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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 $CO_2$". Please find our revised manuscript attached, along with a version highlighting changes using the "Track Changes" feature in Microsoft Word. The revised manuscript now includes 9 tables in the *Supplemental Information*.

We appreciate the constructive feedback from the editor and two reviewers and their 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-by-line comments by both of the reviewers. These changes 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.


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).**

**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 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 replication. We have considered the comments from the second reviewer about pot size and have added a statement calling for future work using a similar experimental approach in natural settings. Additionally, we have considered the alternative statistical approach suggested from the second reviewer. However, we have decided to not implement an information-theoretic approach to simplify models due to the fact that null treatment responses are just as informative in making inferences about whether eco-evolutionary optimality or nitrogen limitation drives plant responses to elevated $CO_2$ as

significant treatment responses. We contend that the minimum adequate model needed to test hypotheses is the full model presented in the manuscript. Finally, we have made efforts to remove redundant keywords as requested from the Editor. We feel these changes have improved the interpretation and 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 nitrogen 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 nitrogen fertilization treatments (including two additional tables in the *Supplemental Information*) and also includes

citations suggested in some of the line-by-line comments (referring to Lines 81-84, 129-145 in the previous submission) 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 $CO_2$ treatment on 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 $CO_2$ are positively correlated with nitrogen acquisition and fits well in this paragraph. We have added a clause to the sentence starting on line 140 to link whole-plant responses to elevated $CO_2$ 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 $CO_2$ across nitrogen availability gradients, especially because whole-plant responses to elevated $CO_2$ 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 were grown in the experiment and were equally divided into 36 treatment combinations (2 $CO_2$, 2 inoculation, and 9 nitrogen fertilization treatments in a full-factorial setup). Thus, four replicates were included in each unique $CO_2$-by-inoculation-by-nitrogen fertilization treatment, which is now explicitly stated on line 196. This replication scheme is sufficient to test our hypotheses with confidence given that the experiment was designed to evaluate nitrogen fertilization treatments as a continuous predictor of leaf and whole-plant responses to $CO_2$ treatments. The slope that explained the effects of nitrogen fertilization on each unique $CO_2$-by-inoculation combination was assessed and drawn using a maximum of 36 data points. However,

we removed 16 uninoculated individuals who had formed root nodules. To explicitly define the replication of treatment combinations, we have added two tables to the *Supplemental Information*. Table S3 summarizes the sample size for each unique $CO_2$-by-inoculation-by-nitrogen fertilization combination (replication ranging from 1 to 4 replicates per combination) and Table S4 summarizes the sample size for each unique $CO_2$-by-inoculation combination (replication ranging from 28 to 36 measurements per trendline). Table S4 clarifies the number of data points used to fit the slope that explained the effects of nitrogen fertilization on each unique $CO_2$-by-inoculation combination. This table is copied below for ease of review. All supplementary tables that follow 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

| $CO_2$ treatment | Inoculation treatment | n |
|---|---|---|
| Ambient $CO_2$ | Uninoculated | 28 |
| | Inoculated | 36 |
| Elevated $CO_2$ | Uninoculated | 28 |
| | Inoculated | 36 |

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

Each experiment iteration lasted for 7 weeks. Gas exchange 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 on line 210 that clarifies 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?**

We used a 16-hour photoperiod, which was decided with the original intent to maximize the amount of time per day that *G. max* could photosynthesize and accumulate biomass. However, *G. max* is also often classified as a short-day crop species, meaning that flowering and the onset of reproduction can be induced with an increase in nighttime duration. Thus, the long

photoperiod also allowed us to inhibit the onset of reproduction, further maximizing biomass accumulation during the growth period by maximizing the time experimental plants spent in their vegetative growth phase. A shorter daylength may have resulted in the experiment needing to be harvested earlier, but we do not suspect that any changes to photoperiod would have modified our results. We have added a sentence to briefly explain this, starting on line 215:

> "This daylength allowed plants to maximize vegetative growth across the seven-week experiment while minimizing the onset of reproduction"

**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 234 by including the total sample size of gas exchange measurements in 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. We have also added a sentence to the end of the paragraph that directs the reader to Table S3 and S4 for a summary of the replication scheme:

> "A summary of the replication scheme after these individuals were removed is included in the Supplemental Information (Table S3-4)."

