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Karen Haubensak, Ph.D.

Subject-matter Editor

*Ecosphere*

Dear Dr. Haubensak,

We are pleased to submit a revised version of *Ecosphere* manuscript ECS18-0757, “Genetic variation in tree leaf chemistry predicts the abundance and activity of autotrophic soil microorganisms”. We have fully addressed each of the three reviewers’ comments in detail below. Our responses immediately follow each reviewer comment, page and line numbers listed in our responses refer to the revised version of the manuscript.

Consistent with ESA’s data policy, we have made our data and R code publicly available at:

https://www.github.com/selmants/CT\_amoA

We expect our paper to be of immediate interest to a wide audience of microbial ecologists, ecosystem scientists, and evolutionary ecologists. We hope you find our revisions and responses to reviewers’ comments sufficient to merit publication of our manuscript in *Ecosphere*.

Sincerely,

Paul C. Selmants, Ph.D.

Reviewer #1 (Comments to the Author):

This is well-written and interesting manuscript from a research group that has done foundational work in this field from the late nineties. Their current findings fit well to the earlier research done in their study system and broadens our understanding of the effects of plant genetic variation in ecosystem functioning. I have only few questions and suggestions throughout, yet I also noted something incoherent especially in statistics/statistical tests. See line-by-line comments below.

Line 155-156: The amplification efficiency seems to be quite low. Ideally, it should be over 95%. I just wonder why did you choose these particular primers as the efficiency is not in ideal range?

Response: We chose these particular primers based on the studies cited in the manuscript. We have added text acknowledging amplification efficiency is less than ideal but similar for both AOA and AOB, which should not affect our conclusions because we are most interested in comparing the relative responses of these two groups of microorganisms (lines 186-188).

Line 157, Data analysis paragraph: Maybe it’s better to write about ANOVA tests first and thereafter the regressions (then it follows the same order as in results section)?

Response: We have re-arranged the text in the Results section so that regression and ANOVA test results follow the same order as in the Methods section (lines 215-219).

Line 161-162: Not sure, but should it be “nitrification potential” instead of “potential nitrification”?

Response: Yes, it should be “nitrification potential”. We have changed the wording here (line 194) and throughout the manuscript.

Line 164-165: Why did you used higher than normal significance level as in your (significant) correlations p is below 0.03?

Response: As stated on lines 164-165 of the original version of this manuscript, we selected a significance (alpha) level of 0.10 because of low power due to small sample sizes. The cutoff for significance is selected prior to running statistical tests to avoid bias, which is what we did here. We have added text to specify that we set alpha = 0.10 prior to running statistical tests (lines 196-199).

Line 168-169: I don’t understand why significance level is set to “unnormal” 0.10 as the effect of tree zone on the ratio of archaeal-to-bacterial amoA was not even near to significant. If I understood right, you have 6 soil samples per 3 stands within each zone. I think that it should be enough to demonstrate significant effects of tree zone (if there are such) even if you use a “normal” 0.05 significance level.

Response: As stated above in response to the previous comment, we set the significance level to 0.10 because of relatively low sample sizes. There is nothing magical about alpha = 0.05, it is an arbitrary convention. Also, the significance level should be selected prior to running statistical tests to avoid bias, and should never be altered after the test has been performed. We have added text stating that we selected the significance level prior to performing statistical tests (lines 196-199 and lines 203-204).

Results:

Line 172: The first part of this sentence belongs to discussion. I think that you can start directly from “Foliar CT concentrations…”

Response: We have re-written this sentence so it begins with “Foliar CT concentrations … “, as suggested by this reviewer (line 207). However, we disagree that pointing to consistency with prior work does not belong in the Results section. Therefore, we still include a phrase pointing out that the trend in foliar CT across the Populus hybridization gradient is consistent with previous research (lines 209-211).

Lines 173-175: You should also present statistical tests here (ANOVA results), right? Now, only the means are presented, but their statistical difference is not tested anywhere.

Response: Our hypotheses relate to whether variation in foliar CT is a significant predictor of variation in nitrification and amoA gene abundance. Our use of statistical analysis was aimed specifically at testing our hypotheses. We list mean and standard deviations here to demonstrate that stand-level foliar CT along this Populus hybridization gradient varied as expected and as demonstrated in previous studies cited in this manuscript. We have made the data available online at https://www.github.com/selmants/CT\_amoA, so readers are free to perform such a statistical test if they see fit. No changes were made in response to this comment.

