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Bc. Tereza Billichová

Effect of long-term drought on plant-associated microbiota
Vliv dlouholetého sucha na mikrobiotu asociovanou s rostlinami

Diploma thesis

Supervisor: prof. RNDr. Zuzana Münzbergová, Ph.D.
Consultant: Dina in ´t Zandt, Ph.D.

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Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 29. 4. 2024

Bc. Tereza Billichová

Abstract

Plant-associated microorganisms are very important for plant growth. Microbiota influence, for example, nutrient uptake, flower and fruit production or biocontrol of pathogens. Microorganisms are found in various plant organs. Each plant part then creates different environments for the microorganisms to live in. This may influence their different functions depending on which plant part they are found in. One such function is to help plants cope with adverse conditions. Drought, as an adverse condition, has a major effect on both plants and micro-organisms. The aim of this thesis is to study effect of long-term drought on the composition of the microbiota in the rhizosphere and leaf and root endosphere.

Long-term drought affected the composition of microbial communities in different plant parts. According to the original hypothesis, the response to drought of prokaryotes differed from that of fungal communities. Fungal communities are more stable and their alpha diversity did not change much during the different durations. The opposite trend in diversity is seen in prokaryotes, where a relative increase in specialists can also be observed.

Regarding specific microbial taxa, the results confirm the previously reported trend of increasing *Actinobacteria* abundance during drought. For fungi, on the other hand, there was a surprising increase in saprophytic taxa. Last but not least, the results also show a difference in the sharing of microbial taxa among the different plant parts. In fungi, a clear division of taxa between the rhizosphere and endosphere of plants was observed. In prokaryotes, on the other hand, we see a partition between the underground parts of the plant and the leaf endosphere. This trend intensified during the drought.

The results therefore show that long-term drought has an impact on the composition, diversity and function of plant-associated microbial communities. This effect was not only evident after two years of drought, but it also intensified during the fourteenth year of drought.

Key words: Drought, plant microbiota, *Festuca rubra,* rhizosphere, plant endosphere

Abstrakt

Mikroorganismy asociované s rostlinami jsou pro růst rostlin velmi důležité. Mikrobiota ovlivňuje například příjem živin, tvorbu květů a plodů nebo biologickou kontrolu patogenů. Mikroorganismy se vyskytují v různých rostlinných orgánech. Každá část rostliny pak vytváří pro mikroorganismy rozdílné prostředí, ve kterých mohou žít. To může ovlivňovat jejich různorodé funkce v závislosti na tom, ve které části rostliny se nacházejí. Jednou z takových funkcí je pomáhat rostlinám vyrovnat se s nepříznivými podmínkami. Sucho jako nepříznivý stav má velký vliv jak na rostliny, tak na mikroorganismy. Cílem této práce je zabývat se vlivem dlouhodobého sucha na složení společenstev mikroorganismů v rhizosféře a endosféře listů a kořenů.

Dlouholeté sucho mělo vliv na složení mikrobiálních společenstev v různých rostlinných částech. Dle původního předpokladu se lišila odpověď prokaryot od odpovědi houbových společenstev na sucho. Houbová společenstva jsou více stabilní a jejich alfa diverzita se během rozdílné délky příliš neměnila. Opačný trend v diverzitě je vidět u prokaryot, kde se dá pozorovat i relativní nárůst specialistů s délkou trvání sucha.

Pokud se jedná o konkrétní mikrobiální taxony, výsledky potvrzují dříve zaznamenaný trend zvyšujícího se zastoupení *Actinobacteria* během sucha. U hub byl naopak pozorován překvapivý nárůst saprofytických taxonů. V neposlední řadě z výsledků vyplývá i rozdíl ve sdílení mikrobiálních taxonů mezi jednotlivými rostlinnými částmi. U hub bylo zaznamenáno jasné rozdělení taxonů mezi rhizosféru a endosféru rostlin. U prokaryot naopak vidíme předěl mezi podzemními částmi rostliny a listovou endosférou. Tento trend se v průběhu sucha prohluboval.

Z výsledků je proto patrné, že dlouholeté sucho má vliv na složení, diverzitu a funkci mikrobiálních společenstev asociovaných s rostlinou. Tento vliv byl patrný nejen po dvou letech sucha, ale jeho efekt se prohloubil i během čtrnáctého roku sucha.

Klíčová slova: sucho, rostlinná mikrobiota, *Festuca rubra,* rhizosféra, rostlinná endosféra

Contents

1 Introduction

Despite their size and possible insignificance in the eyes of humans, microorganisms play a vital role in the functioning of other organisms – plants included. One of the most well-known relationships of microbiota with plants is mycorrhiza, in which fungi, among other things, provide the plant with a better supply of water and nutrients (Gryndler et al. 2004, (Alvarez et al., 2009). The plant in turn provides the fungi with energy and assimilates, and also shelter (Firakova et al., 2007). Additionally, tuber-associated bacteria can also help with nutrient supply by being involved in nitrogen uptake (Alvarez et al., 2009; Read & Perez-Moreno, 2003) and other mutualistic bacteria can also influence the cycling of sulphate or carbon (Knief et al., 2012; Ofek-Lalzar et al., 2014).

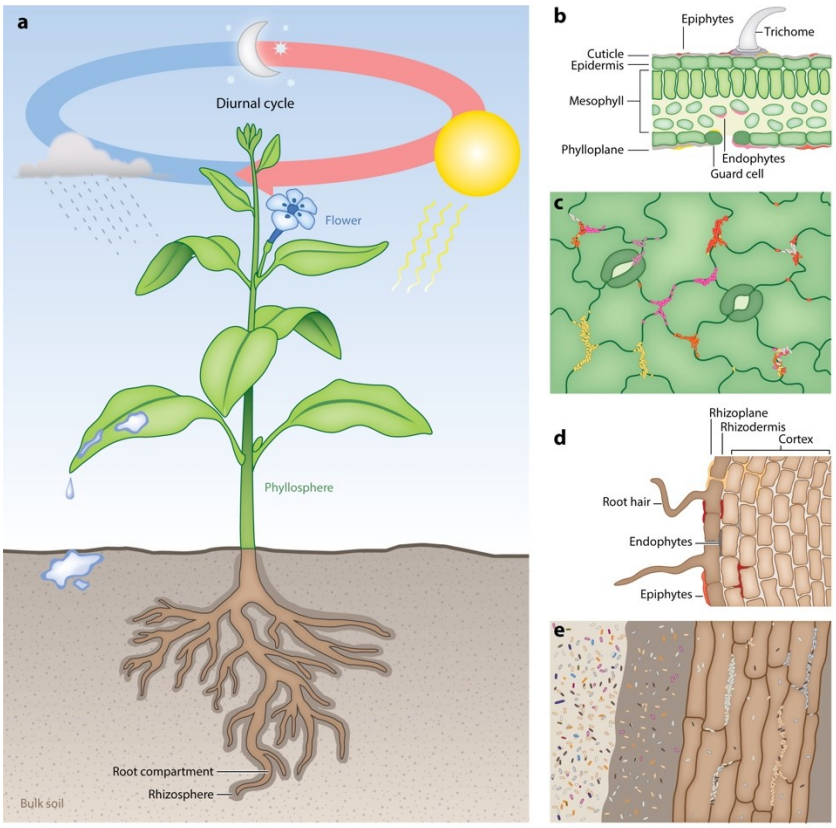
In addition to influencing nutrient cycling in plants, microorganisms can also influence plants through the production of proteins of various functions. For example, catalase produced by bacteria in the root serves as a stress response and may also influence root development (Ofek-Lalzar et al., 2014). Rhizobia also influence root structure and are involved in other processes such as seed germination, seedling development, photosynthesis and stomata activity (Chi et al., 2005).

Microorganisms also have an important effect on the production of plant hormones, which e.g. influence the production of flowers and fruits (Zhu et al., 2022). They also help the plant in biocontrol of pathogens through the production of antimicrobial substances (Basilio et al., 2003) and play an important role in the plant's response to stress conditions (Bashir et al., 2022). However, in addition to the positive effects, microbiota can also have a negative impact on plants in the form of parasites and pathogens, which are equally important for plant functioning (Klironomos, 2002; Zhu et al., 2022).

2 Plant as habitat

Microorganisms can be found in a wide variety of plant parts. In relation to the plant, they can be found in its aboveground (Ehrenfeld et al., 2005; Muller et al., 2016) but also in belowground organs (Muller et al., 2016; Zhu et al., 2022) and of course in the surrounding soil (Muller et al., 2016) (Fig. 1). Particularly important in this context is the role of the endosphere of the root and leaf. These various parts differ from each other in structural and biochemical conditions and thus provide unique conditions for microorganisms to live (Gong & Xin, 2021; Massoni et al., 2021; Muller et al., 2016).

Leaves can be considered the most stressful habitat, as the microorganisms here are much more exposed to environmental influences than those in soil. These effects include in particular UV radiation, herbivory and more frequent fluctuations in temperature and humidity (Delmotte et al., 2009; Muller et al., 2016). The endosphere of the leaf further differs from the belowground parts by its characteristics in terms of photosynthetic metabolic capacity and the large apoplast, which serves as an additional habitat for microorganisms (Gong & Xin, 2021). Plant metabolites can also create various conditions which can differ based on different plant parts. For example, the leaf microbiota is adapted to process more diverse and complex sugars (polysaccharides and leaf cuticle waxes) than those of roots and soils, which obtain nutrients primarily from the rhizodeposits (Bai et al., 2015; Muller et al., 2016).



Müller DB, et al. 2016.
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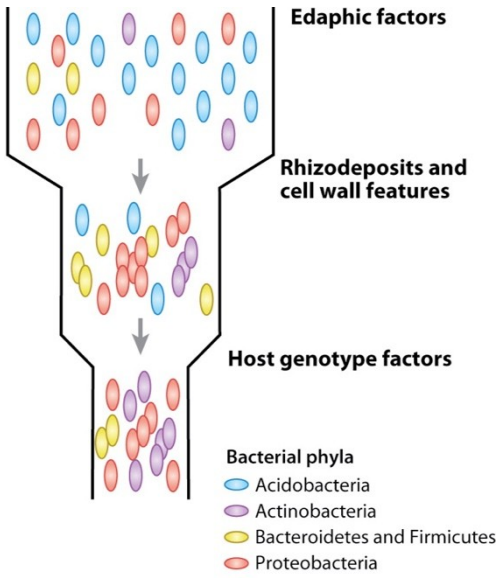
Figure 1. In figure (a) we can see the plant divided into the phyllosphere (i.e. the area of the aboveground organs that is free in the environment and is closer to the environmental influences causing various stress conditions) and the rhizosphere (which is located belowground and is primarily influenced by edaphic conditions such as, soil type or soil moisture). In Figure (b) we see the anatomy of the leaf and its colonization by bacteria (including the so-called phylloplane and intercellular spaces of mesophyll). Leaf characteristics (e.g. cuticle composition and thickness or the presence of stomata and trichomes) can influence the colonization process. In Figure (c) we see colonization of the abaxial part of the leaf. The bacteria colonize the leaf in aggregates between the epidermal cells around the stomata. Figure (d) shows the anatomy of the root showing the progression of colonization from epiphytic regions through the rhizoplane to the endophytic compartments. Figure c) then shows the different composition of the microbial communities and furthermore how the anatomical features of the root influence the colonization itself (Muller et al., 2016).

All these factors have an impact on the specific composition of microbial communities. In general, the diversity of microorganism communities decreases the closer they are to the endosphere. There are many different microbial strains in the surrounding soil, the richness of which is lower in the rhizosphere. The rhizoplane then serves as a sorting gate, allowing only a select few strains into the root endosphere. Which microorganisms eventually enter the endosphere depends on several factors. One is the plant itself, which carries out selection by the already mentioned primary and secondary metabolites (Edwards et al., 2015; Lundberg et

al., 2012; Muller et al., 2016). Plant characteristics such as cuticle thickness or its chemism also play a role in determining which microorganisms will eventually be able to thrive in this environment (Kembel et al., 2014; Muller et al., 2016).

Environmental influences (which affect the composition of the surrounding microbial banks and their dispersal), as well as the competition between the microorganisms themselves and the founder effect, also play an important role (Muller et al., 2016). It has been found out that the largest source of microorganisms for plant roots is the soil, which determines which strains of organisms are available to the plant (Basilio et al., 2003; Bulgarelli et al., 2013; Muller et al., 2016) (Fig. 2). Interestingly, microbiota numbers begin to increase in the endosphere only 24 hours after contact with soil, and stable community composition occurs after two weeks (Edwards et al., 2015).



Bulgarelli D, et al. 2013.
Annu. Rev. Plant Biol. 64:807–38

Figure 2. In the figure we can see a two-step selection model for root microbiota differentiation. The soil environment determines the structure of bacterial communities in soil biomes. The next step in determining the composition of microorganisms is the rhizodeposition and cell wall properties of the plants. The last step involves convergent selection of bacteria that are found on the rhizoplane and further inside the root (Bulgarelli et al., 2013).

Compared to the rhizosphere, the phyllosphere is less complex and differs in the composition of its microbial communities (Knief et al., 2012). In the case of the aboveground parts, the role of soil is substituted by air, which serves as an important source of microbiota.

After the colonisation of the leaf, the communities then evolve gradually, always following a similar pattern, with a difference in the relative abundance of each group, which may vary from plant to plant (Maignien et al., 2014). The general trend in the composition of leaf communities is then independent of geographical or climatic conditions (Knief et al., 2012). In addition to the air, microbiota may enter the leaves from belowground organs (Chi et al., 2005; Grady et al., 2019; Massoni et al., 2021). Up to 90 % of leaf taxa are also found in the rhizosphere (Grady et al., 2019). In addition to migration from the soil, microorganisms can also enter the leaves via insect vectors, wind or rain (Grady et al., 2019; Massoni et al., 2021)

Thus, the function and composition of the microbiota depends on where it is located in relation to the plant (Bashir et al., 2022). Among the bacteria, *Actinobacteria* (especially *Streptomycetaceae*), *Proteobacteria* (*Betaproteobacteria*), *Firmicetes*, *Spirochaetes* are the most dominant in the endosphere, while few *Acidobacteria*, *Gemmatimonadetes* are abundant in the rhizosphere (Bulgarelli et al., 2013; Edwards et al., 2015; Lundberg et al., 2012). In contrast, *Acinobacter*, *Variovorax* and *Pseudomonas* (Maignien et al., 2014) were recorded in the phyllosphere (Fig. 3).

Müller DB, et al. 2016.
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Figure 3. The figure shows the phylogenetic structure of the plant microbiota. Sequence data from several studies (citation numbers in brackets) were clustered into operational taxonomic units (OTUs) and further linked within families. In figure we can see families from root, leaves and in one case grapevine flowers (outer ring) (Muller et al., 2016).