**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 interpretation in this sentence was based on the main effect of $CO_2$ treatment on total leaf area and total biomass showing a general positive effect of elevated $CO_2$ on total leaf area and

total biomass when averaged across nitrogen fertilization and inoculation treatments. This paragraph discusses main effects of elevated $CO_2$ on whole-plant traits, with the subsequent paragraph discussing the stronger positive effects of elevated $CO_2$ with increasing nitrogen fertilization.

The reviewer is correct that there does not seem to be a strong effect of elevated $CO_2$ on total leaf area or total biomass under low N fertilization in the uninoculated plants. Indeed, while pairwise comparisons indicate that elevated $CO_2$ increased total biomass in uninoculated individuals even under 0 ppm N (Tukey: $p=0.005$), there was no effect of elevated $CO_2$ on total leaf area when uninoculated individuals received 0 ppm N (Tukey: $p=0.543$), 35 ppm N (Tukey: $p=0.310$), or 70 ppm N (Tukey: $p=0.135$). We think the null effect of elevated $CO_2$ on total leaf area under low N fertilization is an interesting caveat to the observed responses and thank the reviewer for pointing it out, especially as it relates to the main 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 558:

> "Interestingly, this interaction revealed no effect of $CO_2$ treatment on total leaf area in uninoculated individuals under low nitrogen fertilization, supporting previous work showing that $CO_2$ fertilization effects on traits related to whole-plant growth are often absent under low nutrient availability (Sigurdsson *et al.*, 2013). Similar effects of $CO_2$ treatment on total leaf area under low nitrogen fertilization may have been due to plants being unable to satisfy demand for soil nitrogen similarly between the two $CO_2$ treatments."

**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 $CO_2$. 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 [$CO_2$] 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 the kind words about our manuscript. This question about what eco-evolutionary optimality theory says about nitrogen fertilization outside of $CO_2$ treatments is very important, particularly for understanding how terrestrial biosphere models should link nitrogen cycle dynamics with photosynthesis and growth (as recently reviewed in Stocker et al., 2025).

Eco-evolutionary optimality theory uses a demand-driven scheme for predicting photosynthesis, where investment in photosynthetic enzymes is primarily determined by environmental factors that influence the demand to build and maintain photosynthetic enzymes (e.g., $CO_2$, temperature, light) (Smith *et al.*, 2019; Harrison *et al.*, 2021; Stocker *et al.*, 2025). However, plants must be able to acquire sufficient nitrogen to satisfy this demand while minimizing the costs of acquiring nitrogen, making nitrogen availability a key regulator of patterns expected from eco-evolutionary optimality theory. 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, or when costs to acquire nitrogen relative to water increase. Such positive effects of nitrogen availability on photosynthetic traits are expected to diminish as nitrogen availability begins to approach leaf-level demand for building and maintaining photosynthetic enzymes. In such cases where nitrogen availability exceeds leaf-level demand for photosynthetic enzymes, the theory predicts that plants should allocate excess nitrogen not

needed to satisfy leaf-level demand for photosynthesis toward the construction of other plant tissues, such as additional leaves. This leads to a tradeoff between investment in leaf quality when nitrogen availability is insufficient for satisfying leaf demand for photosynthesis and leaf quantity when nitrogen availability exceeds demand for photosynthesis across nitrogen availability gradients.

As the reviewer pointed out, we observed strong positive effects of increasing nitrogen fertilization on leaf and whole-plant traits in uninoculated plants. This result was expected, as uninoculated plants are nitrogen-limited under low nitrogen fertilization treatments and cannot meet the leaf-level demand for photosynthetic enzymes. Interestingly, we found some evidence for diminishing returns of photosynthetic traits as nitrogen fertilization increased. For example, uninoculated plants demonstrated smaller increases in $V_{cmax25}$ between 350 and 630 ppm N (39% increase) than between 0 ppm N and 280 ppm N (79% increase). This pattern suggests that plants reached a threshold where nitrogen fertilization satisfied leaf-level demand for photosynthetic enzymes. In contrast, nitrogen fertilization effects on photosynthetic traits were absent in inoculated individuals. This pattern was also expected, as inoculated plants were presumably able to satisfy leaf demand for building and maintaining photosynthetic enzymes across the nitrogen fertilization gradient, investing more heavily in symbiotic nitrogen-fixing bacteria under low nitrogen fertilization and increasing investment in direct uptake with increasing nitrogen fertilization.

