METABOLITE ANALYSIS OF RESURRECTION PLANTS

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Abstract

The vegetative tissues of resurrection plants have the unique ability to survive to almost complete intracellular water loss and to recover their full metabolic activity upon rehydration. This phenomenon is called desiccation tolerance and it involves several metabolic changes that are employed by the plant to overcome a number of drought stresses like mechanical destabilization, membrane denaturation and oxidative damage. The multiple responses to these drought stresses comprise both protection mechanisms during dehydration and repair mechanisms upon rehydration. In this study, metabolic changes involved in protection mechanisms will be analysed at three different water contents during the dehydration process in seven different resurrection plants and two desiccation sensitive species. Furthermore, a comparison of the metabolite composition among these plants will be presented. The aim of this study is to detect which are some of the key metabolites involved in desiccation tolerance and which are in common to all the studied resurrection plants. Some species-specific metabolite responses will also be introduced. This study will give a first hint on some protection mechanisms, even though further researches on species-specific responses and on repair mechanisms should be enhanced to get a greater understanding of the complex phenomenon which is desiccation tolerance.

1. Introduction

1.1 Desiccation tolerance

1.1.1 What is desiccation tolerance

Drought tolerance, in general, is considered as the survival of an organism in response to low environmental water availability, while maintaining high internal water abundance (Berjak 2006). Desiccation tolerance, by contrast, is a more specific drought mechanism that can be defined as the ability of an organism to survive the loss (>95%) of most of its intracellular water for extended periods and to fully recover its metabolic competence upon rehydration (Farrant, Brandt, and Lindsey 2007).

Desiccation tolerance (DT) is a common phenomenon that can be found in a few animals, some fungi (mainly yeast) and in plants. In most of the plants it is located in the reproductive structures (pollen, spores and seeds), while is quite a rare trait in vegetative tissues. The majority of desiccation tolerant plants are found in the less complex clades such as algae, bryophytes and lichens, while much less frequently in pteridophytes and angiosperms. There are no known DT gymnosperms (Farrant et al. 2007; Oliver et al. 2000).

1.1.2 What are resurrection plants?

Among the various organisms that are desiccation tolerant are unusual plants called "resurrection plants". Their distinctive characteristic is the ability to tolerate extreme degrees of water loss (up to 95% of cellular water), and in their complete recovery of their normal physiological function on rehydration. In addition, they can survive and recover from multiple cycles of dehydration and rehydration, thanks to their stability of the dry tissue and their longevity in the dry state (Gaff and Oliver 2013). Their desiccation tolerance is a sophisticated phenomenon founded on a wide variety of complex mechanisms which comprise both protection against stresses and repair of the resulting damages (Berjak 2006; Cooper 2002). These mechanisms can vary in relation to the species, the drying rate and environmental conditions such as temperature, light exposure and humidity (Zhang and Bartels 2018) even though some of the responses are often found in common (Farrant et al. 2009).

At least 330 different species of resurrection plant have been discovered to date (Berjak 2006), of which 135 are angiosperms (Costa et al. 2017). These plants are mainly present in seasonally arid subtropical and tropical regions and a rich diversity of them is found in arid and semiarid areas of southern Africa (Berjak 2006; Costa et al. 2017; Moore et al. 2009; Vicré et al. 2004).

1.1.3 Desiccation stress and metabolic responses

Desiccation is an important abiotic stress and induces in plants a variety of changes that threaten their health and survival. The main stresses that plants have to overcome in order to survive desiccation can be classified in three main categories: (i) mechanical stress associated with loss of turgor, which leads to cytoplasmic shrinkage and plasmolysis, (ii) denaturation of proteins and destabilization or loss of membrane integrity, and (iii) oxidative stress related to the disruption of metabolism and production of damaging ROS (Farrant et al. 2007; Vicré et al. 2004). These changes can bring normal plants to death, while resurrection plants can stand and overcome them thanks to a number of specific adaptive mechanisms (Farrant et al. 2009):

a) Dismantling of the photosynthetic apparatus and chlorophyll degradation (poikilochlorophylly) or chlorophyll retention and masking from the light

(homoiochlorophylly) in order to minimize damages related to photo-oxidative stress due to ROS production.

b) Accumulation and upregulation of antioxidants to quench ROS.
c) Accumulation of sucrose and other raffinose family oligosaccharides and cyclitols. These components act with proteins for subcellular protection by vitrification.
d) *De novo* synthesis of stress associated proteins, such as late embryogenesis abundant proteins (LEAs) and small heat shock proteins (sHSPs) for macromolecular and subcellular integrity.
e) Additional vacuoles formation and substitution of the internal lost water with compatible solutes to prevent mechanical stress such as plasmolysis.

All these adaptive mechanisms can vary among resurrection plants, but some similar response's patterns can be found and used to group species. The differences between response in resurrection plant are due to various reasons:

- A first big difference can be encountered between the lower orders (bryophytes and lichens), termed "fully desiccation tolerant" and the higher orders (angiosperms), called "modified desiccation tolerant" (Oliver and Bewley 1997). The main differences between these two groups are found in the adopted protection and repair mechanisms, but also in their sensitivity to the drying rate (Farrant et al. 2009, 2007; Vicré et al. 2004). Less complex plants, like bryophytes, can withstand rapid desiccation (also within an hour) and survive to almost complete water loss. They therefore heavily rely on cellular repair mechanisms, since lot of damages occur after rapid drying (Oliver et al. 2000). More complex plants, such as angiosperms, are more sensitive to rapid water loss and can only withstand desiccation if it occurs slowly (between 12 h and several days). These plants, in contrary, rely in larger part on protection mechanisms and during gradual water loss they have time to produce compounds and activate protection processes that allow them to minimize damage related to drought stress (Oliver et al. 2000).
- Resurrection plants also have two very specific responses of their photosynthetic apparatus during the desiccation process, which splits them in to two clearly different groups: poikilochlorophyllous (PDT) and homoiochlorophyllous (HDT) plants (Vicré et al. 2004). The first group is mainly composed of monocotyledons (Vicré et al. 2004) and is defined by the ability to dismantle the photosynthetic apparatus and to completely degrade chlorophyll, in order to limit photo-oxidative stress, avoiding excessive ROS damage (Costa et al. 2017). Even though the probability of light damage is lowered, the disadvantage for these plants is the necessity to resynthesize *de novo* their photosynthetic system upon rehydration, employing a lot of energy (Vicré et al. 2004). Contrarily, homoiochlorophyllous plants retain their photosynthetic apparatus in the dried state, do not degrade chlorophyll and tend to avoid light damages of their tissue by modifying their morphology (Oliver et al. 2000; Vicré et al. 2004).

Since resurrection plants have different strategies to respond to desiccation stress, a complete individual study and then a detailed comparison between their mechanisms is essential to find out which are the most critical ones that play the main roles in desiccation tolerance. This would contribute to the development of relevant and complete model systems to inform crop improvement efforts.

1.1.4 Evolution of desiccation tolerance in resurrection plants

The evolution of desiccation tolerant plants has been recently reviewed by Costa et al., in 2017. Initially, vegetative desiccation tolerance arose with its transition from aquatic to terrestrial life forms (bryophytes), when likely adverse conditions, the difficulty and costs of survival were high. Over time, plants spread inland and developed more complex structures (tracheid in tracheophytes) that could conduct water from the soil and transport it up to their aerial tissues. There might be lot of reasons why these plants lost DT in vegetative tissues. A hypothesis that could partially explain it, could be that a slow growth, probably related to the high metabolic costs of DT, limited their ability to adapt to a new and complex environment. Therefore, to adapt to it, they might have lost DT in vegetative tissues and developed alternative mechanisms to prevent water loss, confining DT in reproductive structure such as spores, pollen and seeds (Costa et al., 2017; Oliver et al., 2000).

After the loss of desiccation tolerance in vegetative tissues and the redirection of DT to seeds, the trait re-evolved multiple times in separate lineages resulting in the development of new vegetative desiccation tolerant plants called "resurrection plants" (Oliver et al. 2000).

Since modified-desiccation tolerant plants are seed plants, it has been proposed by Farrant and Moore, in 2011, that the evolution of all angiosperm resurrection plants has occurred thanks to the non-deactivation of early stage genes involved in desiccation protection of seeds. The expression of these genes might be deactivated for a while during seed germination and then reactivated during seedling establishment and consequently redirected toward the vegetative tissues. Seeds could therefore be considered as a probable source of genetic programming that allowed the evolution of resurrection plants.

Indeed, resurrection plants have shown to be very similar to desiccation tolerant seeds, called orthodox seeds, in terms of their metabolic responses to drought stress (Costa et al. 2017; Moore et al. 2009).

1.1.5 Why it is important to study resurrection plants

In recent years, desiccation tolerant plants have attracted increasing research interest, since their biology has the potential to inform crop improvement strategies in response to climate change and declining rainfall. "The impact of drought on crop production is of continuous and growing concern as the world struggles to meet food production targets for an increasing global population" affirmed Oliver et al., in 2011. The need to clearly understand the critical mechanisms at the base of desiccation tolerant resurrection plants is of great importance and is essential for developing strategies that could enhance and improve drought tolerance of all major crops (Farrant et al. 2015; Oliver et al. 2011; Vicré et al. 2004).

Climate models forecast an increase in drought and a decline of water resources in the main world's agricultural areas. Since this will happen in the near future, it is highly urgent to develop more drought tolerant crop varieties (Costa et al. 2017; Oliver et al. 2011). This pressure is strongest in developing countries, which lack the necessary resources for the crop improvement required for food security.

1.1.6 Systems biology for understanding resurrection plants

Some good results in engineering dehydration-induced genes in *Arabidopsis thaliana* have been reported (Moore et al. 2009), even though the strategy used, based on single-gene engineering, is limited when trying to reproduce such a complex phenomenon as desiccation tolerance. The

authors suggested that a holistic approach is needed in order to engineer in sensitive species all the most important genes involved in desiccation tolerance and coordinate their expression. This strategy requires a modelling approach which identifies the key elements, like genes, proteins or metabolites, that are responsible for desiccation tolerance. Such an approach, termed systems biology, allows the study of different aspects of biological systems, individually, in concert, and in relation to endogenous and exogenous changes. Since desiccation tolerance is a multi-genic and multi-factorial phenomenon, it is necessary to identify the individual factors involved in DT and their network dynamics. By using the systems biology approach, it would be possible to discover the common and crucial mechanisms responsible for desiccation tolerance. (Moore et al. 2009). This approach depends on multivariate analysis to elucidate components and relationships within the mechanisms of DT and depends on the database produced by the combination of different "omics" technologies: genomic, transcriptomic, proteomic and metabolomic.