Microorganisms adapt to factors such as environmental influences, competition or plant metabolites by physiological changes and especially by the production of various metabolites. The previously mentioned stress conditions of the phyllosphere force bacteria to produce proteins that help them to withstand these conditions. Such proteins include, for example, superoxide dismutase, catalase or DNA protection proteins. In addition,

Gammaproteobacteria produce oxidative stress regulators (Delmotte et al., 2009). *Streptomycetes* in the roots in turn form antimicrobial substances (especially against Gram-positive bacteria but also applicable against fungi and bacteria) (Basilio et al., 2003). In the roots, we also find enzymes of methanogenesis, methane oxidation or nitrogenase (Fig. 4) (Knief et al., 2012; Ofek-Lalzar et al., 2014). Dinitrogen reductase and dinitrogenase are found in the root and leaves (Knief et al., 2012). Plants, together with their microorganisms, form a complex feedback system that is able to compensate for the negative effects of their environment (Lundberg et al., 2012; Muller et al., 2016).

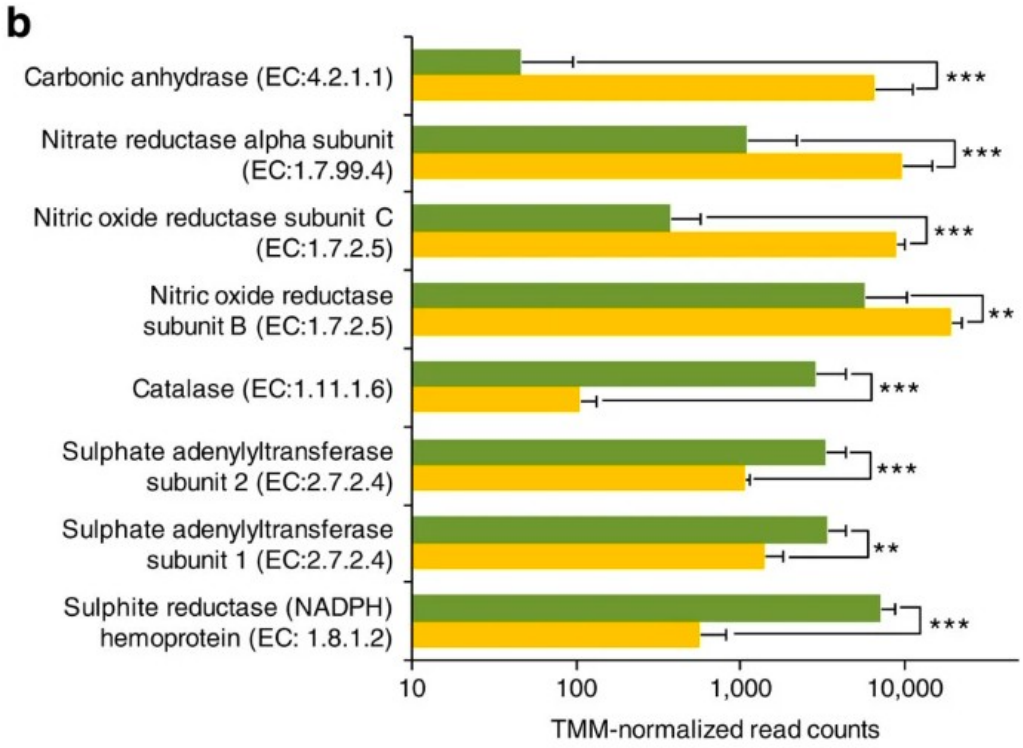

Figure 4. In the picture we can see some other genes than those mentioned above that are expressed by microorganism in plants. Specifically, we can see genes that are expressed differently depending on whether they were in Cucumis sativus (green) or Triticum turgidum (yellow). This shows a significant host effect on bacterial protein production because the composition of the bacterial communities was very similar between these plants (Ofek-Lalzar et al., 2014).

3 Plants, microbiota and drought stress

As mentioned above, in addition to the environmental characteristics provided by the plant itself, abiotic conditions also influence the microorganisms, either directly or through their association with host plants (Ehrenfeld et al., 2005; Firrincieli et al., 2020; Xu et al., 2018). In recent years, due to the increase in anthropogenic emissions, there have been significant changes in climate, including temperature increases and fluctuations in water availability. These climate changes are causing extreme weather events that can lead to disruptions in the water regime, causing e.g. extreme droughts. All of this further has a major impact on the functioning of individual organisms and consequently entire ecosystems (IPCC 2014, IPCC 2018).

Drought has various effects on organisms. For plants these effects are mainly negative and they can be cause of mechanisms, which we can observe on three different levels. On physiological level these are e.g. reduction in turgor, stomatal conductance, photosynthetic activity or growth. On the biochemical in level, changes in Rubisco activity and increases in stress metabolite and antioxidative enzymes appear. Last but not least, we also see changes at the molecular level such as the expression of ABA biosynthetic and responsive genes and specific protein syntheses such as LEA, DSP or RAB (Pinhero et al., 1997). If a plant cannot cope with drought, a reduction in its fitness logically follows – poor growth, fewer flowers and subsequently fewer fruits. Drought stress then, in the worst case, leads to plant death.

The response to drought in microorganisms is not as unified as in plants. In fact, they are much more diversified and complex groups that have very different adaptations and thus may respond differently to drought (de Vries et al., 2018). However, in general, the literature suggests that bacteria respond much more quickly to changes including drought than fungal communities, which appear to be much more stable (Castro et al., 2010; de Vries et al., 2018).

Regarding the effect of drought on the interactions of plants and their microbiota, the microbiota can significantly help plants in the event of drought, even long-term drought. Both ectomycorrhizal symbioses helped seedlings with nutrient supply and reduced the negative impact of drought (Alvarez et al., 2009) and arbuscular mycorrhiza also helped seedlings, which then had much less biomass loss during the drought treatment (Ruizlozano et al., 1995).

In the case of bacteria and belowground organs, the situation is more complex. Plants that have a much closer relationship with their bacteria may paradoxically experience more negative effects of drought than those with a looser relationship (these bacteria had better

survival rates and were capable to help their plants (O'Brien et al., 2018). This is especially true for nitrogen-fixing bacteria, which may not be able to sustain tuber damage due to drought and thus reduce their relationship with the plant (Albrecht et al., 1984; Guerin et al., 1991). On the other hand in leaves, a positive effect of the bacteria was observed under drought conditions. Lau & Lennon (2012) found that plants that were inoculated with microorganisms that had been previously exposed to drought had a higher number of flowers and fruits compared to other treatments. These plants had only a 19 % less decline in flowers and fruits than those plants that grew with the moisture-adapted biota, which had a 59% decline. While the drought reduced the richness of the communities, the bacteria that helped with plant competitiveness remained. Another study further found that bacteria can produce substances (e.g., catalases, peroxidases, or phenolic compounds) that help plants overcome drought-induced oxidative damage (Naveed et al., 2014).

However, drought itself is not the only factor that can influence the behaviour and responses of organisms to it. It is clear from the above that a plant can serve as a diverse habitat with many different parts. This in turn influences the effect of drought on the microorganisms within plants. Studies have shown that organisms in the endosphere of the root respond much more to drought than those in the rhizosphere or soil (Fitzpatrick et al., 2018; Santos-Medellin et al., 2017). Another factor is the length of the drought. It can make a difference to organisms if the drought occurs only once and for a short period, or if the drought is long and then recurs. How microorganisms will behave on longer time scales has been addressed by studies looking at the legacy effect of drought. In general, there were changes in the composition of microbial communities. Bacteria were able to respond quickly to changing treatments, while fungi were not very plastic in their response and were slower to recover from previous conditions (de Vries et al., 2018; Fuchslueger et al., 2016).

Resilience of fungi can also be reflected in the relationship between plants and soil pathogens. The abundance of fungal pathogens can increase with recurrent droughts, due to their highly resistant dormant spores that accumulate in the soil over time (Crawford & Hawkes, 2020; Preece et al., 2019). Microorganisms that were previously in drought treatment (after several generations) were able to compensate for the negative effects of the current drought stress on plants (Lau & Lennon, 2012).

However, most studies to this date that have addressed effect of drought on relationship between plants and their microbiota only been conducted over a relatively short period of time and have not taken much account of the effect of recurrent prolonged drought on the plant-associated microbiota.

3.1 The aim of the work

The aim of the diploma project is to fill the gaps in our understanding of how long-term drought affects microorganisms of the leaf and root endosphere and rhizosphere. Furthermore, I explore what connections we can find between the leaf and root endosphere and the surrounding rhizosphere under these stress conditions and what kind of comprehensive picture we can get from this.

Based on the findings in the literature so far, my hypotheses are:

- A prolonged drought will lead to a reduction in the diversity of microorganism communities. The remaining taxa will be those that are appropriately adapted to drought and may help the plant to cope with drought stress.
- There will be a similar composition of microbial communities between plant compartments, which could also indicate a possible exchange of organisms between these plant parts.

4 Materials and Methods

4.1 Experimental setup

The study site is part of the alpine grassland meadow in the Stubai Valley near Innsbruck (47°07'00.0"N 11°19'00.0"E) at 1850 m above sea level. This meadow is cut annually and fertilized every 3-4 years. The mean annual temperature is 3 °C and the mean annual perception is 1100 mm (Bahn et al., 2006; Fuchslueger et al., 2016).

The summer drought was artificially induced roofing. The roofing system was installed every summer for up to 14 years by team of M. Bahn from Uni. Innsbruck. The three plots were specifically affected for 0, 2 and 14 years (Fig. 5). Each regime was held in four blocks,

My model plant is a common European perennial grass *Festuca rubra*, which is one of the dominant species in the studied meadow (Bahn et al., 2006).

Figure 5. Photos of plots and construction for summer rain shelter.

4.2 Sample collection

From every block we selected three *Festuca rubra* plants and sampled their leaves, roots and rhizosphere (i.e. 36 samples in total for each kind of measurement) and bulk soil. We further cut plant species around each *Festuca rubra* in 20 cm diameter (in summer and then in

autumn when we collected the plants). We transported the samples in a refrigerator. After transport to the Botanical Institute of the CAS in Průhonice we sterilized collected leaves with series of soaking in sterile water and solutions of 75% etOH and 3.25% NaClO. After that we froze all the samples for DNA isolation.

4.3 DNA isolation

After disruption of frozen leaf, soil and root samples in TissueLyser, I isolated DNA from them. This was done using the DNeasy Plant Mini Kit from Qiagen for leaves and roots and NucleoSpin Soil from Macherey-Nagel for soil. I followed both protocols except I did not elute the isolated DNA in 100 µl Buffer AE (SE in case of soil), as written in the protocols, but in 50 µl distilled water. I determined the DNA concentration using a NanoDrop spectrophotometer.

4.4 DNA sequencing and analysis

I characterized the fungal communities by sequencing the *internal transcribed spacer* (ITS2) region, and I characterized the bacterial communities using the 16S ribosomal RNA gene. PCR amplification of fungal ITS2 was performed using labelled primers gITS7 and ITS4 (Ihrmark et al., 2012). Amplification of the V4 region of bacterial 16S rRNA was performed using the labelled primers 515F and 806R (Caporaso et al., 2012). PCR was performed according to Scholer et al. (2017) and Nilsson et al. (2019) in duplicates.

I purified the amplified DNA using QIAquick PCR Purification Kit. I followed the protocol from manufacturer.

Sequencing of PCR products was performed by a commercial company SEQME.

After that, I used the pipeline SEED2 2.1.2 (Vetrovsky et al., 2018) to cluster the fungi and bacteria sequences into *Operational Taxonomic Units* (OTUs). In this pipeline I did merging pair-ends using *fastq-join* 1.1.2 (Aronesty, 2011), quality filtering (I removed sequences with quality lower than 30), sequence trimming and removing ambiguous bases. I did fungal ITS extraction using ITSx 1.0.11 (Bengtsson-Palme et al., 2013). Then I removed chimeric sequencies with VSEARCH 2.4.3 (Rognes et al., 2016). Getting of the representative sequences was done within MAFFT 7.222 (Katoh et al., 2019)). For each cluster thus formed I identified matches at specific taxonomic levels using *blastn* against The SILVA ribosomal RNA database (Quast et al., 2013) and UNITE (Abarenkov et al., 2023).

4.5 Data analysis

I performed statistical data analyses using Rstudio version 2023.12.1.402 (R Core Team, 2023). I used the Redundancy Analysis (RDA) and the Rstudio package *"Vegan"* (Oksanen et al., 2022) for primary detection of patterns in the compositions of microbial communities based on drought duration. From the dataset, I've removed OTUs with frequency less than 5 and further transformed it using the *Hellinger* method. I chose the effect of individual blocks of a given treatment on samples as the covariate. The significance of the model was tested using a Monte Carlo permutation test with 499 permutations and each sample as a "*strata"* type *"free"*.

I calculated and analysed Shannon diversity index with Rstudio packages *"nlme"* (Pinheiro, Bates, R Core Team, 2023) and *"car"* (Fox & Weisberg, 2019). Whether differences between treatments are significant was assessed using estimated marginal means and the *"emmeans"* package (Lenth, 2024).

I obtained the Specialisation index using the packages *"phyloseq"* (McMurdie & Holmes, 2013) and its graphic output with *"ggplot2"* (Wickham, 2016) based on the methodology from Chen et al. (2021) and Devictor et al. (2008).

Another analysis I performed was Differential abundance analysis using the packages *"ALDEx2"* (Fernandes et al., 2013*), "microeco" (Liu et al., 2021)* and "*ggtree"* (Xu et al., 2022). For data with a frequency greater than 5, I first used the *"ALDEx2_kw"* method with family a taxa level and then plotted the top 30 significant OTUs. I then used the *"lefse" method to generate cladograms.*

I determined the taxonomic composition using the packages *"microbiome"* (Lahti et al.) and *"plyr"* (Wickham, 2011). I examined microbiota taxa at the class and family level.

Finally, I created Venn diagrams using the packages *"microeco" (Liu et al., 2021)* and *"ggplot2"* (Wickham, 2016) while overlap were weighted by relative abundance.

Across analyses, the packages *"RColorBrewer"* (Neuwirth, 2022) and *"tidyr"* (Wickham, 2024) were used.

5 Results

5.1 Effect of drought on microbiota composition

From the RDA results (Fig. 6 and Fig. 7), we can see that for most observed plant compartments in both prokaryotes and fungi, there is a distinct differentiation of microbial communities depending on the length of drought (except for the fungi in the leaf endosphere).

In case of prokaryotes, we can see that RDA for all three plant compartments was significant (tab. 1). In rhizosphere and root endosphere there is a relatively noticeable trend in differential according the treatments. In leaf endosphere the division is mainly between 0 and both of the other years (Fig. 6).

