RESPONSE TO REVIEWERS - **egusphere-2025-2078**

Reviewer_2

The article "Managed black truffle-producing systems have greater soil fungal network complexity and distinct functional roles compared to wild systems" by Barou et al. characterizes the fungal community in truffle plantation soils of Spain (with an outstanding sample size) and compares it with the fungal community in the soil of wild truffle-producing forests, while providing in-depth insight into the role of Tuber melanosporum in the soil fungal community networks, as well as into the influence of the most important fungal guilds on soil enzymatic activity. Truffle cultivation has traditionally advanced by observing the ecology of truffle in the wild. However, comparisons of wild and cultivated truffle sites are rare. This makes the study relatively novel. Besides, the information on soil enzymatic activity of the truffle soils is also relatively novel, even more its study in relation to the composition of the soil fungal community. All this information is an incremental knowledge that helps to better understand the role of truffle within the soil fungal community of truffle plantations and to unravel the role of this fungal community into the productivity and sustainability of truffle cultivation. The introduction of the study provides a comprehensive review of the state of the art, as well as clear and specific objectives and hypothesis for the study. The materials and methods are sufficiently described. The results are clearly exposed and provide an exhaustive analysis of the data, delving into ecological relationships with potential agronomic interest. The discussion connects the study objectives and hypotheses with the results, thoroughly exploring the ecological and practical implications of the study.

We thank to Referee #2 for his/her insightful and encouraging assessment. We have answered point by point to his/her comments in blue, as follows:

However, a paragraph with the study limitations, the potential lines for future research and/or the practical implications of the study for truffle cultivation could help emphasizing the relevance and novelty of the study ("Understanding the ecology, including the biodiversity and functioning, of black truffle-dominated soils will provide a stronger foundation for making informed decisions regarding the management of black truffle plantations" may not be the strongest sentence to end the manuscript).

We greatly appreciate this suggestion. Following the recommendations, we have better elaborated the closing sentence of the manuscript by including the practical implications of the study. The new ending for the conclusions paragraph is "*Further research based on pairwise experimental designs that include samplings in/out of the black truffle dominated areas, i.e., the brûlés, would help us to clarify the biotic and abiotic transformations induced by T. melanosporum in soils. The findings of this study on fungal biodiversity and the functioning of black truffle-colonised soils offer valuable practical insights, providing a robust scientific foundation to enhance decision-making and drive more effective management strategies, such as those related to fertilisation and soil microbiome management, for black truffle plantations.*"

However, a few minor issues should be clarified:

1) L125 Which were the dates of sampling? Besides, the final sampling size is 231 (explained in L137), but it is not clear which is the final size for wild/cultivated and for spring/autumn.

The samplings were conducted during October and November 2019 for the autumn campaign, and during April and May for the spring campaign.
For both autumn and spring campaigns 68 trees were sampled in plantations and 48 trees in forests as mentioned in L125, which made a total of 232 samples processed. However, due to a failure in a plantation sample of spring, 231 samples were finally considered for the analysis. We have now added this detail about the plantation-spring sample that failed in the manuscript at the 2.2 section of Materials and Methods.

2) L129 "Soil functioning was proxy through the potential activities" Is this sentence ok?

We have rephrased the sentence for a better understanding, as follows: “As a proxy for soil functioning, the potential activities of eight exoenzymes related with… were calculated.” (2.1 section of Materials and Methods).

3) L189 The tests for hypothesis 2 do not seem to correspond with the hypothesis 2 specified in L101, since no hypothesis talks about soil parameters. Besides, the study aims to find the differences in the fungal community between cultivated and wild truffières (L91). For this, the networks of both types should be compared, or alternatively, it should be tested whether the network for wild sites is different from a random network and whether the network for plantations is different from a random network.

Thank you for this observation. In fact, our second hypothesis is whether *T. melanosporum* is a hub species within the fungal network of plantations and/or forests.
In L189, the impact of the type of truffle productive system and season on the fungal network is linked to hypothesis 1, not to hypothesis 2 as previously stated leading to the misunderstanding pointed by the referee, and we have now corrected this in the revised version of the manuscript.
We also have now restructured the hypotheses at the end of the Introduction to ensure clarity and to explicitly include all the factors and variables analysed in the study.

4) L205. How does ENET methodology deal with proportion data (percentage of reads for a guild)? Proportion data are frequently not normal data (GLMM?). Besides, since 3-4 guilds (including "non classified OTUs") practically dominate the community, the percentage of the main guilds are most likely highly correlated. How do you assess that colinearity does not affect the results of ENET methodology?

Before running ENET, we scaled the OTU matrix to the minimum sequencing depth, filtered out the least abundant OTUs, and then standardized with *decostand* function, allowing for equitable comparison of OTUs. As we mentioned in L206-209, the LASSO and Ridge penalty-based regression modelling incorporated in ENET (Zou and Hastie, 2005) helps to avoid the overfitting and the collinearity between OTUs.

5) L239 Figure S4 seems closely related to the main specific objectives. Why is it not included in the main manuscript? Besides, why did you decide to characterize the alpha-diversity only with richness and not with indices of diversity such as Shannon or Simpson?