1.2 Metabolites analysis in resurrection plants

1.2.1 Metabolomics technology

Among the "omics" technologies, metabolomics is a relatively new field and concerns the detection and quantification of small molecule metabolites in the plants' cells and tissues. This technology could significantly contribute to the study of desiccation tolerance in plants, by tracking different and critical compounds induced during stress metabolism responses (Shulaev et al. 2008; Wishart and Greiner 2007). Metabolomics is continuously developing and includes the following approaches: metabolomic fingerprinting, metabolite profiling and targeted analysis. These can be specifically used depending on the study case, but they can also be combined into a more comprehensive analysis.

Metabolomic fingerprinting is used to identify particular stress responses in metabolic signature or patterns, however, without the identification of metabolites and their precise quantification.

Metabolite profiling instead concerns a simultaneous measurement of all or a set of metabolites. Various techniques are available for this analysis and includes NMR (nuclear magnetic resonance), GC-MS (gas chromatography-mass spectrometry), LC-MS (liquid chromatography-mass spectrometry), CE-MS (capillary electrophoresis-mass spectrometry) and FT-IR spectroscopy. Within these techniques, GC-MS is frequently used for plant metabolite profiling. Using this technique, it is possible to profile multiple compounds belonging to different chemical classes such as sugars, sugar alcohols, organic acids, amino acids and fatty acids. The main limit in this technology is the ability to analyse only volatile compounds or compounds that are volatilized by chemical derivatization. Therefore, for non-volatile compounds, LC-MS and CE-MS can be alternatively used.

As third approach, targeted analysis is used for the determination of specific concentrations of a limited number of known metabolites. This technique has been widely used to identify metabolites involved in specific stress responses and it can also be used for comparative metabolite profiling of a large number of known metabolites (Shulaev et al. 2008).

All these metabolomic approaches can detect and quantify a wide range of different metabolic compounds involved in desiccation tolerance in resurrection plants, but this field is still young and with its future development will hopefully lead to new insights on which are the critical components indispensable for desiccation tolerance.

1.2.2 Primary metabolites involved in desiccation tolerance

The existing literature documents several classes of primary metabolites that play central roles in desiccation tolerance:

Sugars

Different kinds of sugars appeared to be very much involved in desiccation tolerance. These include glucose, fructose, sucrose, raffinose and stachyose, etc. Massive changes in carbohydrate profile have been shown during dehydration in resurrection plants: monosaccharide sugars such as glucose and fructose are usually abundant in the hydrated state, but in late stages of dehydration they seem to decrease. As well as starch, they also might be employed as carbon source and reallocated mainly to sucrose and raffinose family oligosaccharides formation (Suguiyama et al. 2014; Farrant et al. 2015; Berjak 2006).

Some sugars, in particular glucose and fructose, but not sucrose, participate in Maillard reactions and they may cause their glycation by binding with proteins, which would be a damaging reaction for the plant. Therefore, when drying, plants reduce their content in order to limit the damage (Moore et al. 2007). Furthermore, the decrease in monosaccharides abundance may limit respiration and its associated ROS production (Moore et al. 2007). By contrast, sucrose and raffinose are accumulated in the dry state and are likely to be involved in a number of protective and repair functions. They can stabilize the sub-cellular milieu by vitrification. In addition, these sugars can also form hydrogen bonds, to stabilize molecular interactions like in the membranes. Lastly, they can be accumulated in vacuoles, together with other compatible solutes, for a better mechanical stabilization (Farrant et al. 2015; Farrant et al. 2009; Farrant 2000).

Other Compatible Solutes

A wide variety of other compounds have been hypothesised to function as compatible solutes. These include amino acids such as proline, glycine betaine, pinitol and sugar alcohols such as sorbitol, ribitol, arabitol, inositol and mannitol (Suguiyama et al. 2014) and are also used to replace water for membrane or cytoplasmic stabilization (Moore et al. 2007).

Antioxidant Systems

Numerous antioxidant systems have been reported in resurrection plants, which together serve to protect desiccated tissues from the considerable damage that would otherwise kill the plant due to ROS and free radical compounds (Dace 2014). The mechanism of antioxidant protection is activated in the early stage of dehydration by antioxidant enzymes such as ascorbate, GDP mannose-3',5' epimerase, glutathione reductase (GR), superoxide dismutase (SOD), catalase and ascorbate peroxidase (AP) (Farrant et al. 2015). The metabolites involved in the antioxidant system are small molecules: water-soluble compounds such as ascorbate and lipid-soluble compounds like tocopherols (Farrant et al. 2007). Some antioxidants that appear to have particular significance for resurrection plants include glutathione, α-tocopherol (John P. Moore et al. 2007; Oliver et al. 2011) and a wide variety of polyphenols such as 3,4,5-tri-O-galloylquinic acid in *Myrothamnus flabellifolia*. Furthermore, additional antioxidants such as 1-cys-peroxiredoxin, glyoxalase I family proteins, zinc metallothionine and metallothionine-like antioxidants (Moore et al. 2007).

Pigments

Even though poikilochlorophyllous (PDT) and homoiochlorophyllous (HDT) species use different strategies to protect them from light absorption, they both accumulate pigments, such as carotenoids and anthocyanins. In HDT, pigments are firstly employed to prevent light absorption since such pigments act as "sunscreen", masking the chlorophyll from excessive radiation (Vicré et al. 2004). Another function of these pigments, both in PDT and HDT plants, is to act as antioxidants by quenching free radicals, to minimize light damage (Farrant 2000; Vicré et al. 2004).