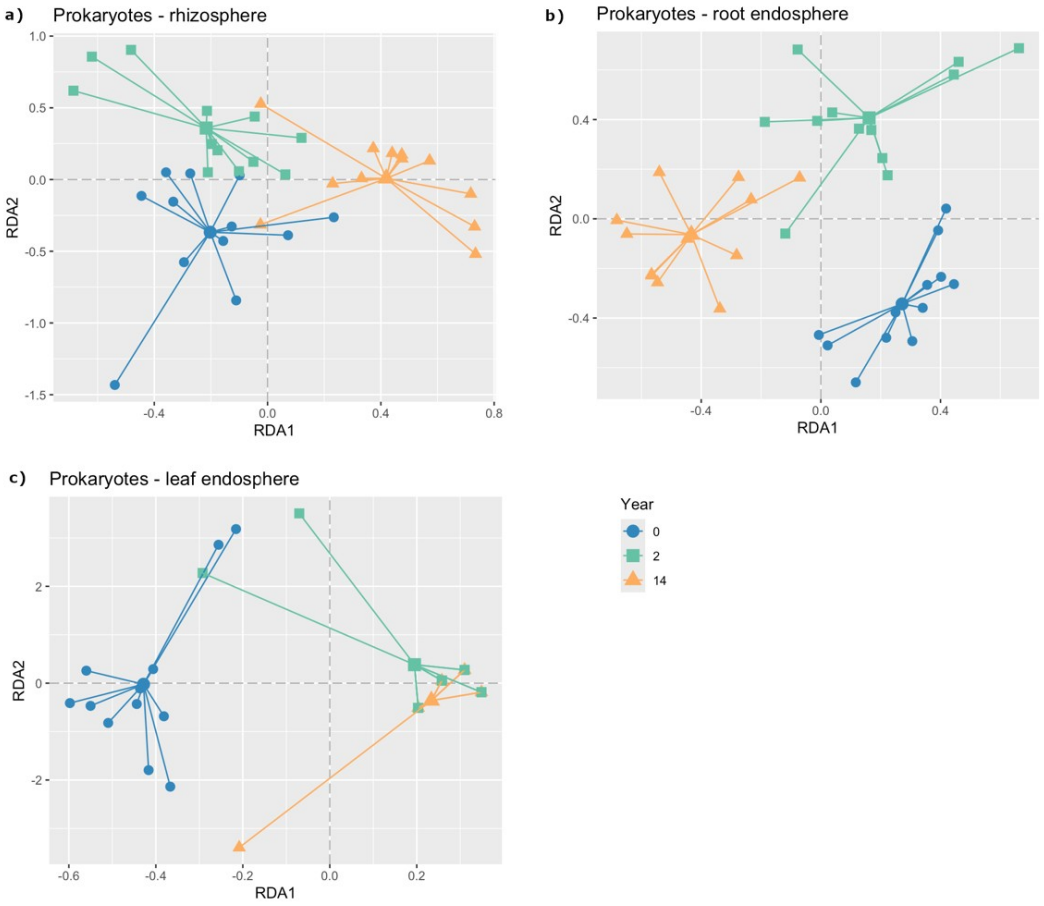

Figure 6. Ordination diagrams of redundancy analysis (RDA). Each diagram represents one of the observed plant compartments. Thanks to the "ordispider" method we can see patterns in the microbial communities based on the drought treatment. a) RDA of prokaryotes in the rhizosphere. The RDA1 axis explains 8.5 % of the variation and the RDA2 axis 2.7 % of the variation. b) RDA of prokaryotes in the root endosphere explains 8.3 % of the variation and RDA2 explains 4.5 % of the variation. c) RDA1 of leaf endosphere explains 42.5 % of

the variation and RDA2 explains 1 % of the variation.

Table 1. Results of RDA testing the effect of drought treatments on prokaryotes communities in different plant compartments.

Plant compartment	total variation explained	P-value
rhizosphere	11.2 %	0.002 **
root endosphere	12.8 %	0.002 **
leaf endosphere	44%	0.002 **

In case of fungi, we can see a significant distribution of microbial communities based on the year but only in rhizosphere and root endosphere (Fig. 7). The RDA of leaf endosphere was not significant (Tab. 2).

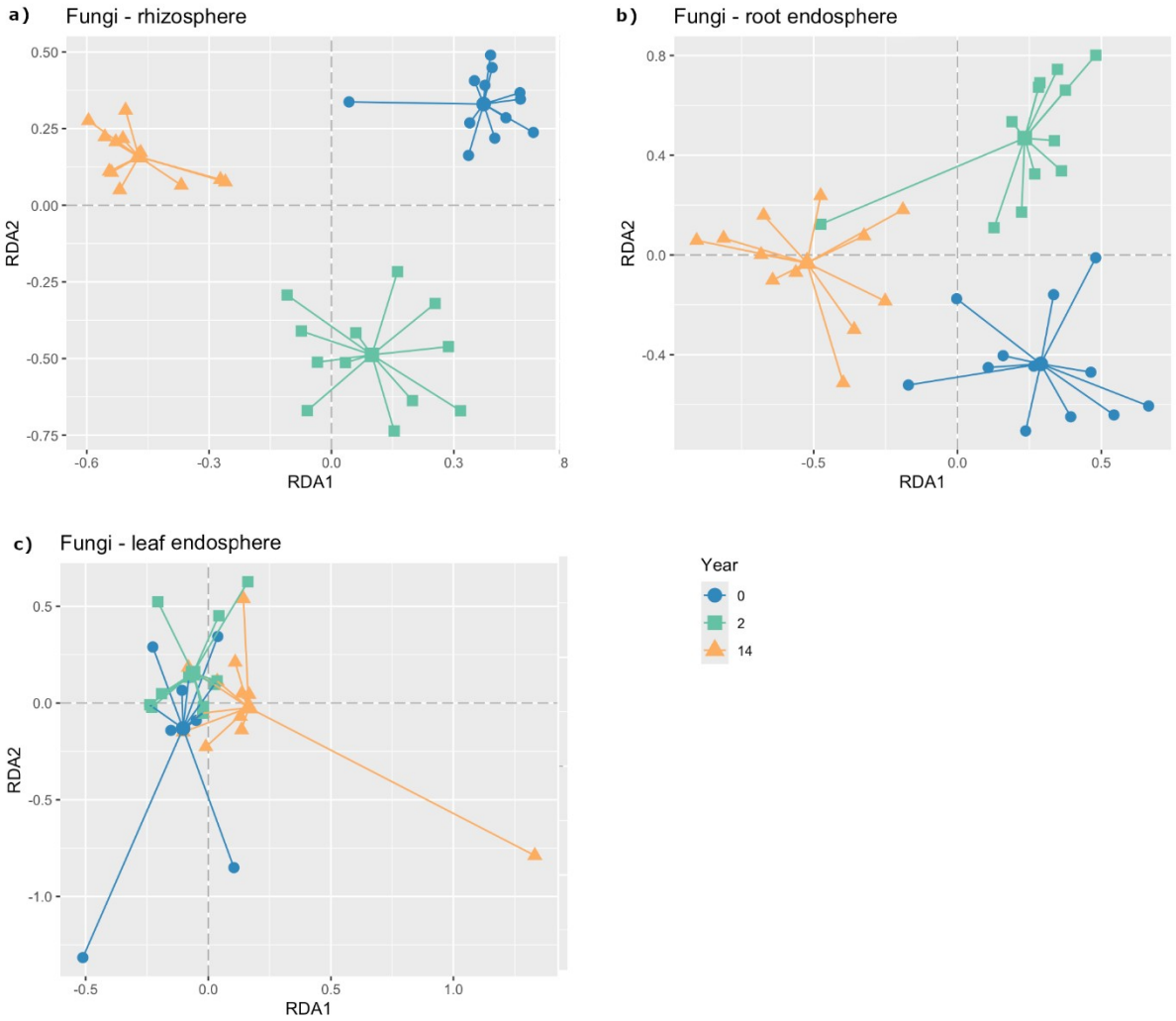

Figure 7. Ordination diagrams of redundancy analysis (RDA) for fungi. Each diagram represents one of the plant compartments. a) RDA of fungi in the rhizosphere. The RDA1 axis explains 12.4 % of the variation and the

RDA2 axis 5. 5 % of the variability. b) RDA of prokaryotes in the root endosphere explains 6.2 % of the variation and RDA2 explains 2.8 % of the variability. c) RDA1 of leaf endosphere explains 5.8 % of the variation and RDA2 1.9 % of the variability.

Table 2. Results of RDA testing the effect of drought treatments on fungi communities in different plant compartments.

Plant compartment	total variation explained	P-value
rhizosphere	17.9 %	0.002 **
root endosphere	9,0%	0.004 **
leaf endosphere	7.7 %	0.154

5.1.1 Taxonomy

In prokaryotes, we can see that the root endosphere is richer in number of the families than the rhizosphere (Fig. 8), with *Bacilliaceae* in particular dominating in the endosphere. The relative abundance of *Bacilliaceae* even increases with drought length. For the root endosphere, we can see that *Micromonosporaceae* or *Streptomycetaceae* are present, among others.

Regarding class representation, we can observe a significant representation of *Actinobacteria* in those plant compartments. The relative abundance also increased with time in both compartments. For the rhizosphere, we have also a significant representation of *Bacilli* and *Thermoleophilia*. Relative abundance increased in both classes with the duration of drought. For the root endosphere, we also see significant abundances of *Alphaproteobacteria* or *Thermoleophilia*, but their relative abundance did not change with time.

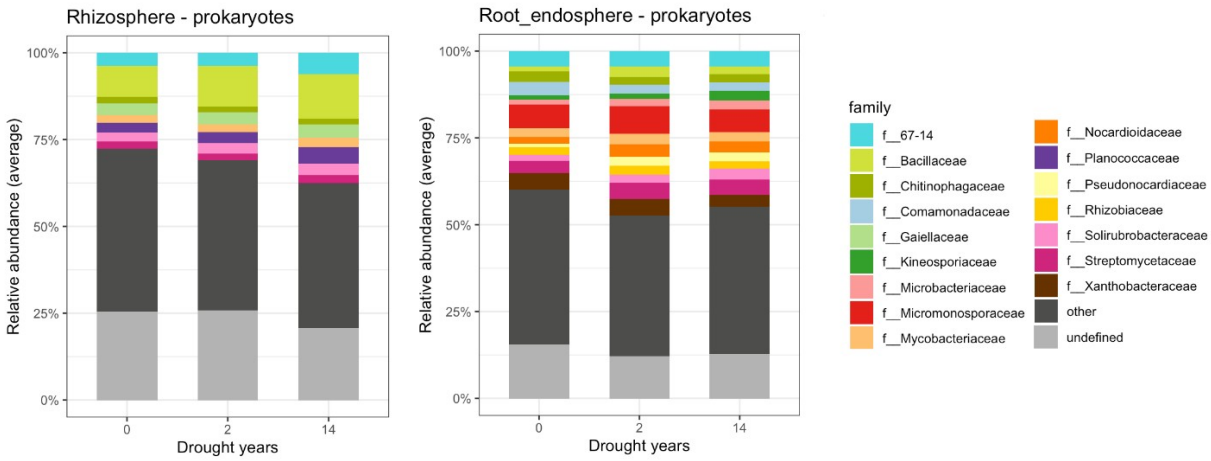

Figure 8. Relative abundance of prokaryote families in rhizosphere and root endosphere.

For the leaf endosphere (Fig. 9), the *Bacilliaceae* recur, and in contrast to underground parts we have a significant representation of *Archoleplasmataceae*, *Planococcaceae,* or *Sphingomonadaceae*.

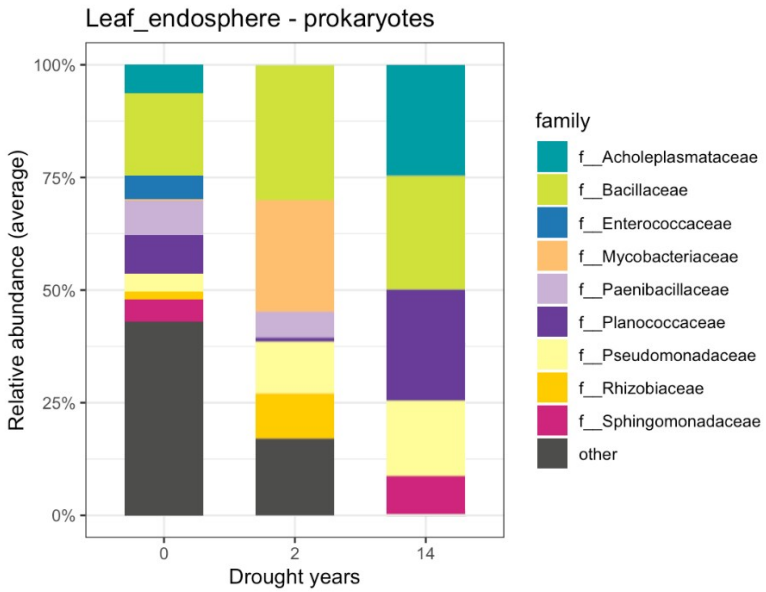

Figure 9. Relative abundance of prokaryote families in leaf endosphere.

On the taxa distribution based on drought duration between the rhizosphere and root endosphere (Fig. 10) we can see a clear partition of fungi families among the different plant parts. In the rhizosphere, we find mostly *Archaeorhizomyces, Aspergillaceae,* or *Piskurozymaceae*. While in the root endosphere, we see a significant representation mainly in *Hyaloscyphaceae, Mycenaceae,* or *Magnaporthaceae*.

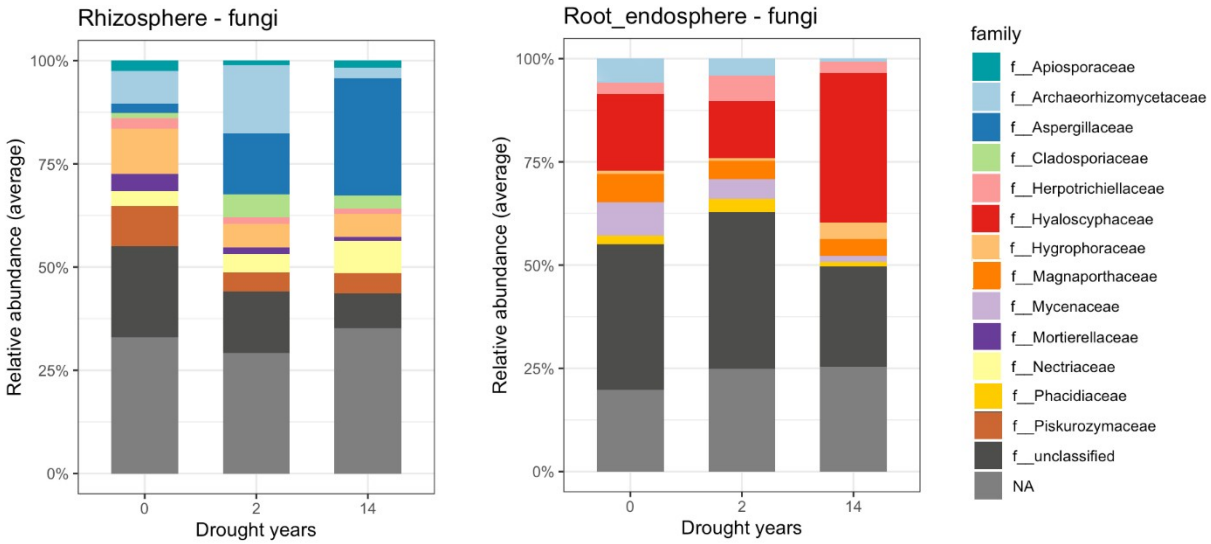

Figure 10. Relative abundance of fungi families in rhizosphere and root endosphere.

