

May 14, 2024

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

Please find our revised manuscript (#24025), now titled *Symbiotic nitrogen fixation reduces belowground biomass carbon costs of nitrogen acquisition under low, but not high, nitrogen availability*, attached. Note that this slight title change is the result of a reviewer comment. Specifically, we have added the words “belowground biomass” to more accurately reflect the findings of the manuscript. We have also included a copy of the revised manuscript with changes noted through the “Track Changes” feature in Microsoft Word..

We thank the Associate Editor and two reviewers for their helpful and constructive feedback, as we feel these changes have greatly improved the manuscript. We have revised the manuscript to now only focus on nitrogen uptake through direct uptake or symbioses with nitrogen-fixing bacteria, have clarified study limitations and modeling implications, have completed the requested changes to figures, and have expanded the body of work cited in the manuscript.

Below, we provide a point-by-point response to 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.

If you have any questions or concerns about our revised submission, please contact us at the contact information listed in the submission portal.

Sincerely,

The Authors (names removed for anonymity per journal guidelines)

Associate Editor Evaluations:

Recommendation: Accept Revision Required

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

Please see the comments from two expert reviewers. I would suggest that the authors frame their research with the extensive body of research conducted in this area and clarify how this research is novel or different than previous research.

Thank you to the reviewers for the helpful and constructive comments on our manuscript. In response to this comment we have emphasized the novelty of our research and tied it more closely to previous studies. We expand on the specifics of this in the responses below.

Referee #1 Evaluations:

Recommendation: Unacceptable

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

Comments and suggestions to the Author(s):

This study aims to test the effects of inoculation and nitrogen fertilization on the carbon cost of nitrogen uptake in Glycine max with a factorial greenhouse experiment. The authors found that only under low nitrogen levels did inoculation reduce the structural carbon cost of nitrogen uptake. This was primarily driven by change in N content rather than decrease in structural carbon toward nitrogen uptake. These results are in concordance with previous work done to understand the abiotic and biotic controls of nitrogen uptake efficiency. Overall, however, the study is very small and not particularly novel, limiting the interest to readers.

There are many, many studies growing N fixers, especially G. max, at different levels of N. This study includes just 64 plants, of an extremely well-studied species, and doesn't appear to have much new to offer in terms of analyses or results. To be clear: the experiment itself seems sound - it's just that we have a lot of similar data from decades of previous research.

Soybean is a model plant that has been studied abundantly with respect to nitrogen fixation and uptake. From these studies, we have a strong understanding of how N availability influences investment in their rhizobial partners. Given the limited scope of the study itself, the setup of the intro based on Earth system models seems out of place. Earth system models and the smaller models used to build them are modeling dynamics with respect to trees, which are vastly different than what is observed in model herbaceous plants such as soybean. So the application of this study to these models is severely limited in this respect. The manuscript does not state 1) if these findings differ from the current assumptions made in these models and 2) how these data could/should be explicitly incorporated given the focus of the introduction.

We thank the reviewer for their opinions on the novelty of the experiment we performed. We performed this experiment to explicitly test how variation in nitrogen availability, modified through fertilizer amounts and the capacity for individual plants to form symbioses with nitrogen fixing bacteria, influence plant carbon costs to acquire nitrogen. We specifically did this using a single species to directly quantify these effects. We did this because Earth System Models are now including explicit nitrogen dynamics. Thus, these models must accurately simulate carbon-nitrogen economics of nitrogen-fixing plants, as the carbon-for-nitrogen exchange of this symbiosis comprises an important link between ecosystem carbon and nitrogen cycles. In contrast to the assumption made by the reviewer, models must be able to simulate carbon-nitrogen economics for plants globally, and so must accurately simulate these dynamics in both herbaceous and woody plants in natural and agricultural systems that acquire nitrogen through a variety of different nitrogen acquisition strategies. Many current-generation Earth system models use plant functional groups that range from trees (e.g., broadleaf deciduous trees) to grasses (e.g., C3/C4 grasses) to crops (e.g., C3/C4 crops) to predict ecosystem carbon and nutrient fluxes (e.g., JULES, CESM), so understanding carbon-nitrogen dynamics in a herbaceous crop species as done here is not trivial to model benchmarking and future model development. In models that incorporate explicit nitrogen dynamics, the carbon costs of nitrogen fixation and direct nitrogen uptake are relatively static. Our study shows that these costs are dynamic and provides an empirical benchmark for further model testing and development. Because we performed our experiment under controlled conditions while also controlling for life form and phylogeny, we

have made it simpler for these models to perform simulations designed to hit these benchmarks. To address this concern, we have provided further emphasis in the manuscript on the novelty and usefulness of the study we performed.

