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February XX, 2025

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

Thank you for your 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, along with a version highlighting changes using the “Track Changes” feature in Microsoft Word.

We appreciate the constructive feedback from the editor and two reviewers and their overall positive remarks on our study. In response to reviewer feedback, we have revised the manuscript to explicitly state the sample sizes used in our statistical analyses. Additionally, we have carefully considered the alternative statistical approach suggested by Reviewer 2. However, given our study’s design and hypothesis-driven framework, we believe our current approach remains the most appropriate for addressing our research questions. 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 detailed point-by-point response to all reviewer comments. Reviewer comments are presented in black, with our responses in red. 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 additional 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 point. Finally, we have made efforts to remove redundant keywords. We feel these changes have improved the interpretation of the paper and 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)**

Thank you for sharing this citation! We have added Sigurdsson et al. (2013) here and have added this citation in the Discussion section to contextualize the observed null effect of CO2 treatment on total biomass and total leaf area under low nitrogen fertilization.

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

Thank you for pointing out this paper to us. This paper shows that ANPP responses to elevated CO2 are positively correlated with nitrogen acquisition and fits well within this paragraph. We have added a clause to the sentence starting on line XX to link whole-plant responses to elevated CO2 with nutrient uptake. We have also added an additional citation to support this statement:

“Therefore, considering nitrogen acquisition strategy is important when examining plant responses to elevated CO2 across nitrogen availability gradients, especially because whole-plant responses to elevated CO2 are often positively correlated with nitrogen uptake (Feng et al., 2015; Stocker et al., 2025).”

**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 (as stated on line XX). This replication scheme is sufficient to test our hypotheses with high confidence given that the experiment was designed to evaluate nitrogen fertilization treatments as a continuous predictor of leaf and whole-plant responses to CO2 treatments. 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 16 uninoculated individuals who had formed root nodules. We have added two tables to the *Supplemental Information*: Table S3 now summarizes the sample size for each unique CO2-by-inoculation-by-N fertilization combination (n ranging from 1 to 4 replicates), and Table S4 now summarizes the sample size for each unique CO2-by-inoculation combination (n ranging from 28 to 36 measurements). Table S4 clarifies the exact number of data points 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. All supplement tables that proceed Table S4 have been relabeled in the *Supplemental Information* and their corresponding in-text citations have been updated.

**Table S4** Replication scheme for each unique CO2-by-inoculation combination

|  |  |  |
| --- | --- | --- |
| **CO2 treatment** | **Inoculation treatment** | **n** |
| Ambient CO2 | Uninoculated | 28 |
| Inoculated | 36 |
| Elevated CO2 | Uninoculated | 28 |
| Inoculated | 36 |

**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 grow 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 can be 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 (n = 144, but 128 data points are included in the analyses after the removal of 16 uninoculated individuals that had significant nodulation) in the experiment were measured for gas exchange. We have clarified this in the text on line XX by including the total sample size of gas exchange measurements in parentheses at the end of the sentence.

**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 acknowledge 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 often 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.

**References from Reviewer 1**

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 wonder 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?**

Thank you for your kind words about our manuscript. This question about what eco-evolutionary optimality theory says about N fertilization outside of CO2 treatments is a very important one, especially in the context of understanding how terrestrial biosphere models should link N cycle dynamics with photosynthesis. At the leaf-level, eco-evolutionary optimality theory predicts that plants should exhibit strong positive effects of increasing nitrogen availability on photosynthetic traits when nitrogen availability is insufficient for satisfying the leaf-level demand for building and maintaining photosynthetic enzymes. However, such positive effects of nitrogen availability on photosynthetic traits are expected to diminish as nitrogen availability begins to exceed leaf-level demand for building and maintaining photosynthetic enzymes. In such cases where nitrogen availability exceeds leaf-level demand for photosynthetic enzymes, nitrogen taken up by plants should be allocated toward other structures, such as toward the construction of additional leaves or toward leaf structural tissue as a strategy to ward off herbivores or prolong leaf longevity. In this experiment, we observe consistent positive effects of N fertilization in uninoculated plants; however, note that there is some evidence of a saturation point for photosynthetic traits. This pattern suggests that plants were increasing nitrogen uptake with increasing N fertilization to satisfy leaf-level demand for building and maintaining photosynthetic enzymes, and this trend diminished as N fertilization approached the threshold where N availability satisfied or exceeded leaf-level demand for photosynthetic enzymes. In contrast, N fertilization effects on photosynthetic traits are absent in individuals that were able to acquire nitrogen through nitrogen fixation. In this scenario, plants are able to capture all of the N needed to satisfy demand, either through N fixation under low N fertilization or through direct uptake with increasing N fertilization. These are very interesting patterns and were one of the main reasons we decided to impose a nitrogen fixation manipulation in the experiment.