Line 183: “because of the high within-zone variability”, can you write like this if you don’t present how high the within-zone variability was? In my opinion, you should give e.g. a range within each zone or something else (supplementary figure?) to describe this data in more detail.

Response: We have added the coefficient of variation for archaeal-to-bacterial amoA ratiosin the hybrid zone (line 242). In addition, all the data are available in an online repository at https://www.github.com/selmants/CT\_amoA.

Fig 1. Could you separate the observations from different zones in these figs? E.g. open circle for Fremont, closed for Narrowleaf and grey for hybrids etc. It’s quite easy to imagine which are from Fremont, but it would be interesting to see how narrowleaf and hybrids are located in these regression lines.

Response: We have altered Fig. 1 so that observations from the different zones are represented by different shapes (circles, triangles, squares) as recommended by this reviewer.

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Reviewer #2 (Comments to the Author):

The paper by Selmants et al. examines the variation and links of leaf condensed tannin (CTs) concentrations, soil nitrification rates and abundance of bacterial and archaeal ammonia oxidizers among stands of two Populus species and their hybrids.

The paper is clear and easy to read, but very shallow in terms of providing new information. Variation of leaf CT concentrations among Populus species and their hybrids and the influence of this variation on soil N transformations have been shown in numerous studies, which leaves the correlative link between leaf CT concentrations and soil bacterial and archaeal oxidizer abundances as the only novel aspect of this study. That the variation of abundance of archaeal oxidizers, but not that of bacterial oxidizers, seems to be linked to the variation of soil nitrification rates among the field sites is an interesting observation as such. However, this link is correlative and in fact, depends heavily on one Fremont site of high archaea abundance. Is such observation worth of publication or should it rather be used as a starting point for experiments that corroborate the finding and reveal the mechanisms behind the observation? Looking at Fig. 1, it seems that the observed trends between bacterial and archaeal ammonia oxidizers and leaf CT concentrations are due to Fremont sites having significantly less bacterial than archaeal ammonia oxidizers, while no big differences appear in other sites. With this data set, isn't it premature to conclude that leaf CT concentrations drive the abundance of archaeal, but not those of bacterial ammonia oxidizers? What if the Fremont sites simply provide a great habitat for archaeal, but not for bacterial ammonia oxidizers and there is no real link between leaf CT concentrations and ammonia oxidizers? I would like to see more work on the subject to support the conclusions before publication.

Given that the title includes "genetic variation" and much of the text is built on this concept, there is little evidence of intraspecific variation in nitrification rates or bacterial and archaeal abundances (Fig. 1). The results suggest that the two species are different and have different effects on soil N transformations, but there is no genetic variation within species (not even the hybrids differ from both parent species). In fact, the reader remains puzzled how intraspecific genetic variation could be tested with the sampling scheme used.

As a minor point, the values of leaf CT concentrations given in the first sentence of results and in Fig. 1 do not seem to fit together.

Response:

We would remind this reviewer that science is incremental, and that our data provide evidence not only that variation in a genetically heritable plant trait is a significant predictor of AOA abundance, but also a significant predictor of soil nitrification potential, which strongly suggests the decline in AOA induced by increasing foliar CT inputs is the mechanism driving reductions in nitrification potential. We agree that our results are correlative, and we would welcome future experimentation to isolate mechanisms driving our trends.

We also agree with this reviewer that “the Fremont sites simply provide a great habitat for archaeal, but not for bacterial ammonia oxidizers”, and have tested whether variation in a number of factors significantly predict variation in both amoA gene abundance and soil nitrification potential. Based on the preponderance of evidence, and consistent with our hypothesis, we concluded that foliar CT concentrations were the primary driver of differences in AOA across sites because other factors that typically drive both AOA abundance and nitrification rates, including soil pH, soil organic C, soil total N, foliar N concentrations, soil moisture, and soil C:N, were not significant predictors of nitrification potential or amoA gene abundance.

The “genetic variation” in the title refers to variation in foliar CT concentrations, which has been shown in prior studies cited in this manuscript to be a genetically heritable trait that varies predictably across Populus spp. hybridization gradients. We did not test this intraspecific variation ourselves. Instead, we have built upon prior knowledge to demonstrate the extended impact of variation in this heritable trait on additional ecosystem components and functions.

