Ectomycorrhizal fungal network complexity determines soil multi-enzymatic activity

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- **RC1**: 'Comment on egusphere-2024-119', César Marín, 26 Feb 2024 reply

  The manuscript entitled “Ectomycorrhizal fungal network complexity determines soil multienzymatic activity”, by Prieto-Rubio and co-authors is a very neat one. The authors examined the effects of habitat (ie. season, plant host, etc) on classical ectomycorrhizal parameters (ie. richness) but also on co-occurrence network parameters, and in turn, the effects of these on a group of enzymatic activities related to the C, N, and P cycles, in two relatively contrasting sites in Spain. They overall found marked differences (patterns) across the sites, and an important effect of habitat on network structure parameters. Keystone ectomycorrhizal taxa were not the main determining taxa influencing enzymatic activity, which is a very interesting result by itself. For me, it was a bit strange that the authors expected that community structure parameters (or increased network complexity) influenced their MAI index (which summarizes all enzymatic activities), as it is not expected that ECM fungi equally affects all 3 nutrient cycles. I also have some (mostly form) more comments below. Other than that, I was quite happy reading this article. This is a very nice example on how proper soil microbial ecology should be done and analyzed.

Abstract

L.19. Change “cycling were” to “cycling were also”.

Done (L.19).

L.22. Change “linages” to “lineages”.

Done (L.22).

L.23. Change “Sebacinales, Pezizales” to “Sebacinales, and Pezizales”.

Done (L.23).

L.24. Change “context-dependent pointing to” to “context-dependent, pointing to”.

Done (L.24).

L.25. Change “functionality,” to “functionality;”.

Not changed in order to keep the phrase sense (L.25).

Introduction

L.38-39. I am not totally sure these models/studies are truly causal. For causal inferences about soil biodiversity on ecosystem functioning, please check DOI: 10.1038/s41467-019-12798-y and DOI: 10.1016/j.scitotenv.2023.163683

As highlighted, we have re-formulated the sentence to clarify the use of correlation/regression-based analyses on community-function studies: *“Some of the most widespread tools to associate community metrics with ecosystem functions are based on correlations across variables, or regression models that allow inferring ecosystem function responses through community structure predictors e.g., species richness, phylogenetic structure (Pérez-Izquierdo et al., 2017; Bastida et al., 2019; Krapu and Borsuk, 2020)”* (L.36-39).

One of the recommended references is included in the manuscript

L.46. And species hypothesis (as per the UNITE database) for fungi.

Included (L.46).

L.60. Change “fungal lineages promoting” to “fungal lineages, promoting”.

Done (L.61).

L.64-65. There is quite a bit of very recent literature on ECM hyphosphere, including several reviews. It might be nice to cite it here.

As suggested, we have incorporated up-to-date literature that reinforces the need to investigate the functionality of soil microbial associated to the mycorrhizal environment. The new references included are:

- Martin, F. M., and van der Heijden, M. G. A.: The mycorrhizal symbiosis: research frontiers in genomics, ecology, and agricultural application. New Phytol., https://doi.org/10.1111/nph.19541, 2024.

- Nguyen, N. H.: Fungal Hyphosphere Microbiomes Are Distinct from Surrounding Substrates and Show Consistent Association Patterns. Microb. Spec., 11(2), e04708-22., https://doi.org/10.1128/spectrum.04708-22, 2023.

L.70. What kind of “spatial-temporal factors” specifically?

As suggested by both reviewers, we have specified those factors within the main text:

"we firstly hypothesized that ECM fungal co-occurrence network structure would be dependent on host plant identity and spatial-temporal factors (habitat and seasonality)" (L.72-73).

L.69-75. These are very nice hypotheses, very well justified.

We greatly appreciate the comment.

L.79. Change "host plant individual and ECM fungal" to "host plant individual, and ECM fungal".

Done (L.80).

Materials and Methods

L.85. Change "as Segura) and Monte la Sierra" to "as Segura), and Monte la Sierra".

Done (L.86).