Class	Examples	Functions
Sugars	Glucose, Fructose, C8 2-Octulose, Sucrose, Trehalose, Stachyose, Maltose, Arabinose, others oligo- and poly-saccharides, Aldoses	• Energy metabolism and storage (storage reallocated as carbon towards phosphoenolpyruvate pathways) • Glass formation in anhydrobiosis for cellular protection and membrane stabilization (vitrification) • Water replacement in anhydrobiosis (replacing for H bonds) • Compatible solutes in anhydrobiosis (osmoprotectant) • Structural polymers • Necessary for the resumption of metabolic activity (could help to avoid enzyme denaturation)
Sugar alcohols	Mannitol, Glycerol, Erythritol, Arabitol, Xylitol, Ribitol, Sorbitol, and myo-inositol	• Compatible solutes in anhydrobiosis and osmoprotectant • Stabilizing agents against thermal denaturation • Vitrification in anhydrobiosis
Fatty acids and lipids	Palmitic acid	• Energy storage • Structural role in membranes as phospholipids, maintain the integrity of water conducing elements • Intermediates in production of many secondary metabolites and volatiles • Limit water loss due to transpiration
Organic acids	Citric acid 3,4,5-tri-O-galloylquinic acid (derivate of quinic acid)	• Primary energy metabolism • Antioxidant function
Amino acids	Alanine, tyrosine, y-glutamyl-isoleucine, y-glutamyl-leucine, proline	• Monomers of proteins • Compatible solutes • Vital components of overall nitrogen metabolism • Some (notably phenylalanine and tryptophan) are critical precursors of secondary metabolites • Upregulation of some amino acids is observed in generalised stress responses

Pigments	Luteolin (flavonoid), anthocyanins, zeaxanthin (carotenoid), vanillate	• Plant pigments • Antioxidant activity: by accelerating the non-photochemical quenching (NPQ) of ROS products • Protection from the sun by acting as a "sunscreen" to mask chlorophyll from the light • Numerous physiological roles

Table 1: Selection of primary metabolites classes with examples and function explanation (Zia et al. 2016; Farrant et al. 2015; John P. Moore et al. 2009; Farrant, Brandt, and Lindsey 2007; Vicré, Farrant, and Driouich 2004; Farrant 2000)

1.3 Numerical analysis

1.3.1 Pre-processing

After the GC-MS analysis, the data obtained are usually messy, noisy and incomplete, therefore several steps of pre-processing are needed to assess and improve data quality (Wehrens et al. 2009).

1. **Noise reduction**: physic-chemical data always contain noise, where the term noise" is usually reserved for small, fast, random fluctuations of the response. The goal of this step is to generate data with higher quality, while decreasing the noise levels.
2. **Baseline removal**: Sometimes it's possible to find a 'background signal' which is far away from the zero level. It is therefore necessary to correct this phenomenon, since it influences measurements like peak heights or peak areas.
3. **Aligning peaks**: Analytical data often suffers shifts in peak positions. In mass spectrometry, this shift is usually uniform over the m/z axis and are usually small, therefore can be easily corrected by the use of standards.
4. **Peak picking:** This step is used in order to avoid misalignment of peaks and transforms spectra into a list of features. In chromatography, peaks are usually described as a modified normal distribution and can be split into several overlapping segments. Peaks can be then identified by peak height or peak area.
5. **Scaling:** Many analytical methods provide data that are not on an absolute scale; the raw data is such a case cannot be directly used to compare different samples. In case the total intensity of a spectra would be sample-dependent, then the spectra should be scaled in a way that the intensities could be compared.

1.3.2 Principle component analysis

Principal Component Analysis (Varmuza & Filzmoser 2009) is a method for exploratory data analysis that has the aim to reduce the dimension of multivariable dataset and to represent it while holding the most information possible. It computes a new orthogonal coordinate system based on latent variables, that represents distances between objects (vectors) in the high dimensional variable space. Those orthogonal latent variables describe the variability within the data set and identify those variables that most contribute to it. By plotting the samples' Principal Components, it is possible to visualise whether the sample sets of interest cluster within the multidimensional data space, and so to determine which principal components (and hence which original variables) best represent the differences among the sample populations. Usually, the majority of the data set variability is accounted for by the first few principal components, nevertheless the number of components is the same as the number of variables of the whole dataset. PCA is indeed a particularly

useful method of analysis for mega-variate datasets with high levels of co-variance, such as those generated in metabolomic experiments.

1.4 Plants under study and objectives of the research

The main aim of this study is to analyse the primary metabolites involved in desiccation tolerance in a variety of resurrection plants and to compare them in order to detect possible similarities.

In this project, plants from different clades and with different anatomy and physiological adaptation mechanisms have been studied, since the comparison between them can facilitate our understanding of the mechanisms underlying DT and can be a first step towards the identification of suitable plants that could be used as model plants for the engineering of DT (Farrant and Moore, 2012; Moore et al. 2009).

Firstly, they have been singularly analysed to observe the changes in metabolites over the dehydration process and to compare their composition between hydrated and desiccated state.

Furthermore, to deeply understand the mechanisms underlying desiccation, metabolites have also been compared between late dehydration and full desiccation, since it is the moment where most changes in the metabolome occur (Zhang and Bartels 2018). Since this comparison has hardly been addressed (Zhang and Bartels 2018)the second main point of this research is to understand and show the different physiological and molecular responses at late degrees of water loss. Indeed, it has been shown that most of the resurrection plants clearly change their metabolite composition and physiological characterization when they reach the boundary between dehydration and desiccation, which is thought to be at approximately 40-30% of RWC (Zhang and Bartels 2018).

