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Dear Dr. Alistair Rogers and the rest of the Editorial Board at *Journal of Experimental Botany*,

Thank you for the positive comments regarding our manuscript (JEXBOT/2024/314669), titled “Nitrogen demand, availability, and acquisition strategy control plant responses to elevated CO2”. Please find our revised manuscript attached. A manuscript copy with changes noted through the “Track Changes” feature in Microsoft Word is also included.

We thank the editor and two reviewers for their constructive feedback and overall positive remarks about the manuscript. The revised manuscript now includes explicit mentions of the sample sizes used for the statistical analyses, although we note that nitrogen fertilization treatments are included as continuous fixed effects due to the high number of fertilization treatments included in the study. We have also considered the alternative statistical approach proposed by the second reviewer, noting that our approach is commonly adopted in previous work in similar topics. We believe that either approach would provide similar results. Finally, we have considered all of the line comments by both of the reviewers and find that these line revisions provide additional context and nuance that strengthens the main message of the paper.

Below, we provide a point-by-point response to all of the 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.

Sincerely,

Evan A. Perkowski, Ph.D.

On behalf of coauthors Ezinwanne Ezekannagha and Nicholas G. Smith

**Response to Editor and Reviewer Feedback**

**Editor Comments**

**Thank you for your submission to JXB. Your paper has been reviewed by two experts in the field. Both reviewers provided strong support for the work and were excited about the paper, as am I. Both reviewers found your statistical approach to be sound but asked for some additional clarity on the rationale for the approach you took, and a clear indication of the replication (please see a recent editorial for additional guidance if necessary**[**https://doi.org/10.1093/jxb/erab268**](https://nam04.safelinks.protection.outlook.com/?url=https%3A%2F%2Fdoi.org%2F10.1093%2Fjxb%2Ferab268&data=05%7C02%7Cevan.a.perkowski%40ttu.edu%7C05a3f2d87b4240143f4d08dd46971bd5%7C178a51bf8b2049ffb65556245d5c173c%7C0%7C0%7C638744337047099682%7CUnknown%7CTWFpbGZsb3d8eyJFbXB0eU1hcGkiOnRydWUsIlYiOiIwLjAuMDAwMCIsIlAiOiJXaW4zMiIsIkFOIjoiTWFpbCIsIldUIjoyfQ%3D%3D%7C0%7C%7C%7C&sdata=wXGjmNkW3gCvpmBH8payTknqAXYK0A00FuHKEX5bkSY%3D&reserved=0)**).  
  
Reviewer 2 raised a question about pot size, but actually making the argument in the opposite direction to what I may have expected. I am comfortable with your current treatment of this issue in the manuscript.  
  
Note that phrases used in the keywords that also appear in the title are redundant so please consider replacing those that are repeated.  
  
Please note that ALL the points raised by the editor and reviewers should be addressed either by modification of the manuscript or by discussion in response to the reviewers, and these changes summarized in your ‘Response to Reviewers’. Revised papers should ideally be received within four weeks, if you feel you will need longer please reply to this e-mail to let the editorial staff know.**

Thank you for the positive assessment of our manuscript and for the useful summary of the reviewer comments – we are excited to see our work discussed positively and constructively by the reviewers. We have addressed all points raised by the reviewers, providing additional context where needed to clarify our statistical approach and the replication design. We have considered the comments from reviewer 2 about pot size and have decided to leave our existing treatment of this issue. Finally, we have made efforts to remove redundant keywords. We feel these changes have improved the interpretation of the paper and have improved the clarity of the paper’s main message. Below, please find our responses to each of the reviewer’s comments.

**Reviewer 1**

**In this study, the researchers used a nitrogen-fixing soybean plant to test two main hypotheses (nitrogen limitation and eco-evolutionary theories) that are used to explain the response of C3 plants to elevated CO2 with respect to leaf photosynthetic and respiratory traits as well as whole-plant productivity. In one group of plants they used non-inoculated plants and the second group of plants was inoculated with nitrogen-fixing bacteria. These two groups of plants received nine levels of nitrogen fertilization, and half of them were exposed to elevated CO2 of 1000 ppm and the second group at ambient CO2. The main findings of the study were that photosynthetic capacity (Vcmax and Jmax) acclimated with elevated CO2 by decreasing, regardless of the nitrogen fertilization treatments, supporting the eco-evolutionary (optimality theory), while at whole plant level, nitrogen fertilization enhanced leaf and biomass production, supporting the nitrogen limitation theory. In my opinion, this study was carefully designed to evaluate thoroughly these two theories that are often used interchangeably to explain both physiological and whole-plant responses to elevated CO2 therefore, I was impressed by this careful experimental design. The statistical models was also appropriate. However, throughout the method sections, the authors did not specify replicates used in N fertilization treatments, making it hard to fully understand the strength of the stats…My overall comments to the manuscript are rather minor and I believe this work will advance our understanding on the responses of plants to elevated CO2 in relation to nitrogen availability.**

Thank you for your positive comments about our paper. We agree that it is important to specify the replicates used in the N fertilization treatments and have included our response to this in one of the line comments below. To summarize our changes, the revised manuscript now includes an explicit explanation of the replication scheme used for the N fertilization treatments and also includes citations suggested in some of the line comments below.