These are very interesting patterns and were one of the main reasons we decided to impose a nitrogen fixation manipulation in the experiment. While there are already implicit comments about eco-evolutionary optimality theory predictions across nitrogen availability gradients in the *Modeling Implications* section, we have added an additional paragraph to this section to explicitly clarify the expected eco-evolutionary optimality response. This paragraph starts on line 634 and is copied below:

> "Increasing nitrogen fertilization increased indices of apparent photosynthetic capacity, but this pattern was only observed in uninoculated plants. Increasing nitrogen fertilization also increased $N_{area}$ and $Chl_{area}$ more strongly in uninoculated plants (Fig. 1). 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 leaf-level demand for building and maintaining photosynthetic enzymes, or when changes in nitrogen availability decrease the relative costs of nitrogen acquisition and use compared to those of water acquisition and use (Wright *et al.*, 2003; Harrison *et al.*, 2021; Stocker *et al.*, 2025). In such cases where nitrogen availability exceeds leaf-level demand for photosynthetic enzymes or costs to acquire nitrogen relative to water increase, the theory predicts that positive effects of increasing nitrogen availability on photosynthesis should diminish, with excess nitrogen not needed to satisfy leaf-level demand for photosynthesis being allocated toward the construction of other plant tissues (e.g., additional leaves). Given this, strong positive effects of increasing nitrogen fertilization on indices of photosynthetic capacity in uninoculated plants were expected, as uninoculated plants were nitrogen-limited under low nitrogen fertilization and could not meet the leaf-level demand for photosynthetic enzymes. We also found some evidence for a diminished positive effect of nitrogen fertilization on photosynthetic traits, with uninoculated plants demonstrating smaller increases in $V_{cmax25}$ between 350 and 630 ppm N (39% increase) than between 0 ppm N and 280 ppm N (79% increase). In contrast, nitrogen fertilization effects on photosynthetic traits were absent in inoculated individuals. This pattern was also expected, as inoculated plants were able to acquire sufficient nitrogen across the nitrogen availability gradient to satisfy leaf-level photosynthetic demand, investing more strongly in microbial symbionts under low nitrogen fertilization and shifting to nitrogen acquisition through direct uptake pathways as nitrogen became more available."

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 poorer fit at lower fertilization levels, likely due to the smaller sample size of uninoculated plants in these treatments due to unintended nodulation. However, the assumptions of linear mixed effects models (e.g., Gaussian distribution of model residuals, homogeneity of variance, etc.) are satisfied and we observe similar patterns 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 $CO_2$. However, as Iversen (2010) largely reviews belowground rooting depth responses to elevated $CO_2$, which was constrained by pot depth in this experiment, we have also added Iversen et al. (2008) in the discussion of root responses to elevated $CO_2$. Iversen et al. (2008) shows that root biomass production doubled under elevated $CO_2$, a pattern that was associated with reduced fine-root turnover and accelerated fine root mortality that increased ecosystem carbon and nitrogen 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 to elevated $CO_2$, 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. These sentences start on line 699:

> "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 $CO_2$. While we caution against using this study to make such extrapolations, a similar experiment conducted under field conditions would help validate the patterns observed here while providing insight into how resource competition within and across species may shape plant responses to nitrogen availability and elevated $CO_2$."