The output of this study is a list of metabolites that are identified in 7 different resurrection species at three different relative water contents (fully hydrated, moderate dehydrated at approx. 50-30% RWC and at fully dehydrated). Secondly, a detailed comparison of the metabolomes of the different species is going to be given, with a possible individualization of footprint mechanisms of DT. Lastly, this research will be a useful database for future testing of hypothesis on resurrection plant and desiccation tolerance.

1.4.1 Studied plants

The studied plants belong to diverse clades (view table 1), from relatively ancient and simple ones like bryophytes to higher and more complex clades such as angiosperms. Within the Angiosperm clade, both monocotyledon and dicotyledon species have been used, in order to give a broader and completer idea of desiccation tolerance and to facilitate the development of model plants that in the future could be useful for the engineering of their genes in to both mono and dicots crops. Moreover, as a control, desiccation sensitive species have been used and their metabolome compared to the DT species, for a better understanding of the differences in constitutive and desiccation induces metabolites.

Species	Clade	Orden	Family	Type
Selaginella sp.	Bryophyte	*Licopodiales*	*Selaginellaceae*	DT
Xerophyta viscosa	Angiosperm	*Pandanales*	*Velloziaceae*	DT
Xerophyta elegans	(monocot)	*Pandanales*	*Velloziaceae*	DT
Xerophyta humilis		*Pandanales*	*Velloziaceae*	DT
Eragrostis nindensis		*Poales*	*Poaceae*	DT
Eragrostis tef		*Poales*	*Poaceae*	DS
Myrothamnus flabellifolia	Angiosperm	*Gunnerales*	*Myrothamnaceae*	DT
Craterostigma pumilum	(eudicot)	*Lamiales*	*Linderniaceae*	DT
Arabidopsis thaliana		*Brassicales*	*Brassicaceae*	DS

Table 2: Plants under study and their phylogenetic classification. Type describes the ability of a plant to tolerate (DT) or not (DS) desiccation.

The most ancient and simplest species that has been studied in this research is a spike moss of Selaginella spp, that hasn't been classified yet and that is physiologically very similar to DS *Selaginella lepidophylla*. It has the ability to 'resurrect' from an air-dried state in a very short time (Yobi et al. 2013) and is the only one of the resurrection plants of this study, that has been analysed only at fully hydrated and fully desiccated states, because of its extremely rapid dehydration cycle (approx. 24 h).

Within the monocot angiosperms, three different Xerophyta species have been studied. The most studied up to now is *Xerophyta viscosa*, and much is already known about its genome and metabolome. Less is known about *Xerophyta humilis*, while *Xerophyta elegans* is poorly known. It is going to be interesting the comparison of the three of them, in order to understand their closest similarities but also their main differences. One element that differs them is that first two species are poikilochlorophyllous, while *Xerophyta elegans* is homoiochlorophyllous.

Included in the monocot angiosperms, *Eragrostis nindensis* has been studied. This grass has already been widely studied by several authors (Balsamo et al. 2005; Berjak 2006; Plancot et al. 2014) and they have shown that one of its main characteristics is the diversity of its inner and outer mature leaves in the response to dehydration. Indeed, it has been shown that inner leaves survive to dehydration and recover upon rehydration, while outer leaves are senescent and die during dehydration (Berjak, 2006). Like *Xerophyta viscosa* and X. *humilis*, it is poikilochlorophyllous.

Eragrostis tef is very similar to *Eragrostis nindensis* but is desiccation sensitive. It has been used as control for the monocot angiosperms.

Within the dicotyledonous angiosperms, two very different species have been studied, both homoiochlorophyllous. One is *Myrothamnus flabellifolia*, which is a unique resurrection plant, since it is the only woody shrub and it is characterized by fan-like leaves which fold upon desiccation (John P. Moore et al. 2007). It is indeed characterized by lignified stems and a bushy phenotype. It is the largest (0.5-1.5 m high) and most widely distributed resurrection plant species (J P Moore et al. 2007).

The other dicot RP is *Craterostigma pumilum*. It is also homoiochlorophyllous, its internal leaves are desiccation tolerant, while its outer leaves become senescent after one dehydration cycle. Despite what is already known of its anatomical adaptations, little is still known about the biochemical and molecular processes associated with the shutdown of the photosynthetic metabolism in such species (Zia et al. 2016).

As dicots angiosperm control, the Columbia wild type of *Arabidopsis thaliana* has been used.

1.4.1 Water loss and boundary between dehydration and desiccation

Water loss in resurrection plants is influenced by environmental factors, which include wind, humidity, temperature and soil water potential. In general, in a standard static condition (greenhouse), it seems that almost all of them have a RWC decrease which follows a nearly linear fashion, when water is withheld (Zhang and Bartels 2018). Nevertheless, Gaff (1977), observed that the dehydration trend is approximately linear initially, where the plant is still able to retain all the water, but as soon as the soil moisture is exhausted, the water content falls rapidly (Gaff and Oliver 2013). In both cases, it is very difficult to define the boundary between late dehydration and desiccation using the RWC as indicator (Zhang and Bartels 2018). However, in order to understand better the mechanisms underlying desiccation tolerance, it is necessary to identify the threshold between moderate and extreme dehydration, where most metabolic changes occur. A comparison of the metabolites between late dehydration and desiccation will be helpful in characterising this turning point. Zhang and Bartels, 2018 showed from their data that the transition occurs around 40% of RWC, but it will be interesting to see through this current study, if this boundary is the same in all species, or if there can be any variability.

1.5 Research questions

This study, through the analysis and the comparison of the metabolite composition of the plants under study, has the aim to answer to the following questions:

1. Are metabolites different among hydrated, dehydrated (50-30%) and desiccated samples in each species?
2. Are desiccation-induced metabolites similar among resurrection plants?