L.90. Change "Arnold and Pinus pinaster" to "Arnold, and Pinus pinaster".

Done (L.93).

L.91. Change "Rosaceae and Lamiaceae" to "Rosaceae, and Lamiaceae".

Done (L.94).

L.94. Change "Q. faginea and 29" to "Q. faginea, and 29".

Done (L. 99).

L.95-97. After removing the organic debris? If so, please say so. Also, I wonder how mono-dominant (or not) are these forests? How do you guarantee that the ECM root tips collected belonged to the specific tree under which the soil core was collected? Please add information to clarify this.

As wondered, litter layers were discarded before collecting roots and the surrounding soil. Regarding ECM root sampling, we did not collect soil cores, but instead holes were opened in soil and roots were always carefully traced to be sure that belonged to the chosen plant individual as detailed in Prieto-Rubio et al. (2022, https://doi.org/10.1007/s00572-022-01083-4).

To clarify the plant community composition, the forests are classified as Mediterranean mixed, recording high canopy and understory plant community diversity, but with few common plant species between the study sites (see the free-access database https://doi.org/10.20350/digitalCSIC/15133 for a detailed list of the woody plant community composition in these forests).

To respond the mentioned questions and better detailing the methodology, we have partially re-formulated the sentences embedded in the section 2.2:

"After removing the litter layer, we collected three sub-samples (10 x 10 x 20 cm holes) with secondary roots tracked from the target plant and surrounding soil, and further combined into a single composite sample for each individual (Prieto-Rubio et al., 2022)" (L.101-102).

L.99. Change "pH and gravimetric moisture" to "pH, and gravimetric moisture".

Integrated in the modification described below.

L.98-99. Explain briefly how these 3 soil parameters were determined.

We have cited the previous paper in which soil physical-chemical analyses were described (Prieto-Rubio et al., 2022) and added methodological information about the physical-chemical analyses:

"Before physical-chemical analyses, soil surrounding roots was recorded and homogenized in each sample, air-dried and 2 mm-sieved. The soil pH was measured in solutions (1:5, w:v in H2O); the gravimetric moisture (GM) was calculated as the difference in soil weight before and after drying the samples at 105 °C for 48 h; and the soil organic matter (SOM) was determined by loss on ignition at 400 ºC for 4 h (Walkley and Black, 1934)" (L.104-108).

Walkley, A., and Black, I. A.: An examination of the Degtjareff method for determining soil organic matter, and a proposed modification of the chromic acid titration method. Soil Sci., 37(1): p 29-38, 1934.

L.103. Change "Q. ilex and Q. faginea" to "Q. ilex, and Q. faginea".

Done (L. 113).

L.104. Change "using UNITE database" to "using the UNITE database".

Done (L. 114).

L.109. Change "C, N and P cycling" to "C, N, and P cycling".

Done (L. 119).

L.110. Change "β-glucuronidase and laccase" to "β-glucuronidase, and laccase".

Done (L. 120).

L.115. Cite vegan correctly.

Done (L.126).

L.116. Mention the package from where the function prcomp comes from, and cite such package. Same elsewhere where functions are mentioned alone.

Done (L.126).

L.142. Cite igraph correctly. Same elsewhere where packages are mentioned but not cited.

Done (L. 151).

L.146. Change “closeness and betweenness” to “closeness, and betweenness”.

Done (L.156).

L.159. Cite lme4 correctly.

Done (L.169).

L.173. Change “level) and seasonality” to “level), and seasonality”.

Done (L.183).

Results

L.227. Change “24 and 28 in Jaén, and 2, 18 and 22 in” to “24, and 28 in Jaén, and 2, 18, and 22 in”.

Corrected (L.243).

L.256. Change “(21.28 %) and Pezizales (12.77 %)” to “(21.28 %), and Pezizales (12.77 %)”.

Done (L.271).

L.269. Add a comma (,) before “and alkaline phosphatase”. Change “laccase and acid phosphatase” to “laccase, and acid phosphatase”.

Corrected by adding a figure legend explaining the enzymatic activities predicted by OTUs per study site.

