Stress adaptions of the resurrection fern *Anemia caffrorum's* microbiome and metabolism across seasons

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Plagiarism Declaration

I know the meaning of plagiarism and declare that all of the work in the dissertation (or thesis), save for that which is properly acknowledged, is my own.

2024/10/15

Signed by candidate

Michael Wittenberg

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Acknowledgements

Before presenting my master's work in this thesis, I need to acknowledge a few people with whom this would not have been possible and who made significant contributions to shaping this project.

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Abstract

Resurrection plants can tolerate desiccation for extended periods of time. So far, *Anemia caffrorum* is the only known resurrection plant which exhibits this phenomenon seasonally. In the wet season, its fronds are desiccation sensitive (DS) whilst in the dry season they are desiccation tolerant (DT) and can survive losing more than 95% of their cellular water content. Its rhizome on the other hand is tolerant all year round and likely regulates the tolerance of the fronds. As such, it provides a unique model to contrast desiccation sensitivity and tolerance within the same plant species. Furthermore, previous transcriptomic and metabolomic analyses on the rhizome suggested that it attempts to modulate its microbiome during desiccation and its response to pathogenic microorganisms during the wet season. Therefore, in this study, *A. caffrorum* was used as a model to identify microorganisms associated with either plant desiccation sensitivity or tolerance and characterize their dynamics in response to fluctuations in water content and across seasons. Furthermore, to identify potential signaling or selection mechanisms between *A. caffrorum* and its associated microorganisms, matching metabolomics was conducted. Additionally, this allowed for the characterization of the metabolic mechanisms of desiccation tolerance employed by *A. caffrorum* and its associated microbes.

To this end, the root endosphere, rhizosphere and bulk soil controls were sampled from *A. caffrorum* in its natural environment across seasons. This captured samples from hydrated DS individuals as well as desiccated and rehydrated DT individuals. In order to characterize their bacterial and fungal compositions, genomic DNA extracts of all samples were subjected to 16S and ITS amplicon sequencing on a PacBio Sequel II platform. In addition, polar metabolites and lipids were measured from these samples using combinations of untargeted Gas and Liquid Chromatography Mass Spectrometry.

The results showed that indeed *A. caffrorum* can enrich its endosphere with beneficial taxa such as symbiotrophs whilst excluding pathogens. Furthermore, *A. caffrorum* can shape its microbiome across seasons and changing water contents to respond to stress. In the hydrated state, particularly in the DS individuals, biotic stress appeared to be prioritized as these samples exhibited numerous pathogenic, antibiotic producing and antibiotic resistant taxa in addition to antibiotic metabolites. In addition, a number of growth promoting bacteria were found to be increased in these hydrated samples which may assist *A. caffrorum* with growth processes during water availability.

In response to desiccation, triacylglycerols and phospholipids were accumulated. Moreover, simple monosaccharides were likely mobilized to more complex desiccation protective di- and trisaccharides. This shift may have selected for an increase of glycolytic and saprobic taxa. Such functionalities may be beneficial to *A. caffrorum* during desiccation and potentially allow for rapid remobilization of nutrients upon rehydration.

Network analysis within the endosphere identified that microbes were generally most linked to lipid metabolism. Here the antibiotic producer *Dactylosporangium* was the top taxa influencing network topology and was exclusively correlated to numerous lyso phospholipids which are known as signaling molecules involved in *inter alia* the plant immune response. Thus, *A. caffrorum* may efficiently regulate its microbiome by acting on influential antibiotic producing taxa. These findings, if validated through further studies, highlight possible mechanisms through which crops may also modulate their microbiomes in response to stress. This could contribute to ensuring food security under increasing climate change driven biotic and abiotic stress.

Chapter 1

Introduction

This thesis investigates microbial and metabolite dynamics during desiccation and across seasons in the resurrection plant *Anemia caffrorum*, in order to explore the possible broader potential and application of such findings for future more drought resistant agriculture and soil remediation efforts. The thesis begins by outlining the global challenges to food security presented by climate change driven drought and land degradation and introduces how this present study on *A. caffrorum* attempts to address these. In the second chapter, the experimental design, site and sample collections are described. In the third and fourth chapters, these samples are subjected to metabolomics and metabarcoding analyses to uncover mechanisms of desiccation tolerance and their associated microbial dynamics. These two data sets are then analyzed jointly in the fifth chapter to identify possible host-microbe communication and selection mechanisms. Finally, these findings and their implications for improving crop drought tolerance and soil remediation efforts are summarized in the concluding chapter.

The interlinked food security, climate change and land degradation crisis

A significant challenge facing humanity is ensuring food security in the face of rising food demands and increasing abiotic stresses on food production. A meta-analyses of 57 food demand projections highlights that by 2050, global food demand will likely increase by 35% to 56% compared to the demand of 2010 (Van Dijk *et al.*, 2021). Furthermore, during this same period, food production is expected to come under increasing stress from anthropogenic driven factors such as climate change and land degradation. Consequently, there is mounting pressure to meet this demand by additional expansion and intensification of agriculture on finite arable land. However, such agricultural expansion and intensification concomitantly contributes to climate change and both agricultural practices and climate change are the primary drivers of soil degradation (FAO, 2020; Sünnemann *et al.*, 2023). As such, alternate agricultural interventions are urgently required to boost crop productivity and tolerance to stress to ensure food security into the future.

While the negative consequences of climate change receive a lot of research attention, the consequences of land degradation are less well-known. Nonetheless, anthropogenic practices are currently degrading 24 billion metric tons of fertile soil per year and globally around one third of all land is already degraded to some extent (UNCCD, 2017; Cherlet *et al.*, 2018). This degradation is evidenced by the decline of soil organic matter (SOM) and more critically, soil biodiversity (Spohn *et al.*, 2023; FAO, 2020). This impact on agricultural soils leads to increased plant stress and thus lower yields. The numerous critical functions that particularly soil microbes play will be discussed below and in chapters 4 - 6, although the numerous roles of SOM, for example in reducing the effect of drought by increasing soil water retention should not be overlooked (Mahmood *et al.,* 2023). Therefore, protecting soil health is paramount to ensuring continued food production under an increasingly stressful environment and this deserves further research attention.

Microbial biostimulants as a solution

Current agricultural practices, namely: the use of mineral fertilizers (Treseder, 2008), pesticides (Thiele-Bruhn *et al.*, 2012), mono-cropping (Figuerola *et al.*, 2015), tilling (Tsiafouli *et al.*, 2015) and the practice of bare-fallow (Vukicevich *et al.*, 2016) are all leading to land degradation and subsequent loss of soil biodiversity. Thus, the discontinuation or reduction of such practices in favour of practices such as no-till, intercropping, improved crop-rotations, cover cropping and mulching are gaining traction as pragmatic ways to increase soil biodiversity, whilst maintaining and often even increasing yields (Triplett & Dick, 2008; Li *et al.*, 2021; Vukicevich *et al.*, 2016; Du *et al.*, 2022). Given that such measures also result in reduced carbon emissions and increased soil carbon sequestration, these measures have the added benefit of slowing climate change. Accordingly, such interventions are a step in the right direction, although further measures are still required to prevent the decline of agricultural soils.

More recently, there is a growing interest in using microbial biostimulants for land restoration efforts and these are expected to take the lead on future soil health interventions (Coban *et al*., 2022). In addition, microbial biostimulants also show major promise in boosting crop productivity and stress tolerance (Liu *et al.*, 2020). Therefore, they have potential to simultaneously address land degradation and food insecurity.

Microbial biostimulants have been applied as single bacterial or fungal isolates, consortia of multiple microbes, or even as whole soil microbiome applications (Middleton *et al.*, 2015). Their benefit to soil and thus plant health can be attributed to their ability to *inter alia* remove soil pollutants (Zhang & Zhang, 2022), chelate heavy metals (González & Ghneim-Herrera, 2021), break down organic matter and increase soil aggregation (Totsche *et al.*, 2008). Furthermore, they can reduce plant stress and increase crop yield by suppressing plant pests and pathogens (Ayaz *et al.*, 2023), prime crops against future biotic and abiotic stresses (Ayaz *et al.*, 2023; Liu *et al.*, 2020), and finally increase the bioavailability of nitrogen and phosphorus for plant growth (Rashid *et al.*, 2016). Collectively, these interventions result in improved crop yields and stress tolerance, whilst permitting the use of less fertilizers and pesticides, which in turn further boost soil biodiversity. Consequently, the use of microbial biostimulants, in addition to the overhaul of agricultural methods, has major potential in restoring soil health and improving crop yields and stress tolerance to meet the ever increasing food demands and environmental stresses of the future.

Focusing on the need for increased crop drought tolerance

One of the most deleterious crop stressors is drought, which if prolonged induces water deficit in plants. Water deficit leads to loss of cell turgor, membrane integrity and oxidative damage in plants and thus negatively impacts plants growth and productivity (Shao *et al.*, 2008). Water deficit can be classified into dehydration stress, which refers to milder water loss experienced during short drought periods, or as desiccation stress, which refers to almost complete water loss (Tardieu, 2012). Plants can respond to these stresses using molecular and physiological mechanisms including stomatal closure, osmotic adjustment, and activation of antioxidant defense systems which can provide a level of drought tolerance or desiccation tolerance (Reddy *et al.*, 2004; Oliver *et al.*, 2020). However, plant desiccation tolerance requires numerous additional physiological and molecular adaptations, which are discussed in chapter three.

Most agricultural plants, however, are particularly susceptible to water deficit. They rely on drought escape strategies which are not suited to prolonged periods of water deficit (Mitra, 2001). This is particularly relevant in rural areas, where small scale subsistence farms primarily rely on only rainfall to irrigate their crops (Lamaoui *et al.*, 2018). Given that approximately 75% of the world's population lives in rural areas, where agriculture is the primary source of income, and that climate change is increasing the incidence and duration of droughts, crop plants need to be equipped with improved drought tolerant mechanisms (Rural Poverty Report 2011).

Resurrection plants as a solution

This need for greater crop drought tolerance has led to the study of so-called resurrection plants. By definition, resurrection plants are desiccation tolerant (DT), meaning that that they can survive losing more than 90% of their cellular water content in their vegetative tissues (Alpert, 2005). Most bryophytes such as mosses are DT. However, less than 0.1% of vascular plants exhibit vegetative desiccation tolerance – although many likely still remain to be classified as such (Stark et al, 2016; Proctor and Pence, 2002; Marks *et al.*, 2021). It has been hypothesized that initially all land plants had this phenotype, but that this trait was then lost in the vegetative tissues of vascular plants, being retained only in seeds and spores, and subsequently re-evolved independently across the various resurrection plant lineages (Oliver et al, 2002; Fisher, 2008; Gaff & Oliver, 2013).

Interestingly, there is a significant overlap between the genes involved in the maturation of DT seeds, which are present in crop plants, and the genes implicated in the DT response (Illing *et al.*, 2005; Costa *et al.*, 2017; VanBuren, 2017). Therefore, an in-depth understanding of the mechanisms of desiccation tolerance, and their induction, could allow for such genes to be similarly expressed in the vegetative tissues of crop plants, affording them enhanced drought and possible even desiccation tolerance (Hilhorst & Farrant, 2018).

Consequently, efforts to impart some of this tolerance to crop plants has focused on genetic engineering approaches. Unfortunately, this has had limited success due to the complexity of these DT mechanisms and the difficulties of working with resurrection plants. Furthermore, the societal concerns around genetic modifications may also hamper such pursuits. On the other hand, drought protective microbial biostimulants do not necessarily require genetic modification and so may be a more viable route to crop improvement, though would likely fall short of imparting full tolerance to desiccation. Accordingly, this has sparked an explosion in plant microbiome studies in recent years to identify microorganism that can improve crop drought tolerance.

Surprisingly, resurrection plant microbiomes remain relatively unexplored. To date, only six studies have investigated the microorganisms associated with resurrection plants in relation to their desiccation tolerance (Jackson *et al.*, 2006; Đokić *et al.*, 2010; Fernandes-Júnior *et al.*, 2015; Tebele *et al.*, 2023; Lozo *et al.* 2023; Silva *et al.*, 2023). Note as an aside, other studies on the resurrection pteridophyte *Pellaea calomelanos* have investigated the medicinal properties of its bacterial endophytes, though have not discussed or examined these in relation to the host plant's desiccation tolerance (Mahlangu & Serepa-Dlamini, 2018; Tshishonga & Serepa-Dlamini, 2019, 2020). Strikingly, only the oldest of these studies by Jackson *et al.*, (2006) investigated how the microorganisms of resurrection plants change in response to desiccation. However this was investigated without the use of Next Generation Sequencing and in this study fungi were not examined. Thus, a comprehensive investigation of resurrection plant microbial dynamics under desiccation is lacking and the relatively unexplored resurrection plant microbiomes present a research gap.

Filling this research gap could allow for the improved design of drought protective microbial biostimulants. This is supported by unpublished work from our lab and two other studies which could use microbial isolates from resurrection plants to improve plant growth and drought tolerance and so demonstrate that resurrection plants do harbour microorganisms with utility for crop improvement (Fernandes-Júnior *et al.*, 2015; Lozo *et al.*, 2023).

Furthermore, resurrection grasses such as *Eragrostis nindensis* and *Oropetium capense* are commonly found in disturbed soils such as on the road side or next to mines (van Oudtshoorn, 1992; personal observation) and much like other resurrection plants, thrive in shallow and inhospitable soils where few other plant species can survive (Porembski & Barthlott, 2000; Porembski, 2007, 2011). This suggests that resurrection plants, along with their associated microbial communities, could additionally play a role in soil remediation efforts by possible preparing damaged soils for the subsequent growth of crop plants.

Consequently, considering the collective evidence of all of the above, further research is required to explore the potential applications of resurrection plants, their molecular mechanisms of desiccation tolerance and their associated microbial communities in agriculture.

Anemia caffrorum as a model to study vegetative desiccation tolerance

The resurrection plant *Anemia caffrorum* presents a distinctive model within which to investigate these research questions further. Unlike all other known resurrection plants, which are inherently DT all year round, *A. caffrorum* is only seasonally DT. During the dry season, it demonstrates DT in its fronds, while in the rainy season, the fronds become fully desiccation sensitive (DS) (Farrant *et al*., 2009; Nadal *et al.,* 2021). This seasonal contrast between tolerance and sensitivity to desiccation within the same species makes *A. caffrorum* an ideal model for studying the underlying mechanisms of desiccation tolerance, its induction, and associated microbes.

However, more recent omics studies on *A. caffrorum* from our group suggested that unlike the fronds, its rhizome retains desiccation tolerance at all times, and likely contributes to the regulation of desiccation tolerance and sensitivity in fronds (Shoko, 2016, Wittenberg, 2021, Khan, 2023). Furthermore, these prior studies of the rhizome pointed to microbial involvement and possibly intervention during responses to desiccation and across relative states of desiccation tolerance. However, these data also implicate the involvement of the roots and rhizosphere in these microbial interactions. Therefore, this study aimed to build on this prior work by for the first time looking at *A. caffrorum's* microbiome within the root endosphere, (hereafter referred to simply as the endosphere) and the rhizosphere via metabarcoding. Furthermore, to uncover potential host – microbe selection or communication mechanisms, metabolomics was also performed within these spheres. This then also allowed for the assessment of the metabolic mechanisms of desiccation tolerance in these so far unexplored spheres. Accordingly, this study had three major research questions, which are stated below:

Research questions:

Within *A. caffrorum's* endosphere and rhizosphere,

1) How do fluctuations in water content, particularly desiccation, influence the microbial community and metabolism?

2) Are there any seasonal microbial or metabolic signatures that may relate to seasonal adaptions such as the relative desiccation tolerance of the fronds?

3) Is there any evidence of host – microbe selection that may also be mediated by specific metabolites?

In answering these questions, it is hoped that *A. caffrorum's* mechanisms of desiccation tolerance and differences in seasonal lifestyles can be understood more holistically at both the level of the metabolome and microbiome. In addition, it is hoped that by analyzing the microbiome and metabolome data jointly, that mechanisms of host microbe selection and interaction are uncovered. All of these will advance the fields of desiccation tolerance and microbiome research within resurrection plants which may ultimately contribute to increasing the drought tolerance of crop plants.

Disclaimer

I would like to acknowledge that during the final proofreading stage of this thesis, a study was published that, similar to this one, examined both bacterial and fungal dynamics during desiccation in a resurrection plant (Sun *et al.*, 2024). However, unlike this study, it did not include a rehydration time point. Due to the timing of the publication, these findings will unfortunately not be discussed in this thesis. Nonetheless, their results were not very impactful to this work, and there were no major contradictions.

Chapter 2

Field collections and sample processing

2.1 Introduction

Most studies on resurrection plants are performed in controlled laboratory conditions, whilst very few to date have been performed in field conditions. This can be attributed to the difficulty in controlling variability in a far more complex field environment and the technical difficulties of applying treatments of interest. Therefore, it may often be easier to carry out experiments and detect statistically significant differences of interest in a controlled laboratory environment.

However, results from laboratory based experiments may not always be as valid and applicable as field based studies. This is particularly true in the microbiome field, where lab and field based experiments often contradict each other (Chen *et al.*, 2023). Given that laboratory based experiments mostly deal with disturbed or treated soil, one would expect this to affect microbial community structure and this may alter research findings. Furthermore, resurrection plants have been reported to lose some of their desiccation tolerance when removed from field conditions into more controlled laboratory conditions (Marks *et al*., 2021). Therefore, the study of desiccation tolerance in these plants may miss some mechanisms that can only be detected in more variable field like conditions. This could be exacerbated if the ecophysiology of the plant in question is not well understood. For example, DT *A. caffrorum* have been observed to switch to DS plants after approximately a month of growth in a conviron under well-watered conditions (personal observations). Unfortunately, to date, no established method exists to transition *A. caffrorum* from DS to DT or even maintain their DT phenotype long-term under laboratory conditions. Therefore, prior laboratory studies on *A. caffrorum* required collecting plants from the field and only giving them a short acclimation period before imposing experimental conditions such as desiccation (Khan, 2023). This undoubtedly disturbs the soil and root environment and therefore, this study attempted to rather answer its research questions in a field environment.

Working in a field environment posed a number of difficulties. Firstly, it necessitated the selection of a field site that would minimize intraspecific variation across replicates within the same treatment. For example, factors such as pH, soil type and co-occurring plants have marked impacts microbial community structure and as such need to be constant or at least measured and controlled for in downstream statistical analyses (Lopes *et al.*, 2021; Garbeva *et al.*, 2004). In addition, it may prove difficult to impose certain treatments such as watering regimes for example, if unexpected rainfall occurs. Therefore, this study did not attempt to capture multiple time points along a conventional dry down curve and rehydration as is commonly reported for other resurrection plant and previous work on this species (Farrant *et al.*, 2009). Instead, this study relied entirely on natural rainfall patterns for irrigation and desiccation treatments. Unfortunately, this would then not allow for the capture of a desiccated DS collection, as *A. caffrorum* is only DS during the rainy season and is thus unlikely to experience desiccation over this period. Note also, that seasons confound the relative desiccation tolerance of *A. caffrorum* and under the current design these effects cannot be separated. Nevertheless, one could still expect to capture desiccated and rehydrated DT collections as rainfall occurs occasionally during the dry season. Collectively, these could allow examination of microbial and metabolite changes across varying water contents and desiccation tolerance and ultimately begin to answer the questions of this study.

Chapter aims:

1) Identify a suitable field site which will minimize intraspecific variation across replicates.
2) Obtain suitable collections with contrasting desiccation tolerance and water contents. Specifically, a hydrated DS collection and desiccated and rehydrated DT collections.
3) Process and evaluate collected samples for downstream omics experiments.

2.2 Methods

2.2.1 Site and experimental design

A site on Signal Hill (-33.92582702636719, 18.399272918701172) in Cape Town, South Africa was chosen and split into four sub-sites spaced at least 10 m apart. In each sub-site, the *A. caffrorum* individuals with at least three non-diseased fronds above 10 cm in height and at least 2 m away from any other prominent vegetation other than grasses and other *A. cafforum* individuals were identified and labelled. Within each sub-site, four of these labelled plants were randomly selected and ordered. Subsequently, these were sampled from each sub-site across four field collections. Since there were also four sub-sites, each of the four collections thus had four biological replicates.

The first collection was made in August 2022 during wet winter conditions three days after rain. By this time of year, all DT “summer” fronds had completely senesced and all *A. caffrorum* individuals only contained DS “winter” fronds.

The next two collections were in early December 2022 during the summer. By this time, all DS fronds had completely senesced and only fully DT fronds remained on all individuals. These collections were made just before and three days after rain to yield desiccated and rehydrated DT individuals.

The final collection was performed in February 2023 in the late summer to capture a second desiccated and DT collection. Here again, no DS fronds were yet present on any individual.

To obtain these collections, permission was granted by the South African National Parks.

2.2.2 Measurement of bulk soil edaphic factors

The percentage sand, silt and clay was determined by the glass jar method. From each of the four sub-sites, three bulk soil replicates between the depth of 5 cm and 20 cm were collected with a spade and passed through a 2mm sieve. Next, for each replicate, 100 g of sieved bulk soil was vigorously mixed with 100 ml of tap water and 10 g of Omo^{TM} dishwashing powder for one minute in a flat bottomed cylindrical glass jar of height 10.5 cm and radius 3.25 cm. This mixture was left to stand at room temperature for three days, which resulted in the formation of three layers. The top layer was regarded as clay, the middle as silt and the bottom as sand. The width of each soil layer was then measured with a ruler and converted to a percentage relative to the total height of soil (all layers) in each jar.

The pH of the collected bulk soil for each replicate was determined as in Kalra & Maynard (1991). Briefly, 10 g of the collected soil was mixed with 20 ml of 0.1M $CaCl_2$, stirred for 30 minutes and then left to stand for 30 minutes. Thereafter, the pH was measured with a Crison basic 20+ pH meter.

2.2.3 Sample collection procedure

From each sampled *A. caffrorum* individual, roots, rhizosphere, a bulk soil control, fronds and adjacent soil moisture measurements were obtained at sunrise across all collections.

Roots with rhizosphere soil attached were sampled by excavating the root system to a depth of approximately 20cm with a spade and shaking the loosely adhering soil off the roots. Approximately, 50% of the roots, with firmly attached rhizosphere soil were cut off at random points across the length of the rhizome with a sterilized scissor. These were placed into a 50 ml sterilin tube and immediately flash frozen in liquid nitrogen. The sampled plant was then replanted.

Some roots with rhizosphere soil attached, were not flash frozen and instead stored in separate sterilin tubes on ice for relative water content (RWC) determination and for separate study in which microorganisms were isolated. As current RWC measurements are not designed for root tissues, at least one frond was also collected from each plant for RWC determination. These were also stored in tinfoil envelopes on ice to prevent water loss before weighing.

In addition, a soil core of diameter 2.2 cm was taken 20 cm away from each plant. Here, the soil between a depth of 5 cm and 15 cm was kept as a bulk soil control and was also immediately flash frozen.

To minimise DNA and metabolite carry over between each individual collected, fresh gloves used at each sampling and the spade, soil core, scissors and tweezers were sprayed with 0.5% hypochlorite followed 70% Ethanol and dried with a clean paper towel.

Lastly, the soil moisture and conductivity was measured within a radius of 20 cm of each individual using five TEROS3 soil moisture probes connected to an EM50 data logger.

2.2.4 Plant relative water content measurements

The frond and root relative water contents for each sample were determined separately from five technical replicates using frond pinnae across the length of the frond and root fragments across the whole root system respectively. Approximately three to four hours after initial sampling, the pinnae and root fresh weight (FW) was recorded using an OHAUS Explorer analytical balance and subsequently left to incubate overnight in the dark at 4°C in deionized H_2O. The following day, these were re-weighed after visible water on the sample surface was removed by gently dabbing with tissue paper to produce the full turgor weight (FTW). Lastly, each sample tissue was dried at 70°C for 48h to obtain the dry weight (DW). The relative water content (RWC) was then measured according to the following formula as in Barrs & Weatherly (1962):

$$\%\, RWC = \frac{FW - DW}{FTW - DW} \times 100\%$$

2.2.5 Compartment Separation for DNA and metabolite extraction

Flash frozen roots with rhizosphere attached were lyophilized using a Virtis freeze dryer (supplied and serviced by United Scientific) for 48h. The air dry samples were then stored at -20°C until compartment separation. The compartment separation was performed in a laminar flow hood at room temperature. Rhizosphere soil that was attached at a distance of no more than five 5 mm from the roots was carefully removed with a tweezer and spread out on a flat bottomed sterile mortar. Next, this rhizosphere was inspected in small increments (around 10 – 20 mg at a time) to remove all visible root fragments with a tweezer and fine paint brush. The roots were then brushed with a clean paintbrush to remove any remaining visible rhizosphere soil. These cleaned roots were defined as endosphere in downstream metabolomics experiments. However, before DNA extraction, this endosphere was washed further as in Edwards *et al.* (2018). Briefly, the previously cleaned roots/endosphere were washed thrice in PBS with vortexing and thrice in PBS with sonication in an ice water bath. Thereafter, the endosphere was frozen and re-dried in a freeze dryer to produce the final endosphere for metabarcoding. All above compartments were stored at -20°C until extraction.

The bulk soil, although containing far fewer roots, was processed identically to the rhizosphere by inspecting it in small increments to manually remove any visible root fragments or small pebbles.

Lastly, during the compartment separation, all surfaces (except sterile tubes) that came into contact with the sample tissues were first soaked in 0.5% hypochlorite for 30 minutes to break down DNA followed by thorough rinsing in Elix water followed by 100% ethanol to facilitate rapid drying.

2.2.6 Data analysis

Randomization to collect samples and determine their collection order was performed in Microsoft Excel 2016. Results were visualized in Microsoft Excel, except the boxplot which was created in R v4.2.2. The one way analysis of variance (ANOVA) and Tukey's Honest Significant Difference post hoc test was performed in R v4.2.2 using the base functions aov() and TukeyHSD() respectively (R Core Team 2023).

2.3 Results and Discussion

2.3.1 Site evaluation

In order to identify a suitable site to examine the questions of this study, it had to fulfill a number of criteria. Firstly, it had to contain a high density of *A. caffrorum* individuals covering an area large enough to allow for all replicates to be spaced at least 10 m apart. This distance was to avoid sampling the same fungus twice (Lindahl *et al.*, 2013). Secondly, the site should be as homogenous throughout as possible to reduce collection wise intra-specific variability. And finally, the site should ideally be comparable to agricultural soils to allow for potentially easier application of resulting microbial isolates to crop plants.

After evaluating a number of sites across Cape Town, a site on Signal Hill was selected based on these criteria. Here, *A. caffrorum* grew most abundant along a spine approximately 50m from a non-perennial river (Fig. 1A). This unfortunately introduced an elevation gradient. Nevertheless, out of all sites inspected with diameter greater than 50 m, it had the highest density of *A. caffrorum*.

Moreover, sub-regions (at least 10m apart) existed, over which *A. caffrorum* was the most abundant plant species and exhibited homogenous co-occurrences with predominantly grasses (Fig. 1C). The most abundant and consistently co-occurring grass possibly belonged to the genus of *Andropogon*. Since most crops are grasses, the influence of these co-occurring species was deemed more acceptable than the co-occurrence with trees for example existing at other sites.

In addition, the site had homogenous soil type and pH throughout. The soil type comprised of 86% sand, 6% silt and 8% clay and was thus classified as a sandy soil according to the Unified Soil Classification System. When comparing soils across sub-sites, no significant sub-site effect could identified (ANOVA p-value = 1) (Fig. 1B). Similarly, the pH was also statistically constant across each sub-site (p-value = 0.69) (Fig. 1D).

Therefore, although a slight slope existed, the site was deemed suitable to answer the questions of this study.

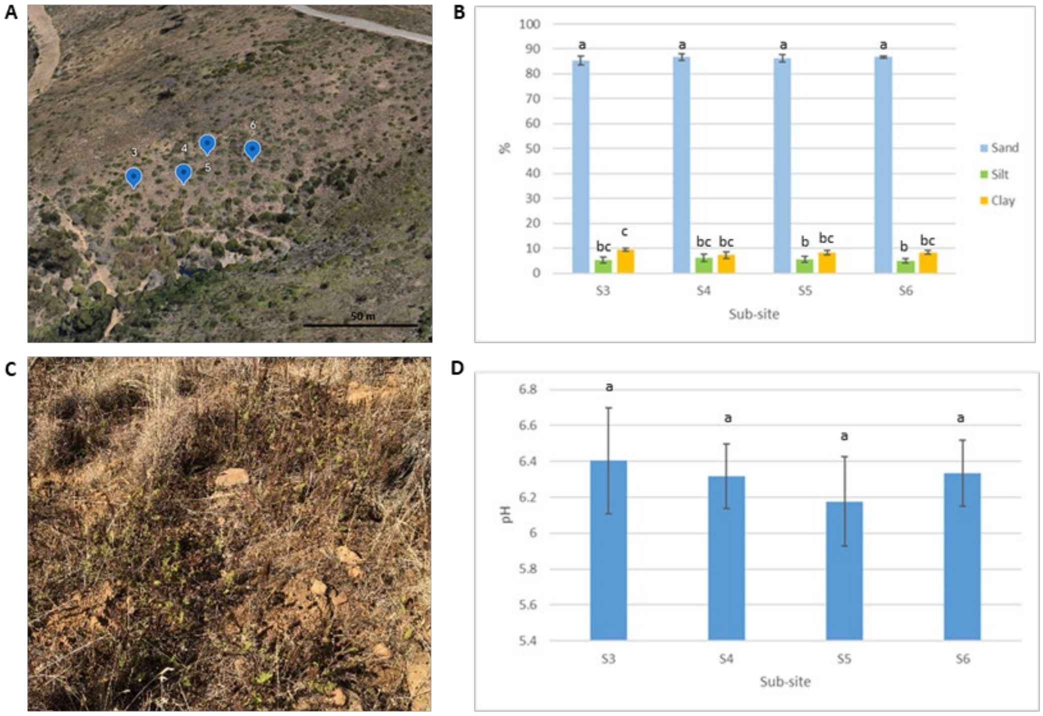

Figure 1 (A-D). Site description. (A) Relative location of the four sub-sites. (B) Percentage of soil types across each sub-site. (C) Representative image of the vegetation type. All green plants are *A. caffrorum*. (D) The pH of each sub-site. Error bars represent the sample standard deviation of the three replicates for each sub-site. Bars that do not have a letter in common, differ significantly at the 5% level after adjusting for multiple comparisons. Statistical significance was determined using ANOVA followed by Tukey's HSD post hoc test in R.v.4.2.2.