For the leaf endosphere (Fig. 11) we see only a limited number of families namely *Clavicipitaceae* and *Ploettnerulaceae*.

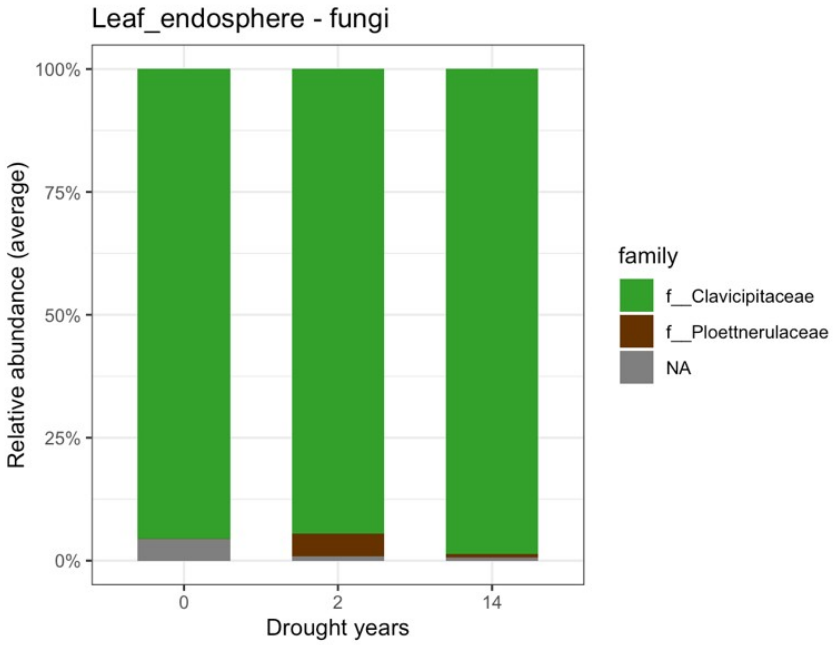

Figure 11. Relative abundance of fungal families in leaf endosphere.

5.1.2 Shannon diversity index

I measured diversity using the Shannon diversity index. In prokaryotes (Fig. 12) we can see that diversity varied depending on the plant compartment. In the rhizosphere, we see a significant decrease in diversity after the 14th year (Tab. 3 and Fig. 12) whereas in the root endosphere, the decrease in diversity took place in the 2nd year and did not change further. For prokaryotes in the leaf endosphere, there were too little data for analysis.

In the case of fungi, I only observed a significant change in the root endosphere and this was between year 0 and year 2 (Tab. 4 and Fig. 13) Neither the rhizosphere nor the leaf endosphere had any significant change in the diversity.

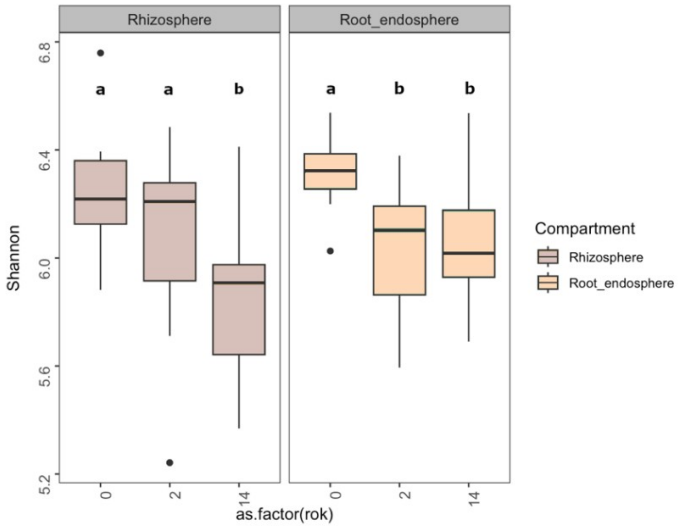

Figure 12. Boxplot of Shannon diversity index of prokaryotes in rhizosphere and root endosphere.

Table 3. Results from pairwise comparison of Shannon diversity index of prokaryotes.

Plant compartment	P-value for 0-2 year	P-value for 0-14 year	P-value for 2-14 year	P-value of the model
rhizosphere	0.9658	0.0002 ***	0.0001 ***	4.011e-06 ***
root endosphere	0.0072 **	0.0099 **	0.9909	0.00107 **

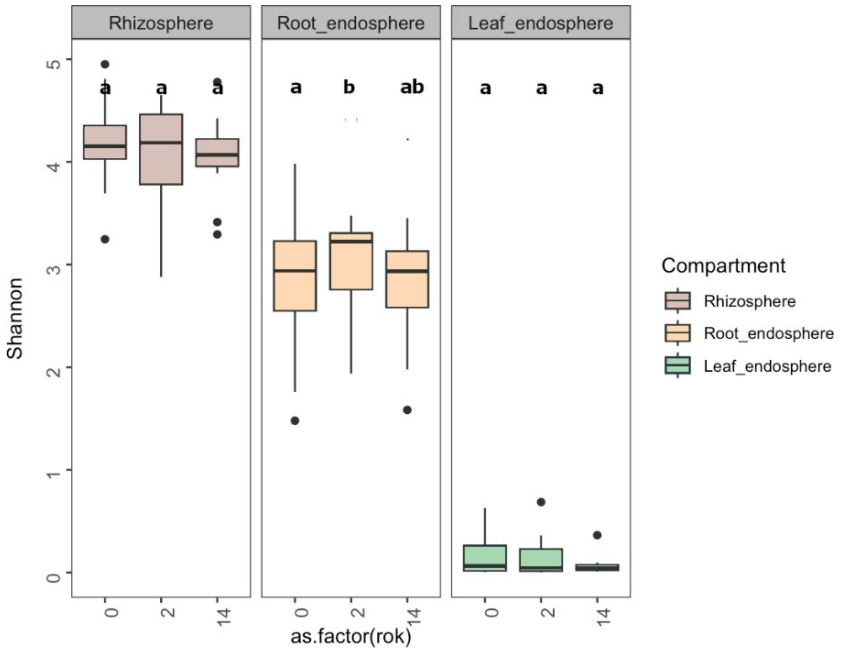

Figure 13. Boxplot of Shannon diversity index of fungi in the rhizosphere and endospheres of roots and leaves.

Table 4. Results from pairwise comparison of Shannon diversity index of fungi.

Plant compartment	P-value for 0-2 year	P-value for 0-14 year	P-value for 2-14 year	P-value of the model
rhizosphere	0.7984	0.7910	0.9999	0.7556
root endosphere	0.0315 *	0.4974	0.2226	0.01699 *
leaf endosphere	0.9163	0.8581	0.5559	0.5389

5.1.3 Specialization index

The results of the Specialisation index showed that prokaryotes of root endosphere (Fig. 14) in the 14th year showed a significant increase in the specialists compared to year 0. For the rhizosphere we then see the same but marginal trend (Tab. 5).

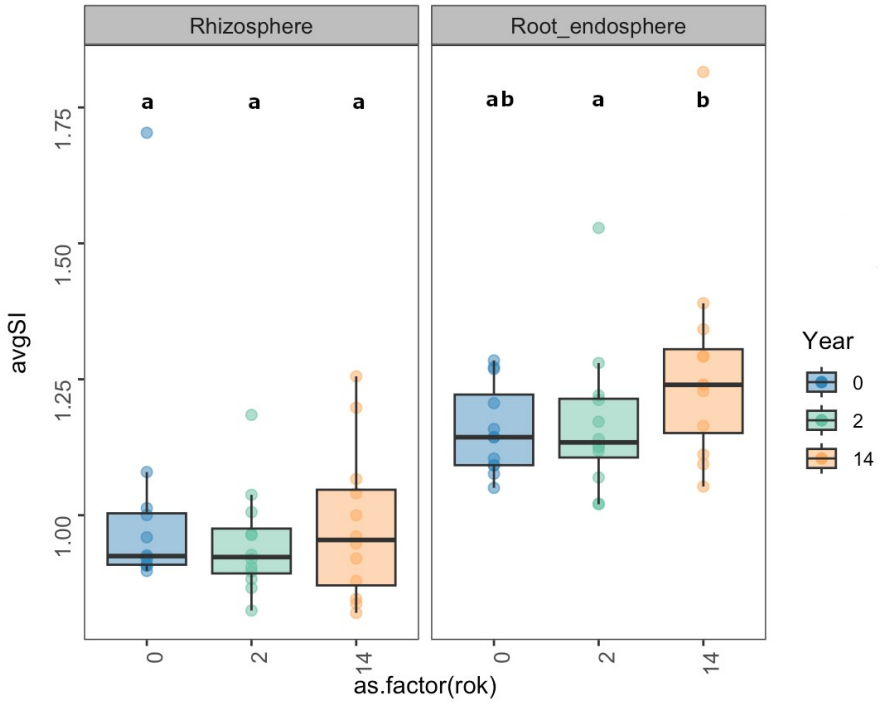

Figure 14. Boxplot of specialization index of prokaryotes in rhizosphere and endospheres of roots and leaves.

Table 5. Results from pairwise comparison of specialization index of prokaryotes.

Plant compartment	P-value for 0-2 year	P-value for 0-14 year	P-value for 2-14 year	P-value of the model
rhizosphere	0.2392	0.0680 .	0.4076	0.05737 .
root endosphere	0.4118	0.1040	0.0053 **	0.000419 ***

For fungi (Fig. 15), we can see a significant change only for the root endosphere – a decrease in specialists between years 0-2 and years 0-14 (Tab. 6).

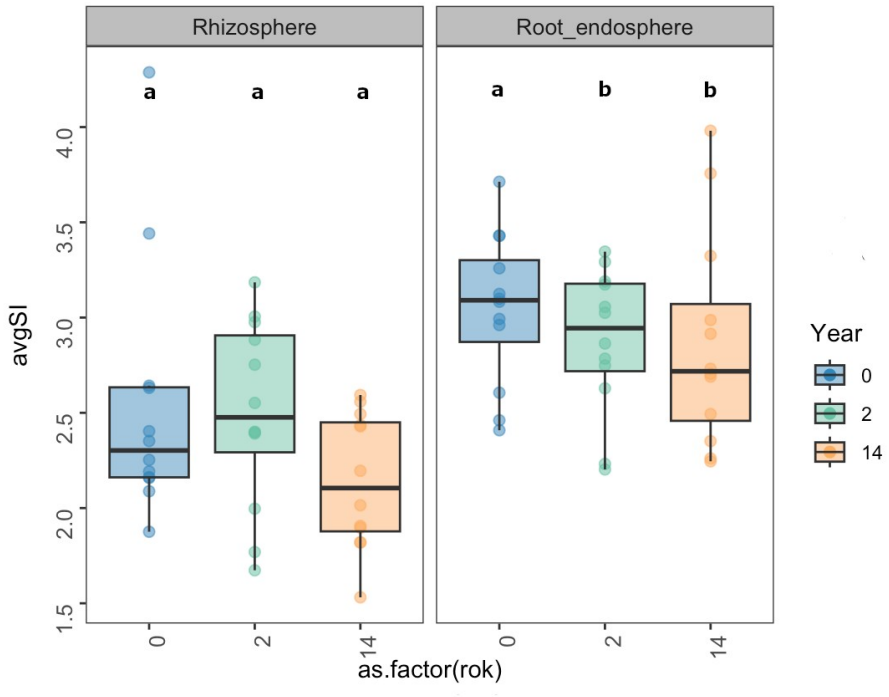

Figure 15. Boxplot of specialization index of fungi in rhizosphere and endospheres of roots and leaves.

Table 6. Results from pairwise comparison of specialization index of fungi.

Plant compartment	P-value for 0-2 year	P-value for 0-14 year	P-value for 2-14 year	P-value of the model
rhizosphere	0.9935	0.1612	0.1956	<2e-16 ***
root endosphere	0.0003 ***	0.0052 **	0.6612	1.748e-05 ***

5.2 Most prominent taxa in relation to drought duration

Using Differential abundance analysis, I identified the taxa that are significantly associated with specific drought legacy. In rhizosphere prokaryotes (Fig. 16) we can see that for the 14th year of drought the most significant families are *Geodermatophilaceae, Micrococcaceae, Solirubrobacteraceae* (from class *Thermoleophilia*), *Mycobacteriaceae*, *Pseudonocardiaceae* (all of them from *Actinobacteria*) and *Peptostreptococcaceae* (*Clostridia*).

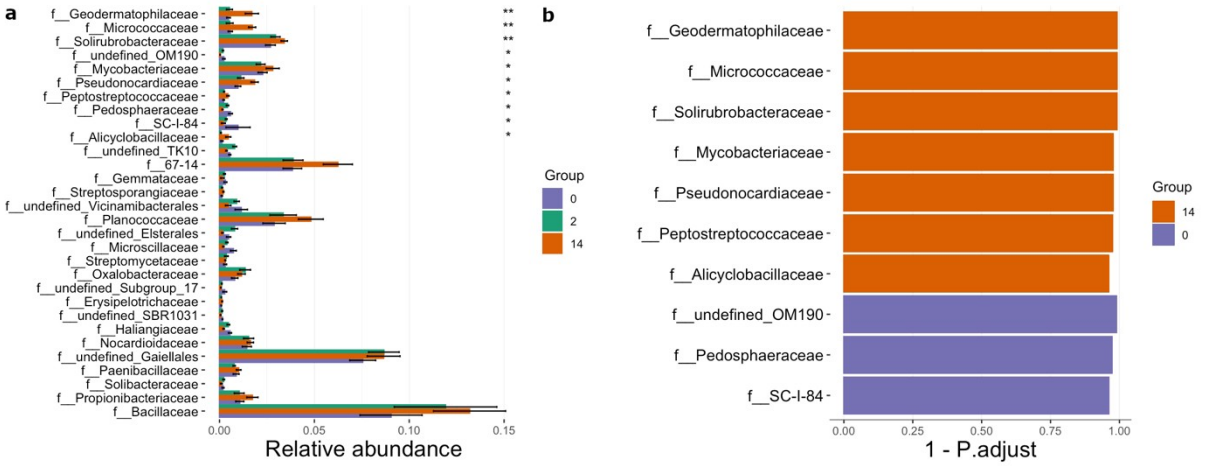

Figure 16. Results of differential abundance analysis in rhizosphere prokaryotes. a) 30 families most strongly affected by drought and their relative abundance in different drought legacy b) bar plot of the indicator families for a given year of drought – based on the results of significant difference in their relative abundance for a given year.