More-detailed comments:

Overall, the introduction is clearly written, even if the focus on earth system models seems strange. However, the third paragraph beginning on line 45 is a little hard to follow. The main point seems to be to describe the biological controls of the carbon cost of nitrogen uptake, but it reads as a series of semi-related scenarios rather than in-depth description of the system. More emphasis on the need to incorporate these different uptake strategies given their different costs would help bring it together.

Thank you for this comment. In response, we have further emphasized the variability in carbon costs of nitrogen acquisition that can occur with varying soil nitrogen availability and nitrogen fixation. As these costs vary across nitrogen availability gradients and likely due to ability to associate with symbiotic nitrogen-fixing bacteria, models that omit variability in costs to acquire nitrogen are likely to bias estimates of plant carbon-nitrogen economics across environmental gradients.

More emphasis on the potential importance of non-structural carbon investment, particularly with respect to the symbiosis pathway beyond the citation offered on line 137, may be helpful. This symbiosis is described as the exchange of fixed nitrogen from rhizobia and sugars, or non-structural carbon from the legume. Discussion about how this exchange could influence the overall results may help add some context.

We thank the reviewer for pointing out this possible confusion. In response to this and a comment from Reviewer 2, we have opted to use the phrase “belowground biomass carbon” to describe the amount of carbon we measured belowground, which includes both structural and non-structural components. As a result, we have replaced “structural carbon cost” with “belowground biomass carbon cost” throughout.

In addition to global systems models, why is the study of structural carbon costs of nitrogen uptake biologically important? Outside of making models better, how does this study add to our knowledge of how plants work?

We thank the reviewer for this comment. As we state in the manuscript, this trait is important for understanding the role that nitrogen fixation plays in determining the effect of nitrogen availability on plant carbon economics. Our results show that, within a single species, nitrogen availability both directly modifies plant carbon economics as well as indirectly modifies these processes through its impact on plant investment toward symbiotic nitrogen fixing bacteria. Variability in these costs across treatment combinations provide important insight into understanding how symbioses with nitrogen-fixing bacteria modify plant nutrient uptake and allocation to aboveground structures across nitrogen availability gradients.

It would also be useful to the reader to introduce the n-fixing symbiosis that is the focus of this study and why this relationship is of particular importance within global systems models. This is alluded to in the abstract (lines 6-10) but a concrete description of this is missing. This will also greatly help the reader understand your hypotheses at the end of the introduction, which we are not currently given any background information to understand why these predictions make sense.

We thank the reviewer for this comment and have added a sentence on line 56 to emphasize the knowledge gap that we are filling with this study.

The paragraph beginning line 81 only focuses on a single study and lacks the detail of the greater body of work that this study fits into. It would be more informative to discuss previous research that is relevant to this study to place this work in a broader context.

We thank the reviewer for this comment. Here and throughout, we have invoked discussion of a greater body of literature to help set up and contextualize our study.

More detail about greenhouse conditions would be good to include, if possible - average temp during day/night; average day length; any supplementary lighting, etc.

We have added mean daytime maximum/nighttime minimum greenhouse temperature details to the Methods. There was no supplementary lighting used.

Figures: It would be more intuitive for the reader to change the x-axis labels from "70" & "630" to "low" & "high" as this is how they are discussed in the main text.

We have made this change as requested.

Referee #2 Evaluations:

Recommendation: Minor changes needed

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

In this study authors conducted a full factorial greenhouse experiment aimed at untangling the role of symbiotic N-fixers in plant N acquisition under different levels of soil N availability. The experiment was very clean and well designed to investigate the questions outlined in the text. Authors found that increasing N availability through fertilization and inoculating seedlings with N fixers (in low, but not high N soils) increased N uptake resulting in greater aboveground biomass. Strikingly, root biomass nor root nodulation responded to N addition or inoculation. Overall, I think this manuscript was based on a sound scientific question and supplied interesting and impactful results that were conveyed relatively clearly. However, I think authors could make significant improvements in the introduction to better outline relevant background information and clarify the importance of this study. Below, I have outlined some general suggestions for each section and some specific line by line comments.

