

August 02, 2024

Dear Dr. Mary Heskell, Dr. Lee Kalcsits, and the Editorial Board at *AoB Plants*,

Please find our revised manuscript (#24025), titled *Symbiotic nitrogen fixation reduces belowground biomass carbon costs of nitrogen acquisition under low, but not high, nitrogen availability*, attached. We have included a copy of the revised manuscript with changes from the previous revision noted through the “Track Changes” feature in Microsoft Word, as well as a clean copy of the revised manuscript for ease of review.

With apology to the first reviewer for our lack of constructive responses, we have made several major changes to the manuscript. First, as requested from both reviewers, we have removed all uninoculated plants that formed root nodules from the analyses. Our results remain unchanged for all traits except for root nodule biomass, the ratio of root nodule biomass to root biomass, and root biomass. Root nodule biomass and the ratio of root nodule biomass to root biomass now each decrease with increasing nitrogen fertilization and root biomass is now greater in inoculated plants. We have also removed our incorrect interpretation of Menge et al. (2023), though still cite the paper in the manuscript to mention that plants can still acquire nitrogen through symbiotic nitrogen fixation under high nitrogen availability. We have also reframed the Discussion to focus less on the cost to acquire nitrogen and more on its component parts per the second reviewer’s suggestion. Finally, we have sifted through the initial comments from the first round of reviewer comments and have made major revisions to the Introduction section, which are summarized below in our response document.

Below, we provide a point-by-point response to the Associate Editor and reviewer comments. We first include the editor/reviewer comment in black-colored font and include our response immediately below each comment in red-colored font. Line numbers, where relevant, are included in our response to reference where major changes were made in the revised manuscript.

Please contact us at the contact information listed in the submission portal if there are any questions or concerns about our revised submission.

Sincerely,

The Authors (names removed for anonymity per journal guidelines)

Associate Editor (Comments for the Author (Required)):

Please see the comments from the two expert reviewers. Reviewer #1 requested that you make sure you have carefully addressed their comments from the original submission and provided two key required revisions before the manuscript can be considered acceptable.

Thank you for the opportunity to resubmit this manuscript and for the patience from you and the rest of the editorial team as well as the two reviewers. We have made the two key required revisions requested from the first reviewer and have made expansive efforts to improve the Introduction section of the manuscript after revisiting comments from both reviewers during the first round of revisions. Please see below for more detailed responses to reviewer comments and suggestions.

Referee #1 Evaluations:

Recommendation: Unacceptable

Referee #1 (Comments for the Author (Required)):

Most of my concerns from the first round of review remain, and I am disappointed in the author's responses to both set of reviews. I won't enumerate my concerns again, but will point out two resolvable issues, both based on the other reviewer's previous comments, that should be addressed.

With apology to the reviewer for the unsatisfactory responses in the last round of revisions, we have re-read the concerns and responses from the last round of revisions from both reviewers. We have made a few additional revisions beyond the two points raised below that stem from the initial review, summarized below, that we hope the reviewer will find satisfactory.

We have made considerable changes to the Introduction, as many of the first and second reviewer's initial comments aimed to clarify this section. First, we acknowledge that the small sample size and number of treatments in the experiment limits the applicability of this experiment to Earth system model development and agree with the reviewer that the mention of Earth system models was out of place. We have removed the first paragraph that puts our work

in this context and have removed most mentions of Earth system modeling throughout the manuscript. The manuscript has broadly been reframed to introduce and discuss the ecology of the observed responses.

Second, we have revised the second and third paragraph of the Introduction that describe the biotic and abiotic controls on carbon costs to acquire nitrogen. The second paragraph now describes expected differences in costs to acquire nitrogen across acquisition strategies and poses possible mechanisms that might explain these differences between species that rely on direct uptake versus species that rely on symbioses with nitrogen-fixing bacteria. We have also included a brief explanation of the symbioses between plants and nitrogen-fixing bacteria as the reviewer suggested. The third paragraph, which initially juggled effects of multiple abiotic factors on carbon costs to acquire nitrogen, now streamlines content by only discussing effects of nitrogen availability on carbon costs to acquire nitrogen. The paragraph finishes by discussing the role of symbiotic nitrogen fixation on modulating variance in costs to acquire nitrogen across nitrogen availability gradients.

Finally, we have streamlined the introduction by removing the paragraph that summarizes results from Perkowski et al. (2021) as the content in this paragraph was redundant to the revised previous paragraph.

First, and most importantly, the nodulated but uninoculated plants should be excluded from the uninoculated data. This is a bummer but it's very common and doesn't represent a mistake so much as just a thing that happens in greenhouses. I can not think of a way in which nodulation in non-inoculated plants is, as the authors say in their response, a product of their treatments. It's very likely just contamination. Given that this represents an unintended treatment exactly opposite of the intended treatment, these points (nodulated but non-inoculated plants) should be removed.

The revised manuscript now reports results from models that do not include any uninoculated but nodulated plants. Effects of treatment combinations on carbon costs to acquire nitrogen, belowground biomass carbon, whole-plant nitrogen biomass, total leaf area, and whole-plant biomass remain unchanged from the previous analyses. There is now a weak negative effect of

inoculation on root biomass ($\chi^2=3.884, p=0.049$), where there was previously a marginal negative effect ($\chi^2=3.268, p=0.071$).