2.3.2 Evaluation of the collections

Next, four field collections were made. In each, four samples were collected, one from every sub-site. The first collection was sampled in the wet season three days after heavy rain when all *A. caffrorum* were DS. All subsequent collections were made in the dry season when *A. caffrorum* were DT and of these, collection three was performed after three days of rain. Here, the purpose of the fourth collection was to evaluate the stability of the desiccated microbiome and metabolome. In order to identify if indeed hydrated and desiccated samples were collected for all replicates within each collection, the relative water content (RWC), soil moisture and soil conductivity were measured.

Interestingly, a disparity in RWC existed between the root and frond tissues (Fig. 2A). In the hydrated collections (one and three), the roots exhibited lower RWCs and in the desiccated collections (two and four) they exhibited higher RWCs compared to the fronds. In *Medicago sativa*, the stems and even more so the roots also exhibited a lower RWCs compared to the leaves under “well-watered conditions” (Luo *et al.*, 2019). Furthermore, these authors also observed that upon dehydration the leaf RWC decreased to a much greater extent compared to that of the stems and roots. However, when the change in biomass over this period was also accounted for, the rate of proline accumulation during stress was equal across all three tissues. Since proline is a marker for plant stress, this suggests that all three tissues were experiencing a similar stress which is not reflected by the measure of RWC. Although not proposed by these authors, this highlights that the measure of RWC may not be comparable to, or as useful for non-leaf tissues. Instead, future studies on *A. caffrorum* should include water potential measurements which are more reliable when comparing across different tissues (Sun, 2002).

Consequently, given also the high variability in the roots, even within the technical replicates of the same individual (Fig. 2B), this study will not consider the root RWCs when assessing the water status of *A. caffrorum*.

Instead, the frond RWC and soil moisture was interrogated to classify the hydration status of each sample and collection. In collection one through to four, the average frond RWCs were: 76%, 4%, 67% and 9% respectively (Fig. 2A). Although there were differences in RWC between the hydrated collections one and three and the desiccated collections two and four, these differences were not significant, even at the 10% level when inspecting their adjusted p-values computed by Tukey's Honest Significant Difference test. This grouping of collection one with three and two with four was even more pronounced in the soil moisture data. Here, collections one and three had soil moistures of 12% and 13% respectively whilst collections two and four had soil moistures of 3% and 4% respectively (Fig. 2C). Hence, collections one and three were given a water status of “hydrated” and collections two and four were termed as “desiccated”.

In addition, the Teros3 soil probe also measures soil electrolytic conductivity (EC). Soil EC is determined by the amount of dissolved ions in soil. The higher the EC, the more anions (e.g. nitrate (NO_3^-) and organic molecules) as well as cations (e.g. Sodium (Na^+) and ammonium (NH_4^+)) are dissolved in the soil. Therefore, EC is also directly related to the amount of nutrients and organic matter in the soil. In addition, EC also correlates to microbial community structure and can be used as a proxy for microbial activity (Kim *et al.*, 2016). The EC measurements again split the collections according to their water status (Fig. 2D). This likely relates to the differences in soil moisture as without much water in the desiccated collections, no ions can be dissolved and as such EC values of zero were recorded. Again however, the hydrated collections have statistically indistinct EC’s which suggests perhaps equal soil nutrient compositions and microbial activity. This also supports the fact that microbial and metabolic activity has fully recovered post desiccation in collection three and thus further cements the grouping of collection three and one.

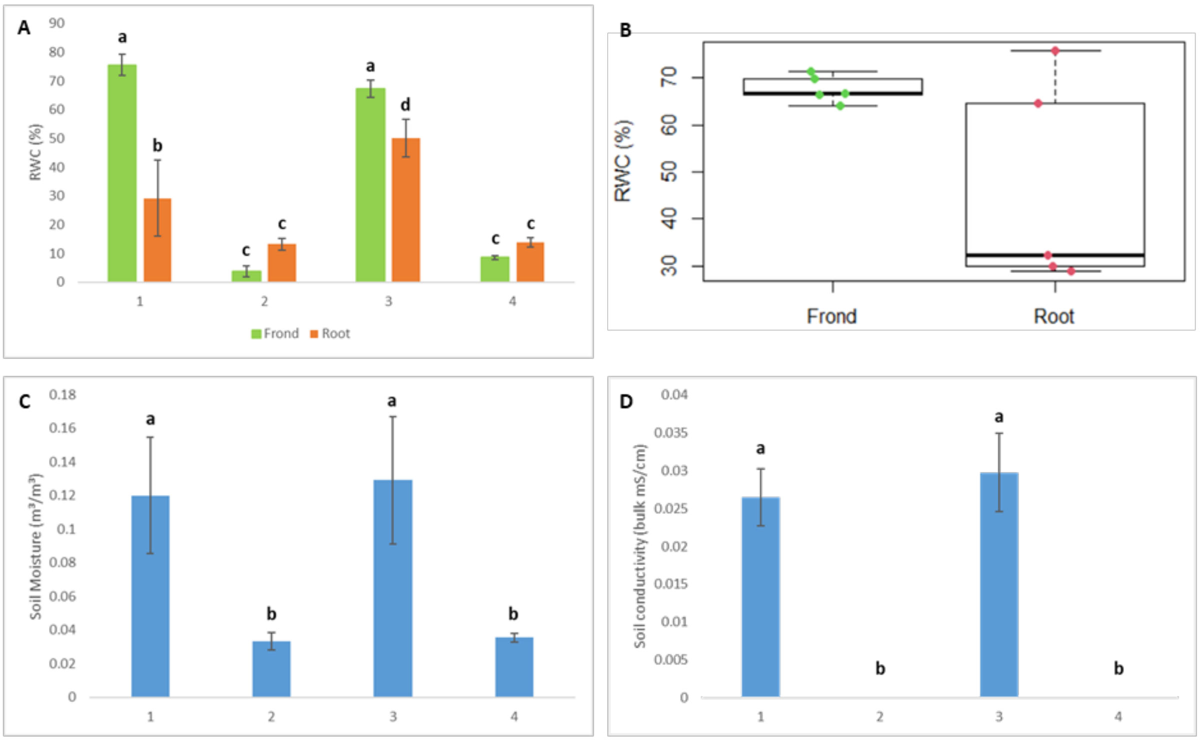

Figure 2 (A-D). Sample physiological parameters measured for collections 1-4. (A) Average relative water content (RWC) measured for fronds and roots across each collection. (B) Technical replicates for sample 1.4 from collection 1. (C) Average soil moisture for each collection. (D) Average soil electric conductivity for each collection. Error bars represent the sample standard deviation of the four replicates for each collection. Bars that do not have a letter in common, differ significantly at the 5% level after adjusting for multiple comparisons. Statistical significance was determined using ANOVA followed by Tukey's HSD post hoc test in R.v.4.2.2.

2.3.3 Optimizing the compartment separation

Finally, in order to prepare the samples for metabolite and DNA extraction, the roots with rhizosphere soil attached had to be separated into endosphere and rhizosphere. Other metabolomics studies within the rhizosphere and more generally within soil research, typically use a 2mm sieve to separate the rhizosphere soil from smaller root fragments (Swenson *et al.* 2015). When this was attempted for *A. caffrorum* however, a number of small root fragments were still visible in the resulting sieved rhizosphere soil. *Anemia caffrorum's* roots, particularly when desiccated, were very brittle and are thus prone to fragmenting into smaller pieces that can pass through the sieve. In addition, it was observed that the wires of the sieves tested, cut root fragments into smaller pieces that could then pass even more easily through the sieve pores and were consequently even harder to remove thereafter. Some of these root fragments were smaller than the larger soil particles and so using a sieve with smaller pores would likely also not work and probably just break up the roots further. Therefore, a more laborious procedure was developed in which the rhizosphere was removed in small increments from the roots which could then be spread out on a flat surface and inspected to remove any visible root fragments or pebbles.

When metabolites were extracted and analyzed by Gas Chromatography Mass Spectroscopy from this optimized non-sieved method in addition to sieved rhizosphere and endosphere originating from the same sample, the optimized non-sieved method exhibited a much smaller sucrose peak when compared to the sieved method (Fig. 3). Since sucrose was by far the largest peak present in the endosphere, it was used here as a proxy for root contamination of the rhizosphere. Trehalose, which was present in both desiccated endosphere and rhizosphere and may be of microbial origin (discussed in chapter 3) serves as a control as it still remains in the rhizosphere processed by the more laborious method and highlights that sucrose is likely of root origin. Therefore, although no replicates were used in this test, the more laborious method without a sieve was selected to obtain root free rhizosphere in this study.

Metabarcoding compartment separation procedures are also not without issues. These typically involve washing the rhizosphere soil off the roots using a water based buffer (Simmons *et al.*, 2018; Edwards *et al.*, 2018). The roots are then further washed to produce the endosphere. This re-wetting of tissues makes it unsuitable for metabolomics experiments as this would undoubtedly alter metabolite profiles. However, when the resulting rhizosphere from such methods was inspected, again numerous small root fragments were visible that would likely alter the rhizosphere microbial composition. Thus, the same laborious method used for acquiring the metabolomics rhizosphere was used for the metagenomics rhizosphere. The roots intended for metagenomics however, were still washed as in Edwards *et al.* (2018) to remove any remaining microbes attached to their surface.

For consistency, the bulk soil was processed identically to the rhizosphere, although it contained far fewer root fragments. However, the bulk soil was not subjected to metabolomics in this study – only metabarcoding.

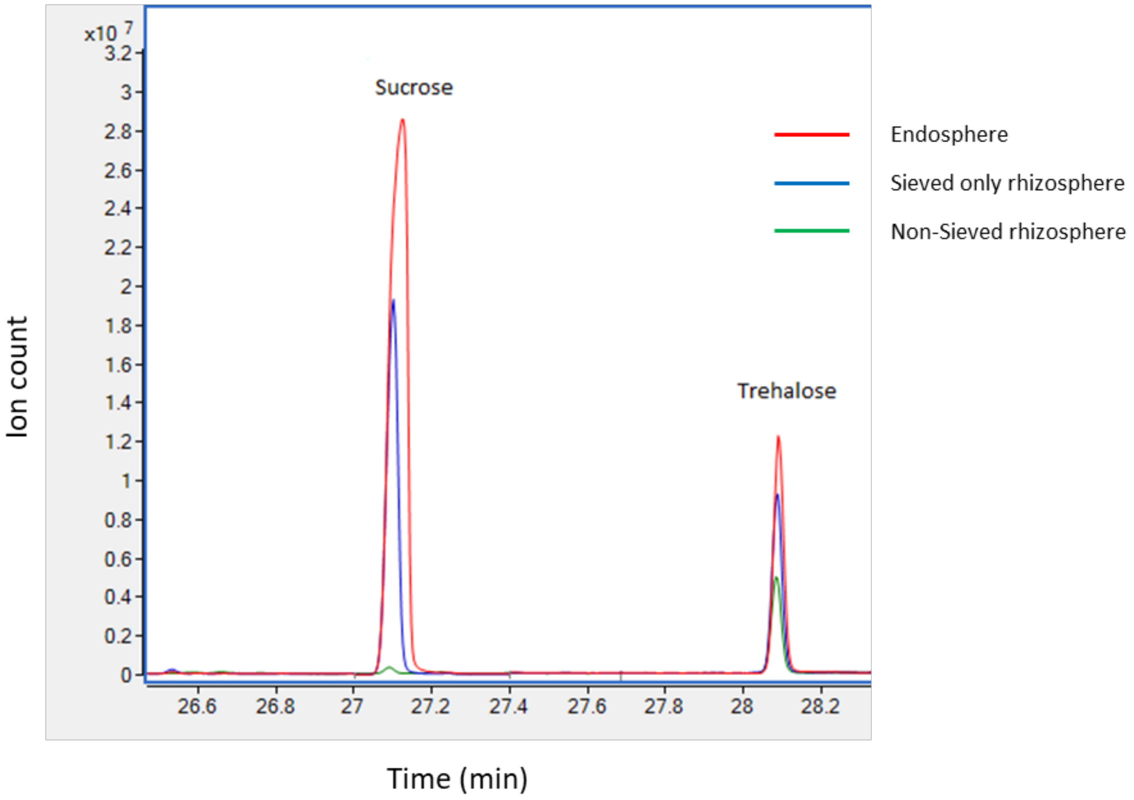

Figure 3. Validation of the compartment separation method. The sucrose and trehalose peaks are shown for metabolite extracts from the same desiccated DT sample. Peaks in red represent endosphere extracts. Peaks in blue represent rhizosphere extracts obtained by sieving only. Finally, peaks in green represent rhizosphere extract from the optimized compartment separation method without sieving.

2.4 Brief Conclusions

In conclusion, a suitable field site was identified that could allow for the sampling of similar replicates across collections. Fortunately, the collections of interest were captured, permitting the investigation of the questions of this study. These were as follows: (1) a hydrated DS collection, (2) a desiccated DT collection, (3) a rehydrated DT collection, and finally (4) an additional desiccated DT collection. Despite highly variable root RWC results, collection one and three and collections two and four could respectively be grouped together as hydrated and desiccated collections based off of their frond RWC, soil moisture and soil EC. However, based off the results and discussion in this chapter, future investigations on *A. caffrorum's* roots should include water potential measurements. Finally, these samples collected could be separated into relatively pure rhizosphere, endosphere and bulk soil, which are suitable for downstream omics analysis.

Chapter 3

The metabolic landscape of *A. caffrorum's* endo- and rhizosphere

3.1 Introduction

The prior chapter describes the experimental design, site and physiology of the samples within this study. This chapter expands on this by characterizing the metabolic landscape of these samples via metabolomics approaches. Collectively, chapters two and three provide the environmental context for interpreting the microbiome data whilst also allowing for the description of seasonal and water content related metabolic adaptations.

To begin with, a background on metabolomics and mechanisms of desiccation tolerance within *A. caffrorum* and microorganisms is provided. Following this, the metabolomics results are discussed with respect to water content and seasonal related patterns, as well as their potential influence in microbial interactions. In conclusion, the findings are synthesized and referenced against the initial problem statements of this thesis.

Introducing the metabolomics approach of this study

Metabolomics describes systems based approaches which attempt to measure the levels of metabolites, which are collectively are known as the metabolome. These can be targeted and only aim to measure the levels of specific metabolites or can be untargeted and aim to detect as many metabolites as possible. This study took an untargeted approach and aimed to identify all metabolites involved in the response to desiccation, seasonal adaptations or the regulation of desiccation tolerance and finally interactions with microorganisms. Unfortunately though, this approach may miss some key signaling compounds such as hormones which only exist in very low abundances and are usually only detected by targeted approaches.

Plants are estimated to produce between 200 000 and 1 million metabolites and so no single metabolite extraction or analysis instrument can measure all of the metabolites present in a given sample (Wang *et al.*, 2019; Fiehn, 2002). Therefore, this study employs multiple metabolite extraction and quantification methods to more comprehensively describe the metabolic landscape within these samples.

Firstly, Gas Chromatography Mass Spectrometry (GCMS) was employed to quantify the smaller and more volatile metabolites that would likely not be resolved very well by Liquid Chromatography Mass Spectrometry (LCMS). For this, a semi-polar extract was used that would allow for the quantification of both polar and non-polar metabolites such as sugars and fatty acids respectively. Secondly, LCMS was employed to quantify larger metabolites that are less volatile and thus not so easily detected by GCMS. Here, to facilitate better resolution, polar and non-polar (lipids) were analyzed separately and so an extraction which partitions these into two phases was applied.

Such metabolomics methods lend themselves well to uncovering mechanisms of desiccation tolerance, as surviving desiccation requires major stimulation of the metabolome in both plants and microorganisms (Oliver *et al.*, 2020; Lebre *et al.*, 2017).

Plant metabolic mechanisms of desiccation tolerance

Within plants, metabolomics studies have found that initially in response to water deficit, small molecules such as proline are accumulated as osmolytes to slow the loss of water (Luo *et al.*, 2019; Tymms & Gaff, 1979). Additionally, antioxidants such as glutathione are produced to respond to the increase in oxidative stress induced by water deficit (Hasanuzzaman *et al.*, 2017; Kranner *et al.*, 2002). However, as water deficit intensifies, DS plants succumb to the stress and undergo senescence. Resurrection plants on the other hand, can offset senescence and survive desiccation by employing a range of additional metabolic responses (Oliver *et al.*, 2020).

In response to desiccation, all resurrection plants accumulate metabolites with free hydroxyl groups that can effectively replace water molecules as they are lost. Primarily non-reducing sugars such as sucrose, raffinose and trehalose are used for this and allow for the stabilization of particularly cell membranes which require a hydration shell to avoid fusion (Oliver *et al.*, 2020). In *A. caffrorum*'s rhizome, sucrose is believed to be the primary sugar accumulated in response to desiccation, although trehalose and raffinose are also accumulated (Wittenberg, 2021). These sugars are believed to transform the cytoplasm to a vitrified state which provides further stabilization and slows the flow of metabolic activity (Buitink & Leprince, 2004). In fact, cross sections of *A. cafforum's* rhizome appear glass like in the desiccated state (Khan, 2023).

Another conserved response to desiccation employed by all resurrection plants is the slow down their metabolism, including the shutdown of photosynthesis (Oliver *et al.*, 2020). This allows them to enter a state of dormancy and wait out a desiccation event. However, if chlorophyll is still exposed to sunlight during this time, reactive oxygen species (ROS) are produced (Oliver *et al.*, 2020). At high enough concentrations, ROS becomes toxic and leads to the damage of intracellular structures, especially cell membranes (Gill & Tuteja, 2010). Therefore, all resurrection plants employ mechanisms to shield their chlorophyll from sunlight (Oliver *et al.*, 2020). *A. cafforum* does this through leaf folding and the accumulation of UV absorbing pigments in abaxial scales (Farrant *et al.*, 2009) whilst some other resurrection plants go so far as to completely dismantle their photosynthetic apparatus (Radermacher et al., 2019; Tuba, 2008; Van der Willigen *et al.*, 2001). In fact, the duplication of early light inducible proteins (ELIPS), which protect chlorophyll from photo-oxidation, is so far the only known conserved genomic feature of resurrection plants, which further highlights the importance of this shared response (VanBuren *et al.*, 2019).

However, this conserved protection of chlorophyll should not occur in the roots or below ground organs as these are not exposed to sunlight. Intriguingly though, prior transcriptomic work on *A. cafforum's* rhizome found that ELIPS were amongst the most abundant transcripts expressed in response to water deficit (Khan, 2023). Moreover, additional photo-protective transcripts were found in the rhizome such as the chloroplastic ACTIVITY OF BC1 COMPLEX KINASE 1 and the chloroplastic chaperone protein ClpB3. Thus, it was speculated that these transcripts were potentially transported to the fronds be translated into functional proteins and implicate the involvement of the rhizome in regulating the frond responses to desiccation.

Further evidence for shoot related signals occurring below ground were identified in the proteome of *A. cafforum's* rhizome (Shoko, 2015). Here, it was found that during desiccation the DT rhizome upregulated potentially protective proteins related to shoot system morphogenesis, whilst the DS rhizome upregulated leaf senescence proteins. Furthermore, metabolomic profiling of the rhizome also suggested protection of only DT fronds by potential translocation of protective metabolites (Wittenberg, 2021). Consequently, taking all the above together, the rhizome likely regulates the desiccation tolerance of the fronds. Currently, the specific responses of roots and their potential involvement in signaling to the fronds is unknown. Nevertheless, the above seemingly shoot specific responses need to be kept in cognizance when interpreting the data.

Metabolic responses of microorganisms to desiccation

Similarly to plants, bacteria and fungi respond to desiccation by accumulating antioxidants, osmolytes and sugars to combat the increased oxidative stress and slow the loss of water respectively (Lebre *et al.*, 2017). In particular trehalose is favoured for these functions in microorganisms. As in plants, trehalose has also been shown to act as a molecular chaperone and lead to the formation of cytoplasmic glasses (Alvarez *et al.*, 2004; Lebre *et al.*, 2017). Moreover, growing some microorganisms in media containing a high concentration of trehalose is sufficient to impart desiccation tolerance to otherwise sensitive species under these conditions (Tapia *et al.*, 2015). Further similarities are exhibited by photosynthetic microorganisms such as cyanobacteria which either degrade their chlorophyll or attempts to shield it via the accumulation of pigments (Hirai *et al.*, 2004). Given the overlap in responses to desiccation between plants and microorganisms, mutually beneficial synergistic responses are a possibility.

However, microorganisms also exhibit unique metabolic responses. Particularly bacteria but also some fungi produce extracellular polymeric substances (EPS) in response to desiccation which allow for the formation of biofilms which can greatly slow the loss of water (Roberson & Firestone, 1992; Breitenbach *et al.*, 2022).

Interestingly, the metabolome of *A. caffrorum's* rhizome contained the metabolite N-acetyl-D-mannosamine (Wittenberg, 2021), which has been shown to be an integral component of both bacterial and fungal EPS (Deo *et al.*, 2019; Oliva *et al.*, 2024). Wittenberg (2021) speculated that this compound may perhaps be exuded into the rhizosphere to stimulate the production of EPS which would slow the loss of water around the roots thus potentially protecting both *A. caffrorum* and its microbiome. Thus, in complex environments, diverse organisms may collaborate with each other to overcome the shared stress of water deficit.

Metabolic responses to drought in the rhizosphere and host microbe interactions

Plants can exude up to 50% of their fixed carbon into their rhizosphere to favourably alter the rhizosphere soil chemistry and interact with *inter alia* microorganisms, nematodes and other plants (van Dam & Bouwmeester, 2016). This occurs via passive and active methods and so most low molecular weight compounds found in the roots will also make their way into the rhizosphere (Oburger & Jones, 2018). Furthermore, the composition of these root exudates is the greatest factor which shapes the associated microbiome in the rhizosphere and so is highly relevant to investigating mechanisms of microbial selection (Bi *et al.*, 2021). For example, an increased exudation of organic acids can lower the pH thereby altering the microbiome (Macias-Benitez *et al.*, 2020). Moreover, certain exudates, especially phenolic acids, can be utilized only by specific microorganisms, promoting their abundance while being toxic to other taxa (Mandal *et al.,* 2010). Hence, by altering the metabolic environment within the rhizosphere, host plants can select for potentially beneficial microorganisms that may be beneficial during desiccation or for seasonal adaptation.

However, so far no method exists to measure native root exudates without introducing a significant bias (Oburger & Jones, 2018; van Dam & Bouwmeester, 2016). For example, some of the most cited studies that measure root exudates of plants under drought, did so by firstly removing the roots from the plant and incubating these in solution for 48 hours or longer (Gargallo-Garriga *et al.*, 2018; de Vries *et al.*, 2019; Ghatak *et al.*, 2022). Since the metabolome can react within seconds to the sudden availability of water and the stress of root excision, these very likely alter the native root exudation profiles of plants under water deficit and so may not be that ecologically relevant (Oburger & Jones, 2018). In addition, a myriad of other methods exist that involve re-wetting or disturbing the root system, such as the use of hydroponics, rhizobox systems or microsuction cups that each come with their own biases and limitations (Neumann *et al.*, 2009). Similarly, the method of directly extracting metabolites from soil applied in this study is limited by the fact that it is impossible to discriminate between metabolites of plant or microbial origin, although at least the methodology does not involve re-wetting or disturbing the root system. Consequently, there is no consensus on how to measure root exudates and consequent responses of exudation profiles to drought are hard to generalize.

Nevertheless, a few trends are evident in response to drought, though responses to desiccation are thus far unexplored in the published literature. In response to drought, plants continue to exude carbon into the rhizosphere, sometimes even at increased rates (Karlowsky *et al.*, 2018; Preece *et al.*, 2018; Brunn *et al.*, 2022). However, microorganisms only metabolize this carbon upon rehydration which leads to an explosion of microbial metabolic activity that can assist plants during recovery via increased mineralization of nitrogen and phosphorus (Karlowsky *et al.*, 2018 and references therein).

In addition, the composition of root exudates is also altered under drought. Gargallo-Garriga *et al.*, (2018) report that under drought 71% of root exudates are secondary metabolites whilst upon rehydration these shift to be 81% primary metabolites. These drought induced shifts in exudate profiles can favourably alter microbial activity in the rhizosphere upon rehydration by increasing microbial respiration whilst inhibiting nitrification and thus the loss of available nitrogen to plants (de Vries *et al.*, 2019; Ghatak *et al.*, 2022). Taking all of the above together, plants appear to alter their rhizosphere chemistry during drought to prioritize rapid recovery of their microbiome and thus resumption of favourable interactions upon rehydration. Hence, simultaneously characterizing the metabolome along with the microbiome in the endosphere and rhizosphere can assist gaining a deeper understanding of host microbial interactions. With this in mind, this chapter's aims are given below.

Chapter Aims:

1) Characterize the metabolic responses to fluctuations in water content - especially desiccation.

2) Identify metabolites associated with seasonal adaptations that may also impact the level of desiccation tolerance in the fronds.

3) Identify metabolites that may facilitate communication/selection between *A. caffrorum* and its associated microbes.

4) Compare these metabolic mechanisms to prior studies on *A. caffrorum's* rhizome and fronds.

3.2 Methods

3.2.1 Metabolite quantification by gas chromatography mass spectroscopy

Root/endosphere samples were processed and lyophilized as described in chapter 1. Before metabolite extraction, samples were ground for 2 minutes at 25 Hz in a Retch MM 400 mixer mill. A mass of 50 mg ground endosphere was extracted with 1 mL of pre-cooled (-20 °C) 4:1 methanol:water spiked with 4 µg/mL ribitol. After the addition of the extraction solvent and brief vortexing, samples were kept on an orbital shaker at 1200 rpm for 45 min at 4 °C followed by a 15 min sonication step in an ice water bath. Subsequently the samples were centrifuged at 10 000 g for 15 min at 4 °C and 340 µL of the supernatant was dried down in a SC110 Savant SpeedVac concentrator (Made in Farmingdale, NY City, USA) and stored at -80 °C until analysis. In addition, a further aliquot of 340 µL was removed for hormone analysis for a separate project. From the remaining supernatant, 20 µL were removed from all tubes except from the blank extract and combined to produce a quality control sample.

Similarly, 50 mg of dry rhizosphere per sample was homogenized for 2 minutes at 25 Hz in a Retch MM 400 mixer mill. However, before extraction an additional overnight fumigation with 0.3 mL chloroform under agitation of 200 rpm at room temperature was performed. Following this, the same extraction as above for the endosphere was followed, with the exception that 600 µL of extraction solvent was used of which 510 µL was removed and dried for analysis. No hormone aliquots were removed from these samples. Finally, from the remaining non-blank extract supernatants 30 µL was used to produce the quality control sample.

The dry extracts of both endosphere and rhizosphere were derivatized by methoximation with methoxyamine hydrochloride in pyridine and silylation with N-Trimethylsilyl-N-methyl trifluoroacetamide (MSTFA) as described in Lisec *et al.* (2006).

Following derivatization, 1 µL of each sample was injected in a randomised order into an Agilent 7890A GC coupled with a 7000C triple quadrupole (MS/MS). A split ratio of 14:1 and 9:1 was used for the endo- and rhizosphere samples respectively using a helium carrier gas at a flow rate of 1.0 mL/min. The injection temperature was set at 240°C and metabolite separation was achieved within a J&W DB-5ms column (30 m x 0.25 mm x 0.25 µm). Here, the oven temperature began with a 30 second hold time at 80 °C before a linear increase until 325 °C where it was held for a final 1.5 minutes. After a solvent delay of 5.7 minutes, ionization was achieved by a 70-eV electron beam and spectra were recorded in a full scan mode at 4.8 scans per second, over the range from m/z 70 to 500. Here, the gain factor at the detector was set at two except for between 27.5 and 27.4 minutes where it was set to one to prevent overloading of the sucrose peak in the endosphere samples only.

Chromatograms were evaluated using Agilent's MassHunter 8.0 programme. Peaks were annotated by matching spectra and retention times to standards recently run with the same parameters. In addition, library searches were conducted to the NIST 14.0 and Fiehn spectral libraries and if retention times were parsimonious to an unambiguous match with a match score above 70, these metabolites were putatively annotated by their match. As these metabolites were not confirmed by a reference standard, these annotations are marked with an asterisk. All other peaks were labelled as “UE” and “UR”, for unknown endosphere and rhizosphere peaks respectively. Nevertheless, some of these could be annotated to class level by inspecting their spectra, library matches and retention time. Metabolites were thus annotated to three levels analogous to the Metabolomics Standard Initiative

(MSI) (Sumner et al., 2007). Finally, only peaks with an average signal-to-noise ratio greater than three within at least one collection group were retained in the final data matrix.