Introduction

- Authors spend a lot of time in the introduction comparing and contrasting different nutrient acquisition strategies, but this study is specifically focused on symbiotic relationships between

plants and N-fixing bacteria. I would suggest focusing the information in the introduction on this topic (e.g. try to introduce the idea of symbiotic N fixation by the end of the first paragraph).

We thank the reviewer for this comment and, as a result, have shifted the focus of the Introduction to place greater emphasis on nitrogen fixation. However, we have chosen to keep a similar structure to the Introduction, where we first introduce the importance of carbon-nitrogen interactions and the role that nitrogen acquisition strategies play in modifying this, but now with a greater emphasis on the nitrogen fixation strategy.

I'm not sure it is necessary to discuss mycorrhizal fungi. Some examples of lines that I think might be unnecessary/could be condensed to make space for background information focused on relationships between plants and N fixing bacteria: L38-44, L50-56, L59-64

We have removed mention of mycorrhizal fungi throughout the main text and only mention direct uptake and symbioses with nitrogen-fixing bacteria.

- By condensing down the initial paragraphs, authors could spend more time introducing studies that have specifically investigated effects of N availability on N fixation and plant growth dynamics. Although lines 81-96 are a nice summary of Perkowski 2021, authors could consider expanding this to encompass background information from different studies.

In response to this and the comments from Reviewer 1, we have added discussion and citation of a greater body of literature to better introduce and contextualize our study.

- The response of N-fixing bacteria to increasing N availability is also an important part of the relationship you are studying (though authors focus on the plant side of the story in this manuscript). It might be important to introduce some past work showing N fixer responses to increasing N availability as well? This study finds that alleviating N limitation through N addition did not drive decreased symbiotic N-fixation in multiple species at multiple latitudes. I'm wondering if this study might be worth introducing here or later in the discussion:

- Menge, Duncan N. L., Amelia A. Wolf, Jennifer L. Funk, Steven S. Perakis, Palani R. Akana, Rachel Arkebauer, Thomas A. Bytnerowicz, et al. 2023. " Tree Symbioses Sustain Nitrogen Fixation Despite Excess Nitrogen Supply." *Ecological Monographs* 93(2): e1562. <https://doi.org/10.1002/ecm.1562>

We thank the reviewer for this comment and pointing us to the Menge et al. (2023) study. We now discuss it in the manuscript. Importantly, the Menge et al. (2023) study corroborates our results by showing a decreasing trend in nitrogen fixation with increasing soil nitrogen availability. We have also added a sentence to the Introduction that summarizes nitrogen-fixing responses to nitrogen availability, starting on line 75 and copied below:

“Indeed, plants that form associations with symbiotic nitrogen-fixing bacteria often exhibit reduced responses to changes in nitrogen availability despite reduced investment toward nitrogen fixation with increasing nitrogen availability (Gutschick 1981; Taylor and Menge 2018; Friel and Friesen 2019; McCulloch and Porder 2021; Menge *et al.* 2023; Schmidt *et al.* 2023).”

Results

- In order to make life easier for readers, it could be helpful to include a figure outlining responses of seedlings under each of the 4 trts (essentially conveying the results from Table 1 in an image).

All four treatments are already conveyed in each of the three figures. Soil nitrogen fertilization treatment is on the x-axis. Inoculation treatment is conveyed as separate colored boxplots and points within each nitrogen fertilization treatment level. Each figure includes compact lettering to show pairwise comparisons where Tukey: $p < 0.05$. General model results (e.g., p -values listed in Table 1) could be added to each panel, but this would be redundant to Table 1.

- Figure 3: There appears to be some not inoculated seedlings with significant nodule biomass. Should these plants have been excluded from the analyses? This point should be addressed in the methods/discussion.

The reviewer is correct that some individual plants had root nodules despite not being inoculated. We have decided to keep these in our analyses, as this was a consequence of our treatment. We have added a sentence in the Methods to indicate that individuals were not screened for level of nodulation prior to analyses. We have also added a sentence to the Discussion to point out that some non-inoculated individuals formed nodules.