**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.**

Thank you for pointing this out. We have accounted for the nonlinearity in the data distribution by imposing a natural-log transformation on the response variable before fitting the model. The model exhibits a poor fit at lower fertilization levels, likely due to the smaller sample size of uninoculated plants in these treatments due to unintended nodulation. However, model residuals suggest a normal Gaussian distribution, and we observe similar patterns emerge when fitting the data with a generalized additive model that includes a smoothing term for nitrogen fertilization.

We have added Iversen (2010) to the paragraph of the Introduction that summarizes leaf and whole-plant responses to elevated CO2. However, as Iversen (2010) largely reviews belowground rooting depth responses to elevated CO2, we find it more appropriate to contextualize the belowground carbon allocation results with Iversen et al. (2008). This manuscript shows that root biomass production doubled under elevated CO2, a pattern that was associated with reduced fine-root turnover and accelerated fine root mortality that increased ecosystem C and N inputs.

Finally, we acknowledge the limitations of extrapolating these findings to natural systems. While this study does not aim to predict large-scale ecosystem responses, we have added a caveat at the end of the Discussion section emphasizing that a follow-up experiment in a natural setting would provide further insight into how the observed patterns might scale at the community or ecosystem level. This sentence starts on line XX:

“Importantly, there are inherent limitations in using a pot experiment to make inferences about how nitrogen availability modifies community- or ecosystem-level responses to elevated CO2. While we caution against using this study to make such extrapolations, a similar conducted under field conditions would help validate the patterns observed here while also providing insight into how resource competition within and across species may further shape plant responses to nitrogen availability and elevated CO2.”

**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.**

We appreciate the reviewer’s thoughtful comments regarding model selection and statistical inference. While information-theoretic approaches can be valuable for identifying environmental covariates that drive trait variation across time and space, we argue that the full model presented in the manuscript already represents the minimum adequate model. Our experimental design was explicitly structured to test two existing hypotheses – the eco-evolutionary optimality and nitrogen limitation hypotheses – that explain the effects of elevated CO2 on leaf and whole-plant traits. Model parsimony was not a design objective; rather, retaining the full model is necessary for determining which of these two hypotheses explains plant responses to elevated CO2. For example, the eco-evolutionary optimality theory predicts that photosynthetic capacity responses to elevated CO2 should be independent of nitrogen fertilization and inoculation treatments, while the nitrogen limitation hypothesis indicates that whole-plant responses to elevated CO2 should be enhanced with increasing nitrogen fertilization. In this context, the null interaction between nitrogen fertilization and CO2 treatment on *V*cmax25 was expected and is as informative for hypothesis testing as the significant interaction between nitrogen fertilization and CO2 treatment on total biomass. Given this, model simplification via stepwise reduction or AIC-based model selection could lead to the omission of null results that undermine our ability to test these two hypotheses.

While effect sizes can be extracted from regression model summaries, we use ‘emmeans’ to facilitate post-hoc comparisons in cases where significant treatment interactions occur. The function accounts for random effects and adjusts degrees of freedom for fixed effects using methods such as those described in Kenward & Roger (1997). Given that interaction effects are central to our experimental design, ‘emmeans’ provides a robust framework for comparing treatment combinations and is commonly used for interpreting pairwise comparisons in linear mixed-effect models.

We appreciate the reviewer’s suggestion and acknowledge the value of alternative statistical approaches. However, given our study’s design and hypothesis-driven framework, we believe the current approach is the most appropriate for addressing our research questions.

**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.**

The reviewer is correct – there is an interaction between CO2 and N fertilization on *N*area and there is limited discussion about this. Eco-evolutionary optimality theory

**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.**

The increase in *J*max25:*V*cmax25 is common in response to elevated CO2

**Ln 163-165 I’m not sure I agree with this in an expanding system. See comment above and Norby 1996; Körner 2006 references.**

**Ln 177 Should we be using sphagnum moss given the role of peatlands in carbon sequestration?**

Indeed, peatlands are a natural carbon sink for the planet and the harvesting of peat is not great for the environment. In hindsight, we ought to have chosen a more ecologically friendly substrate for the soil-less mix; however, peat moss does provide a useful substrate for enhancing water retention and maintaining organic matter in pots without adding supplemental fertilizer amendments as most commercial potting mixes have.

**Ln 626-628 Not sure this sentence is necessary**

Sentence removed.

**Ln 629 Could mention that inoculation did strongly impact responses to N fertilization**

Sure! This has been added to the first sentence of this paragraph. The sentence now reads:

“Inoculation largely had no effect on leaf- or whole-plant responses to elevated CO2, but played a strong role in determining the effect of nitrogen fertilization on measured traits”

**Ln 718-741 Suggest breaking into 3 paragraphs**The conclusions subsection of the Discussion section has been broken into three paragraphs. One paragraph focuses on leaf and whole-plant responses to elevated CO2, one paragraph focuses on the largely null inoculation effects on plant responses to elevated CO2, and the final paragraph discusses overall conclusions.

**References from Reviewer 2**

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>

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>

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>

Norby, R.J., 1996. Forest canopy productivity index. Nature 381, 564–564. <https://doi.org/10.1038/381564a0>