**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 needed to represent our experimental design. Our experimental design was explicitly structured to test two hypotheses – the eco-evolutionary optimality hypothesis and nitrogen limitation hypothesis – that explain the effects of elevated $CO_2$ on leaf and whole-plant traits. Model parsimony was not a design objective, and retaining the full model is necessary for adequately representing the experimental design and for making inferences about which of these two hypotheses explain plant responses to elevated $CO_2$. For example, eco-evolutionary optimality theory predicts that photosynthetic capacity responses to elevated $CO_2$ should be independent of nitrogen fertilization and inoculation treatments, while the nitrogen limitation hypothesis indicates that whole-plant responses to elevated $CO_2$ should be enhanced with increasing nitrogen fertilization. In this context, the null interaction between nitrogen fertilization and $CO_2$ treatment on $V_{cmax25}$ was expected and is as informative for hypothesis testing as the significant interaction between nitrogen fertilization and $CO_2$ treatment on total biomass. Thus, model simplification via stepwise reduction or AIC-based model selection could lead to the removal of important model terms that would 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 and hypotheses, 'emmeans' provides a robust method for comparing treatment combinations and is a common tool used for interpreting pairwise comparisons in linear mixed-effect models.

We appreciate the reviewer's suggestion and acknowledge the value of considering 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 $CO_2$ and nitrogen fertilization on $N_{area}$, and this was only briefly mentioned in the manuscript. This interaction indicated that increasing nitrogen fertilization had a stronger positive effect on $N_{area}$ under ambient $CO_2$, leading to a stronger reduction in $N_{area}$ under elevated $CO_2$ as nitrogen fertilization increased. However, this pattern did not correspond with a similar effect of nitrogen fertilization on leaf-level photosynthetic responses to elevated $CO_2$. These patterns suggest that the stronger reduction in leaf nitrogen content under elevated $CO_2$ with increasing nitrogen fertilization was likely driven by reduced leaf nitrogen allocation to non-photosynthetic pools (e.g., structural tissue, secondary metabolites, etc.), and not a shift in allocation away from investment in photosynthetic tissues. To address this, we have added the following paragraph to the Discussion, starting on line 525:

> "Negative effects of elevated $CO_2$ on mass- and area-based leaf nitrogen content became more pronounced with increasing nitrogen fertilization (Fig. S2a-b). Since nitrogen fertilization did not affect photosynthetic responses to elevated $CO_2$, this decline in leaf nitrogen content may reflect reduced allocation to non-photosynthetic pools, such as structural tissue or chemical pathways that contribute to herbivore defense (Zavala et al., 2013; Onoda et al., 2017; Johnson et al., 2020). While not a primary focus of this study, understanding leaf nitrogen allocation responses to elevated $CO_2$ across nitrogen

availability gradients would help clarify the role of leaf nitrogen allocation on leaf-level responses to elevated $CO_2$.”

**Ln 100-103 Is coordination optimal? Has the increase 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.**

Coordination of $V_{cmax25}$ and $J_{max25}$ is considered optimal and has been consistently observed in $CO_2$ fertilization experiments. The second half of this paragraph addresses this pattern, citing multiple studies that support the hypothesis that $J_{max25}:V_{cmax25}$ tends to increase under elevated $CO_2$ due to a stronger decrease in $V_{cmax25}$ than $J_{max25}$. This acclimation response is thought to enhance nitrogen-use efficiency by minimizing overinvestment in Rubisco under high atmospheric $CO_2$ conditions. One of the more notable citations included in this paragraph is Smith & Keenan (2020), who used a dataset that included 51 species from 33 elevated $CO_2$ sites to show that $J_{max}:V_{cmax}$ generally increases under elevated $CO_2$. For ease of review, we have copied the portion of the paragraph in the manuscript that provides this justification and relevant citations, starting on line 107:

> “The eco-evolutionary optimality hypothesis predicts that plants optimize leaf nitrogen allocation to photosynthetic capacity to use available light efficiently while avoiding over-investment in Rubisco, which has high nitrogen and energetic costs to build and maintain (Evans, 1989; Sage, 1994; Evans and Clarke, 2019). This strategy enhances photosynthetic nitrogen-use efficiency and allows increased net photosynthesis rates to be achieved by increasing the co-limitation of net photosynthesis rates by Rubisco carboxylation and electron transport for RuBP regeneration (Chen et al., 1993; Maire et al., 2012; Wang et al., 2017; Smith et al., 2019). Empirical evidence supports this hypothesis (Crous et al., 2010; Lee et al., 2011; Smith and Keenan, 2020; Harrison et al., 2021; Dong et al., 2022; Cui et al., 2023), though few studies have connected these patterns with concurrently measured whole-plant responses.”