Line by line comments:

L32: missing a word between "however, often"?

“these models” have been added between “however,” and “often”.

L76-80: This statement suggests that seedling inoculated with N-fixing bacteria might not respond to N addition (or would be less sensitive to N-addition compared to uninoculated seedlings). However, your third hypothesis suggest that inoculated seedlings will respond to increased N availability by investing less in N-fixing bacteria. This statement could be clarified or expanded on.

We have added sentences to the end of this paragraph (starting on line 79) to clarify that the expected lesser carbon cost to acquire nitrogen response to nitrogen fertilization in inoculated individuals is likely due to a shift in nitrogen acquisition from nitrogen fixation under low nitrogen fertilization to direct uptake under high nitrogen fertilization. These sentences are copied below:

“While previous work notes that plants can still acquire nitrogen through symbiotic nitrogen fixation under high soil nitrogen availability (Menge *et al.* 2023), resource optimization theory suggests that reduced sensitivity of plant nitrogen uptake to changes in nitrogen availability in nitrogen-fixing plants may stem from preferential investment toward the resource acquisition strategy that confers the lowest carbon cost and greatest nitrogen gain (Bloom *et al.* 1985; Rastetter *et al.* 2001). If true, similar costs to acquire nitrogen in nitrogen-fixing species may be achieved across nitrogen availability gradients

due to a shift away from nitrogen acquisition through nitrogen fixation to direct uptake as costs to acquire nitrogen through direct uptake decrease (Perkowski *et al.* 2021).”

L92-96: This statement suggests a gap in knowledge (are distinct responses of plants to N fertilization due to symbiotic relationships with AM fungi vs N fixing bacteria?). This is not a gap in knowledge filled by this paper. I would suggest reworking this paragraph- taking out discussion/comparison of the two different N acquisition strategies (N acquisition via mycorrhizal fungi is not relevant to this study) and focus on gaps in knowledge related to symbioses with N fixing bacteria.

Thank you for this comment. We removed wording discussing mycorrhizal fungi from this paragraph to keep the focus on understanding the role of nitrogen fixation on carbon costs to acquire nitrogen across a nitrogen fertilization gradient.

L99: Clarify what these seedlings are being inoculated with (i.e. nitrogen fixing bacteria, not mycorrhizal fungi).

We have added “... were either inoculated or not inoculated with symbiotic nitrogen-fixing bacteria in a full-factorial greenhouse experiment” to line 106.

L118: Provide a brief explanation of why *G. max* was the plant species chosen for this experiment.

A brief explanation was added starting on line 129, reading: “The experiment used *G. max* to be able to compare observed responses from previous work that was not able to disentangle species-specific effects from the explicit effects of nitrogen fixation (e.g., Perkowski et al., 2021).”

L131-132: What was your reasoning for choosing these specific levels of N addition (specifically related to your comments in the 'study limitations' section relating to potentially nonlinear response of root nodulation to N addition)?

We chose these nitrogen fertilization levels based on previous work using a larger number of fertilization levels (Perkowski et al., 2021).

L160-161: It might be worth summarizing some of these points in your paper (here or in the discussion) as this might be important in how readers interpret your results.

We thank the reviewer for this comment and, in response, have included text on these points in the Discussion (line 418-424), copied below:

“Finally, the belowground biomass carbon cost to acquire nitrogen metric used in this study does not account for changes in belowground carbon allocation due to root turnover, respiration, or root exudation. It is possible that nitrogen fertilization and inoculation with symbiotic nitrogen-fixing bacteria may modify metabolic pathways that alter carbon investment (e.g., bacterial respiration). Future studies should carefully assess whether these carbon pools should be measured as failure to measure these pools could risk underestimating the belowground biomass carbon cost of nitrogen acquisition.”

L341: Is it possible that belowground biomass did not change between N addition treatments because plants became limited by another nutrient in the N fertilized soils?

It is possible that fertilization treatments used here may have increased growth limitation by other nutrients. However, if true, we expect that increased growth limitation of any nutrient should enhance belowground carbon allocation as a strategy to increase root exploration and promote increased uptake of the limited nutrient. This, in turn, would increase root biomass and likely increase costs of acquiring nitrogen through an increase in belowground biomass carbon. These patterns were not observed here, suggesting that other nutrients likely did not limit growth.