**Line 81 – 84: Please use these results from the Flakaliden experiment that demonstrated this empirically (Sigurdsson et al., 2013)**

Sigurdsson et al. (2013) is now added.

**Line 129 – 145: how does this study (Feng et al., 2015) fits into this discussion?**

**Line 190 – 193: You need to explicitly tell us how many plants received each fertilization treatment to understand the power of your analyses.**

We agree that it is important to explicitly specify the replication of each treatment group. 144 plants total plants were grown in the experiment and were equally divided into 36 treatment combinations (2 CO2, 2 inoculation, and 9 nitrogen fertilization treatments). Thus, four replicates were included in each unique CO2-by-inoculation-by-nitrogen fertilization treatment. This replication scheme is sufficient to test our hypotheses with high confidence given our decision at the start of the experiment to treat the nitrogen fertilization treatments as a continuous predictor of leaf and whole-plant traits. Thus, the slope that explained the effects of nitrogen fertilization on each unique CO2-by-inoculation combination was assessed and drawn using a maximum of 36 data points. However, it is important to note that we removed uninoculated individuals who had formed root nodules. We have added a table to the *Supplemental Information* (Table SX) that clarifies the number of datapoints used to fit each slope that explained the effects of nitrogen fertilization on each unique CO2-by-inoculation combination. This table is copied below for ease of review.

**Line 201 – 206: how long did each experimental iteration run for?**

Each experiment iteration lasted for 7 weeks. Physiology measurements were collected at the beginning of the seventh week and plants were harvested by the end of the seventh week. We have added a sentence to the end of this section that explicitly defines this:

“Each experimental iteration lasted seven weeks, which was sufficient for plants to make it through the majority of their vegetative growth phase without evidence of reproduction.”

**Line 207 – 213: Why did you chose this photoperiod?**

*Glycine max* is often classified as a short-day crop species, meaning that flowering is often induced with an increase in nighttime hours. While we held the number of nighttime hours constant throughout the experiment, the decision to have a long daylength was in part to further inhibit the onset of reproduction. The long daylength maximized the period in which plants could photosynthesize, increasing the amount of carbon assimilated per day and the amount of biomass that could accumulate across the 7-week growing period.

**Line 226 – 242: I think it is important to report the number of plants measured for leaf gas exchange in each treatment, so far it is not clear**

All individuals in the experiment were measured for gas exchange. We have clarified this in the text.

**Line 334: again you need to specify the exact number of replicates used to understand the power of the statistical analyses**

Done! See comment above for how this is implemented **Line 590 – 591: Looking at the Figure 3 it looks like leaf area and biomass did not substantially increase with CO2 in low N fertilization within the   
uninoculated plants. How did you come up with this interpretation? I only see these increases at higher N fertilization treatments.**

The reviewer is correct (pairwise comparisons indicate no difference between CO2 treatments under low N fertilization in uninoculated plants). The interpretation in this sentence was based on the main effect of CO2 treatment on total leaf area and total biomass showing a general positive effect of elevated CO2 on total leaf area and total biomass when averaged across nitrogen fertilization and inoculation treatments. This paragraph discusses main effects of elevated CO2 on whole-plant traits, with the subsequent paragraph discussing the stronger positive effects of elevated CO2 with increasing nitrogen fertilization. We think the null effect of elevated CO2 is an interesting caveat to the observed responses and thank the reviewer for pointing this out, especially because it supports findings from Sigurdsson et al. (2013). We have added a sentence to the second paragraph of this section to explain this response, starting on line XX:

“Interestingly, this interaction revealed no effect of CO2 treatment on total leaf area or total biomass under low nitrogen fertilization, supporting previous work showing that CO2 fertilization effects on traits related to whole-plant growth are absent under low nutrient availability (Sigurdsson *et al.*, 2013).”

**Line 601 – 603: it would be great to also add references to your figures to easily allow the reader cross check the statement of your results with their respective figures. You can do so throughout your discussion section as it makes easier for readers to fully digest your work.**

We agree with the reviewer and have added figure references in the main text of the Discussion.

**Feng Z, Rütting T, Pleijel H, Wallin G, Reich PB, Kammann CI, Newton PCD, Kobayashi K, Luo Y, Uddling J. 2015. Constraints to nitrogen acquisition of terrestrial plants under elevated CO2. Global Change Biology 21(8): 3152-3168.  
Sigurdsson BD, Medhurst JL, Wallin G, Eggertsson O, Linder S. 2013. Growth of mature boreal Norway spruce was not affected by elevated [CO2] and/or air temperature unless nutrient availability was improved. Tree Physiology 33(11): 1192-1205.**