3.2.2 Metabolite profiling by liquid chromatography mass spectroscopy (polar & non-polar)

This analysis was performed only on endosphere samples. Here, secondary and lipid metabolites were extracted simultaneously as in Salem *et al*. (2016). Briefly, 1ml of pre-cooled (-20 ^{o}C) 3:1 methyl tert-butyl ether:methanol spiked with 1mg/ml of both isovitexin and 1,2-diheptadecanoyl-sn-glycero-3-phosphocholine was added to each 25 mg of dried ground endosphere sample and briefly vortexed. Next, the samples were incubated in an orbital shaker for 30 minutes at 4^{o}C followed by 15 minutes in an ice water bath sonicator. Following this, 500 µL of 3:1 water:methanol was added and sample tubes were centrifuged for 10 minutes at 10 000g. Finally, 450 µL from each of the resulting two phases were removed and dried in a speedvac. The upper non-polar phase was used for lipid analysis and the lower polar phase was used for analysis of secondary metabolites.

The aliquots of the vacuum-dried polar and non-polar phases (450 µL) were resuspended in either 300 µL of 50% Methanol or 200 µL 7:3 Acetonitrile:Isopropanol for the polar or non-polar LCMS analysis respectively. Samples were allowed to stand at room temperature for 5 minutes before they were briefly vortexed. Thereafter, the samples were incubated in an ice sonicator bath for 4 minutes during which time the whole straphor was again briefly vortexed to result in complete sample resuspension. Following this, the samples were centrifuged for five minutes at 14 000 rpm and 120 µL of the supernatant was transferred to LC vials with insert for analysis. From the remaining supernatants, 7.5 µL was removed and combined to produce two quality control samples for either the polar or non-polar analysis.

The polar extracts were analyzed on a Waters Acquity UPLC system (Waters, Mildford, MA, U.S.A.) as in Giavalisco *et al.* (2009). This system was equipped with a HSS T3 C18 reverse phase column (100 × 2.1 mm i.d. 1.8 µm particle size, Waters) at a temperature of 40 °C. The mobile phases consisted of 0.1% formic acid in water (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B). The flow rate of the mobile phase was 400 µL/min, and 3 µL of each sample was injected per run. Gradient conditions were: 0−1 min hold at 1% B, 1−13 min linear gradient 1%−35% B, 13−14.5 min linear gradient from 35%−70% B, 14.5−15.5 min linear gradient 70%−99% B, 15.5−17 min hold at 99% B, 17−17.5 min linear gradient to 1% B and 17.5−20 min hold at 1% B. This UPLC instrument was connected to an Exactive Orbitrap-focus (Thermo Fisher Scientific) via a heated electrospray source (Thermo Fisher Scientific). The spectra were recorded using full-scan negative ion-detection mode only, covering a mass range from m/z 100 to 1,500. In addition, the quality control samples were also run in positive mode to assist with downstream manual peak annotation of unknown peaks. The resolution was set to 70,000, and the maximum scan time was set to 250 ms. The sheath gas was set to a value of 60 while the auxiliary gas was set to 35. The transfer capillary temperature was set to 150°C while the heater temperature was adjusted to 300°C. The spray voltage was fixed at 3 kV, with a capillary voltage and a skimmer voltage of 25 V and 15 V, respectively. MS spectra were recorded from minutes 0 to 19 of the UPLC gradient.

The non-polar samples were analyzed using UPLC (on a C8 reverse-phase column) coupled with Fourier transform mass spectrometry (Exactive mass spectrometer; Thermo Fisher, http://www.thermofisher.com) in positive ionization mode only as described in Giavalisco *et al.* (2011).

Following this, for both polar and non-polar samples, the processing of chromatograms, peak detection, and integration were performed using RefinerMS (version 5.3; GeneData). Next, peak picking was performed manually by examining the chromatograms of pooled quality control samples in Xcalibur and selecting suitable features from the GeneData output table in Microsoft Excel 2016. Some of the resulting features were identified by aligning the GeneData output to an in-house reference library. Here, we allowed for 10 ppm mass error, and a dynamic retention-time shift of 0.1. This also retrieved additional peaks that were not initially picked above. Of these, only those with an intensity greater than 10^4 in all replicates within at least one sample group were retained in the final data matrix. These additional peaks are marked with a "_L" which indicates that they are only a library hit and not necessarily visible as a prominent peak on the chromatograms. This may be because they have a lower ionization efficiency and/or because they are less abundant than the visible peaks. The remaining unknown peaks of interest were annotated by inspecting the tandem MS (MS/MS) fragments and searching up masses or candidate compounds in MassBank, HMDB and the resurrection plant literature. Again, these are marked with an asterisk, owing to the lack of a reference standard. Here, the retention time, similarity of the spectra and correlation to compounds of the same class was taken into consideration.

3.2.3 Data processing and analysis

The three annotated data matrices from each chromatographic platform were normalized by their internal standards before combining them and removing multiples of the same feature. In some cases, the same metabolite was detected across multiple platforms. In such cases metabolites (in particular primary metabolites) originating from GCMS were preferred over those detected across the LCMS platforms, as these were generally better resolved by the former. Missing values were then replaced by 1/10th of the minimum value across all samples for a given feature and finally log2 transformed. All downstream statistical analysis (explained in detail in the results and discussion section) was performed in the R statistical computing software (version 4.3.1; R Core Team 2023), with the exception of the pathway analysis which was performed in MetaboAnalyst 5.0 (Xia *et al*., 2009).

3.3 Results and discussion

3.3.1 Feature annotation

Across the three chromatographic platforms, 621 endosphere features passed the quality filtering and were quantified. Of these, 69% were identified by matching retention time and spectra to a reference standard previously run by the same method on the respective platform. A further 4% could be identified by manually comparing a peak's spectrum to reference spectra available in libraries and online databases. Lastly, of the remaining unidentified peaks, an additional 3% could be identified to a metabolite class level only.

In the rhizosphere, 81 features detected by GCMS passed quality filtering. Of these, 30% were annotated by matching spectra and retention time to a reference standard previously run by the same method. A further 16% could be identified by conducting library searches whilst again taking retention time into consideration. The remaining features could not be identified, although around 8% of these could at least be annotated to a metabolite class level.

3.3.2 The effect of water content on the endosphere metabolome

These 621 features allowed for all samples to cluster by their collection when subjected to principal component analysis (Fig. 1A). This indicates that with the exception of the potential outlier 2.3E in collection 2, the metabolome is influenced primarily by factors captured within each collection and not for example by sub-site specific effects.

Across collections, we were specifically interested in how the metabolome is influenced by water content in addition to any seasonal or DT/DS effects. Between these two factors, the effect of water content may be the most influential, as the hydrated and particularly the desiccated samples clustered more closely to another relative to the other hydration state, whilst there is no clear grouping by season (Fig. 1A). Interestingly, the fact that the 95% confidence interval of collection three overlaps with the desiccated samples, suggests that *A. caffrorum* may not have fully recovered at the level of the metabolome by this stage. This is also reflected by the on average lower frond RWC in collection 3 compared to collection 1 (Fig. 2; Chapter 2).

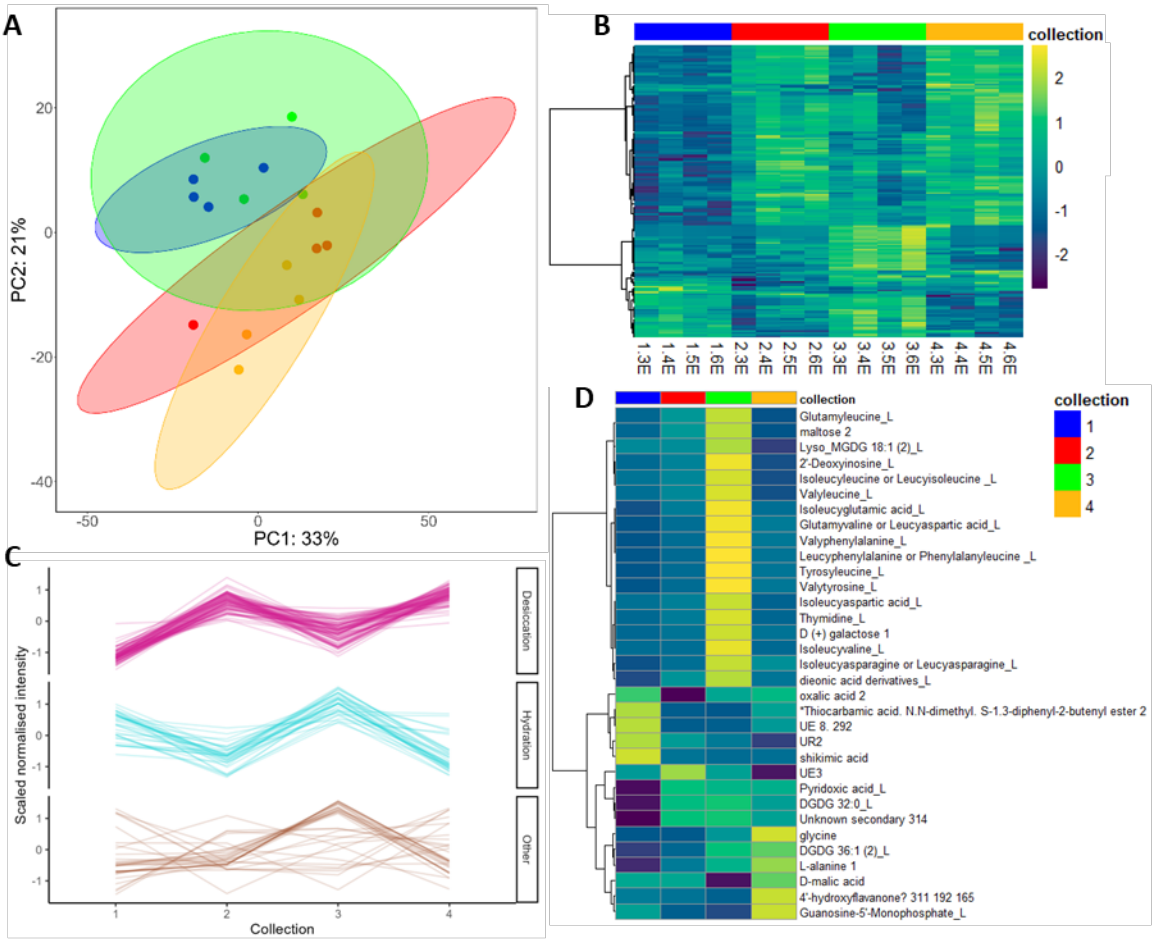

Figure 1 (A-D). Key drivers of metabolite patterns position hydration state as the most influential variable. (A) Principal component analysis of all endosphere features. (B) a heatmap depicting only the significant metabolites identified by ANOVA and (C) their average intensity per collection coloured by their induction pattern. Here desiccation induced metabolites are coloured violet. Hydration induced metabolites are coloured turquoise and all remaining significant metabolites are coloured brown. (D) Collection wise metabolite means of features present in pattern "Other".

In order to identify which metabolites are driving these patterns, analysis of variance (ANOVA) was performed. This yielded 165 metabolites that exhibited differences across collections with a BH corrected p-value of less than 0.05. To visualize their patterning, these were plotted in a heatmap (Fig. 1B). A heatmap with metabolite names is provided in the supplementary section (Supp. Fig. 1). Here, the first two branches of the hierarchical clustering roughly represent features that are either increased in the desiccated (top cluster) or the hydrated state (bottom cluster) (Fig. 1B). This again highlights that the metabolome is primarily patterned by fluctuations in water content.

Nevertheless, other patterns were present within these two clusters. Therefore, in order to extract only patterns of interest and isolate the others, metabolites were identified that were either accumulated in both desiccated tissues or in both hydrated tissues relative to the other hydration state. This was done by searching for differentially abundant metabolites that either followed the trend DOWN-UP-DOWN-UP (desiccation induced) or UP-DOWN-UP-DOWN (hydration induced) in their means across collections 1-2-3-4. These are plotted in Figure 1C and confirm that most differential metabolites followed either one of these two above trends. Interestingly, many hydration induced metabolites appeared to have a marginally higher abundance in the collection three relative to collection one. This could reflect a strong re-activation of metabolism upon rehydration from the desiccated state.

However, additional trends were revealed when inspecting the remaining differential metabolites, which were grouped into the “Other” pattern (Fig. 1C). These metabolites are visualized in another heatmap (Fig. 1D). More than half of these were exclusively increased upon rehydration in collection 3. This spike was mostly attributed to the accumulation of a number of dipeptides. In addition, the sugars maltose and galactose were also accumulated. This pattern might simply reflect the rapid breakdown and turnover of proteins and carbohydrates that may be required to restart metabolism after desiccation.

Thus far, it is evident that fluctuations in water content predominantly shape the metabolome of *A. caffrorum*, with these responses notably intensified following a period of desiccation. Subsequently, pathway enrichment analysis was conducted to investigate if specific metabolic pathways are overrepresented within these water content driven patterns. However, most compounds (particularly the lipids and dipeptides) could not be mapped to a KEGG pathways. Therefore, these patterns were also condensed via enrichment at the metabolite class level. This then also allowed the incorporation of metabolites that could only be identified at a class level.

Intriguingly, metabolic pathways enriched during the hydration induced pattern were also activated during desiccation, albeit in the opposite direction. Notably, 'galactose metabolism' and 'starch and sucrose metabolism' exhibited evidence for enrichment under both patterns, with p-values less than 0.01 (Fig. 2 A&B).

In the hydrated state, metabolites such as glucose, UDP-(glucose/galactose), fructose and glycerol were maintained. However, upon desiccation, these pathways shifted to produce primarily sucrose (which was by far the largest GCMS peak) via its intermediate sucrose-6-phosphate in addition to raffinose, trehalose, sorbitol and sorbitol-6-phosphate. This is in line with a general enrichment of reducing monosaccharides in the hydrated state (Fig. 2C) that are then converted to non-reducing and thus less reactive di- and trisaccharides (via their phosphorylated intermediates) during desiccation. This shift is likely to prevent these reducing monosaccharides from reacting with other metabolites and cellular components leading to the destructive accumulation of reactive oxygen species (ROS) (Van der Toorn & McKersie, 1995).

In addition, these desiccation induced sugars and sugar alcohols likely also serve a myriad of other protective functions. Firstly, these molecules can act as osmolytes and initially slow the loss of water (Oliver *et al.*, 2020). Secondly, their hydrogen bonds can replace water and thus stabilize membranes, proteins and other structures as water is removed and thus also preserve their function (Hoekstra *et al.*, 2001). Thirdly, they have been implicated in forming cytoplasmic glasses which stabilize cell structure and assist in halting metabolism and thus spreading of ROS in the desiccated state (Berjak, 2006). Lastly, many of these have known antioxidant activities and could thus also contribute to preventing oxidative damage by scavenging ROS (Keunen *et al*, 2013). Hence, sugars and sugar alcohols play a major role in combating the deleterious effects of water deficit in *A. caffrorum*.

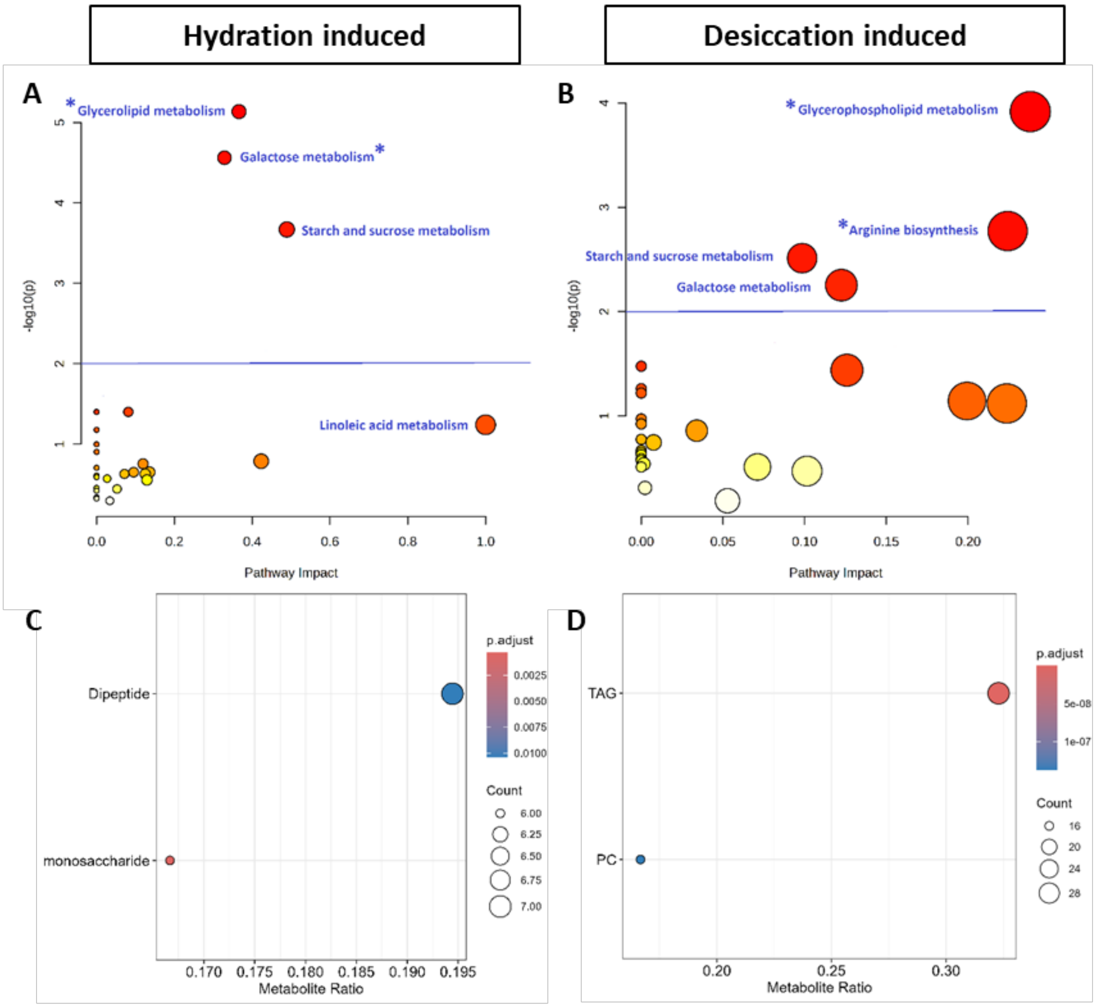

Figure 2 (A-D). Water content modulates lipid, carbohydrate and nitrogen storage
Enriched pathways (A & B) and metabolite classes (C &D) in the desiccated and hydrated roots respectively. In (A & B), the blue line represents a p-value = 0.01, and metabolic pathways with and FDR adjusted p-value < 0.05 are marked with an asterisk.

Similarly, lipid metabolisms related pathways also exhibit a seesaw regulation by water fluctuations. In the hydrated state, glycerolipid metabolism is enriched due to higher abundance of some mono- and digalactosyldiacylglycerols (MGDGs & DGDGs), glycerol, UDP-glucose/galactose and 1-

monopalmitin. In addition, although not part of this pathway, ethanolamine, which is used to synthesize phosphatidylethanolamines (PEs), was also elevated in both hydrated collections (Supp. Fig. 1). Moreover, although their class was not significantly enriched, three prominent fatty acid peaks detected via GCMS were increased in hydrated roots. These fatty acids were palmitoleic acid, oleic acid and linoleic acid (Supp. Fig. 1). Since only the latter could be mapped to a KEGG pathway, the 'linoleic acid metabolism' pathway received a non-significant hit. Nonetheless, this pathway had a maximum impact value and as such has more potential to influence metabolism.

MGDGs and DGDGs are produced in plastids and form the primary components of chloroplastic membranes. As the roots are not exposed to the sun, these are likely produced by non-photosynthetic plastids in *A. caffrorum's* roots, as seen in other studies on *A. thaliana* (Awai *et al*., 2001). Here, their function in roots has been proposed to conserve phosphate reserves by replacing phospholipids in the membranes that facilitate trade with microorganisms (Gaude *et al*. 2004). Interestingly, fatty acids are also a commonly traded carbon source provided by plants to particularly fungi in exchange for phosphate and nitrogen (Jiang *et al.*, 2017). Therefore, a possible explanation for the elevation of these metabolites in the hydrated roots could be related to trade with microbial endophytes which likely assist *A. caffrorum* in growth processes and also in recovery upon rehydration.

Upon desiccation however, this trend is reversed, and instead the Triacylglycerol (TAG) as well as phosphatidylcholine (PC) lipid classes are significantly enriched (Fig. 2D). These comprised nearly half of all metabolites significantly accumulated during desiccation (Supp. Fig. 1). These PCs along with the increase in some PEs as well as their lyso derivatives (LysoPC &LysoPE) resulted in the Glycerophopholipid metabolism pathway being significantly enriched in the desiccated roots (Fig. 2B). Such accumulation of TAGs and phospholipids appears to be a conserved response to desiccation in resurrection plants (Quartacci *et al.*, 1997; Gasulla *et al.*, 2013; Madden, 2019).

This suggests that perhaps *A. caffrorum*, and other resurrection plants, may store carbon in the form of TAGs during desiccation. The accumulation of PCs and other phospholipids could simply reflect the increased membrane surface area and thus phospholipids required to store these TAGs in intracellular vesicles. As in seeds, this storage may provide an energy source to survive prolonged periods of quiescence whilst also allowing for rapid recovery upon rehydration (Harwood & Moore, 1989). Upon rehydration, they could, as in seeds, provide a carbon source before photosynthesis is resumed. In *A. caffrorum*, the TAG 52:2, TAG 54:3 and TAG 54:2 may be the primary forms of storage in roots, as these were all in the top ten largest lipid peaks detected and had peak intensities above 10^9.

TAGs are either stored as cytosolic lipid droplets (LDs) or within plastid plastoglobuli (PGs) – although not much is reported in the latter in belowground organs. The presence of LDs however, is reported in the oil rich DT tubers of yellow nutsedge (*Cyperus esculentus*). Interestingly, this lipid storage within LDs is also associated with the DT of yellow nutsegde, as its sister species, purple nutsedge (*Cyperus rotundus*), does not store lipids and is DS (Niemeyer *et al.*, 2022). Therefore, the below ground storage of TAGs in LDs may be also an essential feature of *A. caffrorum's* DT and should be explored in other resurrection plants.

Moreover, the increase in TAGs may not only relate to energy storage, but may also assist in combating desiccation stress. During plant stress, LDs and PGs can provide unique environments for specialized metabolism that could then in turn assist in the stress response (van Wijk & Kessler, 2017). When comparing the LD proteomes of tubers between the DT yellow nutsedge and the DS purple nutsedge, the proteome of the former was more similar to that of *A. thaliana* seeds (Niemeyer *et al.*, 2022). Given the overlap in the regulation of seed maturation and vegetative desiccation tolerance, this suggests that LDs could participate in the response to desiccation (Illing *et al.*, 2006).

In addition, lipid storage in LD’s may also be involved in mechanical stabilization of membranes. A common phenomenon observed across multiple resurrection plants is increased vacuolation during desiccation. *Craterostigma wilmsii*, *Myrothamnus flabellifolius* and *Xerophyta humilis* accumulate “non-aqueous” cytoplasmic vesicles (i.e. LDs) and plastoglobuli during desiccation in their leaves and rapidly degrade these upon rehydration (Farrant *et al*., 2000; Sherwin & Farrant, 1996). These vesicles were arranged along the cytoplasmic and chloroplastic membranes and thus, the authors proposed that these serve as means of mechanical stabilization against desiccation driven plasmolysis.

Upon desiccation, the accumulation of LDs and PGs were also observed in *A. caffrorum’s* fronds (Farrant *et al.*, 2009). However, their presence could not be detected in the desiccated rhizome (Shoko, 2015). Their absence in the rhizome could however be due to the lower quality of these TEM images. Furthermore, as mentioned in the introduction of this chapter, Khan (2023) identified a number of photosynthesis related transcripts some of which such as ACTIVITY OF BC1 COMPLEX KINASE 1 are localized to PGs. Here this kinase is involved in the stress response by regulating specialized metabolism such as the synthesis of antioxidants within PGs (Martinis *et al.*, 2014; Lundquist *et al.*, 2013). Thus, although LDs or PGs have not yet been visualized in below ground organs of *A. caffrorum*, it is possible that they house the accumulated TAGs and participate in in desiccation protective functions via either mechanical stabilization or specialized metabolism.

The increase in TAGs could also in part be attributed to the decrease in MGDGs and free fatty acids during drying. These more reactive lipids are likely a converted to more inert TAGs to prevent their unfavorable activity during quiescence in a crowded metabolic environment. In many DS plants such as *Arabidopsis thaliana*, MGDGs in particular are known to be converted to TAGs stored in LDs when exposed to drought, heat and salt stresses (Mueller *et al.*, 2015). This is because, during stress, ROS accumulation leads to the cleavage of galactose from MGDGs to produce diacylglycerols (DAGs). DAGs however do not form spherical micelles and so lead the destabilizing of chloroplastic membranes. Consequently, DAG’s are converted to TAGs which can safely accumulate in LD’s without disrupting membranes. This pattern of MGDG and fatty acid degradation during desiccation is consistent across other resurrection plants (Quartacci *et al.*, 1997; Gasulla *et al.*, 2013; Tshabuse *et al.*, 2018; Madden, 2019). Therefore, the reduction of MGDGs and free fatty acids also likely contributes to TAG accumulation in *A. caffrorum* upon desiccating. Upon rehydration however, these TAGs are broken down and likely provide the components to restore MGDGs and fatty acid pools which in turn may facilitate trade with microbes.

Nevertheless, it is important to mention that none of the detected MDGDs were visible as prominent peaks in the non-polar LCMS chromatograms and the only DGDGs that produced clearly defined peaks were not differentially abundant. Thus, other carbon sources such as fatty acids are likely used for TAG synthesis and *A. caffrorum* constitutively maintains DGDGs over MGDGs regardless of water content. In other DS plants, MGDGs are typically more abundant than DGDGs. However, during stress MGDGs are converted to DGDGs as this additional galactose moiety also prevents the MGDG head group cleavage and thus membrane destabilization. Therefore, *A. caffrorum* may constitutively maintain DGDGs over MGDGs to maintain metabolic readiness for desiccation and possible microbial trade across all water contents and seasons.

Finally, three of the amino acids part of the arginine biosynthesis pathway were increased during desiccation (Fig. 2B; Supp. Fig 1). These were L-arginine, L-aspartate and L-ornithine. These all have higher nitrogen to carbon ratios and arginine in fact has the highest such ratio of all amino acids. Therefore, it is ideal for the storage and transport of nitrogen. In seeds, plants and their roots, arginine and glutamic acid are the most abundant free amino acids responsible for nitrogen storage (VanEtten *et al.*, 1963; Nordin & Näsholm, 1997; Bausenwein *et al.*, 2001). Interestingly, arginine appears to be favored over glutamic acid for nitrogen storage during dormancy in poplar trees. In autumn, nitrogen is translocated from senescing leaves to non-senescent perennial tissues and converted to arginine for storage over the winter period (Couturier *et al.*, 2010). During the winter, these trees enter a state of dormancy and as such, arginine may be particularly suited for nitrogen storage during dormancy. In addition, they may also desiccate their perennial tissues to survive freezing. Given that *A. caffrorum* also enters a state of dormancy and desiccation over the dry summer period, it is possible *A. caffrorum* likewise stores nitrogen via this pathway. This higher nitrogen to carbon ratio may help offset senescence in the roots (Chen *et al.*, 2015).

Furthermore, this available nitrogen may be crucial upon rehydration. In the dry season, *A. caffrorum* frequently experiences short periods of water availability through either rain or even mist. In response to this, they briefly open their fronds and likely resume photosynthesis. Therefore, perhaps these mobile nitrogen sources are stored in the dry state so that they may be available for rapid translocation to the fronds upon rehydration. This could allow for the production of nitrogen rich chlorophyll and capitalize on a potentially limited window of water availability.

Taken together, water content fluctuations necessitate major changes to carbohydrate, lipid and nitrogen metabolism and so thus far present the most influential factor shaping the metabolome.

3.3.3 Non-water content related and DS/DT specific patterns in the endosphere

Interestingly, the "Other" pattern visualized in Figure 1C and 1D also revealed that seven metabolites were specific to either DS or DT collections. These may be linked to a DS or DT specific function or may be linked to a functions required during the rainy winter or dry summer seasons. Given the the nature of the metabolites, the latter seems more plausible here.

Collection 1 exclusively contained four organic acids elevated relative to the other collections (Fig. 1D). Small organic acids are known to be exuded by plants to solubilize phosphorus and other minerals in addition to affecting microbial community structure through pH changes and chemotaxis (Jones *et al.*, 2003). These may assist *A. caffrorum* in its growth phase during the wet season.

In addition, "thiocarbamic acid, N,N-dimethyl, S-1,3-diphenyl-2-butenyl ester" may also have insecticidal and antiviral properties (Abdullah, 2019; Aseel *et al.*, 2023). Interestingly, in both these studies this compound was isolated from growth promoting fungi and, in the latter, presented the most prominent peak in the extract of *Trichoderma viride*. Therefore, it is possible that it may in fact be synthesized by endophytic fungi within *A. caffrorum's* endosphere. Nonetheless, these functions may be more important during a consistently wet period when *A. caffrorum* may be more heavily invested in growth, and also experience greater biotic stress. This aligns with findings from the proteome, which showed that processes related to combating biotic stress such as "defense response to fungus, incompatible interaction" were significantly enriched in winter (wet season) rhizomes relative to summer (dry season) rhizomes (Shoko, 2015).