Finally, this reviewer is correct that values of leaf CT concentrations given in the first sentence of the results do not match those in Fig. 1. Values were listed as being in mg/g in both places, but the data in the original version of Fig. 1 were actually in units of percent and mistakenly labeled as mg/g. We have converted the data in Fig. 1 from percent to mg/g so that the values in this figure are now properly labeled, and the values listed in the first sentence of the results (lines 207-209) are now consistent with values displayed in Fig. 1.

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Reviewer #3 (Comments to the Author):

The authors present a well-thought-out study that simply addresses a gap in the literature and establishes a potential mechanism subject to selection whereby plants can engineer soil autotroph microbial communities. The manuscript is well-written and does not require significant modification; however, I do believe that the authors need to further justify their statistical interpretation of their results. I understand the potential necessity for adjusting the significance threshold from 0.05 to 0.1, but in so doing they invite questioning of their results. This is problematic in the context of Figure 1B as it makes one wonder how much of the significance of the depicted relationship could be potentially do to one outlier site where high abundances of AOA gene copies were observed. It may be worth addressing this concern in the text in order to add strength to the author’s interpretation of their results by addressing a potential source of skepticism. I also feel as though the authors have overstated the gradient aspect of their study a bit, as their distribution of sites ends up being somewhat bimodal, especially when looking at the trait they are most interested in determining the impact of (foliar CT concentrations). This is something that should be admitted to in the discussion as a potential pitfall of the design. It does not downplay the effect of their results, however, as they show that hybridization therefore likely does have a significant effect on the traits they measure. Other than those concerns, I find the manuscript acceptable for publication with minor specific comments as follows:

Response: this reviewer is correct that foliar CT does not vary smoothly across the Populus hybridization gradient. This is because F1 hybrids backcross only with P. angustifolia (high CT) and not with P. fremontii (little to no CT), as stated on lines 104-105, and so there is a large gap between foliar CT concentrations of P. fremontii trees and the F1 and backcross hybrids, which have much higher concentrations coming from the P. angustifolia ‘parent’. No changes were made in response to this general comment.

64 – I believe a comma would help this sentence (“previously unknown, large and nearly ubiquitous”).

Response: We have added a comma as suggested by this reviewer (line 69).

89-92 – This addendum is not necessary here, you can add what is novel here to the similar segment at beginning of paragraph or just remove this sentence entirely.

Response: We feel it is helpful to the reader to have an explanation of the novelty and significance of our study in close proximity to our hypotheses. No changes were made in response to this comment.

106 – The term “narrowleaf” has not been defined yet and its meaning must be inferred.

Response: We have altered the text here to explicitly define each of the three zones (lines 122-127).

115 – I might quibble with calling “putting on dry ice” “flash-freezing.”

Response: We have replaced “flash frozen on dry ice” with “placed on dry ice” here (line 136) and also on line 155.

129 – Was soil moisture measured as well? If so it should be included, or reasoning should be provided as to why it was not a relevant parameter.

Response: Yes, gravimetric soil water content was measured on all soil samples, as stated on line 157. We have added text stating that soil moisture was not a significant predictor of nitrification potential or amoA gene abundance (lines 216-219).

142 – You do not have to do so here, but either here or in the discussion some of the pitfalls of using qPCR, particularly to infer function, should be mentioned. That can also be a way in which you assuage any concerns readers might have about the variability in amoA results observed in some of the particularly low CF sites.

Response: We have added text to the discussion section outlining the limitations of using qPCR to infer function, specifically that qPCR cannot distinguish between live, dead, or inactive cells (lines 301-303).

169 – Was there a reason why you still used a cutoff of alpha = 0.1 when you have more replicates to work with for this set as a result of the individual soil sample being your unit of replication?

Response: As stated in the text, we used a cutoff of alpha = 0.1 because of low sample size. Although n = 18 is larger than n = 9, it is still relatively small. No changes were made in response to this comment.

250 – The “although” in this sentence is not needed.

Response: We have re-worded this sentence (lines 324-326) to clarify our point that, despite being adapted to low-N ecosystems, AOA are still sensitive to plant traits that reduce N supplied through mineralization of organic matter.

Table 1 – Why doesn’t this table include the foliar CT concentrations observed? If those methods are detailed then the results should be explicit by treatment group somewhere in the text.

Response: The mean and variance of foliar CT concentrations are listed by zone in the text (lines 207-209) and are displayed by stand in Fig. 1. It would be redundant to include the same data in both a table and a figure. Readers who wish to see the data summarized differently may do so themselves, as we have made all the data available online at https://www.github.com/selmants/CT\_amoA. No changes were made in response to this comment.