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

Tittle: **Managed black truffle-producing systems have greater soil fungal network complexity and distinct functional roles compared to wild systems**

We sincerely thank the Editor for the opportunity to revise our manuscript and the Reviewer for their thoughtful and constructive comments. We are encouraged by the positive assessment of our work and appreciate the suggestions, which have helped us improve the clarity, precision, and overall quality of the manuscript.

As indicated by the editor, we have now modified the end of introduction to make it clearer the objectives and hypotheses of our work.

In response, we have carefully addressed each point raised and made the necessary revisions throughout the text, as indicated in our response. We note that while several improvements have been made, the main findings and conclusions of the study remain unchanged.

We trust that the revised version now meets the requirements for publication, and we hope the manuscript will be found suitable for acceptance.

Reviewer_1

Summary:

In this article (egusphere-2025-2078), the authors set out to investigate the role of black truffle (*Tuber melanosporum* Vittad.) in shaping the fungal community in the soil ecosystem it grows in. They focused solely on the soil fungal community (not considering prokaryotes) and compared the more "natural" forest soil system against the cultivated plantation system. They also compared samples from spring and autumn to gain insights into seasonal effects on the role of *T. melanosporum*. Based on previous studies and general knowledge of fungal ecology, the following hypothesis were put forth:

1. a) soil fungal networks are richer and more complex in forests compared to plantations.
   b) a differential seasonal effect on soil fungal communities can be observed
2. *T. melanosporum* is strongly connected in the soil fungal network, possibly acting as a hub species
3. a) the prevalent functional fungal guilds differ when comparing forest and plantation systems
   b) greater prevalence of ectomycorrhizal fungi vs saprotrophs can be observed in forests compared to plantations

To investigate these hypotheses, topsoil (0-20 cm) from inside the brûlé, i.e. presumably area affected by *T. melanosporum*, was obtained from both systems (four replicates) and in both seasons. The brûlé boundaries were determined visually and multiple samples from within the area were combined to a composite sample, but no negative controls from outside

that area were taken. Some sampling sites were paired (forest and plantation in close proximity), while others were not. Fungal occurrence in the samples was determined by metabarcoding. Co-occurrence networks were created based on this and the role of *T. melanosporum* within these networks was studied. Soil functioning was proxied through the potential activities of eight exoenzymes related to carbon (β-glucosidase, β-cellobiohydrolase, β-xylosidase, β-glucuronidase, and laccase), nitrogen (chitinase and leucine-aminopeptidase), and phosphorus (alkaline phosphatase) cycling. These were measured for the soil samples and the potential role of different fungal guilds in explaining these activities was predicted by modeling.

Forest fungal communities showed significantly greater β-diversity, while α-diversity did not differ significantly between plantation and forest. Based on a single mixed co-occurrence network, OTU links and network complexity appeared significantly higher in plantations compared to the forest system (contrary to expectation from hyp. 1), while no significant difference between seasons was observed. In separately modeled co-occurrence networks for both ecosystems, *T. melanosporum* was not strongly connected to other OTUs and did not appear to act as a hub species (contrary to hyp. 2). Differing abundance of fungal guilds was observed between both systems and ectomycorrhizal were more prevalent in the forest (fitting hyp. 3).

We thank to Referee #1 for his/her insightful and encouraging assessment. We have answered point by point to his/her comments in blue, as follows:

Key limitations of the study:

1. No control samples outside the brûlés were taken, meaning there was no true negative control. The authors themselves identify this limitation (l. 382 – 387), but do not sufficiently address it in their analyses. Co-occurrence works by checking shared patterns of presence or absence. Since only samples from truffle-dominated areas were used which would be expected to almost always contain *T. melanosporum* reads, positive connections would only be expected with other highly abundant taxa, since only these could match the truffles occurrence pattern. Negative connections would also not hold as much informative value in this specific sampling approach, since they would likely mainly depict less abundant / more rare taxa that occur in few samples.

Tuber melanosporum was the most abundant species in all samples (see also Fig. S2 of Barou *et al.*, 2025, where the same study area and samples were assessed). However, an abundance gradient was observed across samples allowing us to explore variations in the relationship with other fungi. We agree that there was no true negative control and future studies including samples in- and outside the brûle are needed to complement our results, which has been now pertinently highlighted in the discussion and conclusions.