2 Materials and methods

2.1 Plant materials and maintenance

2.1.1 Plant growth and maintenance conditions

The plants were collected from different places and then stored in a greenhouse, without being watered for a few months (due to drought problems in Cape Town), at the following conditions:

Temperature: ambient temperature (varying between 10°C to 35°C)
Relative humidity: 50-70% RH
Light intensity: 200-800 µmol/m2/s
Day/night cycle: variable between 10 hrs light:14 hrs dark and 16 hrs light: 8 hrs dark

The drying experiment was executed in a growth room with the following conditions:

Temperature: 25°C
Relative humidity: 70%
Light intensity: 100-200 µmol/m2/s
Day/night cycle: 16 hrs light:8 hrs dark (lights on at 4am, and off at 10pm)

As soon as the plants had been transferred from the greenhouse to the growing room, all of them were watered every day until they were fully hydrated and recovered their optimal physiological functioning. The dehydration process started at different times for each species, in order to avoid an overload of sampling.

2.1.2 Plant materials

Selaginella

The plant was harvested from Pilanesberg, North-West, South Africa, found close to a Myrothamnus.

Three biological replicates were harvested only at fully hydrated and at the desiccated state (5 days after withholding water), since the dehydration process was too quick to catch intermediate water contents.

Notes: The presence of *Myrothamnus* in the same tray can have influenced the drying rate of *Selaginella*. Replicates belong to the same plant.

Leaves were harvested randomly from the whole plant.


Figure 1 :Selaginella spp. Partially hydrated on the left and fully dehydrated on the right

Xerophyta viscosa

Xerophyta viscosa was germinated from seed in growth medium and then transferred to a potting soil mix. Seed was collected from plants growing in the area around Lydenburg, Mpumalanga, South Africa. 10 plants were used as biological replicates, each of which was placed in a different pot. Since these plants had previously experienced other desiccation cycles, the plants already had some die-back of leaf tips, which were removed before the start of the new dehydration cycle. All the replicates took approximately three weeks to reach a relative water content of 80-70%. The 50-30% RWC lasted for 2 days and then the plants reached immediately the desiccation state.

Leaves were harvested from the more central rosette, avoiding too old or too young leaves. This criterion was applied consistently for all the replicates over all the three dehydration stages.

Figure 2: Xerophyta viscosa at fully hydrated stage on the left and fully desiccated on the right.

Xerophyta elegans

Xerophyta elegans plants were collected from Monk's Cowl, Drakensburg, Kwa-Zulu Natal, South Africa. Nine biological replicates were harvested.

Before starting to harvest, all the tips, which were already desiccated and dead due to previous desiccation cycles, were cut off in order to avoid getting confused with the new desiccated tissues.

Notes: The plants situated in the same trays could belong to the same plant, since Xerophyta Elegans grows through stolon. Some plants were bigger than others and had a higher density than others in terms of available surface and competition. This could have influences the drying rate.

Leaves were harvested from the more central rosette, avoiding too old, too young leaves and leaves without tips, too. This criterion has been applied consistently for all the reps over all the three dehydration stages.