The three metabolites exclusively elevated in the DT roots may also not be of great relevance to the acquisition and regulation of DT. Here, pyridoxic acid is an antioxidant commonly accumulated in response to stress (Havaux *et al.*, 2009). The DGDG is of much lower intensity compared the constitutively maintained DGDGs and so is likely not very influential. Lastly, the third DT specific compound was an unknown secondary metabolite which again is hard to interpret.

A likely explanation for why no strong DS signal is observed could be that the roots, just as the rhizome, are tolerant all year round. Shoko observed "well preserved" rhizome cortical cells in both DS and DT plants during desiccation, whilst TEM images taken by Farrant *et al.* of DS fronds showed membrane damage and no recovery upon rehydration (Farrant *et al*, 2009; Shoko, 2015). This demonstrated that unlike the fronds, the rhizome is tolerant all year round. The metabolomics results in this study are akin to the absence of strong DT or DS related signals observed in the rhizome's metabolome (Wittenberg, 2021). Therefore, it is very likely that the roots are also desiccation tolerant across all seasons.

3.3.4 Metabolic profiling within the rhizosphere

Unfortunately, the rhizosphere metabolic profiles were likely altered during storage. Relative to the endosphere, the rhizosphere tissue is inherently more particulate. Although all samples were freeze dried during reduction to an air dry state, the fine particulate rhizosphere can re-absorb water more quickly, particularly when handled at room temperature directly after cold storage. Unfortunately, the only way to effectively remove tiny root fragments contaminating the rhizosphere was to manually pick these out. This compartment separation procedure took approximately 6h per sample and was performed at room temperature. Furthermore, because particularly the DNA extraction required many optimization attempts, samples underwent freeze thaw cycles following this. Given that the samples were supposed to be dry and therefore enzymes would have no solvent to function in, this was assumed safe. Unfortunately, when comparing GCMS chromatograms run from the same sample before long term storage to a few months later, a clear reduction in particularly organic acids and many other metabolites was observed (data not shown). Fortunately though, no difference was seen in the endosphere samples, likely because it was not stored in a ground state. Following this observation, all samples were re-dried which confirmed that all rhizosphere samples, but not endosphere samples, had gained a few percentiles of water. Since many small molecules can be degraded by metabolism in a fraction of a second, it is in hindsight not surprising to find such changes occur over many months. Fortunately though, DNA is less likely to have undergone major changes and in fact metagenomics samples are commonly stored for years at room temperature (Lindahl *et al.*, 2013). Nevertheless, some of the major and perhaps more stable metabolites could still be quantified in the rhizosphere and thus these results are presented and discussed.

The remaining metabolites within the rhizosphere, suggest that water content fluctuations have less of an effect here compared to the endosphere - except upon rehydration (Fig. 3A). This is evidenced by the closer clustering between the desiccated samples and collection 1. Collection 3 however, is discriminated by the first principal component from the others, albeit its 95% confidence interval overlaps with the other collections.

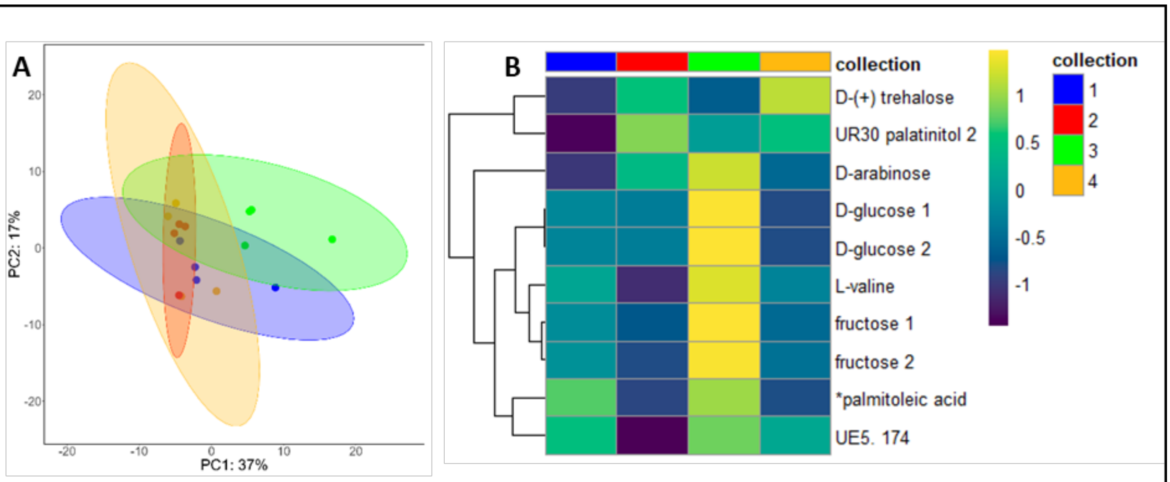

Figure 3 (A & B). Water content, particularly upon rehydration, drives distinct metabolic profiles. (A) Principal Component Analysis of all rhizosphere metabolites detected by GCMS. (B) A heatmap of all significant (BH p-value < 0.05) metabolites identified by ANOVA.

In order to identify if any metabolites differ between collections, ANOVA was performed. Here, only eight metabolites were significant (BH p-value < 0.05) (Fig. 3B). Of these, some exhibited identical abundance patterns to those in the endosphere (r > 0.9 & p-value < 0.05) and are thus likely exuded by *A. caffrorum* into the rhizosphere by the roots. These were: glucose, fructose, palmitoleic acid and trehalose.

Trehalose however, may in fact not be produced by *A. caffrorum*. This is because, in contrast to the endosphere, it is not accumulated alongside sucrose during desiccation. This was in spite of the fact that sucrose was by far the largest endosphere peak in the GCMS chromatograms. Thus, unless the roots (or *A. cafforurm's* endophytes) selectively exude trehalose over sucrose, it may in fact be produced by microbes alongside a putative sugar alcohol labelled “UR30 palatinitol 2”. Both bacteria and fungi accumulate trehalose and sugar alcohols in during desiccation and so this potentially reflects a microbe specific response to desiccation in the rhizosphere (Laskowska & Kuczyńska-Wiśnik, 2020; Niederer *et al.*, 1992; Tapia *et al.*, 2015).

Furthermore, this calls into question the origin of trehalose in the endosphere. A comparative metabolomics study in plants by Dace *et al.* (2023), including seven resurrection plants and two DS species, found that in response to water deficit, all plants either accumulate raffinose or trehalose but never both. Furthermore most plants favour the raffinose production over trehalose (Dace *et al.*, 2023). Therefore the accumulation of both in the endosphere may be the result of microbial trehalose production alongside *A. caffrorum's* production of raffinose.

The remaining metabolites likely exuded by the endosphere (glucose, fructose and palmitoleic acid) are all increased in hydrated tissues, but are particularly abundant upon rehydration. These carbon sources are possibly released by the endosphere upon rehydration to promote microbial activity that may allow *A. caffrorum* more access to phosphorus and nitrogen which in turn may assist *A. caffrorum* in rapidly restarting photosynthesis and growth processes.

Valine was also significantly increased upon rehydration in the rhizosphere although not within the endosphere. Therefore, it is also likely of microbial origin. However, its function here and within the literature remains unclear without further functional testing. For example in the rhizosphere of *Quercus ilex*, it is accumulated during acute drought and after seven days post rehydration relative to control plants (Gargallo-Garriga *et al.*, 2018). Nonetheless, its accumulation here is noted for future studies.

Potentially also of note, was an unknown compound with a prominent peak detected in the rhizosphere whose closest library hit was the antibiotic chloramphenicol. Although, this compound was not differentially abundant across the collections, its relation to an antibiotic potentially has an influence on the microbiome and so is also noted here (Fig. 4A). Furthermore, as antibiotics such as chloramphenicol are highly unstable molecules and so possible significant differences between collections were lost due to the treatment of the rhizosphere samples.

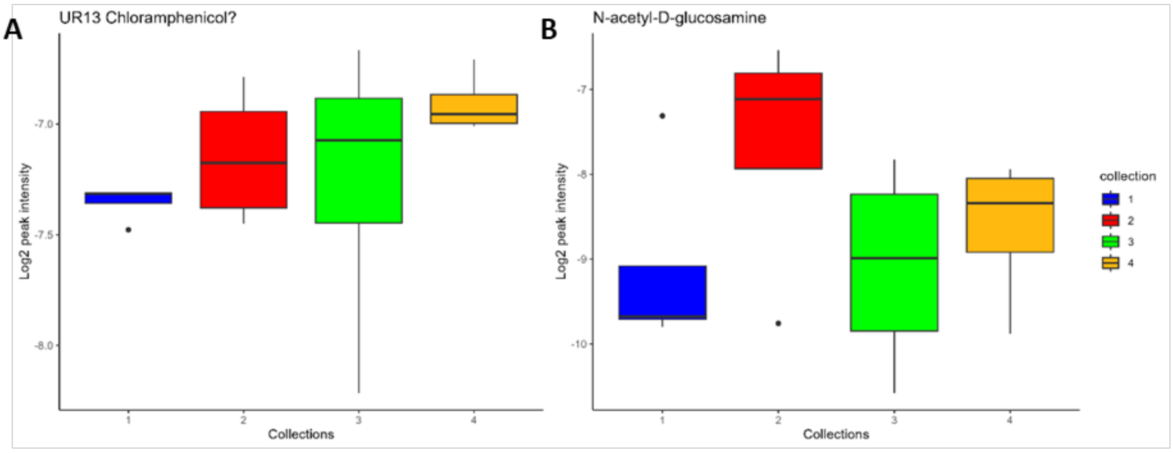

Figure 4 (A & B). Select non-significant metabolites detected in (A) the rhizosphere and (B) the endosphere.

Lastly, a hypothesis of EPS stimulation during desiccation by exudation of a compound annotated as N-acetyl-D-mannosamine (although not verified by a standard) was introduced earlier from prior metabolomics work in the rhizome (Wittenberg, 2021). Unfortunately though, this compound was not detected in the rhizosphere. However, an epimer of this compound, namely N-acetyl-D-glucosamine was detected in the endosphere although not identified as differentially abundant across collections (Fig. 4B). Interestingly though, this compound still appeared to increase in the desiccated collections in line with the original hypothesis and so perhaps the jury is still out on this theory.

3.4 Conclusions

In summary, our analysis of *A. caffrorum's* endosphere metabolome highlights the significant influence of water content fluctuations. Specifically, pathways relating to sugar, lipid and nitrogen storage/metabolism are activated to dynamically respond to hydration changes. In the hydrated roots and particularly in the rehydrated roots, reducing monosaccharide sugars, monogalactosyldiacylglycerols and components of lipids such as free fatty acids, glycerol and ethanolamine and are maintained. These may facilitate trade with microorganisms and growth processes. Upon desiccation however, these are mobilized to desiccation-protective non-reducing di- and tri-saccharides, and larger lipid molecules such as triacylglycerols and phospholipids. Thus, the complexity and size of metabolites increases in response to desiccation. The significance of this shift on the microbiome will be discussed in chapters four and five.

While fluctuations in water content strongly shape metabolic profiles, seasonal effects are limited, likely because the roots, like the rhizome, are also tolerant all year round. Nevertheless, a few metabolites suggest a focus on nutrient acquisition and combating biotic stress during frond desiccation sensitivity in the rainy/winter season, whilst during the dry season the abiotic stress of desiccation is prioritized.

Lastly, although the rhizosphere metabolome is compromised, it still supports the suggestion that sugars and fatty acids are exuded, which may shape microbial community structure. In addition, microbe specific responses to desiccation were also detected and highlight that some prominent metabolites such as trehalose could be of microbial origin.

Overall, these results demonstrate the intricate metabolic adaptations of *A. caffrorum* to fluctuating water availability, seasonal stressors and possible microbial interactions. Illuminating such mechanisms allows for a better interpretation of *A. caffrorum's* microbiome. This will lead to a more holistic understanding of plant responses to stress and so facilitate crop improvement.

Chapter 4

Microbial responses to desiccation and seasonality in association with *A. caffrorum*

4.1 Introduction

The following chapter adds a characterization of microbial dynamics to the prior physiological and metabolic descriptions of *A. caffrorum's* endosphere and rhizosphere. The chapter begins by providing a background on microbiome research methodologies and findings in relation to plant-microbial interactions during water deficit. Next, after describing the methods of this chapter, the results of the bacterial and fungal dynamics during desiccation and across seasons are discussed within the context of the metabolome, although a more complex joint analysis is advanced in Chapter 5. Finally, conclusions are drawn and linked back to the initial problem statements of this thesis; that is, to identify solutions to agricultural improvement.

Introducing metabarcoding and its limitations within this study

The community of microorganisms within an environment is collectively known as the microbiome and can be quantified in various ways. The earliest studies characterized the microbiome via culture dependent methods that typically attempted to isolate as many microorganisms as possible on select growth media. However, whilst these approaches have some advantages such as yielding absolute abundances and only measuring the living taxa, they only capture a small proportion of the total bacterial and fungal diversity and are biased by the choice of the isolation media (Jackson *et al.*, 2006). Moreover, determining the absolute abundance of fungi via culture based methods is complicated by the fact that fungi may differ in their sporulation rates. For example, a fungus that occupies a large proportion of the fungal biomass may only produce relatively few spores and so its absolute abundance would be underestimated in this scenario by culture based methods. Therefore, the use of culture independent methods such as metabarcoding are preferred to acquire the taxonomic profiles of an environment.

Metabarcoding refers to the quantification of specific DNA barcodes from an environmental sample. For bacteria and archaea, the small subunit (SSU) 16S rRNA gene is typically chosen, whilst for fungi the internal transcribed spacer region (ITS) between the SSU and the large subunit (LSU) is most commonly used (Schoch *et al.*, 2012). Individually, these metabarcoding approaches are referred to as 16S and ITS amplicon sequencing respectively. These involve firstly isolating genomic DNA from a sample, followed by the amplification of the specific barcodes from this DNA via polymerase chain reaction (PCR) and subsequently purification of these amplicons. These purified amplicons can then either be used to create a clonal library which can be more laboriously sequenced via first generation sequencing technologies, or these amplicons can be directly sequenced via second or third generation sequencing platforms. Therefore, if truly universal barcode primers are used in the initial PCR step, in theory all microorganisms in an environment with that specific barcode can be detected, provided that the DNA extraction can lyse the cell wall of every microorganism present.

However, 16S and ITS amplicon sequencing are not without their own biases and limitations. Particularly for the latter, truly universal primers do not exist and unfortunately most studies use fungal primers designed in the 1990s when relatively few fungal sequences were available and so have well documented biases and mismatches towards certain taxa (Bellemain *et al.*, 2010; Tedersoo & Lindahl, 2016; White *et al.*, 1990; Gardes & Bruns, 1993). Consequently, primer choice rather than the experimental treatment has the largest effect on determining the microbiome composition and so it is incumbent upon researchers to carefully consider their primer choice in their experimental design and when interpreting their data (Li *et al*., 2020).

Moreover, regardless of primer choice, the PCR step inevitably introduces further biases as particularly the ITS region displays considerable variation in length and so taxa with longer ITS regions are often excluded since PCR preferentially amplifies smaller amplicons (Tedersoo & Anslan, 2019). Therefore, studies that use second generation sequencing platforms that can only accommodate shorter reads typically choose either the ITS1 or ITS2 sub regions as otherwise the full length ITS region is too long for some taxa. However, this introduces further bias as the results of these two sub regions can sometimes contradict each other and currently there is no consensus on which region is better (Tedersoo *et al.*, 2016; Mbareche *et al.*, 2020). Similarly, full length sequencing of the 16S region is not possible via second generation platforms and requires selection of one or more of the nine hypervariable sub-regions where again each hypervariable region introduces its own bias (López-Aladid *et al.*, 2023).

Therefore, this study employed the use of third generation sequencing, also known as long read sequencing, via PacBio's Sequel II instrument. This allows for the full length 16S and ITS regions to be sequenced and was outsourced to Inqaba Biotec^{TM}. However, since particularly primers for the ITS region are prone to biases, we requested they use the recently developed ITS1catta and ITSngsUni forward and reverse primers which critically avoid amplifying an intron present in only *Ascomycota* and thus avoid biasing against this phylum (Tedersoo & Anslan, 2019). Moreover, these primers have the added benefit that they do not amplify plant ITS regions. For the full length 16S amplicon sequencing, we opted for Inqaba's 27F and 1492R primers which are the industry standard although unfortunately they amplify the plant chloroplastic 16S region and do not amplify the archaeal barcodes.

A further limitation of all metabarcoding studies is that the because of the initial amplification of environmental DNA, the data is only compositional and does not provide absolute abundances of taxa. Moreover, the resulting data is often zero-inflated and difficult to normalize leading to challenges in the statistical analysis and inconsistencies across studies (Xia, 2023). Thus, these limitations need to be carefully considered when interpreting all results from metabarcoding studies.

With these limitations in mind, some background on the plant microbiome and its responses during drought/desiccation stress is provided.

Introducing the plant microbiome

Microorganisms colonize all external and internal regions of plants. These regions can be classified into distinct spheres. The phyllosphere refers to all above ground surfaces of the plant, whilst the rhizosphere, which as mentioned in chapter two, refers to the community of soil microorganisms that occur roughly within 5mm of the roots. A subset of the rhizosphere community can colonize the root surface known as the rhizoplane. The endosphere on the other hand, refers to the internal tissues of plants and these microorganisms are referred to as endophytes. These endophytes inhabit primarily the intercellular spaces within plants and specifically in roots, bacteria and fungi are sometimes accommodated in specialized nodule structures which greatly improve plant nutrient acquisition (Lerouge *et al.*, 1990; Valla *et al.*, 1989). Thus, the root endosphere may be the most impactful on plant health.

Consequently, in addition to the rhizosphere, this study specifically targets the root endosphere, although for brevity this region is simply referred to as the endosphere in this thesis. All other microbes in the soil surrounding a plant occur in so called “bulk soil” although in this study this sphere specifically refers to microorganisms at a distance of 20 cm from each plant sampled.

With the exception of the bulk soil, all these regions are reported to be in constant and complex interactions with their host plant. This is not to say though that bulk soil microorganisms have no influence on plants, as these are still involved in soil processes and ecosystem functions that ultimately influence plant health (Orwin *et al*, 2016; FAO, 2020). Still, the bulk soil can serve as a control to assess the effect of plant—microbe interactions as it serves as a proxy for the environment outside direct plant influence.

According to these definitions, numerous beneficial effects of individual bacteria and fungi and the microbiome more broadly were identified and discussed in chapter 1. Such is the magnitude of these effects that plants have been referred to as holobionts and their microbiome as a second genome (Lyu *et al.*, 2021). Given that the ultimate aim of this thesis is to produce knowledge that can lead to crop improvement, in particular to drought stress, understanding the plant microbiome’s involvement in this stress responses is integral to achieving crop improvement and thus guarding against food insecurity.

Microbial dynamics under water deficit and their role in plant health

Under water deficit, including desiccation, the bacterial community structure is usually altered whilst fungi tend to be more stable (Jaeger *et al.*, 2023; Barnard *et al.*, 2013; Naylor *et al.*, 2017). This has been attributed to the fact that compared to bacteria, fungi are generally slower growers, have thicker cell walls that can resist the loss of water and can also distribute water throughout their hyphal network (Guhr *et al.*, 2015 and references therein). These microbial shifts under drought are often accompanied by an increase of saprotrophic microorganisms (Jaeger *et al.*, 2023).

In line with this trend, are the bacterial responses to water availability. Water deficit generally increases the abundance of gram positive bacteria, in particular from phylum *Actinomycetota* (also known as *Actinobacteriota*), which relative to gram negative bacteria tend to be slower growers, have thicker cell walls and have increased saprophytic activity (Barnard *et al.*, 2013; Naylor *et al.*, 2017; Santos-Medellín *et al.*, 2021). Given that these microbial trends occur in both soils and when in association with plants under water deficit, this suggests that these are at least in part unrelated to plant selection mechanisms and likely driven by the inherent differences in their drought/desiccation tolerance which likely relate to their cell wall architecture, growth rates and trophic modes.

Interestingly, such microbial shifts in droughted soils are often associated with increased plant growth when these soils are used to grow plants under well-watered conditions (Ricks & Yannarell, 2023; De Long *et al.*, 2019). Furthermore, rhizosphere soil collected from plants growing in more arid regions produced plants with enhanced plant growth and drought tolerance under well-watered conditions in a greenhouse experiments (Zhang *et al.*, 2022; unpublished findings from our lab). Moreover, when microorganisms are isolated from resurrection plants which experience desiccation, similar plant growth promoting and drought protective effects are observed (Fernandes-Júnior *et al*., 2015; Lozo *et al*., 2023; unpublished findings from our lab). These results suggest that microorganisms associated with drier environments have clear benefits to plants. This raises the question of whether or not there is any host plant involvement in the selection of such microbial benefits.

To answer this question, Ricks & Yannarell (2023) elegantly demonstrated that drought only incidentally selects for this microbial benefit and that this selection occurs independently of plants. However, this study like most, has some limitations. Firstly, since this study was only performed on two species grown in pots under greenhouse conditions, namely *A. thaliana* and *Brassica rapa*, these findings should ideally be tested on other more phylogenetically distant species and in field conditions before they can be generalized. Secondly, no metagenomic sequencing was performed which could have measured the effect of host plants on influencing the microbiome under drought.

If the authors had shown an influence of the host plants on their microbiome, these findings would be more impactful, as this would suggest that although host plants can influence their microbiome, the functional benefits to plants are entirely related to the microbiomes adaptive response to drought. Furthermore, a metagenomics approach would also allow for the quality of such selection to be assessed, as for *A. thaliana* the core microbiome has been well characterized and shows for example a clear selection of *Actinomycetota* in the endosphere (Lundberg *et al.*, 2012). If such enrichment was not observed by Ricks and Yannarell (2023), it would call into question the experimental conditions of their study and so including metagenomics in this case would also have served as a useful control.

Another study however, which investigated the microbiomes of 19 plants under drought, clearly shows that host phylogeny has the greatest influence on determining microbial composition (Naylor *et al.*, 2017). Moreover, these authors show that these grasses can enrich gram positive bacteria, in particular *Actinomycetota*, in their endosphere under drought over and above the enrichment observed in the surrounding soil. This suggests that plants can direct microbial responses to drought although, unlike the study of Ricks and Yannarell (2023), the functional significance of this enrichment was not explored.

Adding to this, Santos-Medellín *et al.* (2021) show that rice can also drive the accumulation of gram positive bacteria over and above that observed in soil. This was evidenced by the accumulation of an unidentified *Streptomyces* of phylum *Actinomycetota* in response to drought, particularly in the endosphere, where under drought it became the most abundant taxon, reaching a relative abundance of 24%. However, upon rehydration, the accumulation of this taxon was rapidly reversed in the soil, although within the endosphere was maintained for weeks post drought. Furthermore, the authors managed to isolate this taxon and when inoculated into rice, they identified that it increased the root length under well-watered and drought conditions compared to their controls (Santos-Medellín *et al.*, 2021). Note however, that this study did not publish measurements of any other physiological traits such as yield, or a measure to showcase the perceived stress such as electrolyte leakage, and so root elongation should not be interpreted as the primary benefit afforded by drought enriched taxa. Nonetheless, unlike the prior studies mentioned, they could to some extent link beneficial function to microbial selection by the host plant and so provide some evidence that plants can indeed drive beneficial microbial dynamics in response to water deficit. However, the mechanisms of such selection as well as the functional benefits require further research in plants.

Accordingly, the aims of this chapter are listed on the following page.

Chapter Aims:

1) Identify microbial changes associated with changes in water content.
2) Identify microbial changes associated with season or relative desiccation tolerance.
3) Evaluate to what extent these microbial changes are influenced by the association with *A. caffrorum*

4.2 Methods

4.2.1 DNA extractions

Genomic DNA was extracted using Qiagen's DNeasy PowerSoil Pro kit. Due to low DNA yields and high impurities, the kit's instructions were followed with a number of modifications. For the endosphere, only 30 mg of root material was used for DNA extraction, except for sample 3.6E for which 90 mg of tissue was used. For the rhizosphere, only 100mg of dry soil was used whilst 250mg was used for the bulk soil samples. In addition, all samples were incubated at 60^{o}C for 10 minutes before the first vortexing step to increase DNA yields. Furthermore, after adding the EA and CD5 wash buffers, the columns were left to stand for five minutes before proceeding with centrifugation to lower contaminations in the final eluate. Lastly, the elution buffer was pre-heated to 37^{o}C before use to further increase DNA yield.

For each collection group within each sphere, aliquots of each kit buffer were prepared to avoid contamination across collections and compartments. Furthermore, a blank extraction was performed to confirm the absence of contamination.

4.2.2 Amplicon Sequencing

The amplicon sequencing was outsourced to Inqaba biotec^{TM}. For 16S amplicon sequencing the 27F (AGRGTTYGATYMTGGCTCAG) and 1492R (RGYTACCTTGTTACGACTT) primers were used to amplify all nine variable regions in the 16S ribosomal small sub-unit. For ITS amplicon sequencing, we requested the use of the recently developed ITScatta (ACCWGCGGARGGATCATTA) and ITS4ngsUni (CCTSCSCTTANTDATATGC) primers to amplify the full ITS region (Tedersoo & Anslan *et al.*, 2019). These two full length barcodes were then sequenced on a PacBio Sequel II instrument and processed to circular consensus sequence reads.

4.2.3 Read processing, clustering and annotation

The full length 16S and ITS reads obtained from Inqaba biotec^{TM} were processed and clustered separately via the Vsearch OTU pipeline implemented in Pipecraft v.0.1.3 (Anslan *et al.*, 2017). Note, when switching from Pipecraft version 0.1.2 to 0.1.3, a bug in the newer version was detected causing errors in the final OTU counts. After consultation with the authors of Pipecraft, we amended one of the backend scripts on our local version by inserting the line "DUC <- unique(DUC)" after line 53 in the ASV_OTU_merging_script.R to resolve this error.

To facilitate the ITS regions downstream detection via ITSx, primers were not removed from the ITS reads before quality filtering using a maximum expected error rate of 2. Next, chimeras were removed by denovo and reference based methods using the default settings and the Uchime reference of 2022. Succeeding this, the full length ITS regions were extracted using ITSx. Note, perhaps because the ITScatta primer binds so close to the start of the ITS1 region, ITSx could not recognize the start of the ITS1 region for most sequences, whilst it could distinguish the ITS2 region very well. Therefore, full and partial ITS regions were permitted in ITSx. These resulting reads were then clustered into operational taxonomic units (OTUs) at a 97% similarity and assigned taxonomy by blastn to the UNIITE 9 general release database (2023) under the default Vsearch parameters in Pipecraft v.0.1.3. Finally, after inspection, singleton OTUs were removed before statistical analysis.

For the 16S reads, primers were removed using CUTADAPT with a minimum overlap of 19 and only linked sequences were retained. The reads were quality filtered using a MaxEE cut off of 12 and only sequences of length between 1300 bp and 1700 bp were retained. Chimeras were removed using both denovo and a reference based method to the SILVA 138.1 SSURef NR99 database using Vsearch. The reads were then clustered to 97% similarity using Vsearch implemented in Pipecraft and singleton OTUs were retained. Finally taxonomy was assigned to genus level using the IDTaxa classifier in R v.4.2.2 trained on the SILVA 138.1 SSURef NR99 database (Murali *et al.*, 2018).

4.2.4 Data analysis

The 16S and ITS data analysis were performed separately in R v. 4.2.2. Stacked bar plots were produced with ggplot2 after pooling reads by either phylum, genus or species. Thereafter, chloroplastic reads and non-bacterial reads were removed from the 16S data and an OTU matrix agglomerated for genus level was used for all downstream analysis. The ITS data was not further processed and left as an OTU count matrix.

For assessment of alpha diversity, the Shannon index was computed with the phyloseq functions estimate_richness() after rarefying without replacement using rarefy_even_depth() (McMurdie Holmes, 2013). Statistical significance of alpha diversities was determined with a Kruskal-Wallis test using the base function kruskal.test() and followed up with Dunn's post-hoc tests using the functions dunnTest() from the FSA package.

To assess the beta diversity, an average dissimilarity matrix after 100 iterations of rarefaction without replacement using the Bray-Curtis distance was obtained between samples by vegan's avgdist() function (Oksanen *et al.* 2022) and plotted as an NMDS plot using phyloseq's ordinate() and plot_ordination() functions. Following this, the significance and R^2 of variables influencing the beta diversity was calculated by vegan's adonis2() function. Here, a model testing the interaction between three variables was tested and was inputted into the adonis2() function as follows: ~ hydration_status*Sphere*DT_status. This analysis was then repeated within each sphere by firstly removing taxa not present in a given sphere before computing the dissimilarity matrix and then applying a model ~ Water_status + Dt_status.

Differential abundance analysis was also performed separately within each sphere with the DESeq2 package using a likelihood ratio test comparing the model (~Collection) to a model with just an intercept (~ 1) (Love *et al.*, 2014). Differentially abundant microbes, with adjusted p-values of either less than 0.05 or less than 0.1, were plotted in a heatmap with the pheatmap package using the Euclidian distance and the ward.D clustering algorithm to cluster microbes.

Functional enrichment analysis was performed with the ITS data only, using the enricher() function from the clusterProfiler package after fungal traits for genera were obtained from the fungal traits data base (Põlme *et al.*, 2020).

Lastly, all plots, except the heatmap, were produced with, or relied on, the ggplot2 package (Wickham, 2016).