In prokaryotes of root endosphere (Fig. 17) we can see significant families even for 2nd year of drought – *Pseudonocardiaceae, Nocardioidaceae*, *undefined_C0119* (class *Ktedonobacteria*) and *Bacillaceae* (*Bacilli*). In 14th year except for those families from rhizosphere (*Geodermatophilaceae, Micrococcaceae, Solirubrobacteraceae)* we can see *Sphingomonadaceae (Alphaproteobacteria), Oxalobacteraceae (Gammaproteobacteria), Devosiaceae (Aplphaproteobacteria)* and *Microbacteriaceae (Actinobacteria).*

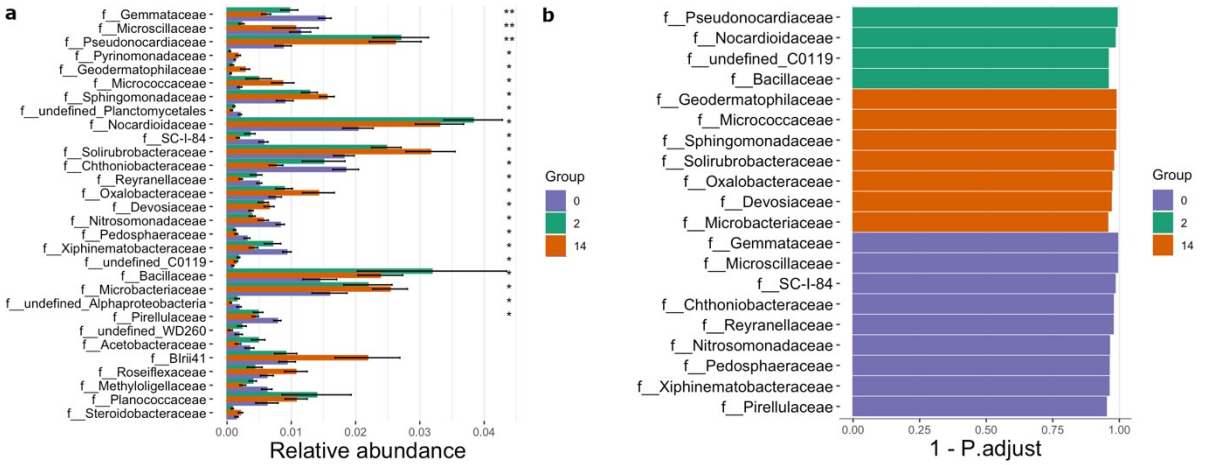

Figure 1. Results of differential abundance analysis in root endosphere prokaryotes. a) first 30 families most strongly affected by drought and their relative abundance in different drought legacy b) bar plot of the indicator families for a given year of drought – based on the results of significant difference in their relative abundance for a given year.

Regarding fungi, in rhizosphere (Fig.18) for the 2nd year of drought there are *Trichosphaeriaceae* (*Trichosphaeriales*), *Serendipitaceae* (*Sebacinales*), *Ceratobasidiaceae* (*Cantharellales*), *Melanommataceae* (*Pleosporales*), *Glomeraceae* (*Glomerales*) and *Apiosporaceae* (*Xylariales*). For the 14th year of drought, we can see *Aspergillaceae* (*Eurotiales*), *Sporormiaceae* (*Pleosporales*), *Nectriaceae* (*Hypocreales*), *Cunninghamellaceae* (*Mucorales*), *Bionectriaceae* (*Hypocreales*), *Phaeosphaeriaceae* (*Pleosporales*) and *Lentitheciaceae* (*Pleosporales*).

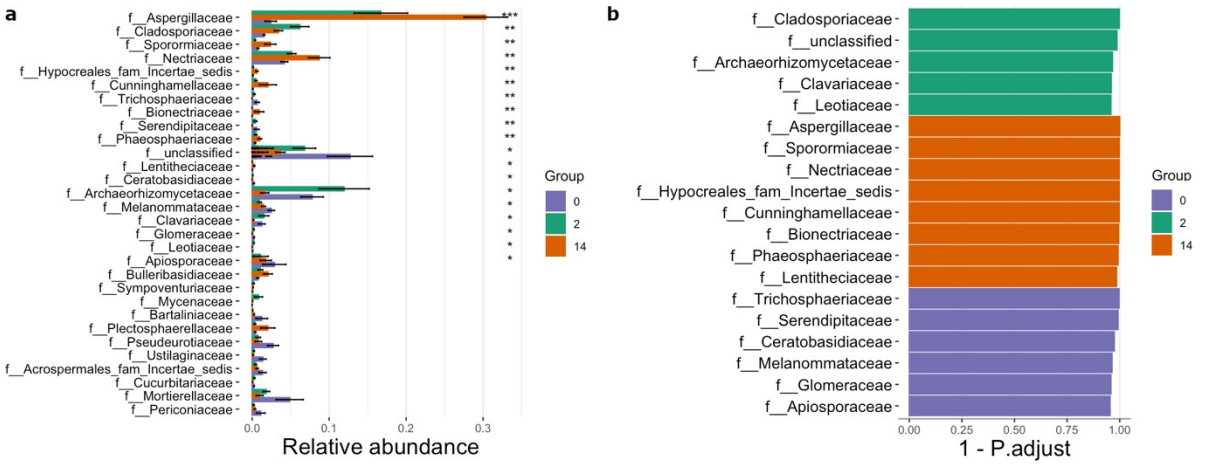

Figure 18. Results of differential abundance analysis in rhizosphere a) first 30 families most strongly affected by drought and their relative abundance in different drought legacy b) bar plot of the indicator families for a given year of drought – based on the results of significant difference in their relative abundance for a given year.

In root endosphere (Fig. 19) fungi there are fewer significant families. For the 14th year of drought, we can see *Aspergillaceae* (*Eurotiales*) and *Cunninghamellaceae* (*Mucorales*) and one family from *Hypocreales* – all of them are even in rhizosphere for 14th year of.

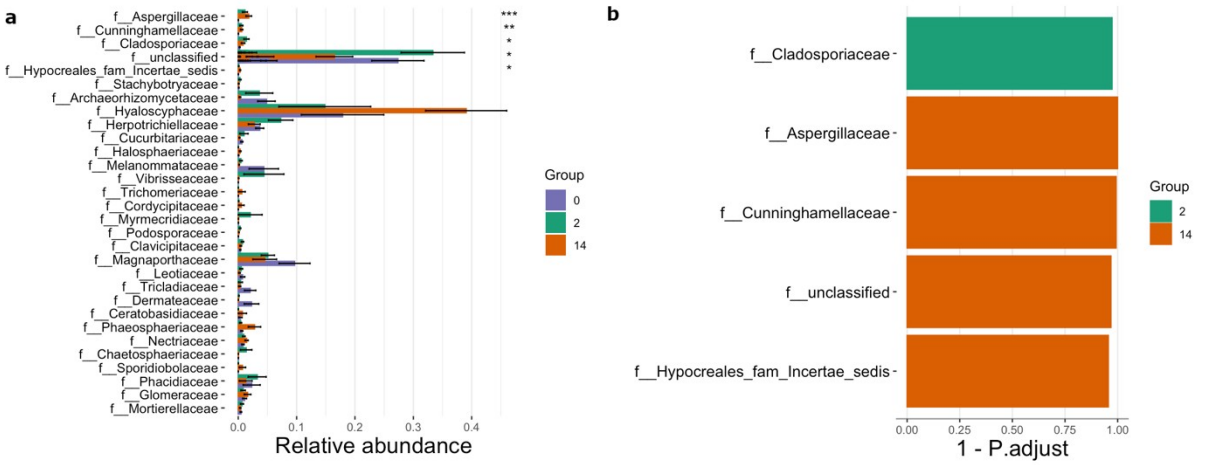

Figure 19. Results of Differential abundance analysis in root endosphere a) first 30 families most strongly

affected by drought and their relative abundance in different drought legacy b) bar plot of the indicator families for a given year of drought – based on the results of significant difference in their relative abundance for a given year.

The cladograms further elaborate results from differential abundance analysis. The cladograms of the prokaryotes (Fig. 20, Fig. 21) clearly show that the taxa most significant for root endosphere years 2 and 14 belong primarily to the class *Actinobacteria*, which is in agreement with previous results of Differential abundance analysis. *Actinobacteria* has been repeatedly found to respond positively to drought treatment compared to other bacterial classes. *Actinobacteria* was also most prominent for the 14th year in the rhizosphere. Then for the rhizosphere 2nd year, I also have representatives of *Alphaproteobacteria* and *Gammaproteobacteria*.

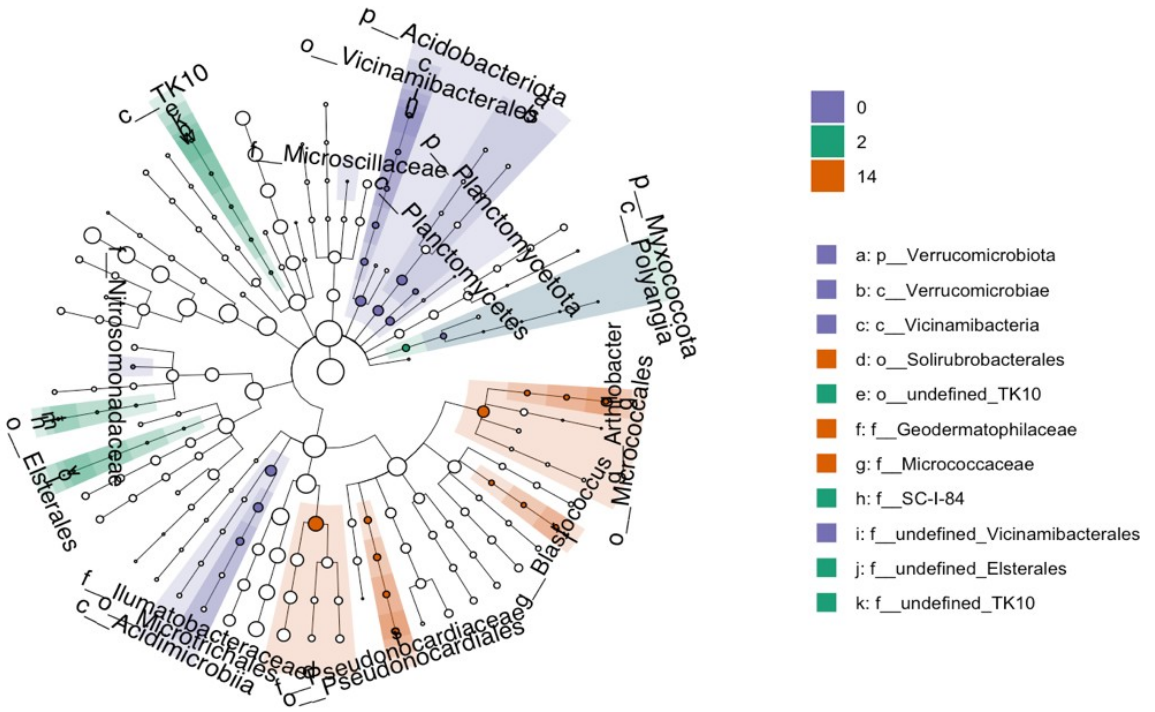

Figure 20. Cladogram of prokaryotes in the rhizosphere and their significance based on the length of the drought.

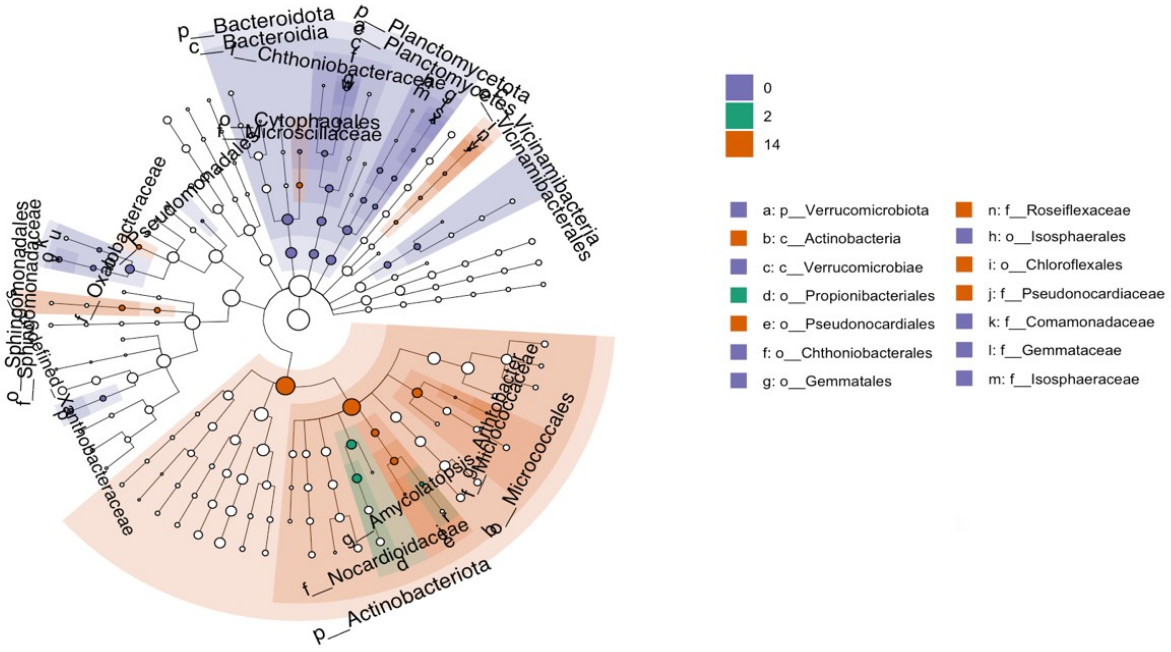

Figure 21. Cladogram of prokaryotes in the root endosphere and their significance based on the length of the drought.

No extensive pattern was observed for prokaryotes in the leaf endosphere due to the low number of identified taxa (Fig. 22).