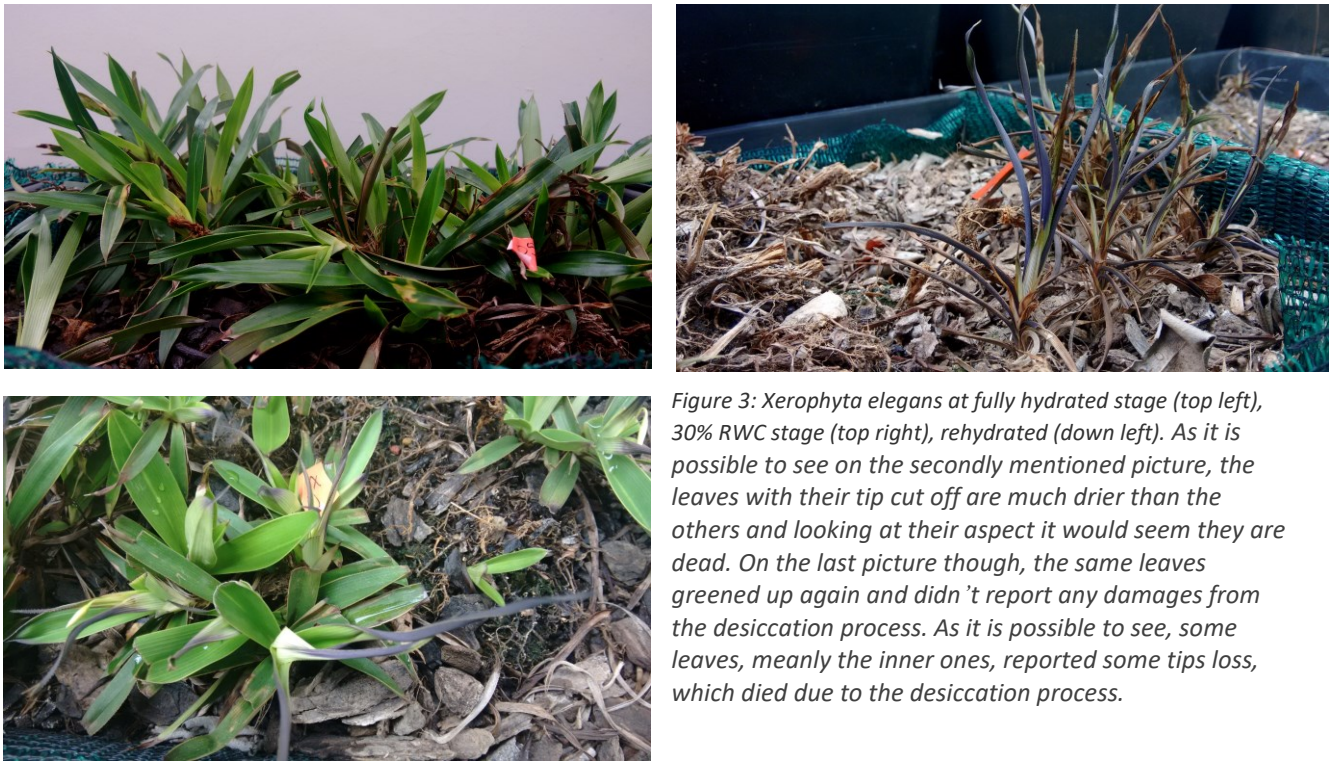

Figure 3: Xerophyta elegans at fully hydrated stage (top left), 30% RWC stage (top right), rehydrated (down left). As it is possible to see on the secondly mentioned picture, the leaves with their tip cut off are much drier than the others and looking at their aspect it would seem they are dead. On the last picture though, the same leaves greened up again and didn't report any damages from the desiccation process. As it is possible to see, some leaves, meanly the inner ones, reported some tips loss, which died due to the desiccation process.

Xerophyta humilis

Xerophyta humilis plants were collected in Borakalalo National Park, North-West, South Africa. Seven biological replicates, placed into two trays, were harvested.

Notes: At the fully hydrated stage, most of the plants were flowering. This could have influenced metabolites composition during the desiccation process. Additionally, the biological replicates were collected from a wild environment where they were all grouped together. Since this species propagate through stolon, the replicates could belong to the same plant.

Leaves were harvested from the more central rosette, avoiding too old or too young leaves. This criterion has been applied consistently for all the replicates over all the three dehydration stages.

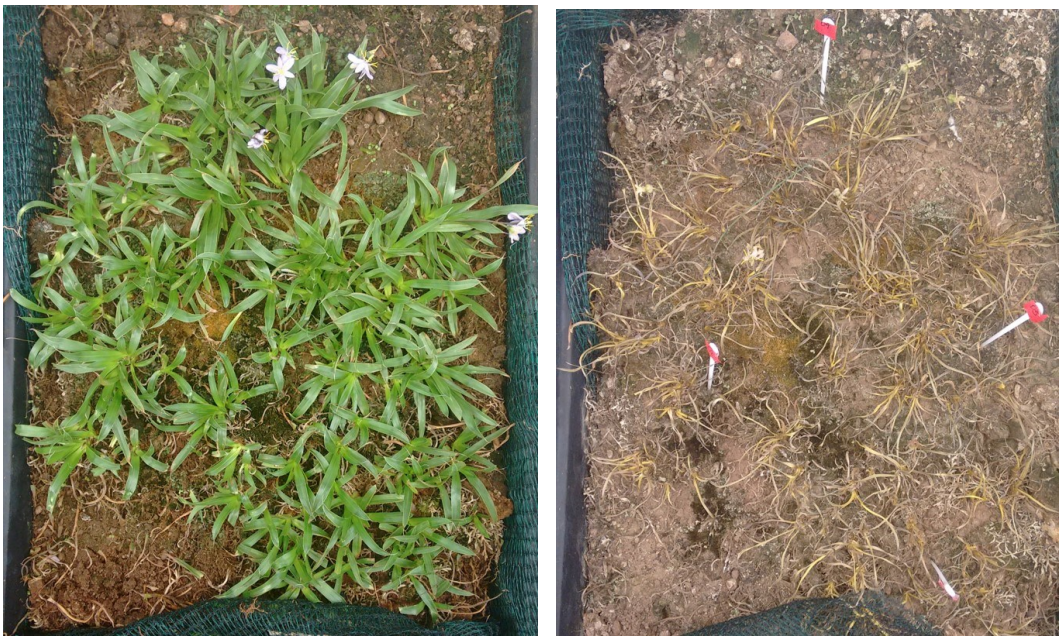

Figure 4: Xerophyta humilis fully hydrated (left picture) and fully desiccated (right picture). Displayed here are the trays with replicates 1,2,3,4.

Eragrostis nindensis

Eragrostis nindensis plants were collected in Aggeneys, Northern Cape, south Africa. 6 different biological replicates placed in independent pots were harvested. Before to start the dehydration process, all the dead leaves were picked up and all the senescent tips were cut off. The range of 50-30% lasted only 2 days, therefore it resulted very difficult to capture it.

Young mature and old leaves have been collected separately since they have a different behaviour to dehydration.

> **How to distinguish old leaves from young mature leaves? (figure 5)** Old leaves (o) are the most external ones of a tiller. Their sheath is slightly opened and detached from the central stem and usually their sheets bend horizontally. Young mature leaves (i) are internal to the old leaves and their state of maturity is defined by the presence of a young new leaf growing inside them. These leaves are much more erect up and their sheath is adherent to their internal new leaf. When sampling, young immature leaves haven't been taken in to consideration, since they are too young yet to have the desiccation mechanisms fully developed**.** Their photosynthesis is not efficient yet and their metabolism is not functioning at the optimum. In order to analyse drought effects, we need mature and well-functioning leaves.

