Opponent's review on the diploma thesis:

Effect of long-term drought on plant-associated microbiota

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Quality and quantity of data collection

This diploma thesis is based on a long-term experiment manipulating water availability in mountainous meadow in Austrian Alps. The experiment as well as sampling of soil and focal plant species (*Festuca rubra*) is very well designed and performed!

Appropriate methodological approaches

All aims of the study focus on changes in plant microbiome composition with particular focus on bacterial and fungal communities. Unfortunately, the Materials and Methods section is poorly described. The information provided there does not allow proper evaluation of applied methods. I am highlighting a few examples:

It is not clear how much material of plant tissue or soil was used for DNA extraction.

It is not clear for how long the surface sterilization of leaves was applied. Did you also applied surface sterilization on collected roots?

You mentioned that you collected rhizosphere and bulk soil samples. What exactly do you mean by rhizosphere samples? How did they differ from the bulk soil samples?

Besides that, I would suggest also providing a bit more detailed information about the molecular methods that were used. I am afraid that reference to Nilsson et al. (2019) does not provide information about PCR conditions necessary to evaluate your analyses, but I might be wrong.

I am not sure what exactly you mean by "removing of ambiguous bases". Can you please explain it? Did you modified the sequences by removing ambiguous bases?

In general, major share of fungal taxa from rhizosphere or root samples was not identified into known families (see Figure 10). This is surprisingly high number of undescribed taxa. Unfortunately, there is not enough information in the Material and Methods to evaluate **taxonomical assignment of identified fungal or bacterial sequences**. Could you please describe in more detail what release of UNITE database was used as a reference data for the blastn search? **What criteria of blastn results were used to assign the representative sequences to taxa in the reference dataset? Did you also identify any non-fungal or non-bacterial sequences in your ITS2 or SSU datasets, respectively? If so, how did you treat them?** Besides that, you mentioned that you "determined the taxonomic composition using the packages "microbiome" and "plyr". The purpose of this analysis and how does it differ from the determination of the taxonomic composition based on blastn results is not clear to me.

Unfortunately, chapter describing statistical analyses does not provide sufficient information either. For example, the fact that drought duration was treated as a factor and not a continuous variable could only be identified by the somewhat odd name of the x-axis in Figure 12 (and a few

others), which was "as.factor(rok)". In addition, it would be very useful to indicate for what purposes the various statistical analyses were used.

It is not clear how the sequencing data were standardized prior to "species richness" analyses (e.g., Shannon diversity index calculation) or did you use raw sequence counts for the calculations? **What was the distribution of sequence numbers per sample in your data and analyses?**

Can you please explain a bit more the Specialization index? It is not clear to me, what exactly do you mean by the specialists. Similarly to the previous analyses, what kind of data was used to calculate the Specialization index? Did you use raw sequence counts or Hellinger transformed data?

Differences in composition of microbial communities among the three studied substrates (rhizosphere soil, root and leaf) was documented by Venn diagrams. Unfortunately, this method does not provide any statistical test, that would evaluate how the observed values differ from a randomly distributed data. Therefore, it is problematic to make solid conclusions based on this analysis. More importantly, **the applied Venn diagram analysis rely on the assumption that sampling effort was similar across the samples and studied substrates. Was this criterion met? Isn't, in fact, the high share of prokaryotic OTUs between root and rhizosphere rather due to low number of sequences, and subsequently low species richness, of leaf samples?** Besides that, which data were used for calculating the Venn diagrams? **Were the diagrams calculated for microbial OTUs or for microbial families?**

Why did you decide to study response of microbial taxa to drought period on family taxonomic level? Wouldn't the identification on species or genus level allow for better biological interpretation, since most species or genera of fungi can be assigned to specific ecological groups such as plant pathogens, endophytes, mycorrhizal fungi or saprotrophs? This issue is then problematic in Discussion, where a single ecological category is assigned to whole fungal family. For example, species from the family Hyaloscyphaceae are known as saprotrophs, endophytes, ectomycorrhizal, ericoid mycorrhizal, mycoparasites, as well as lichen or plant parasites. Similarly, Magnaporthaceae also harbour fungal taxa with various ecologies, not only endophytic fungi (in fact endophytic life-style is rather rare among the genera in Magnaporthaceae).

Please, next time refer to the GPS coordinates of the experimental site. It does not make sense to precise exact GPS coordinates, if they do not refer to your experimental site.

<u>Correct and appropriate interpretation of data (results) in the context of what is known about the problem</u>

While part of the discussion focused on bacterial results is relatively well developed, discussion of fungal results could be more detailed. As mentioned above, this is probably due to suboptimal evaluation of fungal communities using the family level, which does not allow more detailed ecological interpretation of changes in fungal communities. For example, it would be interesting to know more about fungal species or genera and their ecologies, which relative abundance increase in root endosphere of plants exposed to 14 years of drought (e.g., taxa from the Hyaloscyphaceae family).

Could you please specify which analysis or result support your conclusion that there is a gradual change in composition of microbial communities due to drought stress?

Last part of the discussion, focusing on the differences in microbial community composition among the three different studied sources (root, shoot, soil) is relatively poorly developed. The observed results are not compared to any other studies, which also addressed similar questions, although there are numerous.

Personally, I would not use Firakova et al. 2007 as a reference for information that mycorrhizal plants provide organic carbon compounds to their fungal partners.

Adequate and synthesizing choice of presentation tools

The results are well presented.

Figure 6c: This RDA plot shows perfect overlaps of at least four samples from the year 2 and 14. I have to admit that I have never seen such overlap in ordination analyses based on microbial community composition data. Can you please provide number of high-quality sequences of bacteria per these samples? **Did these overlapping sample pairs harbour exactly the same bacterial communities? Did these pairs of samples belong to the same sampling blocks/sites?**

Formal processing of the text

Besides a few spelling mistakes, names of axis in some figures and missing references (e.g., Gryndler et al. 2004), the diploma thesis is well written.

Questions

The most important questions are highlighted in the text above in bold. In addition, I would like to ask which group of microorganisms (fungi or bacteria) may be more important in plant response to drought stress and why.

In Prague 30. 5. 2024   Petr Kohout