The Figure S4 is indeed related with our first hypothesis. We considered presenting Figure 1 in the main text because we thought that it would be useful to have a resume of the fungal composition of our samples at the begging of the results section, and then to introduce the results relative to the first hypothesis. Since this hypothesis has many components, we decided to keep Figure 2 in the main text, as it presents novel results, and move Figure S4 to the Supplementary Material in order to avoid overcrowding the manuscript.
On the other hand, there are many alpha-diversity indexes but, for simplicity, we decided to use just richness in our analyses.

6) L240-241 Significant differences in the PERMANOVA can be related to both differences in the centroid location and in the dispersion of each group samples, which seems to be the case according to Fig. S4b. Contrary to what is said in the manuscript, and according to Fig. S4b, the communities do not seem clearly dissimilar, but only different in dispersion.

Thank you for your observation regarding the apparent discrepancy between the PERMANOVA results and the NMDS plot. We agree that the visual separation in the NMDS is not striking, and this can be primarily due to the inclusion of the factor *site* by the strata fucntion in the PERMANOVA analysis. This is now mentioned in section 2.4 of the Materials and Methods, for clarity.

Through the strata function, PERMANOVA accounts for site variation —in this case, controlling for site-level differences similar to a random factor in LMM—when testing for the significance of our fixed factors type and season. This allows the model to detect subtle but consistent shifts in community composition across groups, even when those shifts are not visually prominent in unconstrained ordination methods like NMDS.

NMDS, by contrast, does not incorporate the strata function or control for site-level variation. It represents overall dissimilarity patterns, which may be dominated by site effects or other sources of variation not related to the factors of interest. As a result, the visual clustering in NMDS may not totally reflect the statistical significance detected by PERMANOVA.

7) L243-244. Wouldn't it be more correct to say that plantations tended to show higher values of pH, K and active carbonate?

While indeed the values of pH, K and active carbonate in plantation samples are higher than in those from forests (it can be also observed in Table S1), the interpretation of the environmental vectors has to be focused on their association with the fungal community structure in the respective truffle producing systems. To make clearer the purpose of the *envfit* function that fitted the environmental vectors, we have now added a small explanation in the 2.4 section.

8) L244-245. Taking into account that both seasons are almost centered in the biplot center, wouldn't it be more correct to say that spring showed more extreme values of pH, OM, Fe, although not always in the same direction?

Yes, indeed. We have now corrected this sentence.

9) L305 Why did you use T. melanosporum No of reads for regressions and not relative abundance of T. melanosporum, which you previously chose as normalized variable (L149)?

We totally agree that *T. melanosporum* relative abundance is a normalized variable and probably is more ecologically meaningful than absolute counts. We applied linear models with this variable and, while the results were quite similar to those of the models done with the number of reads as explanatory variable (and they had almost the same plots), showing a significant relationship with network complexity only in plantation samples, the fitting of the models was not as good. Thus, we preferred to keep the models with the number of reads.

10) Careful with British/American English (e.g. normalised/normalized).

We have now corrected this inconsistency along the manuscript.

11) L321 "To test if soil ecological fungal guilds could explain soil carbon and nutrient cycling". L407 "When the different fungal guilds were further tested for their contribution in soil functioning, saprotrophs did significantly predict most of the soil enzymatic activities tested in both truffle-producing systems". Does the ENET provide correlation values or a partition of the variance? The manuscript suggests the latter. If so, which proportion of the variance in enzymatic activities do the fungal guilds explain?

The ENET is a “feature selection” method that considers all taxa simultaneously and selects those taxa (predictors) that achieve the best prediction of an ecosystem function. The model results in coefficients for each OTU, where non-zero coefficients are used to infer a positive or negative contribution of an OTU to improving ecosystem functioning (Wagg et al., 2019), which in our case is the soil enzymatic activity. Thus, the model does not provide an explained proportion of variance nor a correlation, but causal relations between fungal guilds and the soil enzymatic activity.

12) L346-347 "Our results agree with the differences in β-diversity of soil fungal community previously observed in Mediterranean and temperate climate sites, sampled across four seasons (Piñuela et al., 2024)". How do they agree?

In the study of Piñuela et al. (2024), the fungal community composition appeared more scattered in the samples of wild sites compared to the plantation ones in the NMDS plot (Fig. 1), similarly to our plot, where the forest samples showed a more dispersed fungal community than the plantation samples. We have now added a brief explanation of this result’s agreement in the manuscript (4.1 section of the Discussion).

13) L356 Brûlé in italics?

We initially introduced the term “brûlé” in quotation marks to highlight it as a concept. Given that brûlé is a well-established and widely recognized term among truffle and soil fungal researchers, we chose not to italicize it throughout the manuscript.

14) L370 "Differences in soil fungal network structure among forest and plantations have been, however, regularly explained by the variation in soil properties (Wang et al., 2024b), or different vegetation cover..." Considering this, it would be interesting to discuss a little the differences in vegetation structure between wild/cultivated sites (age of trees, percent canopy cover, percent soil cover by litter, periodic tillage, percent soil cover by herbs/shrubs, etc.).

We agree and, as recommended, we have now added a piece of discussion considering this point at 4.2 section of the Discussion.

REFERENCES

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