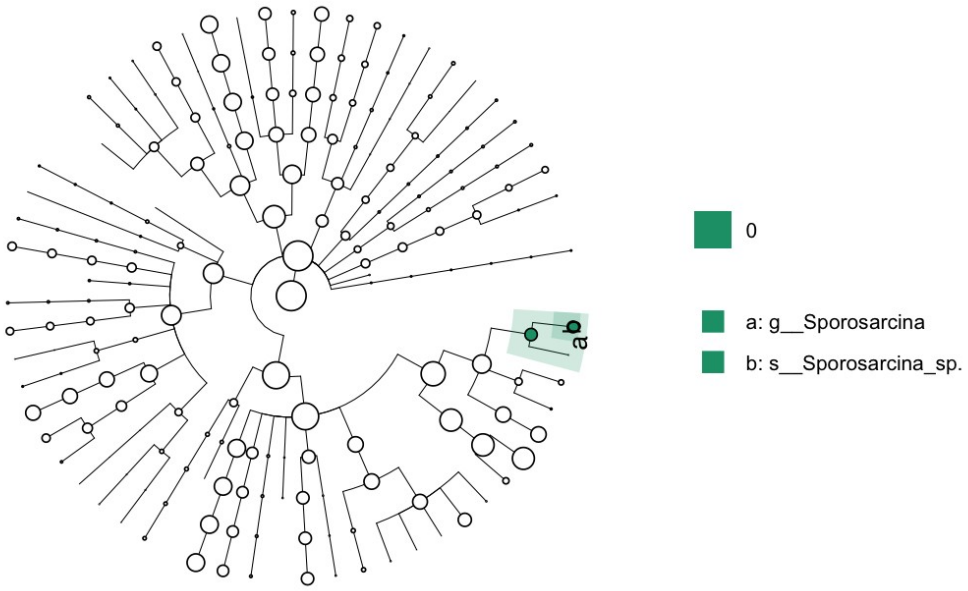

Figure 22. Cladogram of prokaryotes in the leaf endosphere and their significance based on the length of the drought.

In the cladograms (Fig. 23 and Fig. 24) of fungi, we can see that the distribution of the most important taxa based on plant compartments and length of treatment is quite different. The only family that occurred among the plant compartments was the family *Aspergillaceae* (in the 14th year of the drought). For the root endosphere, we can see that *Cunninghamellaceae* is found as an important representative in the 2nd and 14th year.

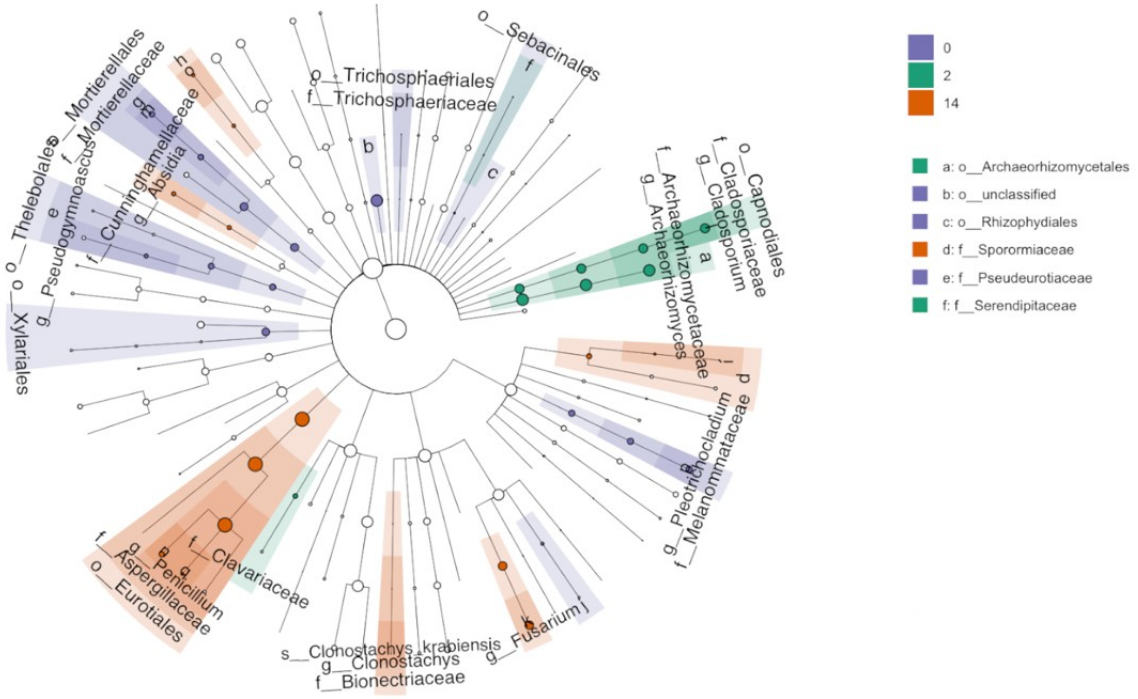

Figure 23. Cladogram of fungi in the rhizosphere and their significance based on the length of the drought.

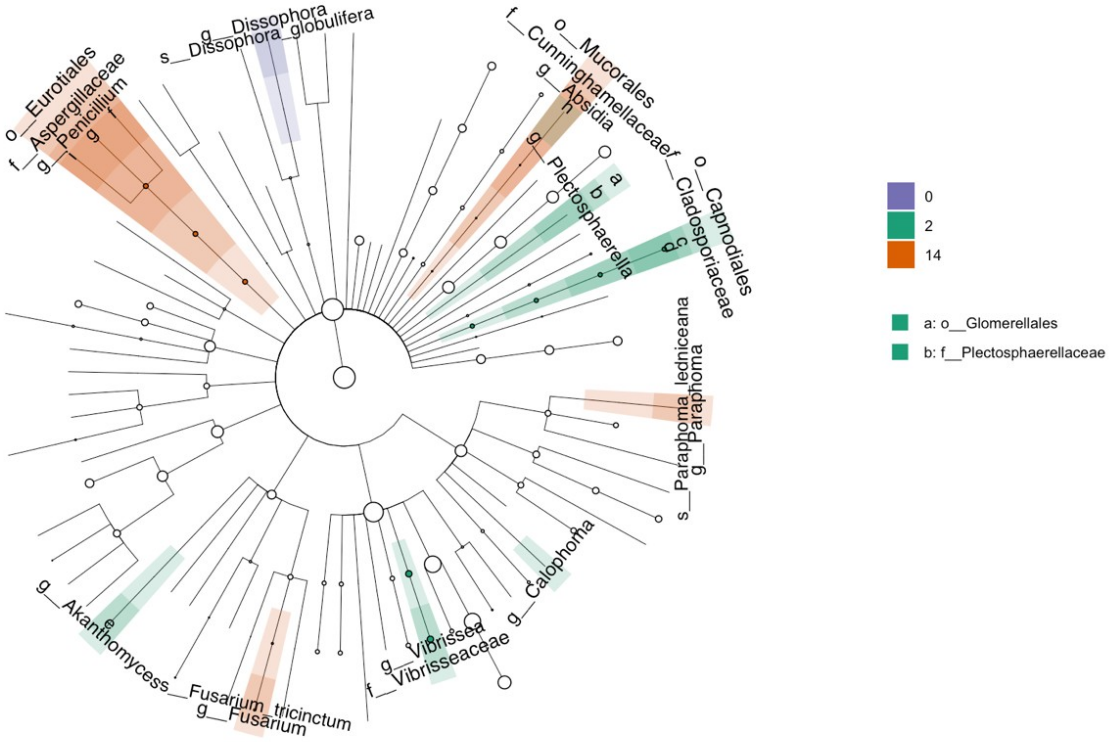

Figure 2. Cladogram of fungi in the root endosphere and their significance based on the length of the drought.

For the leaf endosphere, again we do not see a significant pattern and this is due to the low number of observed taxa (Fig. 25).

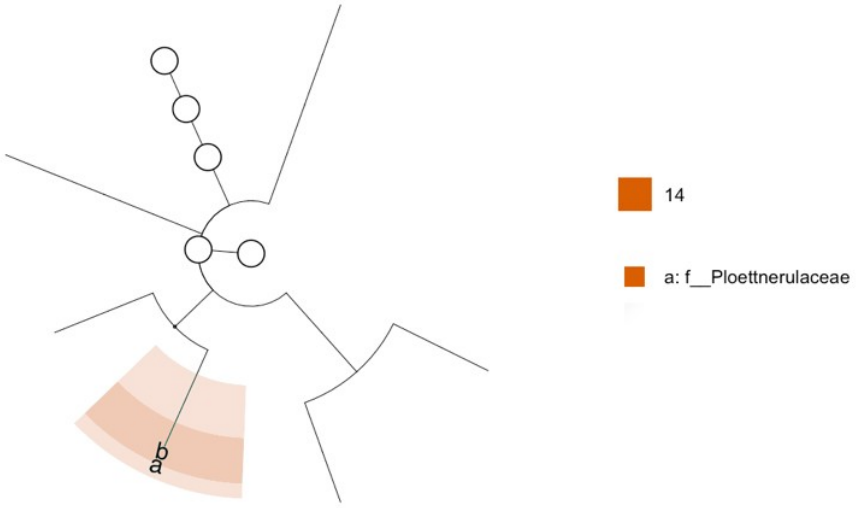

Figure 25. Cladogram of fungi in the leaf endosphere and their significance based on the length of the drought.

5.3 Effect of long-term drought on shared taxa between plant compartments

5.3.1 Venn diagrams

Comparison of taxa composition between plant parts (Fig. 26) then showed two different trends. For the prokaryotes, we can see that the rhizosphere and the endosphere of the root share most of the taxa. In contrast, in fungi, we see a significant partition between the rhizosphere and the root endosphere. On the other hand, fungi tend to share taxa within both root and leaf endospheres, which is not the case for prokaryotes.

Regarding the difference based on drought duration, in prokaryotes, we can see a shift of shared taxa between the rhizosphere and the root endosphere between the treatments. In contrast, for fungi, we see an increase in shared taxa between the leaf and root endosphere.

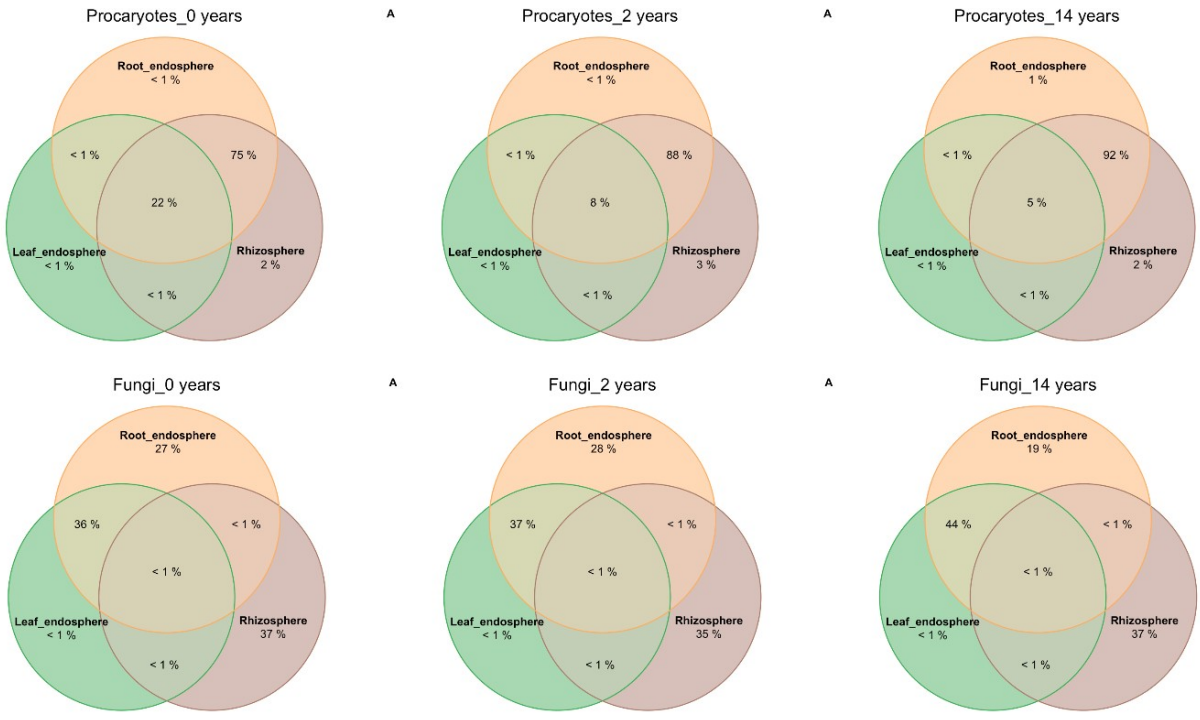

Figure 26. Venn diagrams of shared microbial taxa depending on plant compartment and year treatment.

6 Discussion

6.1 Effect of long-term drought on the diversity of microbial communities

From the results of the redundancy analysis, we see that drought affects the distribution of microbial community composition in most of the plant studied compartments. This, especially for the underground plant compartments, confirms my hypothesis that changes in the composition of microbial communities as a function of drought length are gradual and evolve with the length of drought.

The exception is then the leaf endosphere, where a clear divide is seen between the control and both drought treatments. This suggests that the change in composition took place relatively quickly and has remained so. An explanation for this could be that the above-ground parts of the plant are much more exposed to environmental influences and thus much more susceptible to change. For example, it could be that the overall drought-weakened plant was unable to provide suitable conditions for these microorganisms and they responded rapidly by changing the composition of their communities. A further contributing factor to this effect may be that the leaf endosphere is one of the least diverse of all plant parts and, according to some evidence, the microorganisms present here undergo the greatest selection by the plant (Bulgarelli et al., 2013; Muller et al., 2016). Indeed, the number of taxa in leaf endospheres was poor. This is therefore another thing that could have influenced such a result.

Even in the case of fungi, we can see in the underground parts that the length of the drought explains the distribution of the microbial communities. The exception is again the leaf endosphere, where even there was no significant distribution. The order *Neotyphodium*, an endophytic symbiont of the family *Clavicipitaceae* (Faeth & Sullivan, 2003), was the most abundant order represented in the leaf endosphere. The effect of *Neotyphodium* on plants has been studied since the end of the last century and although it is mainly referred to as a plant-beneficial symbiont, it can also behave as a parasite (Faeth & Sullivan, 2003). *Neotyphodium* has been shown in several studies to have a positive effect on plants in drought (Malinowski & Belesky, 2006). These plants had a greater aboveground biomass and relative growth rate (Morse et al., 2002). On the other hand, the resulting effect of endophytes on plants also depends greatly on the genotype of the plant and other environmental conditions. According to some studies, based on these factors, in addition to the positive effect already mentioned,

endophytes may have no effect or even a negative effect on plants (Cheplick, 2004; Faeth & Sullivan, 2003; Morse et al., 2002), Malinowski & Belesky, 2006). The reason for such a high representation of *Neotyphodium* in my samples is still unclear. It may be the effect of several factors together. Because of the strong attraction between this endophyte and the plant, I believe that it may have become dominant in the plant with an effect on other microorganisms. This may have been further accentuated by a weaker isolation effect.

The fact that there are other changes in the composition of microbial communities depending on the duration of drought is confirmed by the Shannon diversity index. At the same time, its results also follow up on previous studies that report different compositions of microbial communities depending on plant compartments and whether they are prokaryotes or fungi (Bulgarelli et al., 2013; Carbone et al., 2021; Edwards et al., 2015; Hereira-Pacheco et al., 2023; Knief et al., 2012; Maignien et al., 2014; Tkacz et al., 2020). For prokaryotes, this change is evident between the rhizosphere and the root endosphere, with only the 14th year of drought having a significant effect on their diversity, whereas the root endosphere showed a change in diversity after the 2nd year. This may be indicative of several things. First, the composition of prokaryotic communities in the rhizosphere is relatively stable in the short term and only declines significantly in diversity with long-term drought, which goes against the preliminary assumption based on earlier studies that bacterial communities should respond to drought relatively quickly (Castro et al., 2010; de Vries et al., 2018). The decline in diversity in the root endosphere could then be explained by the influence of the plant itself, which may have selected beneficial prokaryotes (Basilio et al., 2003; Bulgarelli et al., 2013; Muller et al., 2016) from early in the drought.