Regarding the network analysis, the applied algorithm SPIEC-EASI relies on transformed data, and its output is the inverse of the covariance matrix, not a correlation matrix. The relative abundances of the OTUs are compositional, meaning they are constrained to sum to a constant (usually 1 or 100%) which introduces a mathematical dependency between components—if an OTU´s abundance increases, others´ abundance must decrease. It does not imply that if one fungus increases, another increases or decreases. To better clarify this, we added some more details in section 2.3 of Material and Methods. The resulting matrix

from SPIEC-EASI allows us to detect positive values, i.e., positive conditional dependence (co-occurrences); negative values, i.e., negative conditional dependence (co-exclusions); or null values, i.e., conditional independence (no edges in the network graph). In this sense, the co-occurrence network-based approach has given us the opportunity to analyse coexistence patterns among fungal species within the brûlé (in natural or managed systems), meaning that the experimental design assumes the presence of *T. melanosporum* in all samples.

One reason for *T. melanosporum* not showing up as a hub species in the analysis could also be that it already modified the microbiome and reduced the abundance of some other fungi.

We agree with this comment, and this limitation has been now emphasized in 4.2 section of the discussion.

Without the outside control, we are unable to compare to the “undisturbed” ecosystem without truffle dominance, which really limits what can be deduced about its actual role in the system.

There is a clear dominance of *Tuber melanosporum* and an abundance gradient of the fungus across samples, which to some extent allows us to assess its effect on the soil. However, the lack of control outside the brûlé and the necessity for new studies has been pertinently emphasized in the 4.2 section of the discussion and also in conclusions. Our objective was to investigate the close environment of *T. melanosporum*, distinct from examining microbiome shifts within and outside the brûlé caused by *T. melanosporum* itself. Specifically, we sought to determine the positive and negative fungal associations with *T. melanosporum*, which required focused sampling within the brûlé because identifying the species associated with the black truffle can be crucial in designing truffle plantation management practices.

In a similar vein, the negative control would have also allowed researchers to rule out environmental filtering as the main driver for co-occurrence, by depicting the community without truffle but in the exact same soil conditions. Some of the sampling sites appear at least somewhat paired, while others are completely singular, which makes it hard to disentangle the actual effect of *T. melanosporum* on the local soil microbiome, compared to differences purely based on abiotic factors. This issue could maybe be circumvented by comparing matched sampling sites. While there can be merit in combining all the data to finder larger underlying trends, some nuance will inevitably get lost by lumping these potentially diverse and unbalanced datasets together.

We agree, and it is precisely to avoid possible environmental confounding factors why we decided to separate network analyses for forests and plantations. Furthermore, the discussion highlights the significant potential of pursuing this analytical approach in future studies (section 4.2), a direction that is already being explored in a complementary ongoing study.

Since detailed per-sample soil parameters are not supplied to reviewers, it is difficult to decide whether this would have been a sensible measure.

In our previous work, Barou et al. (2024), we provided both the methods used to perform soil physical-chemical analyses and the mean ± SE values of a range of soil parameters for plantations and forests, as it is referred in the 2.1 section of Materials and Methods, and also summarized in the present study (see supplementary Table S1).

2. *T. melanosporum* is described as being "dominant" in the brûlé (l. 31 – 33), leading to the distinct and visible vegetation pattern, which also formed the basis for picking sampling spots. Based on this one would expect it to be found in almost every sample, especially since 4 subsamples from each tree were combined. However, fig. 4 shows that for plantations some and for forests a lot of samples appear to have ~0 Tmel reads. This data is only presented as a plot, no table with the exact numbers (sample number + number of Tmel reads) is provided, but based on the figure it seems like a decent chunk of the soil samples per brûlé did not contain any *T. melanosporum* DNA, or not enough to be detected by the metabarcoding approach. This raises the question whether a simple phenotypic determination of truffle-dominated soil is sufficient for actually picking positive samples, or whether amplification efficiency of the ITS region is sufficient. In the current version of the manuscript, the authors do not address the zero Tmel abundance samples at all, which would be a critical point to discuss.

We acknowledge that in the figure, samples with low abundance may appear to have zero reads. However, this is a result of the large scale used in the figure (ranging from 0 to 20,000), which visually compresses the lower values. In fact, no sample had zero Tmel reads; rather, some samples had low read counts, i.e. ≥ 4, as it has been now indicated in the legends of Figs. 1 & 4. In fact, the mean number of Tmel reads per truffle system exceeded 5,600, confirming consistent detection across all samples in both truffle-producing systems. To avoid any confusion, attention to the lack of zero Tmel has been now drawn in the results section (3.1 section). In addition, a piece of discussion has been added to acknowledge the potential limitations pointed by the reviewer (in 4.2. section of the discussion), with which we totally agree.