Discussion

L.285. Change “Sebacinales and Pezizales” to “Sebacinales, and Pezizales”.

Corrected (L.300).

L.297-299. I like this reasoning a lot!

We greatly appreciate the comment.

L.314. Change “variations; by contrast, in” to “variations. By contrast, in”.

Corrected (L.330).

L.317. Change “Sebacinales and Thelephorales” to “Sebacinales, and Thelephorales”. I wonder, though, if Order might be a too broad taxonomic category to analyze this… what about family?

Done (L. 333).

We have justified the use of Order in the figure caption:

“We used Order as was the main resolving taxonomic category to visualize trends of ECM fungi in predicting enzymatic activities within a network context and by incorporating phylogenetic relationships” (L.284-285).

L.324-325. It should be, though? As both mycorrhizal guilds are so different…

In this sentence, we discussed our results with those of Davison et al. (2021) that focused on arbuscular mycorrhizal fungal communities, and arguing that niche space differences might also be variable across the ECM fungal lineages.

L.339. I wonder though, why you expected that all network properties affected MAI. I would expect ectomycorrhizal fungi being more related to some enzymatic activities and overall some nutrients cycling, but not all of them. This is well established in mycorrhizal ecology (ie. MANE framework from 2013).

The aim of these analyses was to evaluate the extent of ECM fungal network structure as potential predictor of MAI outcomes across the studied habitats. As mentioned in the comment, not all extracellular enzymatic activities may be directly affected by ECM fungi, but might do it through soil microbial associates (e.g., saprotrophic bacteria and fungi), hence supporting an indirect effect by this symbiotic fungal guild. As mentioned along the manuscript, this open future and more analytically complex studies by incorporating other soil microbial groups into a network context similar to that proposed in our work.

L.349-350. This makes a lot of sense to me.

We greatly appreciate the comment.

L.354. Change “Pezizales and Russulales” to “Pezizales, and Russulales”.

Done (L. 375).

L.357. Functional redundancy is also one of the expectations of holobiont theory.

We thank for the comment; we have introduced the idea in the text now by re-formulating the main ideas within the discussion section:

“This result supporting the ideas of functional redundancy (i.e., enzymatic activity conservation across ECM fungi, Baldrian and Kohout, 2017) fits well into the holobiont theory (Zobel et al., 2024). Indeed, from the holobiont perspective, this redundancy

ensures that essential functions carried out by microbiota are maintained even if one or more species are lost or disrupted, providing resilience against environmental changes, disturbances, or the loss of specific microbial species (Vandenkoornhuyse et al. 2015). Alternatively, other soil microbial groups could be contributing to P and N mobilization into a much greater extent than ECM in these forest soils (Ward et al., 2021; Xun et al., 2021)" (L.367-372).

Vandenkoornhuyse, P., Quaiser, A., Duhamel, M., Le Van, A., and Dufresne, A.: The importance of the microbiome of the plant holobiont. New Phytol., 206(4), 1196-1206. https://doi.org/10.1111/nph.13312, 2015.

Zobel, M., Koorem, K., Moora, M., Semchenko, M., and Davison, J.: Symbiont plasticity as a driver of plant success. New Phytol., 241 (6), 2340-2352. https://doi.org/10.1111/nph.19566, 2024.

L.358-359. Indeed, this coincides with my previous point (comment for L.339).

L.361-363. Very interesting!

L.363-365. Yes! Especially in the light of all recent ECM hyphosphere studies, showing the crucial role of those bacteria.

We greatly appreciate all these comments.

RC2: 'Comment on egusphere-2024-119'

The Manuscript titled *Ectomycorrhizal fungal network complexity determines soil multienzymatic activity* by Prieto-Rubio et al. was generally nice and an appropriate subject for the journal's scope. The main findings of their first part were that network structuring was context dependant and that more complex networks were influenced by taxon with lower average abundance. Their second part found that more complex networks did not necessarily relate to the activity of several enzymes involved with C, N, and P cycling.