L358-361: Do authors have any thoughts on why or why not these other belowground C investments (root exudates, root respiration, etc.) may or may not be important to consider when quantifying C cost to acquire N. In other words, is there any reason to think that root exudation

or root respiration may change in response to N fertilization or seedling inoculation even though root biomass did not?

It is difficult to speculate about this. However, it is possible that nitrogen fertilization and/or inoculation may alter metabolic processes in a way that modifies belowground carbon investment. For instance, the maintenance of the symbiosis likely requires more carbon than we were able to measure here. High amounts of nitrogen uptake under high nitrogen availability may also require additional carbon investment that we did not measure here. We expand on this in the Discussion, starting on line 396 and copied below:

“If true, greater carbon costs for nitrogen acquisition may have been observed in inoculated plants grown under high soil nitrogen if increased amounts of unquantified plant carbon were allocated toward bacterial respiration. Carbon and nitrogen tracing experiments would be useful for examining this hypothesis.”

L374-378: May be a good point to bring up Menge 2023 showing that symbiotic nitrogen fixation doesn't change as N availability increases. Together with your results, this suggests that association with symbiotic N fixers might increase C cost of N acquisition in higher N soils where plants are investing in bacterial partners (and the energetically expensive N fixation pathway they are undergoing), but not necessarily benefitting from the N they are producing.

Thank you again for pointing us to the Menge et al. (2023) paper. Notably, this paper does show a decreasing trend in fixation with increasing soil nitrogen availability, corroborating our results that show negative trending but insignificant effects of increasing nitrogen fertilization on investment toward symbiotic nitrogen fixation. We now discuss this paper and its relation to our results in the Discussion.

L381: This section should include some more details about the metric used to quantify C cost of N acquisition (unless authors chose to include more details in an earlier section). In general, I think this topic warrants more discussion as mentioned in an earlier comment.

We have added a brief comment on the limitations of this metric - namely that this calculation does not account for root turnover, respiration, or root exudation, which may risk underestimating the carbon cost of nitrogen acquisition. This comment starts on line 418 and is copied below:

“Finally, the carbon cost to acquire nitrogen metric used in this study does not account for changes in belowground carbon allocation due to root turnover or root exudation. Future studies should carefully assess whether these carbon pools should be measured as failure to measure these pools could risk underestimating the carbon cost of nitrogen acquisition.”

Throughout: I might suggest replacing "structural C cost" with "belowground biomass" which will be easier for readers to interpret.

While “belowground biomass” may be easier for readers to interpret, replacing “structural C cost” with “belowground biomass” may mislead readers. Based on the comment from Reviewer 1 about the non-structural component to this measurement, we have opted to use the phrase “belowground biomass carbon” to describe the amount of carbon we measured belowground. As a result, we have replaced “structural carbon cost” with “belowground biomass carbon cost” throughout, including in the revised title.

Throughout: What do you specifically mean by "N uptake efficiency"? I would suggest using a more clear/descriptive phrase to replace "N uptake efficiency" or defining it more clearly early in the ms (ex. L294, 300).

We were using the phrase “nitrogen uptake efficiency” to convey the inverse of the carbon cost to acquire nitrogen metric. That is, nitrogen uptake efficiency was being referenced as the amount of nitrogen returned from a belowground carbon investment. If belowground carbon biomass does not change and whole plant nitrogen biomass increases due to increased nitrogen fertilization, for example, this would entail a reduction in the carbon cost to acquire nitrogen. However, the amount of nitrogen uptake per unit carbon biomass increases, insinuating that

plants increased the efficiency by which they acquired nitrogen. To avoid confusion, we have removed any mention of nitrogen uptake efficiency throughout the manuscript and only discuss observed patterns in reference to the carbon cost to acquire nitrogen.

Throughout: Consider using abbreviations for carbon (C) and nitrogen (N)

We are opting to continue our use of the full element names to avoid any possible confusion.

Figure 1: I might suggest writing out more complete Y-axis titles so that readers don't have to continually reference the figure legend. Also it looks like there is a tiny "text" near the "C" in the third panel.

We have corrected y-axis titles to include each trait's complete name and have removed the tiny "text" that appeared near the third panel label. Quality control on all other figures have also been completed and x-axis values have now been replaced with "low" and "high" per the first reviewer's request.