4.3 Results and discussion

4.3.1 Overview of the 16S and ITS read distribution

The approximately 530 000 16S reads were clustered into 236 868 Operational Taxonomic Units (OTUs), of which only about 5% were non-singleton OTUs. This exceedingly large number of OTUs was resultant of a high proportion of singleton OTUs in the rhizosphere. This is evidenced by the total percentage of reads belonging to singleton OTUs being 88% in the rhizosphere, whilst the endosphere and bulk soil had more acceptable levels of singletons at 37%, and 32% respectively.

The high proportion of singletons is indicative of low sequence quality, as it is unlikely that the rhizosphere would have such high read diversity with almost no repetition of reads. Given that the same DNA extracts gave on average less than 5% singleton reads for the ITS amplicon sequencing results across all spheres, and that the other 16S spheres were less affected, this issue was likely introduced during either the PCR, library preparation or sequencing step. However, since the sequencing was outsourced, this could not be further resolved. A possible explanation for this could be that the rhizosphere samples were processed in a separate batch and somehow during sequencing reads from the same bacterial species were significantly altered such that they were now clustered into individual OTUs. This result was consistent across other read processing pipelines such as the DADA2 pipeline and also clearly illustrated by the quality control tool FastQC (data not shown).

Unfortunately, due to limited remaining rhizosphere soil for some samples and the high cost of sequencing, not all rhizosphere samples could be re-sequenced. However, when two samples were re-sequenced, namely 1.3R and 1.5R to produce 1.3.2R and 1.5.2R respectively, the proportion of singleton reads dropped to below 40%. Importantly though, the proportions of the top phyla roughly mirrored the matching lower quality samples (Supp. Fig. 2). Thus, the effects on sequence quality were considered largely systematic and would likely only lower the sensitivity of downstream analysis. Consequently, the rhizosphere 16S data was still included in this chapter.

Next, in order to identify the distribution of the most abundant phyla and genera for the 16S reads, stacked bar plots were plotted. As a disclaimer, please note that recently a number of prokaryotic phylum names have been changed so that these now all end with an “ota” and so that their names contain part of the name of one of their genera (Oren & Garrity, 2021). These changes have not yet been incorporated into SILVA database which was used to annotate the reads of this study and hence these older names are still used by many publications to this day. Unfortunately, this was only picked up in the final writing stages of this Masters project and so these older phylum names were not corrected throughout this thesis. However, the correct names of phyla mentioned in this study are provided here. Specifically the older phylum names are *Cyanobacteria*, *Proteobacteria*, *Firmicutes*, *Actinobacteriota*, and *Bacteroidetes*, which under the new nomenclature should be *Cyanobacteriota*, *Pseudomonadota*, *Bacillota*, *Actinomycetota*, and *Bacteroidota* respectively.

With that being said, collection and sphere specific trends are evident within the 16S reads (Fig. 1). As expected, the endosphere contained a high proportion of chloroplastic reads (which map to the phylum *Cyanobacteria*) relative to the other spheres (Fig. 1A). This further confirms the effectiveness of the compartment separation demonstrated in Chapter 2. Given that one would not expect to find chloroplasts in roots, these reads possibly originate from other non-photosynthetic plastids.

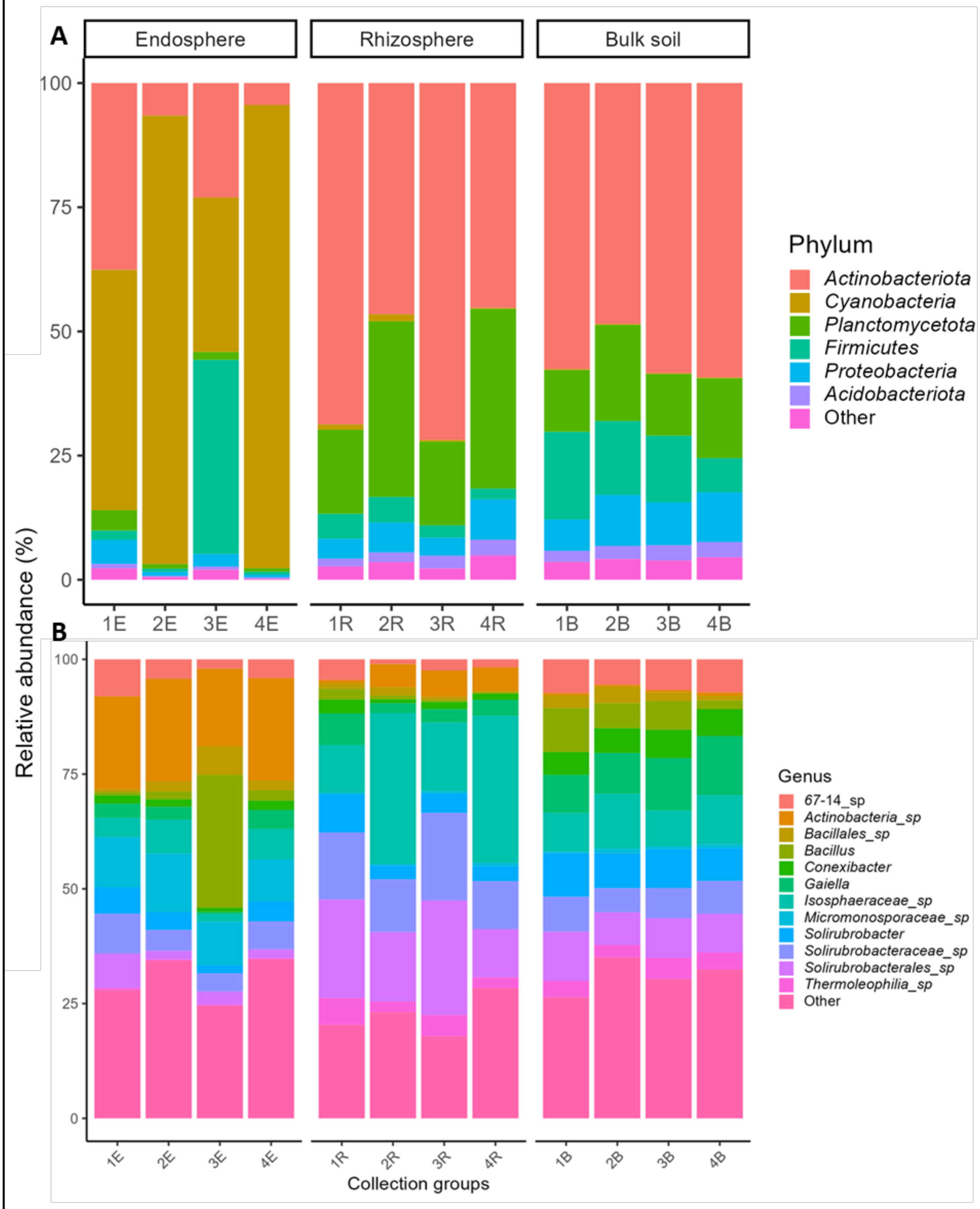

Figure 1 (A&B). Distribution of 16S reads highlighting sphere and collection specific trends. (A) The distribution of all bacterial reads at the phylum level before removal of chloroplastic reads. (B) The distribution of the remaining reads at the genus level, after removal of chloroplastic reads.
Other = the grouped remaining phyla or genera with a median abundance less than 1% or 5% respectively.

Interestingly, the proportion of these plastid reads was higher in the desiccated endosphere relative to the hydrated samples (Fig. 1A). This could either be due to a higher number of plastid sequences during desiccation, or it could simply be that there were fewer bacterial sequences present and thus the proportion of plastid sequences was inflated. This may relate to the increased number of plastid derived plastoglobuli suggested by the lipidomics data in chapter three, although without a quantitative PCR (qPCR) this cannot be confirmed.

Across almost all samples, *Actinobacteriota* were the most abundant bacterial phylum and in the endosphere and rhizosphere, their proportion was greater in the hydrated collections (Fig. 1A; Supp. Fig. 2). Upon rehydration however, *Firmicutes* are on average the most abundant phylum in the endopshere, and do not show such drastic changes in the other spheres. Note though, that only two of the four rehydrated endosphere replicates display this pattern (Supp. Fig. 2). Finally, *Planctomycetota* make up a larger proportion of the reads in the rhizosphere and bulk soil spheres relative to the endosphere. Furthermore, they appear to be increased in the desiccated rhizosphere samples.

Since the chloroplastic reads represented a large proportion of the read count, these were removed to more clearly visualize the distribution of the remaining reads. The remaining 16S reads were then pooled at the genus level to identify the most abundant genera present across the different spheres and collections (Fig. 1B). Note, a number of 16S reads could not be annotated at the genus level and so these were labelled with their next highest taxonomic rank and were pooled with other unknown genera under this rank.

This yielded that most of the abundant pooled taxa were subsets of linked taxonomic ranks. Specifically these included a number of unidentified *Actinobacteriota* belonging to the class of *Thermoleophilia* and within this class the order of *Solirubrobacterales* and within this order the family *Solirubrobacteraceae* and within this family the fully annotated genus *Solirubrobacter* (Fig. 1B). These were highly abundant across all spheres. However, these appear to only be increased by water availability in the endo- and rhizosphere, which already points to a potential influence by *A. caffrorum*. In addition, the genera *Conexibacter* and *Gaiella* fall under the order *Solirubrobacterales* and class *Thermoleophilia* respectively and so are also related to these taxa. The importance of the large presence of these related taxa will be discussed in section 4.3.4, where bacterial taxa exhibiting statistically significant differences across collections were identified and their trends discussed.

In addition, pooling reads at a genus level revealed that the genus *Bacillus* and an unidentified genus within the order *Bacillales* are driving the increased proportion of their phylum (*Firmicutes*) upon rehydration in the endosphere (Fig. 1B). Furthermore, unidentified genera of the family *Isosphaeraceae* mirror the increase of their phylum *Planctomycetota* observed in the rhizosphere and interestingly, appear to have a higher proportion in all desiccated samples. Lastly, the family *Micromonosporaceae* of the phylum *Actinobacteriota* occupied greater than 10% of all bacterial reads in each collection within the endosphere. Notably, they appeared stable across water content fluctuations and so were missed in downstream statistical analysis. Nonetheless, given their abundance and stability across seasons and desiccation, they could very well be an important player in *A. caffrorum's* microbiome. Even so, less abundant members of this family such as *Dactylosporangium* will be discussed in detail in subsequent sections and chapters.

Moving on to the ITS data, the approximately 580 000 ITS reads were clustered into 6685 non-singleton OTUs, of which all were recognized as of fungal origin. This supports the primer choice of this study, as no host plant reads were amplified.

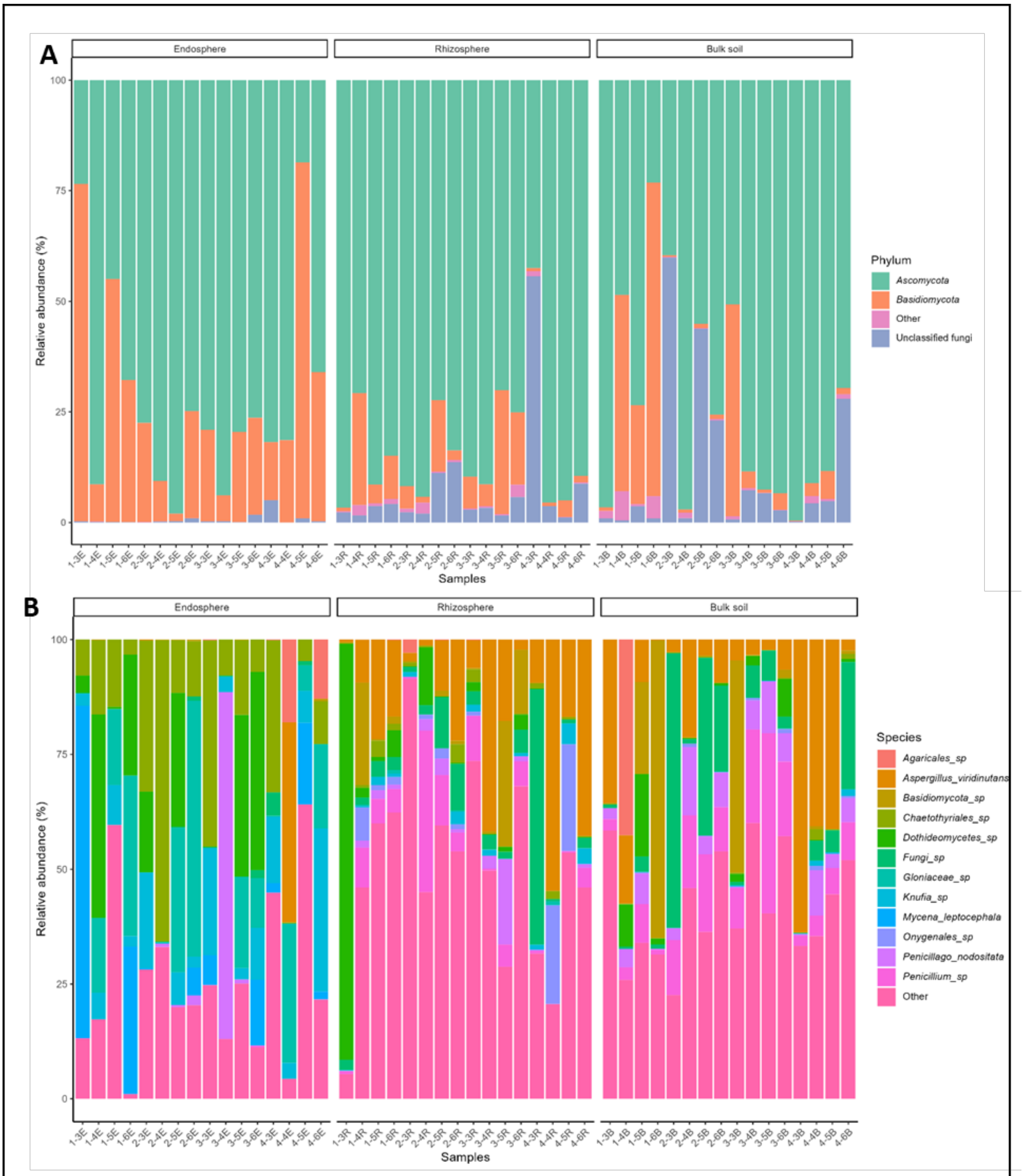

Figure 2 (A&B). Distribution of ITS reads highlighting the sphere specific collection wise intraspecific variation trends. (A) The distribution of all reads at the phylum level (B) The distribution of all reads at the genus level.
Other = the grouped remaining phyla or genera with a median abundance less than 1% or 5% respectively.

Across the three spheres, there was great variation in the composition of phyla and species (Fig. 2A&B). However, a few sphere specific trends were clear. Particularly in the endosphere, most reads belonged to either the phylum of *Ascomycota* or *Basidiomycota* (Fig. 2A). The rhizosphere and bulk soil on the other hand, contained a higher percentage of low abundant phyla and fungal OTUs which could not be confidently placed into a single phylum. This suggests a higher diversity outside the endosphere and implies perhaps that a form of selection is present to enter the endosphere. Generally, the rhizosphere and bulk soil had higher proportions of the species *Aspergillus viridinutans* whilst the endosphere had a greater proportion of the species *Mycena leptocephala*. Furthermore, the endosphere contained greater percentages of unidentified OTUs from the order of *Chaetothyriales*, the family *Gloniaceae* and the genus *Knufia*.

However, none of these show clear and consistent trends across collections, and generally there is great variability. For example, the fungus *Penicillago nodositata* is highly abundant (~75% of all reads) in sample 3-4E, although is only lowly abundant or absent in the other samples. Such variability will likely complicate downstream differential abundance analysis.

Taken together, the above figures highlight which bacterial and fungal taxa are most abundant and likely to drive sphere and collection specific effects. Although these trends have not yet been tested for statistical significance, this overview allows for better interrogation of all downstream analysis, by highlighting the most abundant taxa and their possible patterns. These will be explored further by examining the diversity of the whole community and the patterns exhibited by individual taxa in the subsequent sections. Given that for the 16S data, a number of singletons could still be assigned taxonomy down to a genus level, very often to common genera, all downstream analysis were performed on bacterial reads agglomerated at the genus level to thus partly overcome the singleton issue. This more than doubled the variance explained by the first two axes in principal coordinate analysis although the overall patterns remained the same (Supp. Fig. 3). On the other hand, the ITS data showed no improvement by agglomerating to species level and thus this analysis will simply use OTU read counts that are not binned to a specific taxonomic level (data not shown).

4.3.2 Alpha diversity

To measure the diversity of species within each sample, the Shannon alpha diversity index was separately computed for the 16S and ITS data (Fig. 3). The Shannon index combines the richness (i.e. the number of taxa) and evenness (the allocation of reads across taxa) of a sample by summing the number of agglomerated 16S genera or ITS OTUs in each sample and weighting these by their respective relative proportions in each sample. Thus, a higher Shannon Index indicates greater richness and evenness of taxa within a sample. Such sample specific differences were examined to test for significant effects of sphere, collection, hydration status and DT status.

Analysis using a Kruskal-Wallis test revealed significant effects for only the sphere and collection variables (p-values < 0.01) across both datasets (Fig 3 A&B). However, only when samples were grouped by sphere, did a Dunn's test identify any significant pairwise comparisons with adjusted p-values < 0.05. Here, for the 16S and particularly ITS data, the bulk soil had significantly greater diversity compared to the endosphere. As already suggested above, this indicates that *A. caffrorum* provides an environment within its roots that only accommodates a reduced diversity of fungi relative to the surrounding environment. However, relative hydration and desiccation tolerance or season do not significantly impact the microbial alpha diversity.

The rhizosphere showed contrasting results between the 16S and ITS data sets. The ITS rhizosphere, like the bulk soil, had greater diversity than the endosphere which further supports the notion that *A. caffrorum* only tolerates a subset of fungi from its environment. Conversely, the 16S data exhibited lower diversity in the rhizosphere relative to the other spheres. This was only significant relative to the bulk soil though, due to the much lower diversity observed for the endosphere of collection three.

Alpha diversity metrics, more so than beta diversity metrics, are highly sensitive to the total number of species and are thus are easily skewed by read processing methodology (Willis, 2019). Consequently, this discrepancy between the 16S and ITS data within the rhizosphere may be due to the differences in read quality of the rhizosphere data, as less rhizosphere reads would have passed filtering and so will not be discussed further.

It is worth noting however, that the decrease of bacterial diversity of both the endosphere and rhizosphere in collection three suggests that upon rehydration there may be selective pressures which reduce bacterial diversity. This could relate to the sudden availability of water, though since it is not observed in the bulk soil, it may also be directed by metabolic changes in *A. caffrorum*.

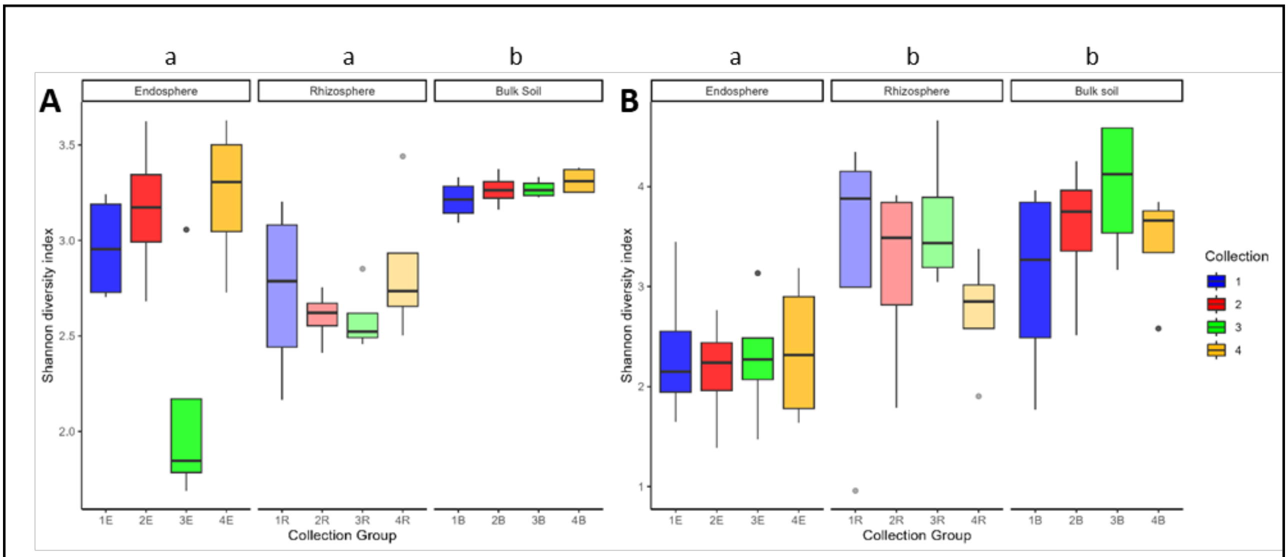

Figure 3 (A&B). Shannon diversity index for (A) the agglomerated to genus 16S reads and (B) the ITS reads. Statistical significance was determined with a Dunn's test for the 16S and ITS spheres separately. Spheres within each panel that do not share a letter in common differ significantly at the 5% level for their adjusted p-values.

Lastly, the effect of sub-site and other potential batch effects such as sequencing run were examined. These were also tested for in all other downstream analysis, though in no case did any of these have significant effects. Thus, the variation observed here, particularly for the fungi, is likely inherent in the environment.

In summary, microbial diversity varies among different spheres, with no detectable influence from relative hydration or desiccation tolerance. Specifically, the endosphere exhibits reduced Shannon diversity, particularly in fungi, compared to its surrounding environment. Furthermore, rehydration within the endosphere leads to a further decrease in bacterial diversity. These observed alterations to microbial diversity may be facilitated by *A. caffrorum* and will be further explored below.

4.3.3 Beta diversity

Next, to investigate differences in diversity between samples, the beta diversity was explored. For this, the Bray-Curtis dissimilarity, which measures dissimilarity between samples based on the abundance of species shared between them, was used. These resultant distances between samples were then visualized using Non-metric multidimensional scaling (NMDS). NMDS attempts to fit all samples into a 2D frame such that the distances between them match their true ecological distances, without attempting to, for example, maximize the variation explained by a particular axis. Therefore, it serves as a dimension reduction and allows assessment of intra- and interspecific variation across samples within grouping variables of interest. In order to obtain metric values for the effect sizes and significance of variables of interest, permutational multivariate analysis of variance (PERMANOVA) was performed.

This showed that for both metabarcoding datasets, sphere significantly (p-value = 0.001) influenced the beta diversity and had the largest effect size out of the variables measured (Table 1). Furthermore, this effect of sphere was larger within the 16S data, where it explained 40% of the total variation compared to 21% for the ITS data. This is also illustrated in the NMDS ordination where for the 16S data all three spheres appear to be separated from another whilst for the ITS data only the endosphere is clustered separately (Fig. 4 A&B).

Unlike the alpha diversity, the effect of water content was significant (p-value < 0.05) at influencing the beta diversity in both data sets (Table 1). With the exception of two 16S endopshere outliers in collection three, which as mentioned in section 4.3.1 had a greater abundance of *Bacillus*, the hydrated and desiccated samples tended to cluster separately from each other (Fig. 4 A&B). Moreover, the effect size of water content was around one third larger for the 16S data (4.9%) compared to the ITS data (3.7%). This may be due to the fact that fungi are generally more stable to changes in water content than bacteria as mentioned in the introduction of this chapter (Jaeger *et al.*, 2023). However, these effect sizes were still marginal compared the effect of sphere and so underscores the influence that *A. caffrorum* has on shaping the microbiome compared to abiotic environmental effects.

Furthermore, only the 16S data contained a significant interaction effect between sphere and hydration status with an R^2 of 5.5% (Table 1). Moreover, for the 16S data only, sphere also interacted significantly with DT status/season, although DT_status was non-significant in both data sets (Table 1). These interaction effects with the sphere variable suggest that *A. caffrorum* can possible alter bacterial dynamics across fluctuations in water content and seasons.

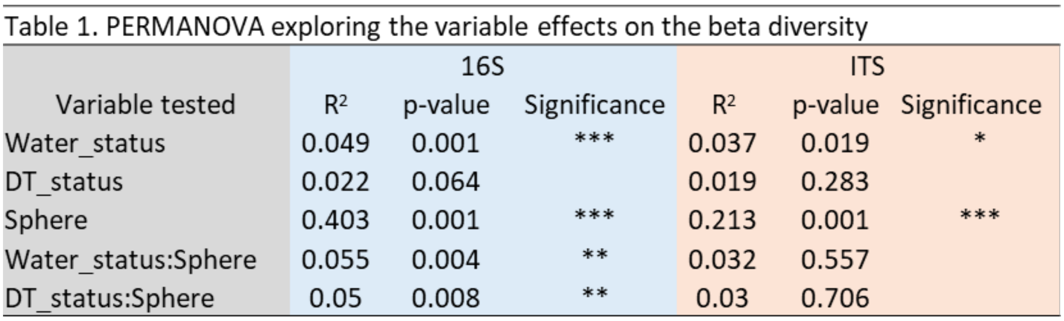

Table 1. PERMANOVA exploring the variable effects on the beta diversity

	16S			ITS		
Variable tested	R^2	p-value	Significance	R^2	p-value	Significance
Water_status	0.049	0.001	***	0.037	0.019	*
DT_status	0.022	0.064		0.019	0.283	
Sphere	0.403	0.001	***	0.213	0.001	***
Water_status:Sphere	0.055	0.004	**	0.032	0.557	
DT_status:Sphere	0.05	0.008	**	0.03	0.706	

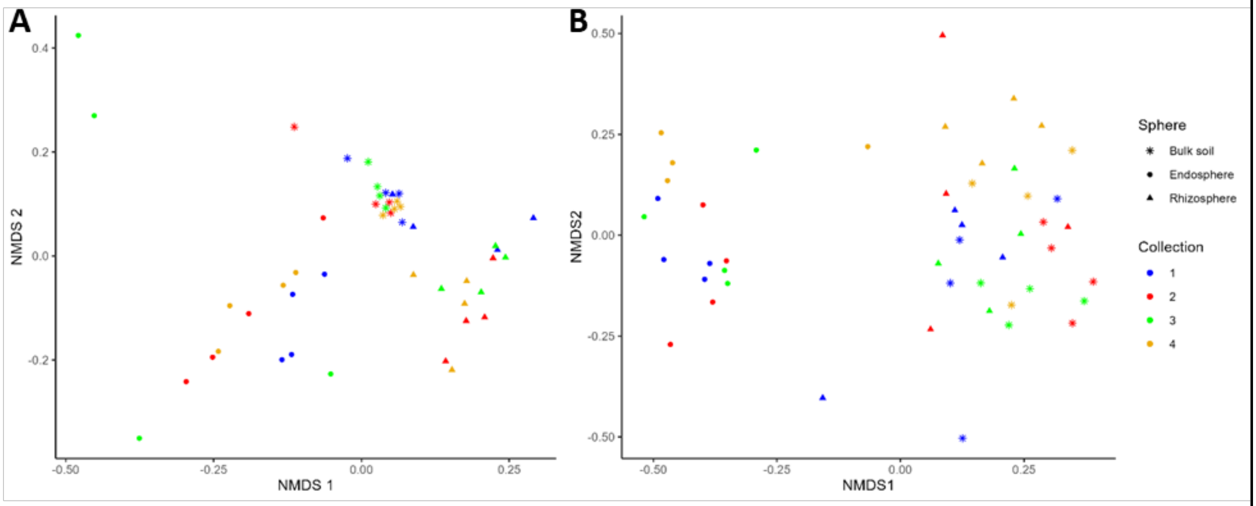

Figure 4 (A&B). Non-metric multidimensional scaling of (A) the 16S data and (B) the ITS data

Consequently to explore these interaction effects, the effect sizes of “Water_status” and “DT_status” were computed separately within each sphere (Table 2). This showed that indeed “Water_status” and “DT_status” had different effects in each sphere for the 16S data. The effect of “Water_status” was largest in the endosphere, marginally smaller in the rhizosphere and non-significant in the bulk soil. This suggests that *A. cafforum* likely attempts to modulate its associated bacteria in response to fluctuations in water content such as desiccation. Such increasing selection for increasing closeness to the plant has also been observed in other plant studies, particularly in response to stresses such drought or heavy metal toxicity (Santos-Medellín *et al.*, 2021; Sun et al., 2021).

Interestingly, the effect of DT_status was significant only within the rhizosphere (Table 2). This suggests that bacterial dynamics across seasons are relatively stable in the bulk soil and within the roots, though are altered within the rhizosphere. This may relate to the lower read quality and thus overall diversity within the rhizosphere. However, as will be discussed later, this may also suggest that *A. caffrorum* mobilizes rhizospheric shifts in its microbiome for seasonal adaption, whilst shifts in the endosphere community are possibly directed to respond to changes in water content.

Table 2. PERMANOVA within each sphere individually

Data set	Sphere	Variable tested	R^2	p-value	Significance
16S	Endosphere	Water_status	0.21	0.005	**
		DT_status	0.09	0.114	
	Rhizosphere	Water_status	0.17	0.02	*
		DT_status	0.2	0.005	**
	Bulk Soil	Water_status	0.08	0.268	
		DT_status	0.11	0.095	
ITS	Endosphere	Water_status	0.07	0.348	
		DT_status	0.05	0.697	
	Rhizosphere	Water_status	0.09	0.101	
		DT_status	0.05	0.811	
	Bulk Soil	Water_status	0.1	0.046	*
		DT_status	0.08	0.179	

To sum up this section, the microbial composition appeared to be primarily influenced by its relative association with *A. caffrorum*, followed by fluctuations in water content and lastly DT/season. Furthermore, the influence of fluctuations in water content on the microbiome was greater in closer proximity *A. caffrorum* and the seasonal effects were strongest in the rhizosphere. Lastly, all these three variables have larger effects on the 16S data relative to the ITS data.