I appreciated "box 1" for explaining the many parameters used in the network analysis. In particular I think they raise a good point in our lack of understanding on the interactions between mycorrhizal fungi across different environments and community assemblage processes. Linking specific ectomycorrhizal fungi to their role in soil processes and overall ecosystem functions is particularly challenging, and we still have few methodological options for studying these questions. I am not surprised they find little to no patterns between enzyme activities and the ECM community and structure. They study only a small portion of the organisms that produce these enzymes on a spatial scale that doesn't really have sufficient resolution to capture the heterogeneous nature of both enzymes and the fungi producing them.

Abstract:

L.18. I would make it more clear that two *Quercus* species are focused on (spp. can imply any number of species greater than one) and which species are studied.

As suggested, we have integrated the name of Quercus species in the abstract (Q. faginea and Q. ilex).

Introduction:

L.33. Could you be more specific in terms of the ways through which species are assembled – which processes?

Thank you for pointing out this question. We have specified in the main text the processes that mainly govern community assembly *"The ways through which species are assembled, e.g., via environmental filters, disturbances, or even by demographic stochasticity,…"*

L.40-41. True – often not considered and difficult to study

We agree.

L.56. Re-word "ECM fungi mainly interact with trees and shrubs" – they form symbioses with trees and shrubs but as is the topic of the paper they also interact with other fungi and bacteria etc.

As indicated, we have re-formulated the sentences in order to highlight the symbiosis formed by ECM fungi with host plants:

"ECM fungi mainly form symbiotic interactions in the roots of tree and shrub lineages" (L.56).

L.70. specify in more details what is meant by spatial-temporal factors

As suggested by both reviewers, we have better detailed the factors we seek in our study: *"we firstly hypothesized that ECM fungal co-occurrence network structure would be dependent on host plant identity and spatial-temporal factors (habitat and seasonality)"* (L.71-72).

Materials and methods:

L.84-91. These sites have been described and used for many other publications. I think it is appropriate you more explicitly mention this so that reader can connect to more information and, in general, for better transparency.

As mentioned, we have included information describing the timeline of studies in these protected areas in Southern Spain:

"*These protected areas have been largely monitored by plant-plant and plant-microbe interactions to better understand topics concerning the Mediterranean forest community dynamics (from Siles et al. 2008, to Garrido et al. 2023, and Pajares-Murgó et al. 2023*" (L.87-89).

Siles, G., Rey, P. J., Alcántara, J. M., and Ramírez, J. M.: Assessing the long-term contribution of nurse plants to restoration of Mediterranean forests through Markovian models. J. App. Ecol., 45(6), 1790-1798. https://doi.org/10.1111/j.1365-2664.2008.01574.x, 2008.

Garrido, J. L., Alcántara, J. M., López-García, Á., Ozuna, C. V., Perea, A. J., Prieto, J., Rincón, A. and Azcón-Aguilar, C.: The structure and ecological function of the interactions between plants and arbuscular mycorrhizal fungi through multilayer networks. Func. Ecol., 37(8), 2217-2230. https://doi.org/10.1111/1365-2435.14378, 2023.

Pajares-Murgó, M., Garrido, J. L., Perea, A. J., López-García, Á., and Alcántara, J. M.: Biotic filters driving the differentiation of decomposer, epiphytic and pathogenic phyllosphere fungi across plant species. Oikos, 2023(5), e09624. https://doi.org/10.1111/oik.09624, 2023.

L.89-95. There was also *Quercus ilex* L. and *Q. faginea* Lam. present at Segura? It seems this way based on Prieto-Rubio 2022. Please make it more clear what species were in each sites and whether or not there was even sampling of each tree species across the two sites. Also what is the distances between different host trees? Is there any chance that root tips from other ectomycorrhizal plants (other oaks or pines) are in the samples assigned to one of the three tree species you studied?

To solve the proposed questions and to better clarify the experimental design and sampling, we have re-formulated the sentences concerning these questions:

"To compare ECM fungal community network-functionality outcomes across the study sites, we selected those ECM plants that were common in both study sites (i.e., Cistus albidus L., Q. faginea and Q. ilex)." (L. 95-96).

