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.

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.

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.

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?

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.

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.

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

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.

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). Im 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

• 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.

• 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. Im 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](https://nam04.safelinks.protection.outlook.com/?url=https%3A%2F%2Fdoi.org%2F10.1002%2Fecm.1562&data=05%7C02%7CEvan.A.Perkowski%40ttu.edu%7C73cebff04e4343cf332c08dc65f8e912%7C178a51bf8b2049ffb65556245d5c173c%7C0%7C0%7C638497366934345215%7CUnknown%7CTWFpbGZsb3d8eyJWIjoiMC4wLjAwMDAiLCJQIjoiV2luMzIiLCJBTiI6Ik1haWwiLCJXVCI6Mn0%3D%7C0%7C%7C%7C&sdata=O90i%2FyJIrdOvP3Hg%2FjvEck9jqWh0WC7Ec2KsRbHxxQ0%3D&reserved=0)   
  
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).

Suggesting single figure?

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

Line by line comments:

L32: missing a word between "however, 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.

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.

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

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

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)?

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.

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?

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?

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.

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.

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

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).

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

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.