In contrast, we do not see overall changes in fungi diversity. This too agrees with previous discoveries that fungal communities appear to be more stable under drought stress (Castro et al., 2010; de Vries et al., 2018). Surprisingly, even the 14th year of drought does not affect their diversity. Why fungal communities are so stable has not yet been determined with certainty. One reason may be already mentioned highly resistant spores which can support fungi communities across time thanks to their accumulation (Crawford & Hawkes, 2020; Preece et al., 2019).

The thought that the stability of fungal communities may be mainly due to general physiological resistance rather than to different specializations may be suggested by the result of the Specialisation index. In the results of this analysis, we see a decrease in specialists at year 14 relative to the control. According to my hypothesis (as far as the general response of

the microbiota is concerned), we should rather see an increase in specialists as a selection of the plant on organisms beneficial to it.

However, in agreement with my hypothesis, we see this trend within the prokaryotes root endosphere – an increase in specialists in year 14 relative to the control. This could confirm both hypotheses. Firstly, that the influence of the plant is stronger in the endosphere, and secondly, that the plant selects for self-beneficial specialists.

6.2 Changes in the taxonomic composition of microbial communities

As far as specific prokaryote taxa are concerned, here too my results are in agreement with previous studies (Naylor et al., 2017; Santos-Medellin et al., 2017; Simmons et al., 2020; Xu et al., 2018), Among the most abundant prokaryote classes are the *Actinobacteria*, *Bacilli*, *Alphaproteobacteria*, and *Gammaproteobacteria*. On top of that in my data I observed *Thermoleophilia*, which was abundant in both underground compartments. Regarding the effect of long-term drought, we see that in both underground compartments, there is a relative increase in the abundance of *Actinobacteria* based on the duration of the drought. For the rhizosphere, then, there are also increases in *Bacilli*.

Actinobacteria appears to be the only class of prokaryotes that has a stable response in increase to drought across the literature. This is in contrast to other classes where responses to drought vary quite a bit depending on the studies (Naylor et al., 2017; Simmons et al., 2020; Xu et al., 2018).

In the rhizosphere, the *Bacilliaceae* is particularly dominant and the relative abundance of *Bacilliaceae* even increases with drought duration. Members of the genus *Bacillus* are well known for their positive effect on plants. From the production of phytohormones, to their role in nutrient uptake or antibiotics and other substances that serve as protection against pathogens (Saxena et al., 2020; Shafi et al., 2017). Some species even play a positive role in the fight against drought (Gagné-Bourque et al., 2016; Vardharajula et al., 2011). For the root endosphere we see represented, among others, e.g. *Micromonosporaceae* or *Streptomycetaceae*, which have a positive impact on plant thanks to their antimicrobial substances (Basilio et al., 2003).

For the leaf endosphere, we see a surprising decline in *Actinobacteria*, and conversely an increase in *Bacilli* and *Gammaproteobacteria*. It is possible that presented members of *Bacilli* and *Gammaproteobacteria* were much more suitable for extreme condition in the

leaves. In addition in phyllosphere we can see mostly competition rather than cooperation (Carlstrom et al., 2019). It is also possible that more competition capable taxa easily won over the less successful one.

In fungi the rhizosphere is predominant by saprotrophic (Polme et al., 2020) *Archaeorhizomyces*, *Piskurozymaceae*, and *Aspergillaceae,* increasing over the years. While in the root endosphere, we see a significant representation, especially in saprotrophic *Mycenaceae* and *Hyaloscyphaceae* and endophytic *Magnaporthaceae (Polme et al., 2020)*, which show an increase – especially in the 14th year. An increase in saprophytic fungi during drought, especially in the root, has been already observed (Hereira-Pacheco et al., 2023). Saprotrophs allow the plant and other microorganisms to access otherwise unavailable nutrients (Chen et al., 2020). Theoretically saprotrophs and their functioning can be supported by plant itself or maybe even by other microorganisms via various feedbacks.

6.3 Similarities in microbial community composition between compartments

Regarding taxon sharing and possible migration, the results suggest that a trend can be observed in prokaryotes whereby as drought progresses, taxa are shared more in the underground parts of the plant rather than between all compartments. As already mentioned this result may be partly due to the overly strong influence of harsh and different conditions of the above-ground parts or strong competition between microbiota. Another factor may be that the diversity recorded in the leaf endosphere is too low, because of the strong presence of plant-selected or highly competitive taxa.

For fungi, on the other hand, we observe a different effect, namely that there is an increase in shared taxa between the leaf and root endospheres. In contrast, the rhizosphere appears to be completely decoupled in this respect (as suggested by the results from the taxonomic analysis). Thus, it is possible that there is some form of migration within the plant endosphere. This sharing is likely to occur within the order *Hypocreales* and *Helotiales*, which were present the most in leaf endosphere. *Neothyphodium* itself is from *Hypocreales* and *Lachnum*, which was significant for leaf endosphere and for 14th year of root endosphere is from *Helotiales.* Order *Helotiales* is one of the larges ascomycetous group with broad range of ecological roles (Wang et al., 2006). Is it therefor uncertain what role *Helotiales* might play in case of the long-term drought stress. In any case, both of these taxa experienced at least a slight increase in relative abundance during drought in both endospheres studied.

7 Conclusion

In recent years, attention has increasingly turned to microbiota and their impact on other organisms. In addition to the relationship between humans and their microbiome, for example, the relationship between plants and their microbiota is also being explored. It is evident from these studies that this relationship between the plant and its associated microbiota plays an important and often irreplaceable role, such as in the case of drought stress.

The results of our experiment built on those of previous short-term studies answer the question of how long-term drought affects plant associated microbiota. In general, different drought lengths had an effect on the composition of both prokaryotes and fungi communities. These changes are then seen predominantly in the rhizosphere and root endosphere. In these two parts, the harsher environmental conditions do not have such a strong influence and the microorganisms have a rather positive relationship with each other (as opposed to increased competition in leaves).

The hypothesis that prokaryotes and fungi would differ in their response was also confirmed. For prokaryotes, we see a decline in the diversity of the root endosphere communities after only two years of drought, when selection of specific taxa by the plant may have occurred. In contrast, we see almost no change in fungi in response to drought at all. This may be due to the physiological characteristics of the groups rather than the relationship with the plant itself. This is in line with the result of the specialization index, where we see an increase in specialists only in prokaryotes and rather a decrease in fungi.

Our experiment also confirmed the previously observed trend of a relative increase in *Actinobacteria* under drought. I also recorded classes such as *Bacilli, Alphaproteobacteria, Gammaproteobacteria* or *Thermoleophilia*. For fungi, I then noted a surprising increase in saprophytic taxa predominantly in the root endophyte.

Last but not least, my work has also provided insight into the sharing of microbial taxa between plant compartments. Prokaryotes share most of their taxa in the rhizosphere and root endosphere whereas fungi share their taxa mostly between the root and leaf endosphere. Both of these trends were deepened by the length of the drought.

This work has provided answers to the question of how a prolonged drought will affect plant-associated microbial communities. However, what specific effects these changes in the microbiota have on plants and other organisms remains unanswered and it is worth addressing these questions in the future.

8 Sources

Abarenkov, K., Nilsson, R. H., Larsson, K. H., Taylor, A. F. S., May, T. W., Froslev, T. G., . . . Koljalg, U. (2023). The UNITE database for molecular identification and taxonomic communication of fungi and other eukaryotes: sequences, taxa and classifications reconsidered. *Nucleic Acids Research*. https://doi.org/10.1093/nar/gkad1039

Albrecht, S. L., Bennett, J. M., & Boote, K. J. (1984). RELATIONSHIP OF NITROGENASE ACTIVITY TO PLANT WATER-STRESS IN FIELD-GROWN SOYBEANS. *Field Crops Research*, *8*(1-2), 61-71. https://doi.org/10.1016/0378-4290(84)90052-2

Alvarez, M., Huygens, D., Olivares, E., Saavedra, I., Alberdi, M., & Valenzuela, E. (2009). Ectomycorrhizal fungi enhance nitrogen and phosphorus nutrition of Nothofagus dombeyi under drought conditions by regulating assimilative enzyme activities. *Physiologia Plantarum*, *136*(4), 426-436. https://doi.org/10.1111/j.1399-3054.2009.01237.x

Aronesty E. (2011). ea-utils : "Command-line tools for processing biological sequencing data"; https://github.com/ExpressionAnalysis/ea-utils

Bahn, M., Knapp, M., Garajova, Z., Pfahringer, N., & Cernusca, A. (2006). Root respiration in temperate mountain grasslands differing in land use. *Global Change Biology*, *12*(6), 995-1006. https://doi.org/10.1111/j.1365-2486.2006.01144.x

Bai, Y., Muller, D. B., Srinivas, G., Garrido-Oter, R., Potthoff, E., Rott, M., . . . Schulze-Lefert, P. (2015). Functional overlap of the Arabidopsis leaf and root microbiota. *Nature*, *528*(7582), 364-+. https://doi.org/10.1038/nature16192

Bashir, I., War, A. F., Rafiq, I., Reshi, Z. A., Rashid, I., & Shouche, Y. S. (2022). Phyllosphere microbiome: Diversity and functions. *Microbiological Research*, *254*, Article 126888. https://doi.org/10.1016/j.micres.2021.126888

Basilio, A., Gonzalez, I., Vicente, M. F., Gorrochategui, J., Cabello, A., Gonzalez, A., & Genilloud, O. (2003). Patterns of antimicrobial activities from soil actinomycetes isolated under different conditions of pH and salinity. *Journal of Applied Microbiology*, *95*(4), 814-823. https://doi.org/10.1046/j.1365-2672.2003.02049.x

Bengtsson-Palme, J., Ryberg, M., Hartmann, M., Branco, S., Wang, Z., Godhe, A., . . . Nilsson, R. H. (2013). Improved software detection and extraction of ITS1 and ITS2 from ribosomal ITS sequences of fungi and other eukaryotes for analysis of

environmental sequencing data. *Methods in Ecology and Evolution*, *4*(10), 914-919. https://doi.org/10.1111/2041-210x.12073

Bulgarelli, D., Schlaeppi, K., Spaepen, S., van Themaat, E. V. L., & Schulze-Lefert, P. (2013). Structure and Functions of the Bacterial Microbiota of Plants. *Annual Review of Plant Biology, Vol 64*, *64*, 807-838. https://doi.org/10.1146/annurev-arplant-050312-120106

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Huntley, J., Fierer, N., . . . Knight, R. (2012). Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *Isme Journal*, *6*(8), 1621-1624. https://doi.org/10.1038/ismej.2012.8

Carbone, M. J., Alaniz, S., Mondino, P., Gelabert, M., Eichmeier, A., Tekielska, D., . . . Gramaje, D. (2021). Drought Influences Fungal Community Dynamics in the Grapevine Rhizosphere and Root Microbiome. *Journal of Fungi*, *7*(9), Article 686. https://doi.org/10.3390/jof7090686

Carlstrom, C. I., Field, C. M., Bortfeld-Miller, M., Müller, B., Sunagawa, S., & Vorholt, J. A. (2019). Synthetic microbiota reveal priority effects and keystone strains in the Arabidopsis phyllosphere. *Nature Ecology & Evolution*, *3*(10), 1445-1454. https://doi.org/10.1038/s41559-019-0994-z

Castro, H. F., Classen, A. T., Austin, E. E., Norby, R. J., & Schadt, C. W. (2010). Soil Microbial Community Responses to Multiple Experimental Climate Change Drivers. *Applied and Environmental Microbiology*, *76*(4), 999-1007. https://doi.org/10.1128/aem.02874-09

Chen, W. Q., Wang, J. Y., Meng, Z. X., Xu, R., Chen, J., Zhang, Y. J., & Hu, T. M. (2020). Fertility-related interplay between fungal guilds underlies plant richness-productivity relationships in natural grasslands. *New Phytologist*, *226*(4), 1129-1143. https://doi.org/10.1111/nph.16390

Chen, Y. J., Leung, P. M., Wood, J. L., Bay, S. K., Hugenholtz, P., Kessler, A. J., . . . Greening, C. (2021). Metabolic flexibility allows bacterial habitat generalists to become dominant in a frequently disturbed ecosystem. *Isme Journal*, *15*(10), 2986-3004. https://doi.org/10.1038/s41396-021-00988-w

Cheplick, G. P. (2004). Recovery from drought stress in Lolium perenne (Poaceae): Are fungal endophytes detrimental. *American Journal of Botany*, *91*(12), 1960-1968. https://doi.org/10.3732/ajb.91.12.1960

Chi, F., Shen, S. H., Cheng, H. P., Jing, Y. X., & Dazzo, F. B. (2005). Ascending migration of endophytic rhizobia from roots to leaves inside rice plants. *Biological Nitrogen Fixation, Sustainable Agriculture and the Environment*, *41*, 381-382.