"A total of 92 adult plant individuals were sampled, accounting 32 for Q. ilex, 31 for Q. faginea, and 29 for C. albidus (47 in Jaén and 45 in Segura, and 48 in autumn and 44 in spring), at least 10 m-distanced among within the given plot. After removing the litter layer, we collected three sub-samples (10 x 10 x 20 cm holes) with secondary roots tracked from the target plant and surrounding soil, and further combined into a single composite sample for each individual (Prieto-Rubio et al., 2022)." (L.99-103).

L.95. Different trees were sampled in spring and autumn?

They were, as the experimental design included a random selection of host plant individuals across the study habitats and seasons.

L.89-90. Why were pine roots not sampled? They are also ectomycorrhizal and I would assume that a piece of the picture regarding of ECM communities and their assemblage is missed by ignoring these. Based on previous descriptions of the sites pine is dominant? I think it would be good to mention this either here or in discussion.

As mentioned, pines were common occurring trees, hence likely promoting part of the ECM fungal taxa pool. Nevertheless, different *Pinus* species were found in the two studied mountain systems, which impeded us to arrange full comparisons across the habitat types.

L.98.99. Were these parameters determined from the same soil samples as which the root tips were removed from?

Correct, the edaphic parameters were determined from the soil fraction that was surrounding root tips.

We have clarified this point in the main text: "*Once in the lab, roots were separated from the surrounding soil, washed with tap water over 2 and 0.5 mm sieves, and all ECM root-tips collected under a Carl-Zeiss Stemi 2000 stereomicroscope (Rincón et al., 2014)".* (L.103-105).

L.124-125. change "we adjusted the methodology described by Wagg et al. (2019) that …" to "we adjusted our methodology as described by Wagg et al. (2019), which…".

Done (L.134-135).

L.126. That is a large difference in terms of minimum sequencing depth – nearly 10 fold. Was the sequencing depth on average similar between sites? The Mean OTU richness was higher in Segura than Jaen based on supp figure S2 so I assume that saturation was reached?

Exactly, mean sequencing depth was similar across samples, and richness saturation was reached in all cases (see details in Prieto-Rubio et al. 2022). Indeed, in this preliminary study based on phylogenetic and compositional structure of ECM fungal communities found similar community responses to those observed in co-occurrence network structure were revealed. We have added this information to the text in L.137-138.

Prieto-Rubio, J., Garrido, J. L., Pérez-Izquierdo, L., Alcántara, J. M., Azcón-Aguilar, C., López-García, A., and Rincón, A.: Scale dependency of ectomycorrhizal fungal community assembly processes in Mediterranean mixed forests. Mycorrhiza, 32, 315-325. https://doi.org/10.1007/s00572-022-01083-4, 2022.

L.177-180. Each soil enzyme activity was used as response in separate models, correct? Clarify or explain the ENET a little bit more.

This is right: each soil enzymatic activity was treated as response variable in separate models.

As suggested, we have briefly described the use of ENET regularization modelling and how it is applied in our study system:

"*Briefly, the ENET regularization combines LASSO and Ridge penalty-based regression modelling, allowing to avoid overfitting and potential correlation effects between predictors (i.e., the OTUs embedded in the co-occurrence networks), as well as minimizing the influence of those predictors that lowly explain the variations in the response variables (i.e., each of the soil enzymatic activities) (Zou and Hastie, 2005). As a previous step before model training, the abundance matrix of ECM fungal OTUs by host plant was standardized*" (L.187-193).

Results:

L.192-193. How can you say that seasonality was a main driver of the differences – these are not the same locations or even tree being sampled at different times and the difference very well could be spatial differences because communities are so heterogeneous? In addition, relic DNA can persist for some time so it is not very conclusive that fungal communities sampled in spring and fall are actually connected to those time periods.