3. Only the fungal perspective is considered, despite bacteria likely making up a large part of the soil microbial community, especially at the alkaline pH found at the sampling sites. This means that only the interactions with the small fungal subset of the soil microbiome are considered. While additional amplicon sequencing for bacteria would have likely exceeded the scope of this study, some less complex methods like a comparison between general 16S vs ITS qPCR could at least have helped quantifying how much of the overall community is not included in this analysis.

We fully agree, and in fact, this is the natural progression of our ongoing work. It would also be incredibly interesting to investigate the functional implications of the complete trophic network (i.e., including fauna) within these unique systems. This will be the focus of future research by our group.

Conclusion:

We understand that some of the mentioned limitations are hard to address without extensive resampling or sequencing, but we strongly urge the authors to reconsider which conclusions can be drawn from their data and which questions go beyond their scope. Especially the title

(l. 1 – 3) as well as the statements about a stronger negative influence of black truffle on the fungal network in plantations (l. 382 – 387) should be carefully reevaluated and potentially rephrased.

Following the recommendations, we have now tone down the paragraphs where potential negative influence of *T. melanosporum* was addressed all along the manuscript (e.g. “spreads”, “colonising”, “presence” instead of “dominates”, “dominating” and “dominance” in the Introduction and Discussion sections). For consistence with this, the title has been also re-evaluated: “Soil fungal network complexity and functional roles differ between black truffle plantations and wild-producing forests”.

Without the negative controls that would depict the undisturbed network, these conclusions do not just require confirmation but lack strong proof altogether, especially since members of the community that might have been fully suppressed by *T. melanosporum* are not accounted for here. An approach of only using paired sites to counteract some of the study design limitations could be promising to investigate the influence of abiotic conditions, as well as forest vs plantation on the fungal community. The issue of brûlé samples without any detectable *T. melanosporum* reads should also be further investigated and put into the focus of the revision.

We sincerely thank the Reviewer for this comment. We agree that clarifying these specific research questions strengthens the novelty and scientific contribution of our work. Following the advice, we have revised the entire discussion section to more explicitly emphasize the key points raised. Soil brûlés have been considered as the best indicator of *T. melanosporum* presence because of the well-known allelopathic effects of truffles on the surrounding vegetation (Streivlová et al. 2012). Also, the size of the brûlé has been related with truffle productivity (García-Montero et al 2007). However, in some cases other soil fungal allelopathy may produce similar effects on plant growth as those observed in the truffle brûlés (Osivand et al. 2018). This could explain why some well-formed brulés show a very low amount (but always above 0) of *T. melanosporum* reads in our study.

In any case, the varying levels of Tmel abundance in well-developed brûlés in our study raise relevant questions about whether a simple phenotypic assessment of truffle-dominated soil is sufficient to identify positive samples, whether higher pooling efforts in soil sampling and/or DNA extraction should be done to minimise soil spatial/temporal heterogeneity or if amplification efficiency of the ITS region alone is adequate, issues that warrant further investigations. As indicated, all these limitations and the continuity of the work have now been thoroughly addressed in the revised version of the manuscript. The need of additional studies and designs has been also highlighted in the discussion and the conclusion sections.

References:

Streiblová E, Gryndlerová H, Gryndler M. Truffle brûlé: an efficient fungal life strategy. 2012-FEMS Microbiol Ecol. 80(1):1-8. doi: 10.1111/j.1574-6941.2011.01283.x

García-Montero, L.G.; Manjón, J.L.; Pascual, C.; García-Abril, A. 2007. Ecological patterns of *Tuber melanosporum* and different *Quercus* Mediterranean forests: Quantitative production

of truffles, burn sizes and soil studies. For Ecol Man 242 (2-3): 288-296. doi: 10.1016/j.foreco.2007.01.045

Osivand, A.; Araya, H.; Appiah, K.S.; Mardani, H.; Ishizaki, T.; Fujii, Y. Allelopathy of Wild Mushrooms—An Important Factor for Assessing Forest Ecosystems in Japan. 2018. Forests *9*, 773. https://doi.org/10.3390/f9120773