4.3.4 Differentially abundant bacteria across collections

Next, to more comprehensively and more formally determine which microbes are driving the effects described above, differential abundance analysis was performed with DESeq2. Here statistical significance across collections within spheres was determined with a likelihood ratio test in DESeq2 relative to a model with only an intercept. Furthermore, only the most abundant taxa were used for this analysis to firstly improve data fit to a negative binomial distribution and secondly avoid some of the potential effects introduced by the lower read quality in the rhizosphere.

For the 16S data, 16, 10 and 2 agglomerated genera were differentially abundant within the endosphere, rhizosphere and bulk soil respectively. However, particularly within the rhizosphere, a number of potentially important taxa narrowly missed the 5% adjusted p-value cut off. These included for example the most abundant rhizosphere family, namely the *Isosphaeraceae* (adjusted p-value = 0.07), in addition to the nitrogen fixing order *Frankiales* of phylum *Actinobacteriota* (adjusted p-value = 0.07) along with the known symbiont order *Rhizobiales* (adjusted p-value = 0.09). Thus, this criterion was relaxed to the 10% level and these additions were marked with an asterisk (Fig 4.5B). To avoid bias, this cut-off was therefore also extended to the collection-wise contrasts in the other spheres and the ITS data. This also had the added benefit of increasing the strictness of the bulk soil control. Under the 0.1 adjusted p-value cut-off, an additional 4, 4 and 11 genera were identified for the 16S endo-, rhizosphere and bulk soil respectively (Fig. 5 A-C).

4.3.4.1 Differentially abundant endophytic bacteria

Within the endosphere, the genera *Kineosporia* (phylum = *Actinobacteriota*), *Burkholderia-Caballeronia-Paraburkholderia* (phylum = *Proteobacteria*), *Paenibacillus* (phylum = *Firmicutes*) and more generally its family *Paenibacillaceae*, the family *Gemmataceae* of phylum *Planctomycetes* and the order *Elev-1554* of phylum C*hloroflexi* were more abundant in the desiccated tissues relative to the hydrated tissues (Fig. 5A). In addition, the genus *Acidothermus* (adjusted p-value = 0.06) of order *Frankiales* and the *Subgroup 7* of phylum *Acidobacteriota* (adjusted p-value = 0.07) also follow this trend, although not significantly at the conventional 5% cut-off for their adjusted p-values.

There is limited information on *Kineosporia*, but it is noted that four of its endophytic species lack the ability to utilize cellulose (Kudo *et al.*, 1998; Li *et al.*, 2009). However, all of the other taxa increased during desiccation are recognized for their glycolytic or cellulolytic capabilities and will be discussed below.

Although not much is known about the *Elev-1554* and *Subgroup 7* orders, their phyla have been shown to contribute meaningfully to cellulolytic activity in grassland soils (Berlemont et al., 2014). The *Burkholderia-Caballeronia-Paraburkholderia* and *Paenibacillus* on the other hand, contain various members with known cellulolytic activity and can degrade fungal cell walls leading to their application as anti-fungal biostimulants (Madhaiyan *et al.*, 2021; Grady *et al.*, 2016). However, the above mentioned phyla and genera contain a large diversity of species and so without functional tests it is hard to ascertain that these indeed have such activity in *A. caffrorum's* endosphere.

More conclusively, the *Gemmataceae* and *Acidothermus,* have been extensively documented to exhibit glycolytic activities (Kulichevskaya *et al.*, 2020a; Kulichevskaya *et al.*, 2020b; Ravin *et al.*, 2018; Barabote *et al.*, 2009). So far, only one species has been described within the genus of *Acidothermus*, namely *Acidothermus cellulolyticus*. Therefore, it is possible that this species is also present in *A. caffrorum's* endosphere, or else, this is a new species within this genus. As the name suggests, the genome of this species contains a sizable fraction of cellulolytic and glycolytic enzymes that can degrade plant and fungal cell walls and metabolize complex carbohydrates and sugars such as trehalose (Barabote *et al.*, 2009).

Similarly, the sequenced members of the *Gemmataceae* all possess large genomes that harbor a diverse array of carbohydrate-active enzymes, including numerous unclassified putative glycoside hydrolases and thus have a large glycolytic potential across both terrestrial and aquatic ecosystems (Kulichevskaya *et al.*, 2020a; Kulichevskaya *et al.*, 2020b; Ravin *et al.*, 2018). Two of these have also specifically been described to have cellulolytic capacity and can utilize trehalose (Kulichevskaya *et al.*, 2012; Kulichevskaya *et al.*, 2020a). Furthermore, this family has been observed to increase in the rhizosphere of *Zea mays* under drought (Zhang *et al.*, 2022). Thus, taken together, this suggests that during desiccation, bacteria which have increased glycolytic or cellulolytic activity are selected for within *A. caffrorum's* endosphere.

The increased proportion of bacteria with such activities within the endosphere suggests an increase of available biomass or glycolytic substrate to decompose. This could be in response to the elevated levels of more complex di- and trisaccharides such as trehalose and raffinose versus monosaccharides such a glucose which were identified in chapter three as accumulated during desiccation (Supp. Fig. 1). The significance of this correlation will be explored further in chapter five.

This is supported by a study on the phyllosphere microbiome of the resurrection fern *Polypodium polypodioides* (Jackson *et al.*, 2006). In this study it was found that desiccation leads to increased phenol oxidase activity, which is an enzyme involved in lignin degradation. Immediately upon re-wetting however, the phenol oxidase activity was reduced and was replaced by a spike in beta-glucosidase activity which then subsequently diminished in the days following re-wetting (Jackson *et al.*, 2006). This observation aligns with our findings, suggesting that desiccation promotes microorganisms capable of degrading recalcitrant substrates. Upon rehydration, these substrates may be converted into more accessible compounds, thereby inducing a succession of hydration-specific microorganisms. Such findings are also observed more generally in soils under drought, where enhanced litter decomposition and glucosidase activity was speculated to free up nutrients that facilitate a rapid rise in microbial activity and succession upon rehydration (Manrubia *et al.*, 2019). Thus, the increase of these glycolytic and cellulolytic activities during desiccation may be advantageous to *A. caffrorum* by perhaps facilitating a rapid recovery upon rehydration. Moreover, this increased enzyme activity may simultaneously degrade fungal cell walls and so be protective against fungal pathogens during desiccation and directly upon rehydration.

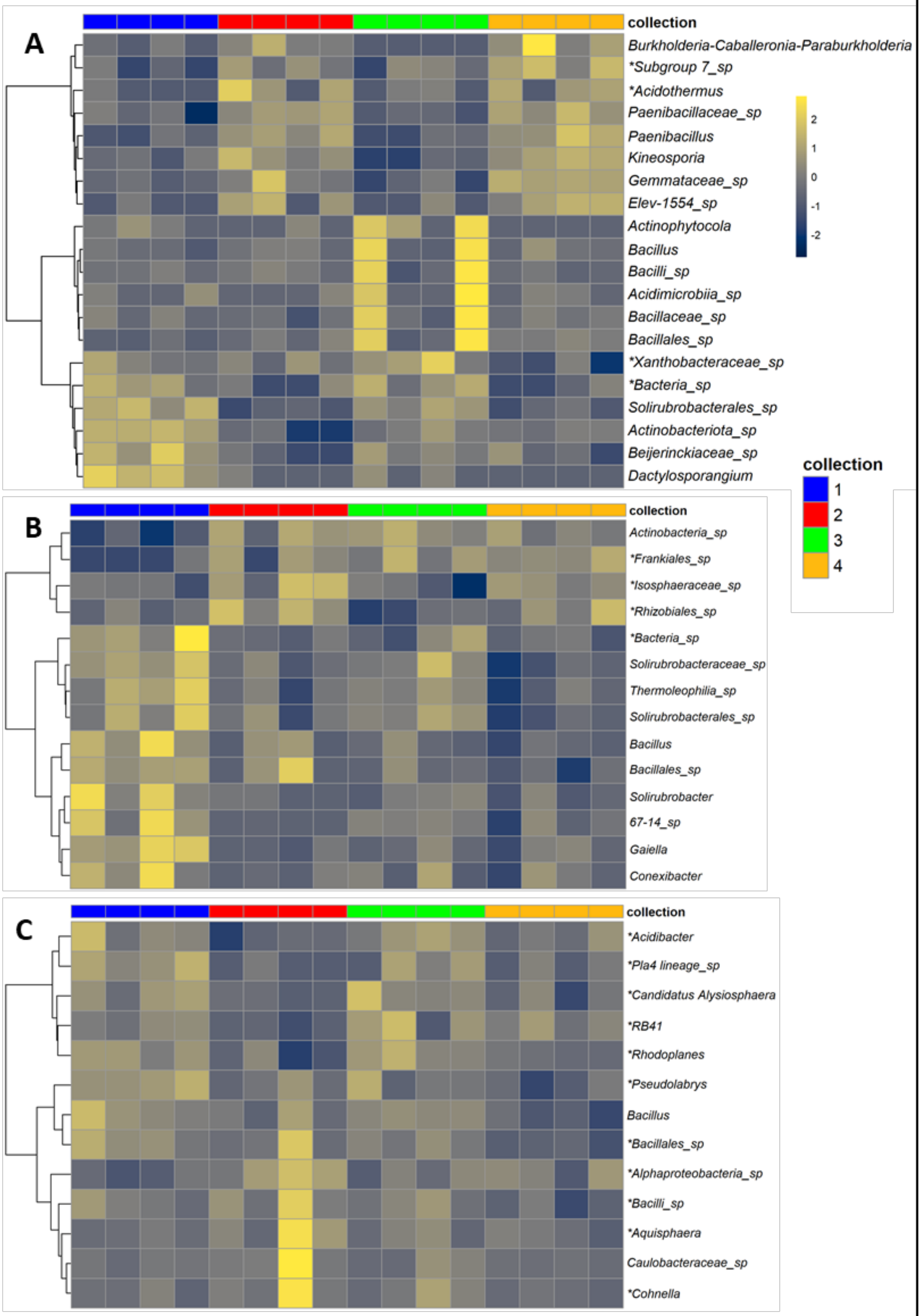

Figure 5 *(A-C)*. Differentially abundant bacterial genera within the (A) endosphere, (B) rhizosphere and (C) bulk soil. Genera with an asterisk have an adjusted p-value between 0.05 and 0.1 whilst all others have adjusted p-values less than 0.05. Intensities represent the variance stabilized abundance and is separately scaled for each genus.

The *Burkholderia-Caballeronia-Paraburkholderia* and *Paenibacillus*, in addition to their ability to degrade fungal cell walls, contain members that can promote plant growth directly, through for example, nitrogen fixation and phosphate solubilisation, or indirectly by reducing plant stress (Madhaiyan *et al.*, 2021; Grady *et al.*, 2016). This is particularly true for the *Paenibacillus* genus, which has been shown to increase the drought tolerance of *A. thaliana*, via priming the induction of two "Response to Desiccation" genes called RD29A and RD29B, which promisingly did not result in a loss of yield (Liu *et al.*, 2020). When these plant genes induced by *Paenibacillus polymyxa* CR1 were deactivated by T-DNA insertions, *A. thaliana's d*rought tolerance was subsequently reduced (Liu *et al.*, 2020). Therefore, such bacteria, and particularly *Paenibacillus*, may also assist in coordinating the desiccation response in *A. caffrorum*, and possibly explains their increase during desiccation. Furthermore, members of this genus and more generally unidentified bacteria the family *Paenibacillacea* were also isolated from the phyllosphere of the resurrection fern *Polypodium polypodioides,* which underscores the potential of drought protective plant growth promoting bacteria harbored by resurrection plants (Jackson *et al.*, 2006).

Some taxa on the other hand, were more abundant in the hydrated endosphere collections. These included a number of *Actinobacteriota* such as the order *Solirubrobacterales* and the genus *Dactylosporangium*, in addition to the family *Beijerinckiaceae* of phylum *Proteobacteria* which were elevated in collection 1 and to a lesser extent in collection 3. Strikingly, collection three exclusively contained greatly increased levels of *Bacilli* or more generally the family *Bacillaceae*, in addition to other *Actinobacteriota* in only two out of the four replicates. Lastly, collection 3 also featured elevated levels of the family *Xanthobacteraceae* of phylum *Proteobacteria* (adjusted p-value = 0.099), however this was only significant at the 10% level (Fig. 5A).

A number of these taxa have growth promoting traits. The families *Beijerinckiaceae* and *Xanthobacteraceae* belong to the root symbiont order of *Rhizobiales*. This order is best known for its ability to form symbiotic relationships with a broad range of host plants where these bacteria fix atmospheric nitrogen in exchange for carbon sources (Garrido-Oter *et al.*, 2018). Interestingly, the majority of isolates from the rehydrated resurrection fern *P. polypodioides* belonged to the *Beijerinckiaceae* and so this plant growth promoting family may similarly participate in the microbial succession and growth promotion post desiccation in *A. caffrorum* (Jackson *et al.*, 2006).

Furthermore, *Bacillus* is probably the genus that shows the most promise as a microbial biostimulant with well described plant growth promoting traits including nitrogen fixation and increasing plant nutrient availability (Yousuf *et al.*, 2017; Cherif-Silini *et al.*, 2016). Given its greatly increased abundance in two of the rehydrated samples, it may assist *A. caffrorum* in growth and recovery related processes upon rehydration. However, this is likely not be a conserved response as this taxon was observed in only half of the rehydrated samples.

In addition to plant growth promoting taxa, the microbiome within the hydrated endosphere also suggests an influence of antibiotics. The genus *Dactylosporangium* has long been recognized for its production of novel antibiotics, with ongoing discoveries contributing to this reputation (Shomura *et al.*, 1980; Cavalleri *et al.*, 1988; Kumar *et al.*, 2023). Thus, it is possible that the genus here may exert an antimicrobial effect in the hydrated endosphere. Supporting this notion is the observed increase in the most abundant bacterial order, namely the *Solirubrobacterales*. This order is frequently associated with antibiotic resistance genes (ARGs) in various environmental settings, including agricultural soils, deserts, contaminated soils, and freshwater ecosystems (Jauregi *et al.*, 2021; Yang *et al.*, 2022, 2024; Thomas *et al.*, 2020; Navarro *et al.*, 2023). Collectively, these findings suggest that increased antibiotic effects and resistance to these may govern bacterial community structure in the hydrated endosphere, particularly in the DS collection 1. The increased antibiotic effect in the roots may be in response to increased biotic stress (in-stead of abiotic stress) potentially experienced by *A. caffrorum* and its microbes during the consistently wet winter period. This so far aligns with the metabolomics data of this study and proteomics and metabolomics data of the rhizome which suggested a shift to focus on biotic stress in hydrated sensitive individuals relative to desiccated individuals (Shoko, 2015; Wittenberg, 2021).

4.3.4.2 Differentially abundant bacteria in the rhizosphere and bulk soil

As indicated by the beta diversity analysis, the rhizosphere contained DT/DS specific effects in addition to water content related effects. In the rhizosphere, unidentified *Actinobacteria*, and those identified to the order of *Frankiales* were increased in the DT collections relative to the DS collection. In addition, the *Isosphaeraceae* of phylum *Planctomycetota* and *Rhizobiales* were increased in the desiccated collections only and mirror the results of similar taxa observed in the roots.

The *Frankiales,* and as previously discussed for the *Rhizobiales,* are best known for their ability to fix atmospheric nitrogen and their beneficial association with plants (Garrido-Oter *et al.*, 2018; Huss-Danell, 1997). The *Isosphaeraceae*, as previously discussed for other *Planctomycetota*, are well known for their ability to degrade complex carbohydrates across diverse environments including soil (Ivanova, *et al.*, 2017; Wang *et al.*, 2015). Therefore, just as in the endosphere, nitrogen fixation and increased glycolytic potential may be important during desiccation and more generally in the DT state. This may explain the increased nitrogen storage observed in the desiccated roots (Chapter 3).

In the rhizosphere of DS *A. caffrorum,* and to a lesser extent the hydrated rhizosphere, the class *Thermophilia* and more specifically members of order *Solirubrobacterales* were more abundant. Again, as previously discussed in the endosphere, these could relate to an increased antibiotic effect and biotic stress present during water availability. Such resistance to biotic stress may not be required or prioritized by *A. caffrorum's* bacterial community during abiotic stress such as desiccation and thus they may reduce in abundance during drying and more generally during the DT summer period in *A. caffrorum's* endo- and rhizosphere. Nevertheless, they still maintained relatively large proportions in the desiccated collections (Fig. 1 A&B).

The *Solirubrobacterales*, although relatively rare, are wide spread across extreme habitats such as Antarctic and desert soils (Chong *et al.*, 2012; Saul-Tcherkas *et al.*, 2011). Furthermore, their diversity and abundance has been shown to be enriched in disturbed agricultural soils (Shange *et al*. 2012). Therefore, these bacteria appear to favour more disturbed and perhaps extreme habitats.

This trend is also true for the differentially abundant *Solirubrobacterales* noted in this study which were further annotated to a genus level, namely: *Conexibacter* and *Solirubrobacter*. In addition the related *Gaielles,* which are in their same class, also followed this trend. Interestingly, like in this study, these were all part of the top ten genera detected in the soil of a pasture suffering from die-back (Whitton *et al.*, 2022). The authors of this study proposed that the order of *Solirubrobacterales*, given their association with extreme environments, are an indicator of poor soil health. However, when this same study remediated this pasture soil with a biostimulant, *Solirubrobacter* and *Conexibacter* further increased in abundance and stood out in downstream analysis as the most significant genera associated with the improved soil quality. Hence, these are likely to not simply be an indicator of degraded soil. Instead, this association with degraded soils and extreme environments could indicate that members of this order and more specifically these genera, could play a role in bioremediation by acting as a pioneer species which prepare the environment for other lifeforms. In *A. caffrorum*, this functionality may be important to thrive in the notoriously nutrient poor soils of its Western Cape where other local flora have been described to also have specialized adaptions to this nutrient poor geological substrate (Cowling & Cambell, 1980; Coetsee *et al.*, 2015). In line with this is the fact that these genera, and more generally the *Solirubrobacterales,* are best isolated on nutrient poor media (Seki *et al.*, 2015).

A further point of interest with these taxa is that two of them are highly abundant in the rhizosphere soils of the resurrection plants *Ramonda nathaliae* and *Ramonda serbica* (Lozo *et al.*, 2023). Specifically, the species *Gaiella occulta* and *Solirubrobacter ginsenosidimutans* were in the top five most abundant species in both of these species. These plants were sampled from their natural habitat and according to the authors “thrive in the crevices of the north-facing steep cliffs, growing in the shallow soil” which suggests reduced access to nutrients. Such growth is typical for many resurrection plants. Thus, the presence of such unusual taxa in these species and *A. caffrorum* may be in response to a shared nutrient poor soil substrate, and supports the notion that perhaps resurrection plants more generally have may have utility for soil remediation efforts.

In addition, the *Solirubrobacterales* were associated with increased plant growth under well-watered and water limiting conditions in *Zea mays* (Moore *et al.*, 2023). Therefore, perhaps the *Solirubrobacterales* also have a growth promoting effect either directly or indirectly by allowing other perhaps more directly beneficial microbes to play their roles and could explain their increased abundance during *A. cafforum’s* growth phase in winter.

A contrasting pattern to the endosphere was exhibited by *Bacillus*. These were enriched in collection 1 in the rhizosphere instead of collection 3 as in the endosphere. However, *Bacillus* also exhibits this pattern in the bulk soil and thus this enrichment of *Bacillus* in the winter may be in response to variables affecting all the soil more broadly such as temperature etc. and not governed by *A. caffrorum*. Nevertheless, this further highlights the differential responses of the roots and surrounding environment and thus cements the role that *A. caffrorum* may play in this.

A number of the remaining bulk soil bacteria showed hydration responsive patterns, although none of these were significant at the 5% level for their adjusted p-values and importantly none are differentially abundant in any of the other spheres. Therefore, the patterns observed in the endo- and rhizosphere are possibly influenced by their association with *A. caffrorum*.

Finally, the canonical enrichment observed in other studies of gram positive monoderms, particularly of phylum *Actinobacteriota*, upon water deficit, and gram negative diderms in response to water availability, was not observed in the endosphere and rhizosphere in this study (Barnard *et al.*, 2013; Naylor *et al.*, 2017; Santos-Medellín *et al.*, 2021). On the contrary, *Actinobacteriota* such as the *Solirubrobacterales* and other gram positive bacteria such as *Bacilli* were more abundant in the hydrated collections, whilst gram negative *Planctomycetota* and *Proteobacteria* were increased in response to desiccation (Fig. 1B; Fig. 5 A&B). In the bulk soil however, all bacteria elevated in the hydrated collections were gram negative in line with the literature discussed. Therefore the lack of clear monoderm or diderm enrichment during respective water deficit or water availability may be related to the specific environment provided for by *A. caffrorum*, which further supports that a selective force is at play.

4.3.5 Differentially abundant fungi across collections

Although the fungi exhibited large intraspecific variation within groups, analysis of the beta diversity detected a small effect of hydration status. Therefore, in order to examine if any specific fungi consistently follow this trend, each sphere was, as with the 16S data, separately subjected to differential abundance analysis.

As expected, the patterns within the differentially abundant taxa were less consistent across replicates within groups and were less affected by water content compared to the 16S data (Fig. 6). For example in the endosphere, *Penicillago nodositata* was elevated in collection 2 and 3 and had lower abundance in collections one and four. In addition, the most abundant OTU, which was classified as an unknown *Dothideomycete*, appeared to progressively decrease in abundance from collection one through to four in the endo- and rhizosphere. In the bulk soil, although most differentially abundant taxa were only significant at the 10% level when adjusted for multiple comparisons. Here, the most striking trend was the difference between collection 1 and 4 suggesting potential gradual shifts over time. Taken together with the stacked bar plots, these results suggest that the distribution of fungi may be "patchy" across the site and so replicates show less resemblance to another.

Nevertheless, a few patterns related to water content or DT status are worth noting. In the endosphere only, *Schizosaccharomyces pombe* and two unknown species within the orders *Atractiellales* and *Trechisporales* tend to be increased in the desiccated collections (Fig. 6A). The genus *Trechispora* was particularly enriched in collection 4 and *Blumeria graminis* was increased in only collection 1. Besides the genus *Cladophialophora*, no endophytic fungi were increased in both the hydrated collections.

Conversely, the rhizosphere exhibits a number of fungi increased in the hydrated collections and only one species, namely *Spegazzinia parkeri*, was increased in both desiccated collections (Fig. 6B). The hydration specific OTUs in the rhizosphere again contained the genus *Cladophialophora* in addition to an unknown *Basidiomycota* and the species *Penicillium janczewskii*, *Periconia circinata* and *Neurospora cratophora*, although the last two were only significant at the 10% level. Furthermore, *Penicillium sexuale* and another unknown species from this genus, and more generally its family the *Aspergillaceae* were increased in the DS rhizosphere, whilst in the bulk soil members of this genus and family increased only in collection 4 (Fig. 6 B&C). Lastly, *Arxotrichum wyomingense* was also elevated in the rhizosphere of collection 1 only.

These patterns of desiccation responsive fungi in the endosphere, and DS or wet season responsive fungi in the rhizosphere and bulk soil, mirror the 16S data. This suggests *A. caffrorum* directs its associated microorganisms to respond to desiccation in the endosphere and seasonal adaptations in the rhizosphere.

All of the differentially abundant fungi across the three spheres that were identified to at least a genus level could be annotated with their proposed trophic modes through the FungalTraits database (Põlme *et al.*, 2020). Of these, four were classified as plant pathogens under their primary lifestyles. These were: *Blumeria graminis*, *Phoma*, *Periconia circinata* and *Fusarium neocosmosporiellum.* With the exception of the latter, these were all differentially abundant in either the endosphere or rhizosphere (Fig. 6). Here, these were either increased in collection 1 or both hydrated collections, and so suggests that during winter, or more generally in the hydrated state, *A. caffrorum* experiences greater biotic stress. This aligns with the observations of this study so far and the rhizome's proteome where "defence response to fungus, incompatible interaction" was enriched in the wet DS season (Shoko, 2015).

One exception to this was the pathogenic genus *Phoma*, which was elevated in the desiccated collection 4 in addition to collection 1. However, *Phoma* is one of the largest fungal genera and may contain as many as 3000 species which are widespread across the globe (Montel *et al.*, 1991). Whilst this genus is best known for containing many phytopathogens, many of its species are harmless soil saprobes, or are pathogenic only when they encounter suitable hosts (Aveskamp *et al.* 2008). Furthermore, this genus also contains beneficial fungi such as *Phoma herbarum*, which promotes plant growth and produces gibberellin (Hamayun *et al.*, 2009). Therefore, without a species level annotation it is unclear if indeed this OTU is a plant pathogen and so this exception should not take away from the overall trend of greater pathogenic fungi observed in the hydrated samples, especially those from collection one.

Blumeria graminis on the other hand, which ranks as the 29th most abundant OTU detected here, is the causative agent of powdery mildew in grasses and is within the top 7 most destructive biological agents causing loss of yield in wheat (Savary *et al.*, 2019). Interestingly, the host ranges of powdery mildews are restricted to angiosperms and have so far have never been described in ferns (Takamatsu, 2004). Therefore, the presence of this species in *A. caffrorum* marks a first report for occurrence in ferns. Given that it is an obligate biotroph, it may also be acting as a pathogen here. Similarly, *Periconia circinata* is the cause of milo disease in grasses such as *Zea mays* and *Sorghum bicolor* (Leukel & Johnson, 1948). However, given that the pathogenicity of these two fungi are primarily reported in grasses, *A. caffrorum* may harbour these fungi as a form of biocontrol against its predominantly grass neighbours and thus lower the competition experienced by *A. caffrorum* during its growth phase in winter. Either way, the presence of these phytopathogens in collection one and three support the notion that biotic stress, including perhaps competition, is greater during the wet season and perhaps more generally in the hydrated state.

Possibly in response to increased pathogenicity experienced by *A. caffrorum*, an unidentified species within the genus of *Cladophialophora* also increases in abundance in the hydrated endo- and rhizosphere (Fig. 6 A&B). This genus belongs to the dark septate entophytic fungi and these have been shown to have antifungal activity against other pathogenic fungi such as *Fusarium oxysporum* (Harsonowati *et al.*, 2020). Furthermore, three members of the genus *Pencillium* also increase during either one or both of the hydrated collection in the rhizosphere. This genus, and particularly *Penicillium janczewskii*, has long been known for its ability to produce bacteriostatic and fungistatic compounds that can also elicit systemic acquired resistance, and thus could directly or indirectly contribute to controlling plant pathogens in winter and more generally the hydrated state (Fleming, 1929; Curtis & Grove, 1947; Madi & Katan, 1998).

The *Atractiellales*, *Trechisporales*, *Schizosaccharomyces pombe* and *Spegazzinia parkeri* all increase in response to desiccation (Fig. 6). None of these have been reported to specifically increase during water deficit, although all display saprobic lifestyles and potentially have beneficial associations with plants. Members of these orders *Atractiellales* and *Trechisporales*, have been associated with plant growth promotion and ectomycorrhizal lifestyles, although are generally considered saprobic (Kottke *et al.*, 2010; Bonito *et al.*, 2017; Aime *et al.*, 2018; Vanegas-León *et al.*, 2019). Furthermore, *Spegazzinia parkeri* was first consistently isolated from the rhizosphere of wheat inoculated with arid soil and according to FungalTraits database is a wood saprotroph (Sivasithamparam, 1974; Põlme *et al.*, 2020). *Schizosaccharomyces pombe*, was classified as a nectar/tap saprotroph by the FungalTraits database although very little is known about its function in plants. However, other yeasts have been shown to have plant growth promoting properties, in particular the alleviation of plant stress (Rezki & Noureddine, 2020; Ramos-Garza *et al.*, 2016) Together, these highlight the research gaps for studying such taxa, and that *A. caffrorum* may harbour unknown plant growth promoting fungi. Nevertheless, all of these are generally saprobic and given that the bacteria also exhibited an increase in such functionality, it may play a crucial role during desiccation or in preparation for rehydration.