As highlighted, seasonality was not a driver of ECM fungal networks at the same extent than habitat characteristics. We have traced the text to correct any mention of it. Indeed, the relationships between spatial-temporal factors with the ECM fungal community structure were previously tested in Prieto-Rubio et al. (2022), confirming the higher effect of spatial factors compared with seasonality on ECM fungal community composition. This result points to a lack of influence of relict DNA. In addition, we relied in a high number of replicates to avoid any potential influence of spatial heterogeneity in our results (15 *C. albidus*, 16 *Q. faginea* and 16 *Q. ilex* in Jaén; 14 *C. albidus*, 15 *Q. faginea* and 16 *Q. ilex* in Segura). Nevertheless, beyond its habitat dependency, the use of network approaches revealed that seasonality did influence on ECM fungal network outcomes across sites.

In addition, and beyond the persistence of relict DNA, we included spring and autumn in the experimental design as being those seasons that allow us to record the peaks of potential biological activity within the ECM symbiotic environment in Mediterranean mixed forests (see, e.g., Richard et al. 2011. Annals Forest Sci. https://doi.org/10.1007/s13595-010-0007-5). This experimental context allowed us to relate structure of ECM fungal communities (on a network basis) with the extracellular enzymatic activities in the surrounding soil.

L.210. varied "neatly" ? what is meant by this.

We have eliminated this typing error.

L.240. This test with Positive and Negative OTUs feel circular. There are relationships between the number of OTUS which have an effect (positive/negative) on enzyme activity and the multi-enzyme activity index… wouldn't it be obvious that positive/negative OTUs derived from the ENET be correlated with the MAI? The fungal links and complexity I understand is can relate the co-occurrence structure to function. What is gleaned from these four model (Positive OTUs/Negative OTUs/detrended Postive OTUs / detrended negative OTUs)? Are those predictors necessary?

Thank you for this appreciation. The goal of these model tests was to investigate if the "functional fraction" of the OTUs co-occurrence networks could explain the multi-enzymatic activity index out from the fraction of the "overall" OTUs co-occurrence network parameters (i.e., including all OTUs occurring in the networks, see results in Table S2). Since ECM fungal OTUs positive- or negatively estimating enzymatic activities were determined from ENET regularization, identifying fungal co-occurrences integrated by, at least, one functional OTU - i.e., positive/negative fungal links and network complexity- did allow us to extend previous ENET outcomes into the co-occurrence network approach.

We have included the next information in the main text to clarify the use of de-trended network parameters:

"In addition, as fungal network properties can be affected by the species/OTUs number, especially when diminishing (Berry and Widder, 2014), the de-trended network parameters by ECM fungal OTUs' richness allowed us to evaluate how the co-occurrence network structure yielded on multi-enzymatic activity responses by discarding the variance intrinsically derived from the OTU richness."

Berry, D., and Widder, S. Deciphering microbial interactions and detecting keystone species with co-occurrence networks. Frontiers in microbiology, 5, 90985. https://doi.org/10.3389/fmicb.2014.00219, 2014.

L.225. What is the relevance of the positive correlations between the centrality metrics? I do not think this information in Figure 2 adds to the paper and you do not mention this in the results text. Either explain or perhaps change figure to better represent the main result – negative correlations between average abundance and OUT-level network centrality metric.

We used the battery of centrality metrics described in the Box 1 as each could inform us about the different structural roles of ECM fungal OTUs embedded in the network. The correlation results revealed that, beyond the negative relationship with OTU abundance, network metrics were positively correlated across the study sites, notably into a greater extent in Jaén than in Segura. Hence, we decided to include this figure to contrast network parameter relatedness across study sites and to understand community assembly on the network basis.

We have now included this information in the Results section (L.241-242).

L.226. I think hub score should be defined in a more detailed way in box 1 – it is important for the results and conclusions so it should be easier to understand how this is determined.

We appreciate this suggestion. Done (in Box 1).