**Reviewer 2**

**Perkowski et al’s manuscript present the results of a growth chamber study of the CO2 responses of soybean under varying nitrogen fertilization rates and inoculation or not with N-fixing symiotic bacteria. The study comprised 144 plants planted in 6 l pots and treated in 6 growth chambers. The goal was to evaluate three hypotheses related to eco-evo optimality versus nitrogen limitation hypotheses:   
“1) Leaf photosynthetic responses to elevated CO2 will be independent of nitrogen fertilization and inoculation treatment. Instead, elevated CO2 will decrease Vcmax more than Jmax, increasing the ratio of Jmax to Vcmax. This response will increase net photosynthesis rates under growth CO2 conditions by allowing rate-limiting steps to approach optimal coordination while enhancing photosynthetic nitrogen-use efficiency.   
2) Following the nitrogen limitation hypothesis, increasing nitrogen fertilization will enhance the positive effects of elevated CO2 on total leaf area and total biomass. This response will be due to increased belowground carbon allocation and nitrogen uptake and with increasing nitrogen fertilization that will be stronger under elevated CO2. Biomass responses to elevated CO2 will be driven by a greater increase in belowground biomass than aboveground biomass, as plants will invest in resource acquisition strategies to meet the increased whole-plant nitrogen demand for building new tissues.   
3) Following the nitrogen limitation hypothesis, inoculation with nitrogen-fixing bacteria will enhance positive whole-plant responses to elevated CO2. These responses will be strongest under low nitrogen availability, where inoculated plants will invest in nitrogen uptake through symbiotic nitrogen fixation over more costly direct uptake pathways. However, these patterns will diminish with increasing nitrogen fertilization as plants acquire more nitrogen through increasingly less costly direct uptake pathways.”  
  
Overall the study is well designed, executed, and fairly reported. I have a few overall questions/comments and several minor comments/questions.   
I like the comparison and evaluation of the eco-evo optimality hypothesis with the nutrient limitation hypothesis and I find the leaf-scale vs plant-scale results compelling. I woinder though, what does eco-evo optimality theory say about N fertilization outside of CO2 treatment? Uninoculated treatments show increases in photosynthetic capacity across board with N fertilization (Fig & table 2). How would this influence the discussion in the “Modeling implications” section?   
  
The root:shoot response to N fert is not linear, especially for the uninoculated treatment (Fig 3) – can this be accounted for in the statistical analysis and how does that modify interpretation of the root:shoot response? Should probably cite Iversen 2010 when mentioning the root response to CO2 literature. Also while I do find these results compelling, they are likely influenced by the fact that they are in pots and can expand resource capture volumes both above and below ground which can influence responses compared to more closed, less disturbed systems (Norby 1996; Körner 2006). This isn’t a deal breaker, just needs some discussion about how this might influence results and how to make inferences at a greater scale / more natural systems.   
While well presented and executed, I find the statistics quite clunky. There are a huge number of individual statistical tests, in the region of 100, and all the inferences are based on these tests. A more robust approach would be to use model simplification and inference based on the minimum adequate models (Burnham & Anderson 2002; 2014). Further I’m not sure why the emmeans package is necessary when all information on effect sizes can be calculated from the lmer models – is that how emmeans works? This suggested method doesn’t need to replace the existing method but I would like to hear why the current method is used over others and it would be good to at least test how the suggested method influences results.   
  
Minor questions/comments:  
There is an interaction of CO2 and N fert on Narea. I’m not sure I saw this mentioned / interpreted in the context of the hypotheses.   
  
Ln 100-103 Is coordination optimal? Has the reduction in Jmax:Vcmax predicted by eco-evo optimality been shown / quantified anywhere? Citation needed and ideally a comparison of the predicted J:V reduction to results obtained here.   
  
Ln 163-165 I’m not sure I agree with this in an expanding system. See comment above and Norby 1996; Körner 2006 referneces.   
  
Ln 177 Should we be using sphagnum moss given the role of peatlands in carbon sequestration?  
  
Ln 626-628 Not sure this sentence is necessary  
  
Ln 629 Could mention that inoculation did strongly impact responses to N fertilization  
  
Ln 718-741 Suggest breaking into 3 paragraphs**

**Burnham, K., Anderson, D., 2014. P values are only an index to evidence: 20th-vs. 21st-century statistical science. Ecology 95, 627–630.**[**https://doi.org/10.1890/13-1066.1**](https://doi.org/10.1890/13-1066.1) **Burnham, K.P., Anderson, D., 2002. Model Selection and Multi-Model Inference, 2nd ed. Springer, New York, NY.  
Iversen, C.M., 2010. Digging deeper: fine-root responses to rising atmospheric CO2 concentration in forested ecosystems. New Phytologist 186, 346–357.**[**https://doi.org/10.1111/j.1469-8137.2009.03122.x**](https://doi.org/10.1111/j.1469-8137.2009.03122.x) **Körner, C., 2006. Plant CO2 responses: an issue of definition, time and resource supply. New Phytologist 172, 393–411.**[**https://doi.org/10.1111/j.1469-8137.2006.01886.x**](https://doi.org/10.1111/j.1469-8137.2006.01886.x) **Norby, R.J., 1996. Forest canopy productivity index. Nature 381, 564–564.**[**https://doi.org/10.1038/381564a0**](https://doi.org/10.1038/381564a0)