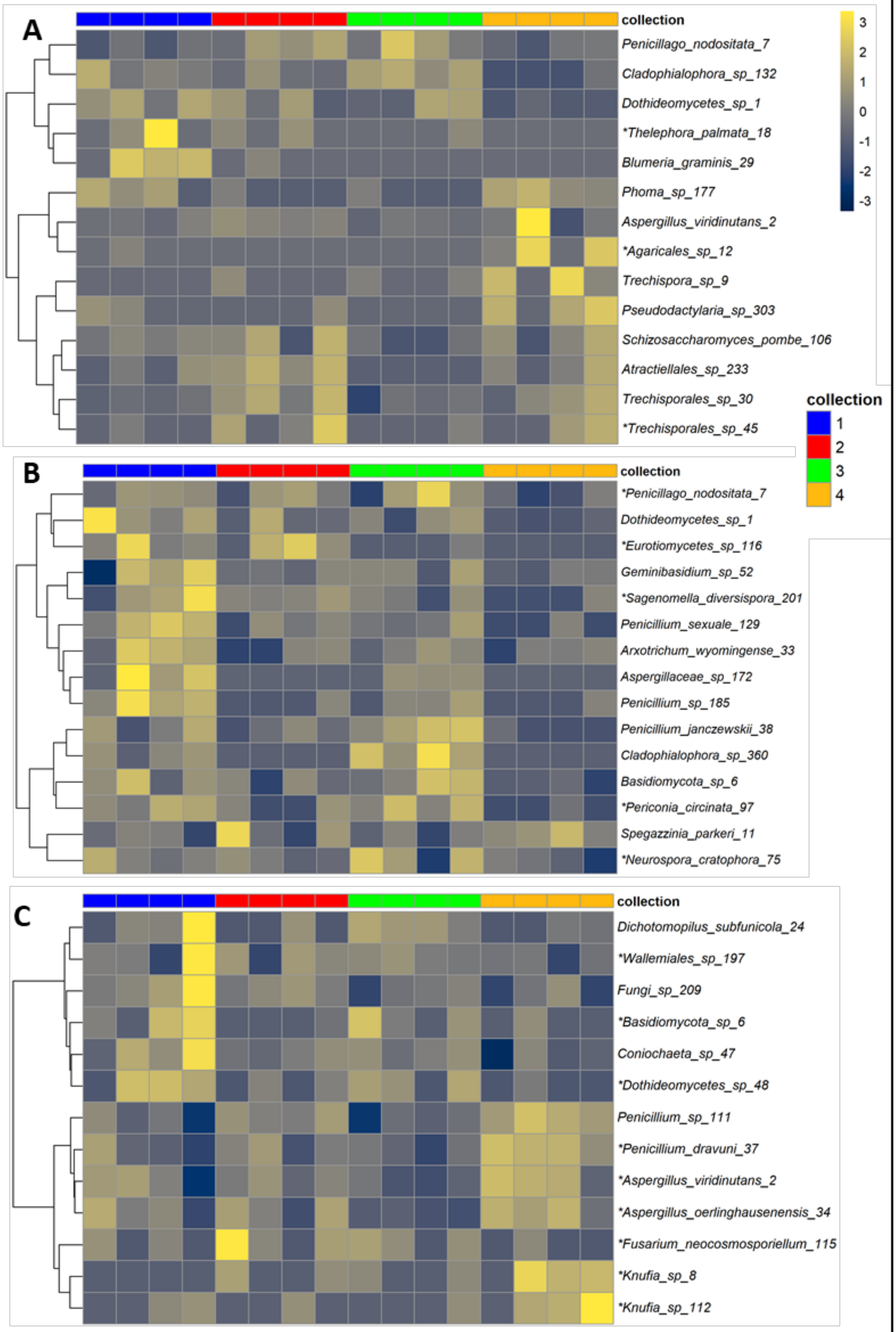

Figure 6 *(A-C)*. Differentially abundant fungal OTU's within the (A) endosphere, (B) rhizosphere and (C) bulk soil. OTU's with an asterisk have an adjusted p-value between 0.05 and 0.1 whilst all others have adjusted p-values less than 0.05. OTU's are numbered by their total abundance. Intensities represent the variance stabilized abundance and is separately scaled for each OTU.

4.3.6 Functional enrichment of fungi between spheres

Given that fungi exhibited high intraspecific variability and that their individual roles were difficult to parse across collections, functional enrichment comparisons across spheres were conducted on a presence and absence basis. Similar to the beta diversity analysis, a Venn diagram shows that the endosphere shares less taxa in common than the rhizosphere and bulk soil (Fig. 7).

Here, taxa only present within the endosphere were functionally enriched for the litter and nectar saprotrophic modes in addition to terms associated with plant growth promotion, namely: ectomycorrhizal, root-associated and root endophyte dark septate.

Taxa only present within the rhizosphere contained a number of other saprotrophic modes such as wood-saprotroph in addition to the pathotrophic modes: “Plant pathogen”, “Mycoparasite” and “Wood pathogen”. Furthermore, it also contained the plant associated terms: “Unspecified symbiotroph”, “Root endophyte”, “Arbuscular mycorrhizal” and “Lichenized”. The bulk soil on the other hand was only enriched for the term “Root-associated”, although under the plant pathogenic capability template, and so indicates an enrichment for root associated pathogens.

These results again point towards the capacity of the endosphere to reduce pathogen entry from the rhizosphere and bulk soil. This may in part be achieved by the presence of other microbes such as dark septate root endophytes, which were discussed above. Furthermore, it underpins *A. caffrorum’s* ability to select for growth promoting trophic modes relative to the bulk soil. For example, ecto- and arbuscular mycorrhizal fungi are well known to increase availability of vital nutrients such as phosphorus to plants (Smith & Read, 2008).

In addition, this analysis shows that saprotrophic modes such as nectar and wood saprotrophs may be important for *A. caffrorums’s* biology since these were induced by desiccation within the endo- and rhizosphere as mentioned in the previous section.

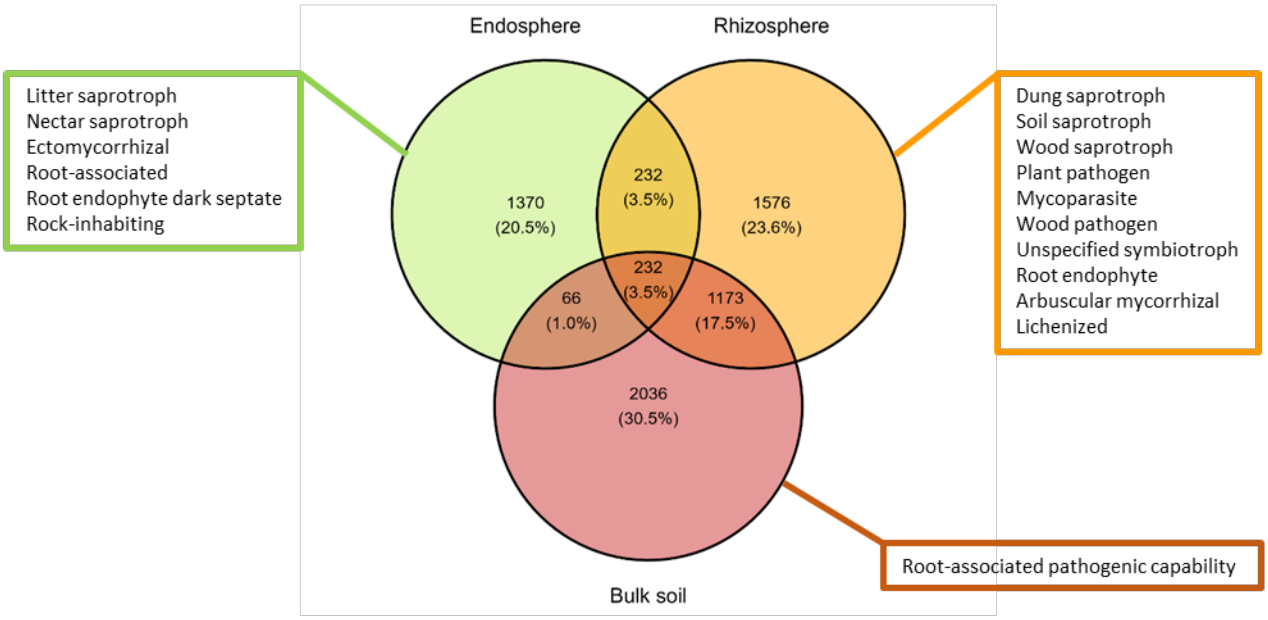

Figure 7. A. caffrorum selects against pathogens in the endosphere. A Venn diagram depicting fungal OTU‘s unique and shared across the spheres on a presence and absence basis. The functionally enriched terms of unique taxa for each sphere are indicated in text boxes and were obtained from the fungal traits database.

4.4 Brief conclusions

To sum up, the bacterial reads exhibited lower quality than the fungal reads and had a larger proportion of singletons, particularly in the rhizosphere. However, this could in part be overcome by agglomerating the reads to a genus level. This revealed that the bacterial and fungal communities at the site of this study differed greatly across spheres and were markedly influenced by their association to *A. caffrorum*. Generally, endophytic microbes, and to a lesser extent rhizospheric microbes, exhibited greater responses to changes in water content than bulk soil microbes. Here, bacteria were more responsive to water content driven changes whilst fungi exhibited smaller effects due to greater intraspecific variation within sample groups.

During desiccation, microbes with greater saprotrophic capacity were generally enriched, and in the hydrated state, growth promoting microbes were enriched. Interestingly, an increase in chloroplastic reads was also observed during desiccation and this may link to the metabolomics data and other studies on *A. caffrorum* which consistently show photosynthesis or plastid related processes in below ground organs during desiccation. The significance of this should be explored further in a separate study.

Moreover, evidence for increased biotic stress from particularly pathogenic fungi was detected in the DS winter collection but also within the rhizosphere of collection 3. Nevertheless, *A. caffrorum* can prevent many of the pathogens in its environment from entering its endosphere and evidence of protective bio-control species was detected alongside potential pathogens in association with *A. caffrorum*.

Finally, a number of species harbored by *A. caffrorum*, including the most abundant bacteria such as the *Solirubrobacterales*, have soil bioremediation potential and could allow for *A. caffrorum* to grow in its nutrient poor environment. Taken together, all these findings indicate that *A. caffrorum* likely exerts selective pressure on its microbiome and that this may play a critical role in surviving desiccation and maximizing growth in response to water availability. Given that many of the microbes reported here are relevant to agricultural crops or soil, these observations also lend themselves to the design of stress protective or plant growth promoting microbial biostimulants.

Chapter 5

Joint metabolite and microbe analysis

5.1 Introduction

Previously chapters two and three contextualized the sample environment with respect to *A. caffrorum's* physiology, soil edaphic factors and its metabolomics landscape within the endosphere and rhizosphere. These allowed for the grouping of sample collections into hydrated (one and three) and desiccated (two and four) collections, although smaller rehydration and DS specific signals still existed between the hydrated collections. Changes in water content generally resulted in the accumulation of more complex carbohydrates and lipids during desiccation. Upon rehydration, the monosaccharides and free fatty acid constituents comprising these complex reserves were more abundant, implying mobilisation thereof.

Within this context, the changes in bacterial and fungal taxa across collections were described in chapter four within *A. caffrorum's* endo- and rhizosphere as well as the surrounding bulk soil. This identified that relative proximity to *A. caffrorum* influenced microbial community structure across water contents, particularly for bacteria. Here, desiccation favored microbes with greater glycolytic and cellulolytic capacity whilst the hydrated samples contained more plant growth promoting taxa. In addition, this data pointed towards greater biotic stress during water availability, particularly during the winter collection 1.

This led to a number of hypotheses of how the metabolomics and metabarcoding datasets may be interacting with another. In chapter three, the increase of glucose, fructose and fatty acids in the hydrated state, but particularly upon rehydration in both the endosphere and rhizosphere suggested that these could serve as plant derived carbon sources available for trade with microbes. In addition, the presence of mono- and digalactosylacylglycerols were speculated to be involved with the formation of symbiosomes with endophytic microbes. Lastly, the increased levels of organic acids in collection one were speculated to alter microbial community structure through pH changes for example.

Chapter four also raised a number of hypotheses. Firstly, the presence of taxa with greater glycolytic capacity was proposed to potentially be selected for by the elevated levels of di- and trisaccharides. Secondly, *Paenibacillus* was speculated to influence the desiccation response via a signaling mechanism. Thirdly, since disruptive rehydration specific effects were also detected in the metabarcoding data, particularly for the bacteria, a possible link to rehydration specific metabolites was proposed. Fourthly, taxa such as *Dactylosporangium* were proposed to produce antibiotics that could maintain the levels of antibiotic resistant taxa such as the *Solirubrobacterales*. Finally, a number of pathogenic taxa were detected and so it was speculated that these may have significant interactions with potentially protective taxa or metabolites.

Therefore, in order to explore these hypotheses, the metabolomics and two metabarcoding data sets were analyzed jointly. This was approached by firstly comparing matching samples in a reduced dimensionality via procrustes and protest analysis and then followed up by correlating all metabolites, pooled bacterial genera and fungal OTUs to another. The significant correlations were used for construction of unipartite (containing all correlations) and bipartite (containing only correlations between microbes and metabolites) networks. This allowed assessment of the above hypotheses and also identified the most influential microbes/metabolites which impacted network topology.

However, given that both the metabolomics and the 16S results were of lower quality for the rhizosphere relative to the endosphere, this joint analysis was ultimately pursued only for the endosphere samples. This analysis revealed support for some of the above hypotheses and advanced understanding of host – microbe selection mechanisms.

Chapter aims:

1) Explore hypotheses raised in previous chapters

2) Identify additional interactions between microorganisms and metabolites

3) Identify major hubs linking the metabolomics and metagenomics data

5.2 Methods

5.2.1 Procrustes and protest analysis

Sample distance matrices for procrustes and protest analysis of metabolomics and metagenomics data sets were obtained from the PCA and NMDS analysis performed in chapters three and four respectively. These were inputted into the procrustes() and protest() functions from the vegan package (Oksanen *et al.*, 2022) and plotted using base R v4.2.2's plot function.

This analysis was then repeated on differentially abundant features only. These features were identified by ANOVA and LRT tests in chapters three and four respectively. For this analysis, euclidian distance matrices between samples were obtained with vegan's rda() function before analysis with procrustes() and protest() as before.

5.2.2 Network analysis

Differentially abundant metabolites and microbes obtained as described in the sub-section above were correlated using Spearman correlation with the rcorr() function from the Himsc package and filtered for correlations with an absolute value greater than 0.7 and a p-value less than 0.05. These were then uploaded to cytoscape to visualize the unipartitite network under the default layout algorithm.

The bipartite network was obtained by filtering the above network for only metabolite – microbe connections. This network was visualized by selecting the Edge-weighted Spring Embedded layout algorithm guided by correlations in Cytoscape (Shannon *et al.*, 2003). Resultant correlation clusters were subsequently manually adjusted so that the labels of each node were legible. In addition, the Integrated Value of Influence (IVI) score was computed for each node using the function ivi() from the influential package (Salavaty *et al.*, 2020).

5.3 Results and discussion

5.3.1 Contrasting omics data sets via procrustes and protest analysis

Commonly, a first approach to multi-omics analysis is to assess to what extent two or more data sets exhibit similar trends across their features. Procrustes analyses does this by reducing the dimensionality of the sample space of the datasets in question and then compares these resultant sample configurations pairwise by rotating one configuration to maximum similarity with another. This then allows visual pairwise comparisons of how these data sets differ in influencing the sample space. The function protest() from the vegan package can then be used to test the non-randomness or significance between two configurations in a given contrast. This approach was applied here to firstly identify if any contrasts between the 16S, ITS and metabolomics data were worth pursuing further and secondly to assess whether or not any samples or sample groups exhibited consistent discordance across all three data sets and were thus not suitable for downstream analysis.

When the unfiltered 16S, ITS and metabolomics data sets were compared by procrustes and protest analysis, none of the pairwise comparisons yielded significant p-values (Supp. Table 1). However, when only the differentially abundant metabolites, bacteria and fungi were used to ordinate their respective sample space configurations, the 16S and to a lesser extent the ITS configurations exhibited significant correlations with the metabolomics sample space configuration (Table 1). Therefore, only the differentially abundant taxa and metabolites were used for subsequent network construction. Since the two microbial data sets did not show significant correlation with another, microbe – metabolite interactions were specifically prioritized by exploring these in a bipartite network.

Table 1. PROTEST analysis contrasting data matrices comprising of only differentially abundant metabolites/bacteria/fungi

Contrast	Procrustes Sum of Squares (m12 squared)	Correlation in a symmetric Procrustes rotation	PROTEST p-value
Metabolites vs 16S	0.4356	0.7513	0.001
Metabolites vs ITS	0.5658	0.6589	0.002
16S vs ITS	0.8308	0.4113	0.162

When inspecting the Procrustes rotations, no sample was consistently stretched more than the others and so for downstream analysis, no outliers were removed. However, for the metabolite and 16S comparison, the rehydrated samples exhibit less consistency between the two datasets and so some of these rehydration specific patterns may not be linked between these two datasets (Fig. 1A). Similarly, for the metabolite – fungi contrast, the desiccated samples appear to be stretched further than the hydrated samples and so desiccation specific patterns may be less coupled between fungi and metabolites (Fig. 1B). Nevertheless, all three contrasts still separate the hydrated from desiccated samples and so downstream analysis is expected to reveal significant associations linked to this pattern (Fig. 1A-C).

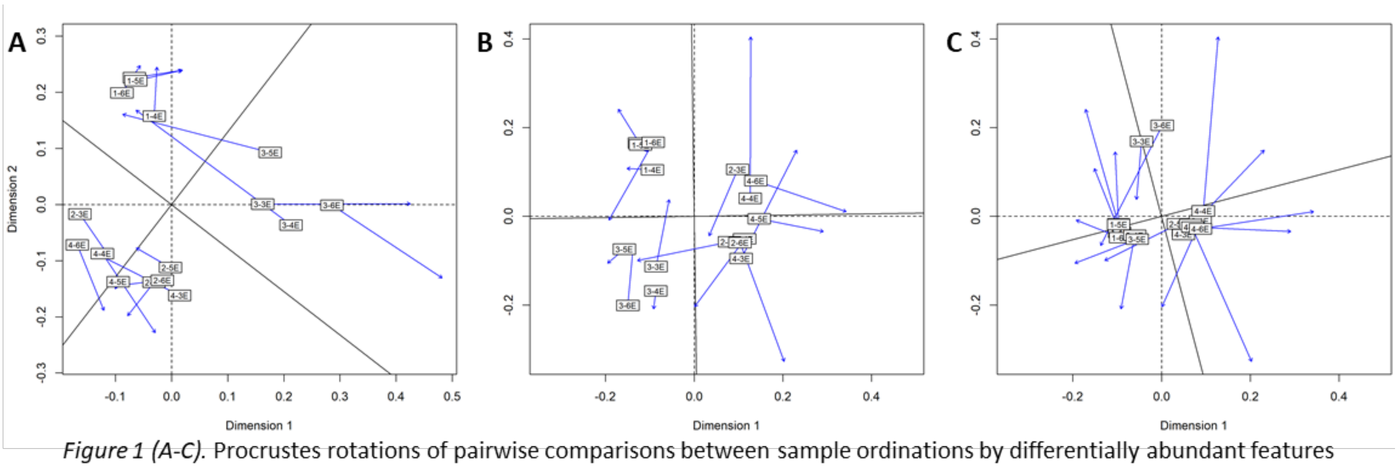

Figure 1 (A-C). Procrustes rotations of pairwise comparisons between sample ordinations by differentially abundant features across the metabolomics, 16S and ITS data sets. (A) Metabolomics data vs 16S data, (B) Metabolomics data vs ITS data and (C) the 16S data vs ITS data.

5.3.2 Identifying associations between features via network analysis

In order to identify associations between features and visualize their relative connectedness, all differentially abundant features were correlated to another using Spearman's rank correlation and the significant correlations greater than |0.7| were plotted in a unipartitite network (Fig. 2). This confirmed the procrustes analysis by illustrating that unidentified bacteria within the class *Bacilli* and genus *Bacillus* are not significantly correlated to any other taxa or metabolites. Given that these were highly abundant only in two rehydrated endosphere samples, these are the likely cause for the disparity in rehydration specific signals observed between the 16S and metabolomics data.

Moreover, this unipartite network illustrates that secondary metabolites and lipids form roughly two separate clusters that appear to be linked by the primary (GCMS) metabolites (Fig. 2). Furthermore, bacteria appear to be more embedded towards the lipid cluster relative to the secondary metabolites. Some fungi are also associated with the lipid cluster, although mostly have reduced connections and so are positioned more towards the outskirts of the unipartite network. This confirms prior observations that bacteria, more so than fungi, display interactions with the metabolome, and more specifically with lipids. Lipids such as phospholipids form the membranes that interface interactions between endophytes and their host plants (Siebers *et al.*, 2016). Thus, these may mediate communication and selection mechanisms between *A. caffrorum* and its microbes and could explain their overall closer association with lipids.

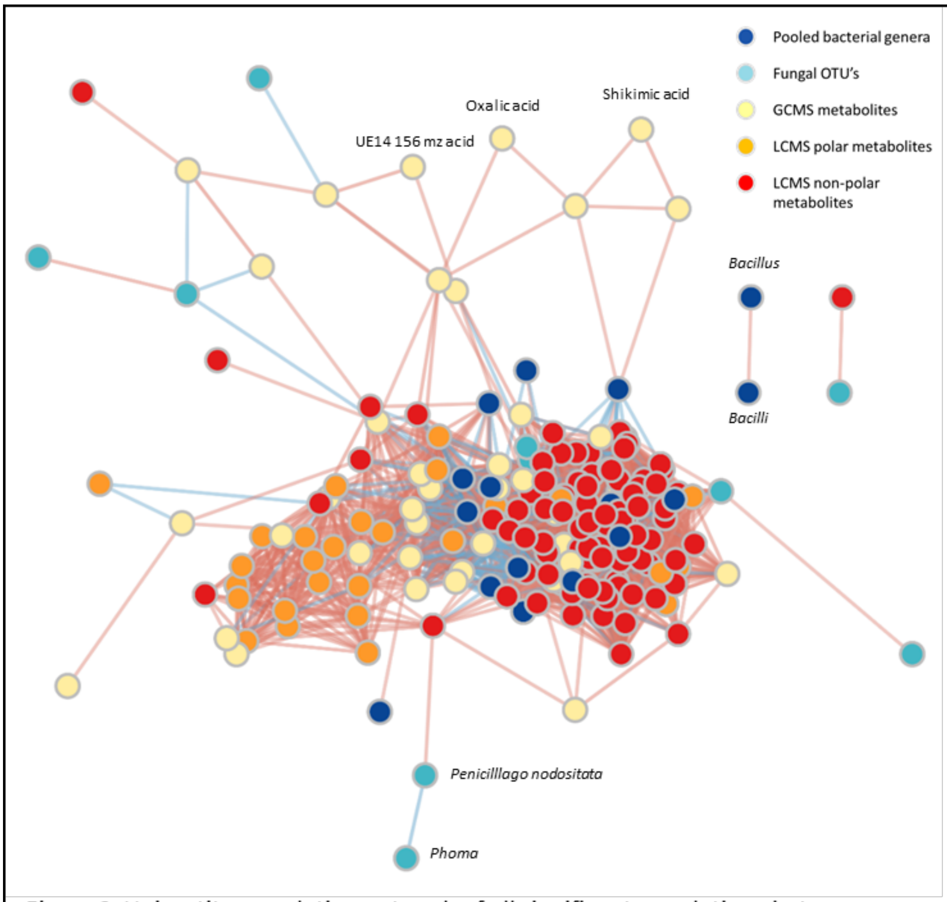

Figure 2. Unipartite correlation network of all significant correlations between differentially abundant bacteria, fungi and metabolites. Nodes are coloured by the origin of their sequencing or chromatography platform. Edges are coloured by the direction of their correlation (Spearman rho), where red edges represent positive correlations and blue edges represent negative correlations. Two nodes have been manually annotated

Therefore, to more closely examine such potential microbe – metabolite interactions, the above unipartite network was filtered for connections between microbes and metabolites only to yield a bipartite network (Fig. 3). The nodes within this new network were then manually re-positioned to allow for their labels to be legible, and therefore their relative positioning is less meaningful here. To identify which nodes had the greatest influence on network topology, the recently developed IVI score was computed for each node. This score ranks the nodes by taking into consideration their relative connectivity with other nodes in combination with their relative ability to link together neighboring network hubs (Salavaty *et al.*, 2020).

This identified eight prominent microbial hubs, of which only one was fungal (Fig. 3). In order of influence, these taxa were: bacteria of genus *Dactylosporangium*, unidentified bacteria within the family *Paenibacillaceae*, unidentified bacteria within the order *Solirubrobacterales*, bacteria of genus *Burkholderia-Caballeronia-Paraburkholderia*, bacteria of genus *Kineosporia*, bacteria of genus *Paenibacillus*, an unidentified fungus within the class of *Dothideomycetes* and finally, unidentified bacteria within the family *Gemmataceae*.

The desiccation induced bacterial hubs *Paenibacillaceae*, *Paenibacillus*, *Burkholderia-Caballeronia-Paraburkholderia*, *Kineosporia* and *Gemmataceae* were all positively correlated to primarily desiccation induced phospholipids such as PE 36:1 and PC 36:1 (Fig. 3). In fact, these were the two most influential metabolites and ranked the 9th and 10th overall following the above mentioned microbes. Moreover, *Paenibacillaceae* was correlated to numerous other phospholipids, including lyso phospholipids in addition to triacylglycerols and other desiccation induced metabolites such as sucrose and raffinose.

These desiccation induced metabolites exhibited negative correlation to the remaining prominent microbial hubs, which were increased in the hydrated endosphere (Fig. 3). These constituted the most influential node, namely *Dactylosporangium*, along with the most abundant bacterial and fungal taxa, namely *Solirubrobacterales* and *Dothideomycetes* respectively. Of these, only the *Solirubrobacterales* exhibited numerous positive correlations to the hydration induced metabolites such as the sugars glucose and fructose and lipids such as MGDG 36:6 (1)/(2), DGDG 36:6 (2) and 1-Monopalmitin. These hydration induced metabolites in turn, exhibited negative correlations to the desiccation induced microbes.

Taking the above together, this bipartite network is clearly shaped by responses to water content fluctuations. Unfortunately, this study did not capture microbial or metabolite changes independent of changes in water content. Therefore, all associations between microbes and metabolites may simply be in response to the water availability of the environment. Nevertheless, given that the results of chapter four suggest that *A. caffrorum* does exert an effect on microbial alpha and beta diversity, such associations between microbes and metabolites will be explored as a possible mechanism of this influence.

One possible mechanism that could be driving microbial changes is changing availability of growth substrates. For example, in the hydrated endosphere, the monosaccharides glucose and fructose are highly abundant and could allow the growth of bacteria that can rapidly utilize these. Upon desiccation however, increasing levels of more complex di- and trisaccharides such as sucrose, trehalose and raffinose may favour the growth of bacteria with greater glycolytic capacity. As discussed in chapter four, all the desiccation induced bacterial hubs here, with the exception of *Kineosporia*, are known for their increased glycolytic ability. Nevertheless, *Kineosporia* is positively correlated to trehalose here and can likely utilize this as a carbon source given that it has been shown to exhibit trehalose phosphorylase activity (Fig. 5.3) (Aisaka & Masuda, 1995). Furthermore trehalose was also positively correlated to the *Gemmataceae* and *Paenibacillaceae* and the latter also exhibited positive correlations to sucrose and raffinose (Fig. 3). Therefore, differential accumulation of metabolites across water availability may select for specific microbes, even if only inadvertently.

Although the accumulation of these water responsive metabolites can be attributed to desiccation protective roles or involvement in normal growth processes, it is possible that some of these are also used to intentionally mediate microbial interactions. For example, trehalose performs numerous desiccation protective functions, although it has previously also been observed to play a crucial role in the health of symbiotic root nodules, where plant derived carbon is traded for microbial nitrogen (Müller *et al.*, 1994; Streeter & Gomez, 2006). Furthermore, trehalose was the primary metabolite induced in *Solanum lycopersicum* when treated with plant growth promoting bacteria (Kalozoumis *et al.*, 2021). Therefore, these studies implicate trehalose as a carbon source associated with symbiotic bacteria. Consequently, given that transcriptomic data on *A. caffrorum's* rhizome suggested an increased association with microbes upon desiccation, it is possible that metabolites such as trehalose play a convenient dual role in protecting *A. caffrorum* and its microbes during desiccation as well as assisting in establishing symbiosis (Khan, 2023).

Another possible mechanism through which metabolites could alter microbial profiles, is through signaling that modulates the plant immune response or facilitates symbiosis. Here, the derivatives of phospholipids, such as lyso phospholipids could play a crucial role. During stress or microbial interaction, phospholipids are cleaved by phospholipases into products such as lyso phospholipids, phosphatidic acids and oxylipins which can be further utilized in the formation of hormones such as jasmonic acid (Siebers *et al.*, 2016). All of these can trigger signaling cascades which can modulate the immune response against phytopathogens (Siebers *et al.*, 2016). In addition, lyso phospholipids are a key signal involved in establishing plant symbiosis with arbuscular mycorrhizal fungi and so may also facilitate beneficial host – microbe relationships more generally (Drissner *et al.*, 2007; Vijayakumar *et al.*, 2016).

Of the above mentioned phospholipid degradation products, only lyso phospholipids were detected in the lipidomics data. Interestingly, *Dactylosporangium*, exhibited solely negative correlations to the desiccation induced metabolites including many exclusive correlations to lyso phospholipids such as LysoPC 16:0 and LysoPE 16:0 (Fig. 3). Lyso PCs and lyso PEs can both trigger plant immune responses to clear select bacteria from the endosphere (Wi *et al.*, 2014; Völz *et al.*, 2021). Therefore, it is possible that these are involved in a suppression mechanism of *Dactylosporangium* abundance. Alternatively, given that lyso phospholipids are also associated with establishing symbiotic relationships, it is possible that the absence of these rather perhaps limits *Dactylosporangium* abundance (Drissner *et al.*, 2007; Vijayakumar *et al.*, 2016).

Moreover, given that *Dactylosporangium* was identified as the most influential node in the bipartite network, such signaling by lyso phospholipids has greater potential to alter microbial community structure (Fig. 5.3). For example, a reduction of *Dactylosporangium* may in turn result in lower levels of taxa such as the *Solirubrobacterales*, which as discussed in chapter four, may be favoured in environments with more antibiotic producers (such as *Dactylosporangium*) due to this order being associated with antibiotic resistant genes (See chapter 4). However, differentially abundant antibiotics were not detected in the roots – possibly because these may also have been at such low concentrations that these would not have passed the filtering criteria of this study. Therefore, a follow up study could investigate this further by applying targeted mass spectrometry to measure if indeed such compounds can be detected in the endosphere and whether or not these are produced by *Dactylosporangium*.