We have slightly revised the models used to explain the effects of the treatments on root nodule:root biomass and root nodule biomass. Models now include only inoculated individuals and include only nitrogen fertilization treatment as a categorical fixed effect, with block included as a random intercept term. We did not include uninoculated individuals or an inoculation fixed effect into models because it seems trivial to include these factors when all remaining root nodule biomass values in the uninoculated treatment are zero. However, it is worth noting that exploratory analyses incorporating uninoculated individuals and an inoculation treatment fixed effect yield the same results. The analyses indicate that increasing nitrogen fertilization decreased both root nodule:root biomass ($\chi^2=4.663, p=0.031$) and root nodule biomass ($\chi^2=6.391, p=0.011$), contrasting the previous manuscript version that reported null effects of nitrogen fertilization on both traits (root nodule:root biomass: $\chi^2=1.291, p=0.256$; root nodule biomass: $\chi^2=1.364, p=0.243$). These findings suggest that individuals decreased investment in symbiotic nitrogen fixation with increasing nitrogen fertilization, consistent with our expectation and many previous studies.

Altogether, the removal of the uninoculated but nodulated individuals did not constitute a major change to the main results besides the new negative effects of increasing nitrogen fertilization on root nodulation. Tables, figures, and the main text of the Results section have all been updated to reflect these changes. Starting on line 360 of the unmarked manuscript, we have modified the Discussion subsection that interprets root nodulation data. We have also fixed our misinterpretation of Menge et al. (2023). The manuscript now discusses that root nodulation responses to nitrogen fertilization are consistent with previous work, but note that Menge et al. (2023) finds that individuals can still acquire nitrogen through nitrogen fixation under high fertilization. This subsection is copied below for ease of review:

“Consistent with our hypothesis, root nodulation and plant investment toward symbiotic nitrogen fixation decreased with increasing nitrogen fertilization in inoculated plants. These patterns corresponded with diminished effects of inoculation treatment on

belowground biomass carbon, whole-plant nitrogen biomass, and total leaf area with increasing nitrogen fertilization. These results are consistent with previous results showing that plants decrease reliance on nitrogen-fixing symbionts as soil nitrogen availability increases (Vitousek *et al.* 2002; Perkowski *et al.* 2021). Though recent work suggests that plants can still acquire nitrogen through symbiotic nitrogen fixation under high nitrogen availability (Menge *et al.* 2023), these patterns indicate that inoculated individuals likely shifted their relative mode of nitrogen acquisition away from nitrogen fixation and toward direct uptake pathways with increasing nitrogen fertilization.”

Second, the authors seem to mischaracterize the Menge et al 2023 paper that reviewer 2 suggests they include. The general results of that paper contrast with the current manuscript, but the authors here seem to be selectively choosing results that support their findings while ignoring the main point of the paper.

Our interpretation of Menge et al. (2023) has been corrected. This was accomplished by removing “even if investment in the nitrogen fixation pathway trends in a negative direction” at the end of the sentence starting on line 367. We have also taken care throughout the rest of the manuscript to ensure that Menge et al. (2023) is correctly referenced.

Referee #2 Evaluations:

Recommendation: No changes needed

Referee #2 (Comments for the Author (Required)):

Overall, the authors did a great job addressing my initial concerns. Findings from this study were interpreted fairly without overselling - shortcomings and caveats were clearly articulated. Below is a list of small concerns for authors to consider:

- It would be more direct and clear to readers to say inoculation "increased plant N uptake" rather than "reduced C cost of N acquisition". The latter statement suggests that C allocation patterns differed after inoculation (which is not what you found). Authors have clearly thought about

terminology in detail - just wanted to mention that the phrases can be wordy and slightly difficult to interpret when reading through for the first time.

Thank you for your kind words about our revision. We have changed this wording throughout the Discussion to clarify these responses. This has comprised a slight shift in the framing of the Discussion, as the original Discussion primarily centered its focus on carbon costs to acquire nitrogen as the integrated trait. Regardless, we have made these changes and hope that it improves the readability of the Discussion. It should be noted that we have not changed these terms in the Introduction, as establishing the variance in costs to acquire nitrogen across acquisition strategies and nutrient availability gradients is a central part of our story setup and experiment motivation. However, we have added a sentence that explicitly explains what carbon costs to acquire nitrogen are (line 35 of the unmarked manuscript) and have added greater emphasis on its component parts (belowground carbon allocation, whole-plant nitrogen biomass) where relevant to understand the mechanisms that drive variance in these costs. These changes throughout were done with the hope that this will make observed patterns and their interpretations easier to digest and understand.

- Authors did a great job addressing comments in the introduction overall. I would still suggest that some of the information in the first two paragraphs (i.e. different N acquisition strategies) could be streamlined as it is not directly related to the specific study.

We thank the reviewer for their kind words. In accordance with suggestions from the first reviewer's initial comments, we have largely reorganized the Introduction to streamline content and provide relevant background needed to understand experiment motivations. A summary of these changes is presented in our response to the first reviewer.

- L132-137: Not sure this level of detail is necessary in the introduction. You might consider explaining this phenomenon (as is done in the previous paragraph) and citing this study rather than outlining the results of this study.

We agree and have removed this paragraph from the manuscript as it was redundant to the end of the previous paragraph.

- L400-402: This sentence is confusing - it reads as though you are drawing conclusions about N addition within each N addition treatment separately?

We agree with the reviewer. This sentence has been removed from the Discussion as part of our reframe away from discussing carbon costs to acquire nitrogen and toward discussing the component parts of this trait.