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

Our experiment was not designed to capture patterns in expanding and dynamic systems. However, previous studies have reported similar leaf and whole-plant responses to elevated $CO_2$ in field settings as was observed in this study (e.g., Crous *et al.*, 2010; Lee *et al.*, 2011; Smith & Keenan, 2020). The hypothesis referenced in the lines mentioned by the reviewer predicts that biomass responses to elevated $CO_2$ would result from stronger increases in belowground biomass than aboveground biomass. This prediction is supported by a meta-analysis (Stocker *et al.*, 2025) that synthesized findings from field $CO_2$ fertilization experiments where treatments were imposed for at least three years. Results from the meta-analysis show that positive biomass responses to elevated $CO_2$ were driven by stronger increases in belowground biomass than aboveground biomass, which increased the root-to-shoot ratio. These responses indicate that these allocation patterns may indeed be captured in expanding systems.

**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 sphagnum moss is an unsustainable anthropogenic action that negatively affects ecosystem carbon sequestration. In hindsight, we ought to have chosen a more ecologically friendly substrate for the soil-less mix; however, peat moss provides a useful substrate for enhancing water retention and maintaining organic matter in pots without adding supplemental fertilizer amendments found in most commercial potting mixes.

**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 $CO_2$, 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 $CO_2$, one paragraph focuses on the null inoculation effects on plant responses to elevated $CO_2$, 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

**Response References**

**Crous KY, Reich PB, Hunter MD, Ellsworth DS**. **2010**. Maintenance of leaf N controls the photosynthetic $CO_2$ response of grassland species exposed to 9 years of free-air $CO_2$ enrichment. *Global Change Biology* **16**: 2076–2088.

**Harrison SP, Cramer W, Franklin O, Prentice IC, Wang H, Brännström Å, de Boer H, Dieckmann U, Joshi J, Keenan TF, *et al.* 2021**. Eco-evolutionary optimality as a means to improve vegetation and land-surface models. *New Phytologist* **231**: 2125–2141.

**Iversen CM**. **2010**. Digging deeper: Fine-root responses to rising atmospheric $CO_2$ concentration in forested ecosystems. *New Phytologist* **186**: 346–357.

**Iversen CM, Ledford J, Norby RJ**. **2008**. $CO_2$ enrichment increases carbon and nitrogen input from fine roots in a deciduous forest. *New Phytologist* **179**: 837–847.

**Kenward MG, Roger JH**. **1997**. Small sample inference for fixed effects from restricted maximum likelihood. *Biometrics* **53**: 983.

**Lee TD, Barrott SH, Reich PB**. **2011**. Photosynthetic responses of 13 grassland species across 11 years of free-air $CO_2$ enrichment is modest, consistent and independent of N supply. *Global Change Biology* **17**: 2893–2904.

**Sigurdsson BD, Medhurst JL, Wallin G, Eggertsson O, Linder S**. **2013**. Growth of mature boreal Norway spruce was not affected by elevated $[CO_2]$ and/or air temperature unless nutrient availability was improved. *Tree Physiology* **33**: 1192–1205.

**Smith NG, Keenan TF**. **2020**. Mechanisms underlying leaf photosynthetic acclimation to warming and elevated $CO_2$ as inferred from least-cost optimality theory. *Global Change Biology* **26**: 5202–5216.

**Smith NG, Keenan TF, Prentice IC, Wang H, Wright IJ, Niinemets Ü, Crous KY, Domingues TF, Guerrieri R, Ishida FY, *et al.* 2019**. Global photosynthetic capacity is optimized to the environment. *Ecology Letters* **22**: 506–517.

**Stocker BD, Dong N, Perkowski EA, Schneider PD, Xu H, de Boer HJ, Rebel KT, Smith NG, Van Sundert K, Wang H, *et al.* 2025**. Empirical evidence and theoretical understanding of ecosystem carbon and nitrogen cycle interactions. *New Phytologist* **245**: 49–68.

**Wright IJ, Reich PB, Westoby M**. **2003**. Least-cost input mixtures of water and nitrogen for photosynthesis. *The American Naturalist* **161**: 98–111.