Intriguingly, *Paenibacillaceae* was positively correlated to a number of lyso phospholipids such as LysoPC 18:2 and LysoPE 18:2 (Fig. 5.3). These tended to be longer and less saturated than the lyso phospholipids that were correlated to *Dactylosporangium* and so may be involved in a different signaling response. Lyso phospholipids, in addition to stimulating plant defenses against biotic stress, have also been reported to increase under abiotic stresses such as drought and freezing in plants (Zheng *et al.*, 2021; Welti *et al.*, 2002). Furthermore, under salt stress, unsaturated lyso phospholipids such as LysoPE 18:2, but not saturated lysophopholipids increase in *Hordeum vulgare* (barley) (Yu *et al.*, 2020). This highlights that these unsaturated lyso phospholipids may similarly be involved in *A. caffrorum's* desiccation response.

Furthermore, in chapter four it was proposed that the desiccation induced taxa, in particular *Paenibacillus*, may be involved in stimulating the desiccation response given that this genus can induce desiccation responsive genes in *A. thaliana* (Liu *et al.*, 2020). However, the results here suggest that an unidentified bacterium within perhaps a different genus but under the same family of *Paenibacillaceae* may be responsible for this signaling in *A. caffrorum*, as this taxon is positively correlated to all the core desiccation induced metabolites such as sucrose and raffinose (Fig. 3). Taken together with the above, the unsaturated lysophopholipids may mediate this signal between *Paenibacillaceae* and *A. caffrorum*.

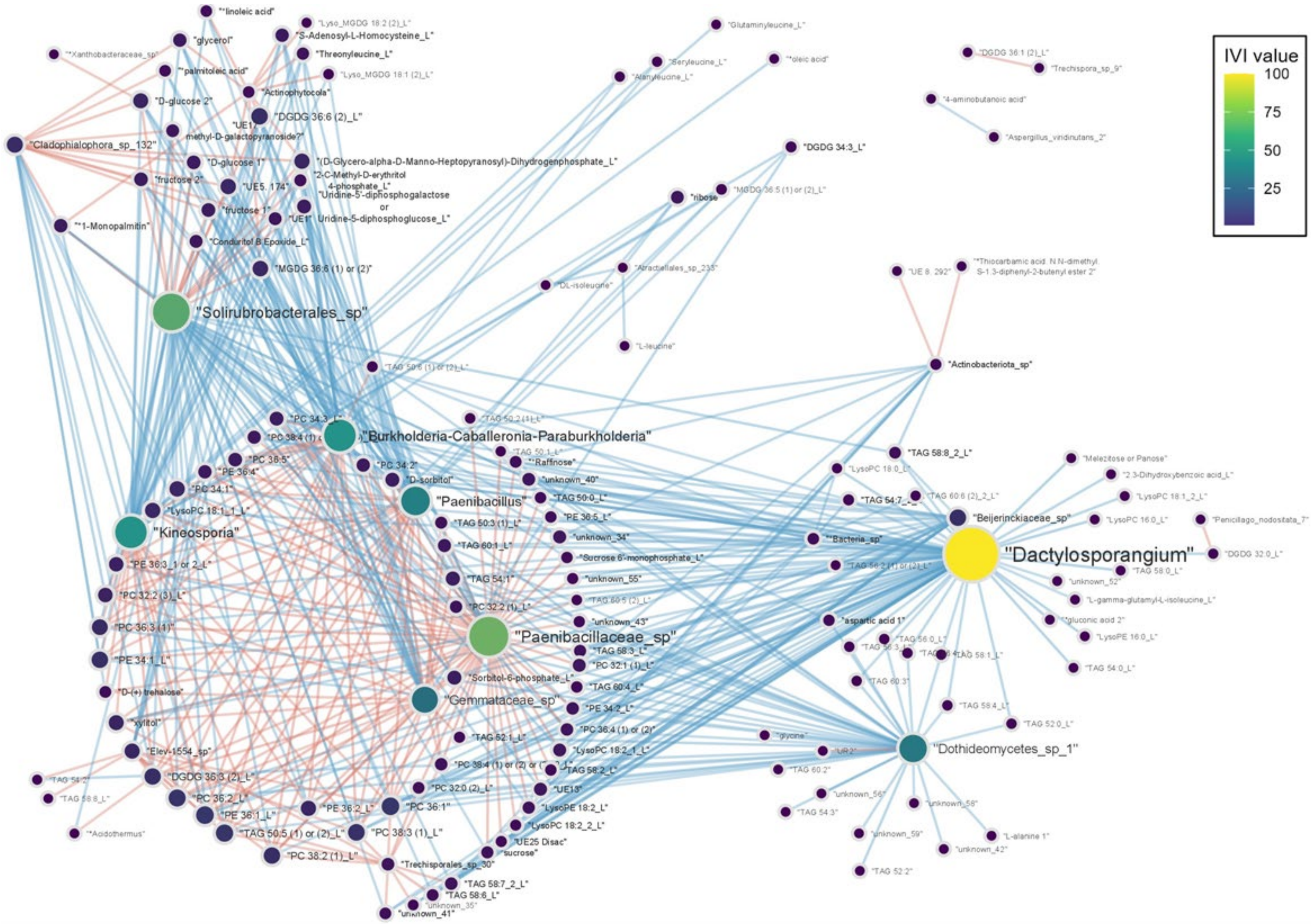

Figure 3. Bi-partite correlation network of significant microbe-metabolite correlations. Nodes are coloured by their relative influence according to the IVI score. Edges are coloured by the direction of their correlation (Spearman rho), where red edges represent positive correlations and blue edges represent negative correlations

Lastly, to investigate some of the remaining hypotheses raised in preceding chapters, the above networks were searched for specific taxa or metabolites. Firstly, in order to investigate potential interactions amongst plant pathogens, connections to *Blumeria graminis* and *Phoma* were identified, as particularly the former was suggested to be acting as pathogen in chapter four. However, only the latter displayed a single negative correlation to the fungal species *Penicillago nodositata* (Fig. 2). *Penicillago nodositata* in turn, was only correlated to a single other feature, namely DGDG 32:0 (Fig. 3). Therefore, in comparison to the effects of substrate availability and potentially signaling by lyso phospholipids, these do not greatly impact network topology and so pathogenic fungi may not be selected against by specific mechanisms. Nevertheless, pathogens are generally still excluded from the endosphere relative to the other spheres (Chapter four, Fig. 7).

As an aside, the presence of digalactosyldiacylglycerols in the roots was speculated in chapter three to be involved in the formation of a peribacteroid membrane or symbiosome that engulfs endophytes within root cortical cells (Gaude *et al.*, 2004). The most abundant DGDGs were not differentially abundant and so may be used to constitutively maintain such membranes. However, given that DGDG 36:6 (2) and DGDG 36:3 (3) were amongst the most influential metabolites correlated to the hydration and desiccation clusters respectively, *A. caffrorum* may also have species specific DGDGs for its various endophytes. Similarly for *Penicillago nodositata*, which under its former name was shown to form symbiotic myconodules in *Alnus* roots, DGDG 32:0 may be specific for the plant membranes interfacing intracellular infection by this species (Valla *et al.*, 1989). Potentially, this mechanism is disrupted by the pathogenic *Phoma* (Valla *et al.*, 1989).

Finally, the elevated presence of some small organic acids such as shikimic acid in the DS collection were proposed in chapter three to perhaps influence the microbial community via pH changes. However, these did not have a marked influence on the microbial community, as these were mostly positioned towards the outskirts of the unipartite network and had no significant connections to microbes (Fig. 2). Thus, their association with DS fronds may relate to another mechanism, such as the solubilization of phosphorus required for plant growth (Vyas & Gulati, 2009).

5.4 Brief Conclusions

In conclusion, when using only differentially abundant features, significant relationships existed between the metabolomics data and the two metabarcoding datasets – especially with the 16S data. Here, microbes were primarily associated with lipids, implicating these in host microbe communication and selection. Specifically, saturated lyso phospholipids may modulate *A. caffrorum's* immune response to alter microbial community structure by acting on influential antibiotic producing taxa such as *Dactylosporangium*. Furthermore, *Paenibacillacea* may influence the core desiccation response via the signaling of unsaturated lyso phospholipids. In addition, this analysis revealed significant correlations between the hydration induced taxa and monosaccharides in addition to the desiccation induced taxa and di- and tri-saccharides. Therefore, this analysis supports the prior hypothesis that the presence of more complex carbohydrates could be driving the selection of more glycolytic bacteria during desiccation. Lastly, unique digalactosyldiacylglycerols were associated with hydration or desiccation induced taxa which may allow for specialized symbiotic relationships across hydration states. Taken together, although these findings could not be separated from water content specific effects, and correlation is not causation, they nevertheless support evidence from previous chapters and speculate on host plant selection mechanisms that should be investigated further.

Chapter 6

Overall conclusions and outlook

In conclusion, metabolomics and metabarcoding of *Anemia caffrorum's* endosphere and rhizosphere revealed additional metabolic mechanisms of desiccation tolerance and highlighted the influence that microorganisms may play in these and seasonal adaptations.

In response to desiccation, the trends of the most abundant metabolites showed an increase in "metabolite complexity". This was evidenced by the conversion of simpler molecules to more complex structures. For example, monosaccharides such as glucose and fructose were likely mobilized to di- and tri saccharides such as sucrose, trehalose and raffinose. Similarly, free fatty acids, glycerol and ethanolamine, abundant in the hydrated endosphere, were replaced by triacylglycerols and phospholipids during desiccation. These results are in line with other studies on resurrection plants (Quartacci *et al.*, 1997; Gasulla *et al.*, 2013; Tshabuse *et al.*, 2018; Madden, 2019) and so were discussed for their known protective functions during desiccation. Taken together, these metabolite dynamics suggested that the roots, much like the rhizome, are also tolerant all year round.

A less familiar functionality discussed in this thesis was the contribution of root plastids to the desiccation response. Evidence for their involvement was seen in changes of chloroplastic specific lipids and plastid DNA during desiccation. Taken in context with other data on *A. caffrorum*, which showed major transcriptomic or proteomic changes of chloroplastic related genes or proteins in the rhizome, this research highlights the importance of considering the impact of these supposedly leaf specific processes in below ground organs. Moreover, this study discusses metabolite changes, such as those of trehalose, in terms of potential microbial contributions, which are mostly overlooked in other studies on resurrection plants. As such, these results contribute our understanding of what is required for desiccation tolerance and so may ultimately assist transferring such tolerance via genetic engineering to crops to increase their drought tolerance.

The metabarcoding data could show that indeed *A. caffrorum* exerts a selective pressure on its microbiome by influencing the diversity and functionality of its associated microorganisms. For fungi this selection appears to be favourable as symbiotic trophic modes were enriched in the endosphere whilst pathogenic fungi were enriched in the surrounding soil. Nevertheless, some obligately pathogenic fungi were present particularly within the wet season in the endosphere. These along with greater proportions of antibiotic producing bacteria and fungi as well as elevated levels of antibiotic metabolites suggest that *A. caffrorum* faces and adapts to biotic stress in the wet winter season.

Generally, bacteria more so than fungi were responsive to changes in water content and only the shifts of the latter appeared to be directed by *A. caffrorum*. In response to water availability, growth promoting bacteria were enriched in *A. caffrorum's* endo- and rhizosphere but not bulk soil across both seasons. These may assist in growth processes by for example fixing atmospheric nitrogen. In addition, the *Solirubrobacterales*, which are commonly found in nutrient poor soils, were the most abundant bacterial order and were consistently elevated in the hydrated collections. These may be an adaptation to the nutrient poor soil that resurrection plants typically grow in and have also been observed to be prominent members of the microbial communities of two other resurrection plants (Lozo *et al.*, 2023). As discussed in chapter four, this work suggests that the *Solirubrobacterales* may be potential candidates for future soil remediation efforts.

In response to desiccation, the proportion of glycolytic and cellulolytic bacteria such as those from phylum *Planctomycetota* were significantly increased. This functionality may allow for nutrients to be rapidly remobilized upon rehydration. Thus, this work marks glycolytic taxa as potentially important players during desiccation, and suggests their inclusion in microbial biostimulant research in order to uncover the potential significance of their role.

This increased glycolytic functionality may be selected for by the accumulation of more "complex" metabolites during desiccation in the endosphere. This was partly supported by the positive correlation between one of these desiccation induced taxa (an unidentified *Paenibacillacea*) and the core desiccation induced metabolites. Given that one of the species from this family can prime "desiccation responsive" genes in *A. thaliana* (Liu *et al.*, 2020), this taxon was also discussed to have a potential regulatory role in the desiccation response. Thus, this work adds further support for its inclusion in microbial biostimulants.

All of the above suggests that *A. caffrorum* can favourably alter microorganisms, especially, to assist with growth processes and overcome biotic and abiotic stresses. Network analysis within the endosphere implicated lipid metabolism as mediating this mechanism of selection. Specifically a number of lyso phospholipids may participate in this process, as these exhibited exclusive correlations to the antibiotic producer *Dactylosporangium* which was also identified to have the greatest influence on network topology. Although this finding requires further validation, it is tempting to speculate that this could present an efficient way to modulate the microbiome by only acting on influential antibiotic producing taxa that could in turn result in larger shifts in microbial community structure. If validated, such mechanisms could allow crop plants to perhaps similarly modulate their microbiomes to allow for improved growth and tolerance to biotic and abiotic stresses such as drought.

Future Outlook

A limitation of this work is that it is restricted in assessing the functional significance of the microbial dynamics reported here. These could be examined in greater detail through metatranscriptomics and Eco plate readings to respectively explore further host microbe interactions and confirm the increased glycolytic capacity of microorganisms as well as assess their substrate specificity.

Furthermore, within this study hundreds of bacteria and fungi were isolated from *A. cafforum's* rhizosphere, although not all could be screened within the time frame of this master's research project. However, future studies could screen these for interactions with lyso phospholipids to establish whether or not the correlation in this study is causal. Furthermore, these could be screened for plant growth promoting or drought protective qualities and the most promising isolates could then directly be tested on crop plants as a microbial biostimulant. As a proof of concept, 16 *Actinobacteriota* and select fungi were tested for their production of the plant hormone indole acetic acid (Supp. Fig. 4 & Supp. Fig. 5). Although some isolates looked promising, the top isolates of each could not increase root elongation in *Z. mays* seedlings (data not shown). Nevertheless, these additional functional experiments are required to impactfully address food insecurity through such research.

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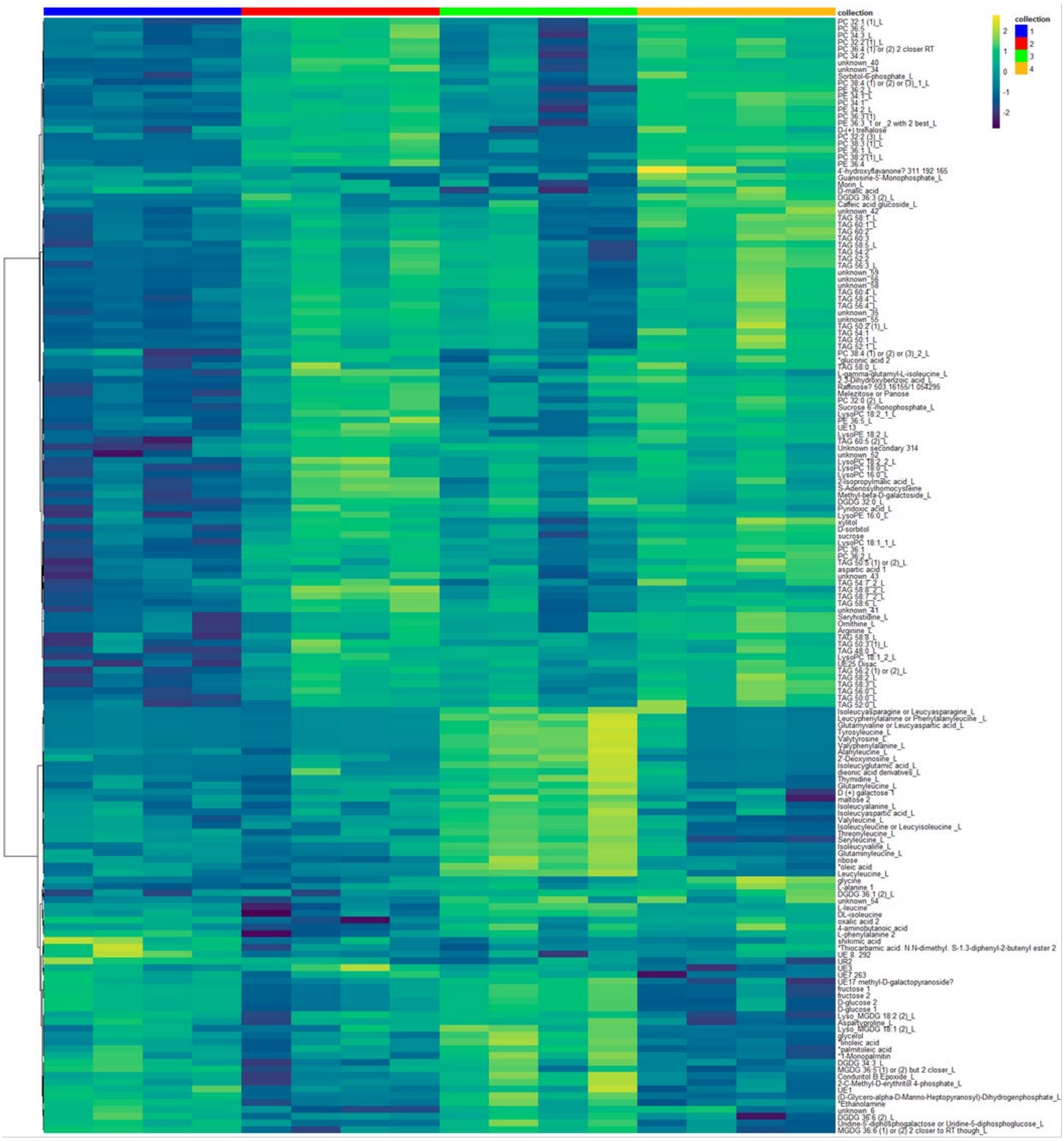
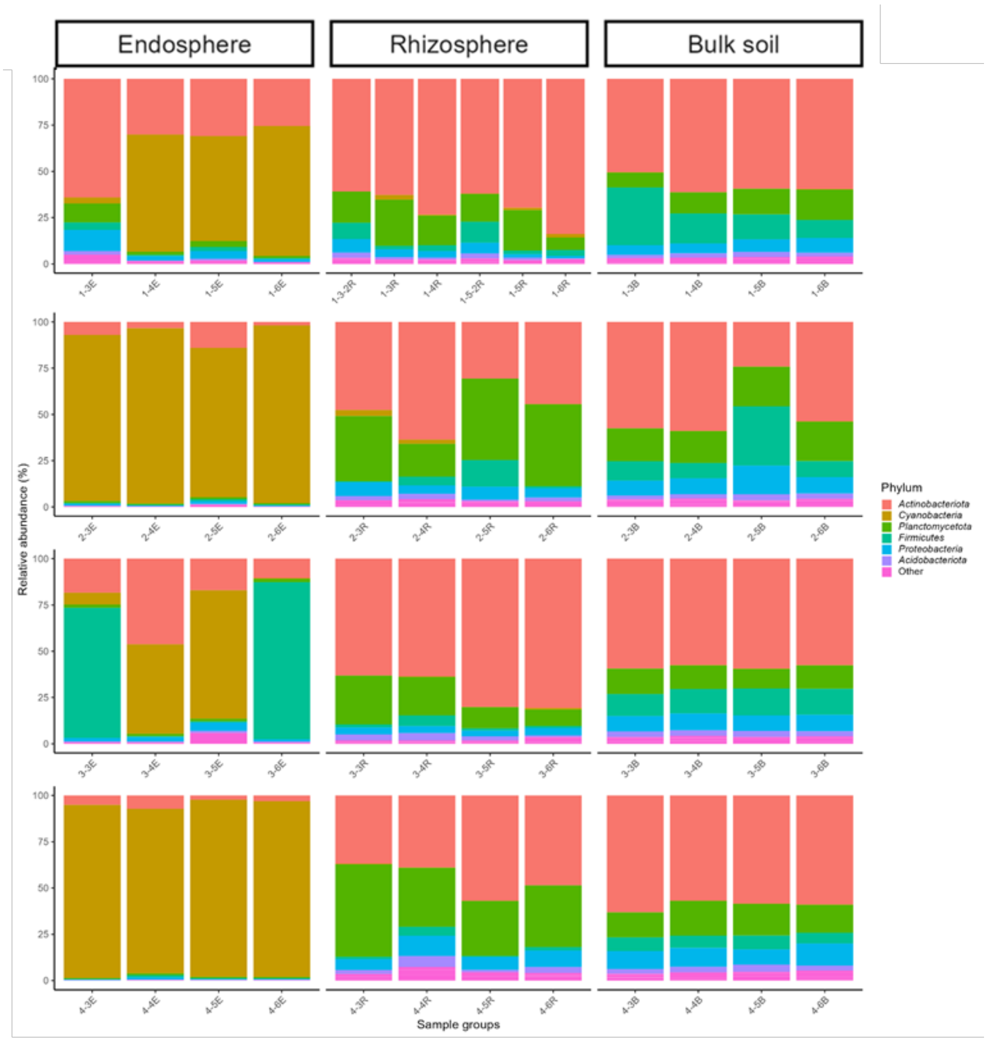
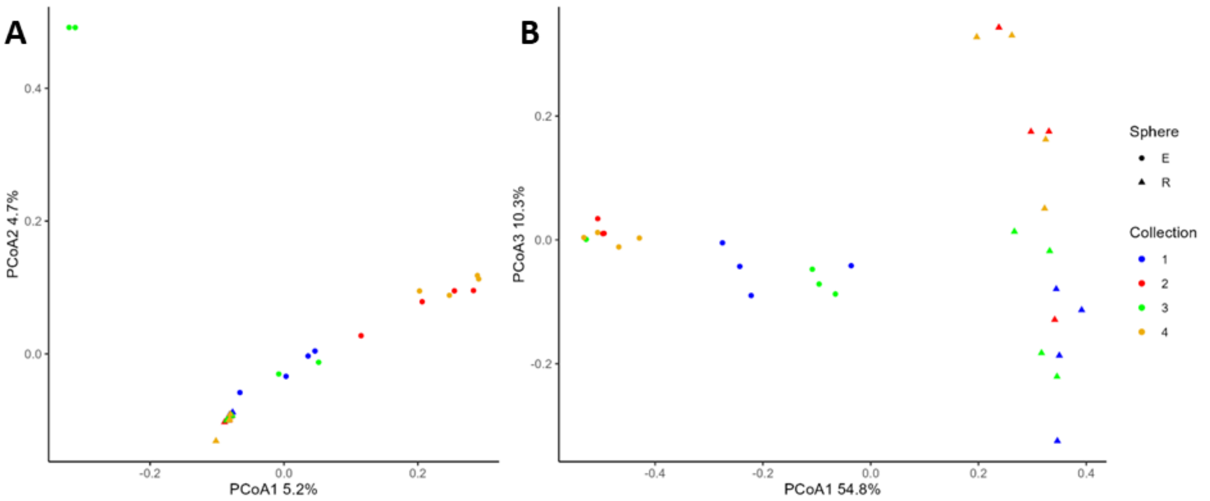
Supplementary figures



Supplementary Figure 1. All endosphere metabolites identified as significant (BH p-value < 0.05) by ANOVA.



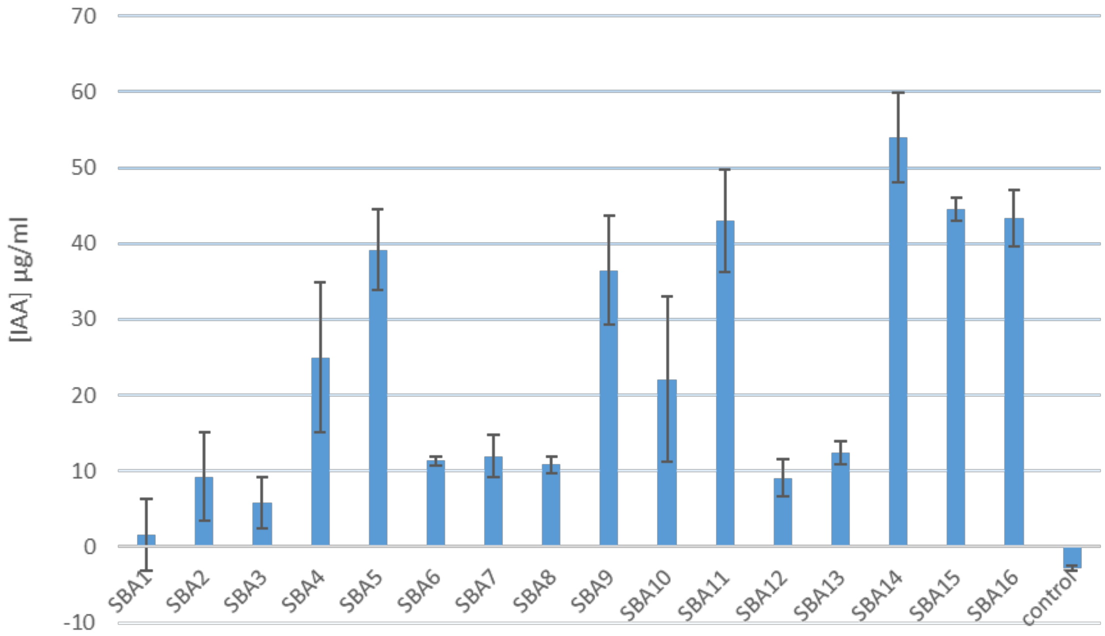
Supplementary Figure 2. Distribution of 16S reads within each sample.
Other = the remaining phyla with a median abundance less than 1%.



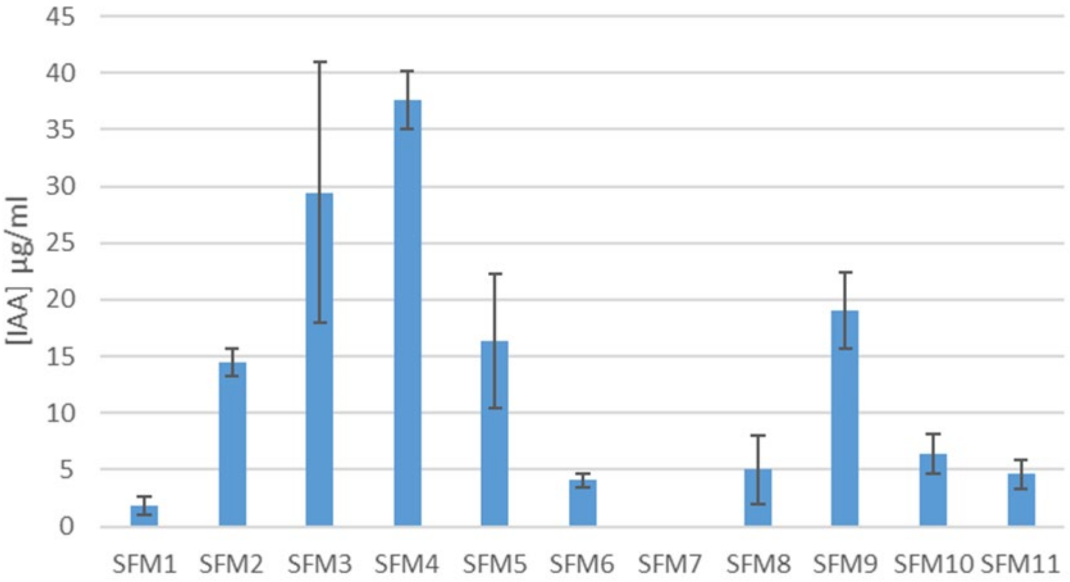
Supplementary Figure 3. Principal Coordinate Analysis of (A) the 16S OTU count matrix and (B) the 16S OTU counts grouped by genus within each sample. This analysis was performed as part of an exploratory data analysis to optimize the workflow and identify patterns of interest in the endosphere and rhizosphere and so does not include the bulk soil.

Supplementary Table 1. PROTEST Analysis of unfiltered data

Contrast	Procrustes Sum of Squares (m12 squared)	Correlation in a symmetric Procrustes rotation	PROTEST p-value
Metabolites vs 16S	0.5950	0.6364	0.156
Metabolites vs ITS	0.4757	0.7241	0.635
16S vs ITS	0.6737	0.5712	0.868



Supplementary Figure 4. IAA production of select *Actinomycetota* isolates. The error bars represent the standard deviation of three replicates for each isolate. IAA was quantified with a spectrophotometer after incubation with Salkowski's reagent after six days of growth on ISP2 broth. The control was inoculated with sterile ISP2 broth.



Supplementary Figure 5. Production of IAA of select fungal isolates. From each isolate, two fungal agar plugs were inoculated in triplicate into Czapex broth amended with 5 mM L-Tryptophan and grown at 30 °C with shaking for 6 days. Thereafter, the concentration of IAA was measured with a spectrophotometer after incubation in Salkwoski's reagent. The error bars represent the standard deviation of three replicates for each isolate. Data for SFM7 is not available, as this isolate did not grow in the pre-inoculation step.