L.258. Does the connection to Laccases in Pezizales make sense? There is not really evidence that species in Pezizales have genes for laccases (AA1_1) – Miyauchi et al. (2020)

Indeed, it seems rather an indirect connection, as it is further discussed. As indicated, the analyses of Miyauchi et al (2020) mainly revealed that ECM fungal taxa, such as those belonging of Pezizales, lacked of overall extracellular enzymatic activity, but when contrasted with the phylogenetically-closest saprotrophic fungal taxon or lineage that are expected to show greater extracellular enzymatic machinery. These results argue with the assumptions discussed later, in our manuscript, on the need to incorporate other soil microbial guilds in ENET regularization, to understand the role of these ECM fungal taxa (e.g., predicting laccase activity by decoupling the role of the ECM symbiont from that of supporting specific saprotrophic fungal and bacterial pools).

L.264. Figure is good at summarizing a lot of results. If possible, I think it would be good to improve contrast of correlation estimates in rings (some that are close to zero are near impossible to see) and make it easier for reader to tell what enzyme each "ring" is because they are different between sites which is stated in figure caption.

Thank you for the recommendations. We have better specified which ring correspond to each enzymatic activity by plotting a legend per study site.

As mentioned, several estimate values are closed to zero, hence impeding to clearly contrast this relationship within the heatmap and indicating that specific OTUs lowly predict a given enzymatic activity. To solve this issue, we encourage in the figure caption to see the estimate values listed for each OTU per study site in the supplementary table S3.

Discussion:

L.279. change "deepening" to "investigating further" or something similar

Done (L.294).

L.280. I would also like to know how frequent they are across samples not just the average abundance or some idea of the variation in abundance (show standard deviation on the average in Table s1?)

As suggested, we have incorporated the occurrence frequency of OTUs in samples in Table S1.

L.292-293. Would be nice to have an idea of the actual variation not just the range – in addition present somewhere the mean and the standard deviation.

Done: *"Co-occurrence networks revealed differential responses to soil pH -ranging from 6.9 to 8.3 (averaging 7.7 ± 0.1) and from 4.9 to 8.2 (averaging 6.6 ± 0.2), in Jaén and Segura respectively-"* (L.308-309).

L.312. The estimate and direction of Network complexity and De-trended network complexity were the same in Jaen – how did it help "fine tune"?

As abovementioned, the de-trended network complexity allowed us to determine that network structure was also independent to OTU richness, and to find that seasonality (i.e., fall *vs* spring) related network structural outcomes.

L.315. Was there a post-hoc test done to investigate the difference among hosts in their de-trended network complexity? This is not stated in methods or results but is mentioned in discussion here that there is only difference at genus level i.e. between *Quercus* and *Cistus*.

The differences among host plants (at Genus or species levels) were evaluated through Anova tests. To better explain this approach, we have reformulated the information on the analytical procedure (sec. 2.5.1).

As de-trended network complexity was the only variable affected by host plant identity, we did not add figures in Supp. Information and pointed Anova results in the main text (L.209-210).

L.340-341. please clarify this sentence – what is meant by conducting?

We have reformulated the sentence for clarity (L.357).

L.355. again I am not sure that this is really true of laccases in Pezizales. But I suppose that maybe saprotrophic fungi, not investigated here, that tend to co-occur with Pezizales would likely produce laccases to degrade C-rich OM in the hyphosphere. One of your conclusions is that it would be important to expand to understand co-occurance among other soil microorganisms, I think the text could benefit from a little more discussion on this.

This comment can be justified with that for comment in L.258, and totally agreed: next methodological approaches should integrate other soil microbial guilds (bacteria and fungi) that may be key to understand these soil functions.

L.363-365. Yes, I agree! It is important to look at the wider picture because ECM interact and impact the assemblage of saprotrophic fungi and bacteria. ECM fungi are also only a fraction of the organisms that produce the enzymes you study.

Totally agree. This is a very topical subject that deserves further investigation.

Supplemental figures and tables:

All figures and tables in supplement I think are relevant and well presented.

I think the supplementary methods needs a bit more detailed on how the soils were prepared for enzyme assays even if it is in another publication. How much soil was used? How were they homogenized?

As suggested by both reviewers, we have included more details about soil processing and enzymatic activity characterization in the main text and Sup. Information.