2020). Additionally, the reduction in leaf nitrogen content allowed increased *A*net,gc to be achieved with greater photosynthetic nitrogen-use efficiency. These patterns are consistent with our expectations and previous studies that have investigated leaf photosynthetic responses to elevated CO2 (Drake *et al.*, 1997; Ainsworth *et al.*, 2002; Ainsworth & Long, 2005; Ainsworth & Rogers, 2007; Crous *et al.*, 2010; Lee *et al.*, 2011; Smith & Dukes, 2013; Poorter *et al.*, 2022; Cui *et al.*, 2023).

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.

**Response**: The reviewer is correct that *A*net,gc were measured under different CO2 conditions; however, these CO2 conditions were reflective of the growth CO2 conditions. *A*net,gc was quantified at 420 μmol mol-1 for individuals that received the ambient CO2 treatment, while *A*net,gc was quantified at 1000 μmol mol-1 for individuals that received the elevated CO2 treatment. We disagree with the reviewer’s suggestion that *A*net,gc does not reflect the effect of CO2 treatment, as these were the realized net carbon assimilation rates each plant was performing while in their respective CO2 treatments. Greater net carbon assimilation rates under elevated CO2 were associated with increased plant allocation to aboveground tissues, which increased total leaf area and fostered greater biomass accumulation compared to individuals grown under ambient CO2. These patterns were observed despite the reduction in apparent photosynthetic capacity and stomatal conductance under elevated CO2. However, we agree with the reviewer that *A*net,gc does not give much information about how CO2 treatments altered investment toward photosynthetic enzymes. We reported effects of elevated CO2 on apparent photosynthetic capacity by referencing patterns associated with *V*cmax25 and *J*max25 in this paragraph, assuming that the reader would be more interested in understanding the mechanisms underpinning the photosynthetic responses to elevated CO2, but have clarification for these changes in the paragraph copied in the previous response starting on line 567 in the main text.

**References**

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