Crawford, K. M., & Hawkes, C. V. (2020). Soil precipitation legacies influence intraspecific plant-soil feedback. *Ecology*, *101*(10). https://doi.org/10.1002/ecy.3142

de Vries, F. T., Griffiths, R. I., Bailey, M., Craig, H., Girlanda, M., Gweon, H. S., . . . Bardgett, R. D. (2018). Soil bacterial networks are less stable under drought than fungal networks. *Nature Communications*, *9*, Article 3033. https://doi.org/10.1038/s41467-018-05516-7

Delmotte, N., Knief, C., Chaffron, S., Innerebner, G., Roschitzki, B., Schlapbach, R., . . . Vorholt, J. A. (2009). Community proteogenomics reveals insights into the physiology of phyllosphere bacteria. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(38), 16428-16433. https://doi.org/10.1073/pnas.0905240106

Devictor, V., Julliard, R., & Jiguet, F. (2008). Distribution of specialist and generalist species along spatial gradients of habitat disturbance and fragmentation. *Oikos*, *117*(4), 507-514. https://doi.org/10.1111/j.2008.0030-1299.16215.x

Edwards, J., Johnson, C., Santos-Medellin, C., Lurie, E., Podishetty, N. K., Bhatnagar, S., . . . Sundaresan, V. (2015). Structure, variation, and assembly of the root-associated microbiomes of rice. *Proceedings of the National Academy of Sciences of the United States of America*, *112*(8), E911-E920. https://doi.org/10.1073/pnas.1414592112

Ehrenfeld, J. G., Ravit, B., & Elgersma, K. (2005). Feedback in the plant-soil system. *Annual Review of Environment and Resources*, *30*, 75-115. https://doi.org/10.1146/annurev.energy.30.050504.144212

Faeth, S. H., & Sullivan, T. J. (2003). Mutualistic asexual endophytes in a native grass are usually parasitic. *American Naturalist*, *161*(2), 310-325. https://doi.org/10.1086/345937

Fernandes, A. D., Macklaim, J. M., Linn, T. G., Reid, G., & Gloor, G. B. (2013). ANOVA-Like Differential Expression (ALDEx) Analysis for Mixed Population RNA-Seq. *Plos One*, *8*(7), Article e67019. https://doi.org/10.1371/journal.pone.0067019

Firakova, S., Sturdikova, M., & Muckova, M. (2007). Bioactive secondary metabolites produced by microorganisms associated with plants. *Biologia*, *62*(3), 251-257. https://doi.org/10.2478/s11756-007-0044-1

Firrincieli, A., Khorasani, M., Frank, A. C., & Doty, S. L. (2020). Influences of Climate on Phyllosphere Endophytic Bacterial Communities of Wild Poplar. *Frontiers in Plant Science*, *11*, Article 203. https://doi.org/10.3389/fpls.2020.00203

Fitzpatrick, C. R., Copeland, J., Wang, P. W., Guttman, D. S., Kotanen, P. M., & Johnson, M. T. J. (2018). Assembly and ecological function of the root microbiome across angiosperm plant species. *Proceedings of the National Academy of Sciences of the United States of America*, *115*(6), E1157-E1165. https://doi.org/10.1073/pnas.1717617115

Fuchslueger, L., Bahn, M., Hasibeder, R., Kienzl, S., Fritz, K., Schmitt, M., . . . Richter, A. (2016). Drought history affects grassland plant and microbial carbon turnover during and after a subsequent drought event. *Journal of Ecology*, *104*(5), 1453-1465. https://doi.org/10.1111/1365-2745.12593

Gagné-Bourque, F., Bertrand, A., Claessens, A., Aliferis, K. A., & Jabaji, S. (2016). Alleviation of Drought Stress and Metabolic Changes in Timothy (Phleum pratense L.) Colonized with Bacillus subtilis B26. *Frontiers in Plant Science*, *7*, Article 584. https://doi.org/10.3389/fpls.2016.00584

Gong, T. Y., & Xin, X. F. (2021). Phyllosphere microbiota: Community dynamics and its interaction with plant hosts. *Journal of Integrative Plant Biology*, *63*(2), 297-304. https://doi.org/10.1111/jipb.13060

Grady, K. L., Sorensen, J. W., Stopnisek, N., Guittar, J., & Shade, A. (2019). Assembly and seasonality of core phyllosphere microbiota on perennial biofuel crops. *Nature Communications*, *10*, Article 4135. https://doi.org/10.1038/s41467-019-11974-4

Guerin, V., Pladys, D., Trinchant, J. C., & Rigaud, J. (1991). PROTEOLYSIS AND NITROGEN-FIXATION IN FABA-BEAN (VICIA-FABA) NODULES UNDER WATER-STRESS. *Physiologia Plantarum*, *82*(3), 360-366.

Hereira-Pacheco, S. E., Estrada-Torres, A., Dendooven, L., & Navarro-Noya, Y. E. (2023). Shifts in root-associated fungal communities under drought conditions in Ricinus communis. *Fungal Ecology*, *63*, Article 101225. https://doi.org/10.1016/j.funeco.2023.101225

Ihrmark, K., Bodeker, I. T. M., Cruz-Martinez, K., Friberg, H., Kubartova, A., Schenck, J., . . . Lindahl, B. D. (2012). New primers to amplify the fungal ITS2 region – evaluation by 454-sequencing of artificial and natural communities. *Fems Microbiology Ecology*, *82*(3), 666-677. https://doi.org/10.1111/j.1574-6941.2012.01437.x

Katoh, K., Rozewicki, J., & Yamada, K. D. (2019). MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. *Briefings in Bioinformatics*, *20*(4), 1160-1166. https://doi.org/10.1093/bib/bbx108

Kembel, S. W., O'Connor, T. K., Arnold, H. K., Hubbell, S. P., Wright, S. J., & Green, J. L. (2014). Relationships between phyllosphere bacterial communities and plant functional traits in a neotropical forest. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(38), 13715-13720. https://doi.org/10.1073/pnas.1216057111

Klironomos, J. N. (2002). Feedback with soil biota contributes to plant rarity and invasiveness in communities. *Nature*, *417*(6884), 67-70. https://doi.org/10.1038/417067a

Knief, C., Delmotte, N., Chaffron, S., Stark, M., Innerebner, G., Wassmann, R., . . . Vorholt, J. A. (2012). Metaproteogenomic analysis of microbial communities in the phyllosphere and rhizosphere of rice. *Isme Journal*, *6*(7), 1378-1390. https://doi.org/10.1038/ismej.2011.192

Lau, J. A., & Lennon, J. T. (2012). Rapid responses of soil microorganisms improve plant fitness in novel environments. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(35), 14058-14062. https://doi.org/10.1073/pnas.1202319109

Liu, C., Cui, Y. M., Li, X. Z., & Yao, M. J. (2021). microeco: an R package for data mining in microbial community ecology. *Fems Microbiology Ecology*, *97*(2), Article fiaa255. https://doi.org/10.1093/femsec/fiaa255

Lundberg, D. S., Lebeis, S. L., Paredes, S. H., Yourstone, S., Gehring, J., Malfatti, S., . . . Dangl, J. L. (2012). Defining the core Arabidopsis thaliana root microbiome. *Nature*, *488*(7409), 86-+. https://doi.org/10.1038/nature11237

Maignien, L., DeForce, E. A., Chafee, M. E., Eren, A. M., & Simmons, S. L. (2014). Ecological Succession and Stochastic Variation in the Assembly of Arabidopsis thaliana Phyllosphere Communities. *Mbio*, *5*(1), Article e00682-13. https://doi.org/10.1128/mBio.00682-13

Malinowski, D. P., & Belesky, D. P. (2006). Ecological importance of Neotyphodium spp. grass endophytes in agroecosystems. Grassland Science, 52(1), 1-14.

Massoni, J., Bortfeld-Miller, M., Widmer, A., & Vorholt, J. A. (2021). Capacity of soil bacteria to reach the phyllosphere and convergence of floral communities despite soil microbiota variation. *Proceedings of the National Academy of Sciences of the United*

States of America, *118*(41), Article e2100150118. https://doi.org/10.1073/pnas.2100150118|1of11

McMurdie, P. J., & Holmes, S. (2013). phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *Plos One*, *8*(4), Article e61217. https://doi.org/10.1371/journal.pone.0061217

Morse, L. J., Day, T. A., & Faeth, S. H. (2002). Effect of Neotyphodium endophyte infection on growth and leaf gas exchange of Arizona fescue under contrasting water availability regimes. *Environmental and Experimental Botany*, *48*(3), 257-268, Article Pii s0098-8472(02)00042-4. https://doi.org/10.1016/s0098-8472(02)00042-4

Muller, D. B., Vogel, C., Bai, Y., & Vorholt, J. A. (2016). The Plant Microbiota: Systems-Level Insights and Perspectives. *Annual Review of Genetics, Vol 50*, *50*, 211-234. https://doi.org/10.1146/annurev-genet-120215-034952

Naveed, M., Mitter, B., Reichenauer, T. G., Wieczorek, K., & Sessitsch, A. (2014). Increased drought stress resilience of maize through endophytic colonization by Burkholderia phytofirmans PsJN and Enterobacter sp FD17. *Environmental and Experimental Botany*, *97*, 30-39. https://doi.org/10.1016/j.envexpbot.2013.09.014

Naylor, D., DeGraaf, S., Purdom, E., & Coleman-Derr, D. (2017). Drought and host selection influence bacterial community dynamics in the grass root microbiome. *Isme Journal*, *11*(12), 2691-2704. https://doi.org/10.1038/ismej.2017.118

Nilsson, R. H., Anslan, S., Bahram, M., Wurzbacher, C., Baldrian, P., & Tedersoo, L. (2019). Mycobiome diversity: high-throughput sequencing and identification of fungi. *Nature Reviews Microbiology*, *17*(2), 95-109. https://doi.org/10.1038/s41579-018-0116-y

O'Brien, M. J., Pugnaire, F. I., Rodriguez-Echeverria, S., Morillo, J. A., Martin-Usero, F., Lopez-Escoriza, A., . . . Armas, C. (2018). Mimicking a rainfall gradient to test the role of soil microbiota for mediating plant responses to drier conditions. *Oikos*, *127*(12), 1776-1786. https://doi.org/10.1111/oik.05443

Ofek-Lalzar, M., Sela, N., Goldman-Voronov, M., Green, S. J., Hadar, Y., & Minz, D. (2014). Niche and host-associated functional signatures of the root surface microbiome. *Nature Communications*, *5*, Article 4950. https://doi.org/10.1038/ncomms5950

Pinhero, R. G., Rao, M. V., Paliyath, G., Murr, D. P., & Fletcher, R. A. (1997). Changes in activities of antioxidant enzymes and their relationship to genetic and paclobutrazol-induced chilling tolerance of maize seedlings. *Plant Physiology*, *114*(2), 695-704. https://doi.org/10.1104/pp.114.2.695

Polme, S., Abarenkov, K., Nilsson, R. H., Lindahl, B. D., Clemmensen, K. E., Kauserud, H., . . . Tedersoo, L. (2020). FungalTraits: a user-friendly traits database of fungi and fungus-like stramenopiles. *Fungal Diversity*, *105*(1), 1-16. https://doi.org/10.1007/s13225-020-00466-2

Preece, C., Verbruggen, E., Liu, L., Weedon, J. T., & Penuelas, J. (2019). Effects of past and current drought on the composition and diversity of soil microbial communities. *Soil Biology & Biochemistry*, *131*, 28-39. https://doi.org/10.1016/j.soilbio.2018.12.022

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., . . . Glöckner, F. O. (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Research*, *41*(D1), D590-D596. https://doi.org/10.1093/nar/gks1219

Read, D. J., & Perez-Moreno, J. (2003). Mycorrhizas and nutrient cycling in ecosystems – a journey towards relevance? *New Phytologist*, *157*(3), 475-492. https://doi.org/10.1046/j.1469-8137.2003.00704.x

Rognes, T., Flouri, T., Nichols, B., Quince, C., & Mahé, F. (2016). VSEARCH: a versatile open source tool for metagenomics. *Peerj*, *4*, Article e2584. https://doi.org/10.7717/peerj.2584

Ruizlozano, J. M., Azcon, R., & Gomez, M. (1995). EFFECTS OF ARBUSCULAR-MYCORRHIZAL GLOMUS SPECIES ON DROUGHT TOLERANCE – PHYSIOLOGICAL AND NUTRITIONAL PLANT-RESPONSES. *Applied and Environmental Microbiology*, *61*(2), 456-460. https://doi.org/10.1128/aem.61.2.456-460.1995

Santos-Medellin, C., Edwards, J., Liechty, Z., Nguyen, B., & Sundaresan, V. (2017). Drought Stress Results in a Compartment-Specific Restructuring of the Rice Root-Associated Microbiomes. *Mbio*, *8*(4), Article e00764-17. https://doi.org/10.1128/mBio.00764-17

Saxena, A. K., Kumar, M., Chakdar, H., Anuroopa, N., & Bagyaraj, D. J. (2020). Bacillus species in soil as a natural resource for plant health and nutrition. *Journal of Applied Microbiology*, *128*(6), 1583-1594. https://doi.org/10.1111/jam.14506

Scholer, A., Jacquiod, S., Vestergaard, G., Schulz, S., & Schloter, M. (2017). Analysis of soil microbial communities based on amplicon sequencing of marker genes. *Biology and Fertility of Soils*, *53*(5), 485-489. https://doi.org/10.1007/s00374-017-1205-1

Shafi, J., Tian, H., & Ji, M. S. (2017). Bacillus species as versatile weapons for plant pathogens: a review. *Biotechnology & Biotechnological Equipment*, *31*(3), 446-459. https://doi.org/10.1080/13102818.2017.1286950

Simmons, T., Styer, A. B., Pierroz, G., Gonçalves, A. P., Pasricha, R., Hazra, A. B., . . . Coleman-Derr, D. (2020). Drought Drives Spatial Variation in the Millet Root Microbiome. *Frontiers in Plant Science*, *11*, Article 599. https://doi.org/10.3389/fpls.2020.00599

Tkacz, A., Bestion, E., Bo, Z. Y., Hortala, M., & Poole, P. S. (2020). Influence of Plant Fraction, Soil, and Plant Species on Microbiota: a Multikingdom Comparison. *Mbio*, *11*(1), Article e02785-19. https://doi.org/10.1128/mBio.02785-19

Vardharajula, S., Ali, S. Z., Grover, M., Reddy, G., & Bandi, V. (2011). Drought-tolerant plant growth promoting Bacillus spp.: effect on growth, osmolytes, and antioxidant status of maize under drought stress. *Journal of Plant Interactions*, *6*(1), 1-14. https://doi.org/10.1080/17429145.2010.535178

Vetrovsky, T., Baldrian, P., & Morais, D. (2018). SEED 2: a user-friendly platform for amplicon high-throughput sequencing data analyses. *Bioinformatics*, *34*(13), 2292-2294. https://doi.org/10.1093/bioinformatics/bty071

Wang, Z., Binder, M., Schoch, C. L., Johnston, P. R., Spatafora, J. W., & Hibbett, D. S. (2006). Evolution of helotialean fungi (Leotiomycetes, Pezizomycotina): A nuclear rDNA phylogeny. *Molecular Phylogenetics and Evolution*, *41*(2), 295-312. https://doi.org/10.1016/j.ympev.2006.05.031

Wickham, H. (2011). The Split-Apply-Combine Strategy for Data Analysis. *Journal of Statistical Software*, *40*(1), 1-29. https://doi.org/10.18637/jss.v040.i01

Xu, L., Naylor, D., Dong, Z. B., Simmons, T., Pierroz, G., Hixson, K. K., . . . Coleman-Derr, D. (2018). Drought delays development of the sorghum root microbiome and enriches for monoderm bacteria. *Proceedings of the National Academy of Sciences of the United States of America*, *115*(18), E4284-E4293. https://doi.org/10.1073/pnas.1717308115

Xu, S. B., Li, L., Luo, X., Chen, M. J., Tang, W. L., Zhan, L., . . . Yu, G. C. (2022). Ggtree: A serialized data object for visualization of a phylogenetic tree and annotation data. *Imeta*, *1*(4), Article e56. https://doi.org/10.1002/imt2.56

Zhu, Y. G., Xiong, C., Wei, Z., Chen, Q. L., Ma, B., Zhou, S. Y. D., . . . Duan, G. L. (2022). Impacts of global change on the phyllosphere microbiome. *New Phytologist*, *234*(6), 1977-1986. https://doi.org/10.1111/nph.17928