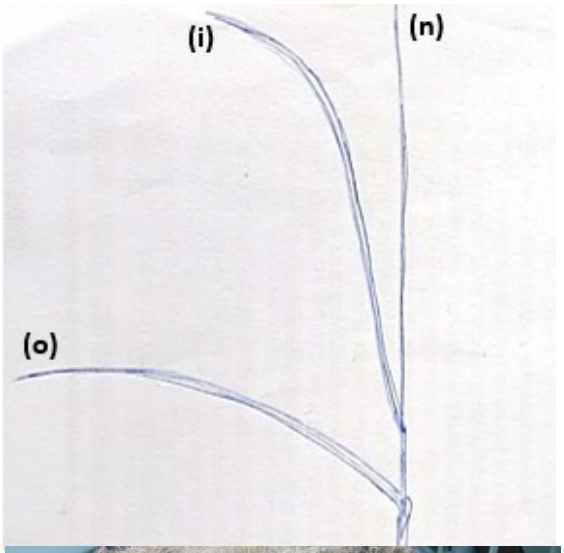

Figure 5: representation of an Eragrostis nindensis tiller: inner non-mature leaf (n), inner mature non-senescent leaf (i), outer senescent leaf (o).

Figure 6: Eragrostis nindensis at fully hydrated (top right), 50-30% RWC stage (low left), fully desiccated stage (low right).

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Craterostigma pumilum

Craterostigma pumilum plants were germinated from seeds in growth medium and then transferred to a potting soil mix. Seeds was collected from plants growing in the Borakalalo National Park, North-West, South Africa. 10 different biological replicates, placed each in a different pot, were harvested the dry tips (edges) that died in previous drying cycles), mainly present in outer and older leaves, were cut off before to start the new dehydration process. The healthiest and biggest leaves from the central rosette, were collected, trying to avoid leaves which showed to be damaged, like with senescent spots or dried/yellow edges. The older outer leaves were not considered for the harvesting, since they are senescent.

Note: at the fully hydrated state most of the plants were flowering, and during the dehydration process, flowers died. This has to be considered as a cause for a possible different metabolites' composition and behaviour to dehydration.

How to distinguish central rosette leaves from the senescent ones?

Leaves that are part of the central rosette are smaller and very closed to each other and are the ones that own desiccation tolerance. Senescent leaves are the outer ones; they are bigger, with a longer petiole that departs from the base of the plant and are horizontally oriented just on the ground surface.

Figure 7: Craterostigma pumilum at fully hydrated stage on the top pictures. In the higher picture on the right is possible to individualize the central rosette (i) from the outer senescent leaves (o). The lower picture on the left shows the 50-30% RWC stage, while the lower picture on the right shows the desiccated stage. It is possible to see in this last one that the outer senescent leaves are indeed dead.

Myrothamnus flabellifolia

Myrothamnus flabellifolia plants were collected from Kunene and Erongo provinces, Namibia. Five biological replicates, placed in separate trays, were harvested.

Notes: some of the plants flowered when the dehydration process started. An additional variable that could have influenced the drying rate could be the different gender of the replicates: replicate 4 was a male, while all the others were female. Leaves were harvested without following a specific criterion, they have been picked randomly from different branches of the plant.

Figure 8: Myrothamnus flabellifolia at fully hydrated stage (left picture), 50-30% RWC stage (middle picture) and desiccated stage (right picture).

Eragrostis tef

Since this species was not present in the greenhouse, it was grown from seeds purchased from Agricol (Pty.) Ltd., Western Cape, South Africa. The seeds were placed in a petri dish with a sterile paper and were left in the growing room for a few days. After germination, the seedlings were placed in individual pots with vermiculite soil. They were planted and grown for 1 month. Eight biological replicates were harvested only at fully hydrated state and then when they died.

Figure 9:Eragrostis tef along its growth. The last picture on the right shows the plants' size when the harvesting started.

Arabidopsis thaliana

Since this species was not present in the greenhouse, it was grown from Col-0 seeds. The seeds were placed in a petri dish with a sterile paper, were left in a fridge for 2 days to satisfy their cold requirement and were left in the growing room for a few days. The seeds germinated in 3 days and the seedlings were transferred in peats. 6 different replicates were used and harvested just before flowering.

Figure 10: Arabidopsis thaliana when planted and at its latest stage before harvesting.

2.2 Sampling procedures

For each species, several biological replicates (previously specified in chapter 2.1.2) were used, depending on the availability of the number of plants that were present at UCT. For each plant, it was picked a specific amount of leaves that would correspond to 50 mg (depending on the leaves size and species) at fully hydrated, 50-30% RWC and fully dehydrated stages, necessary for the subsequent metabolites' analysis. A second collection has been executed with a much smaller amount of leaf's material for the identification of the RWC at the three stages of collection. Desiccation sensitive species (*Arabidopsis thaliana* and *Eragrostis tef*) were harvested only at fully hydrated and at approximately 60% RWC only, when they died.

Species (fully hydrated state)	Amount of leaves harvested for metabolite analysis	Amount of leaves harvested for RWC
Selaginella	1 branch	Half a branch
Xerophyta viscosa	1-2 leaves	Half a leaf
Xerophyta elegans	1 leaf	Half a leaf
Xerophyta humilis	2 leaves	Half a leaf
Eragrostis nindensis	4 leaves	2 leaves
Craterostigma pumilum	1 leaf	1 small leaf
Myrothamnus flabellifolia	5 leaves	2 leaves
Eragrostis tef	2 leaves	Half a leaf
Arabidopsis thaliana	4 leaves	1 leaf

Table 3: Amount of leaves harvested per species at fully hydrated state. For metabolites analysis, the amount corresponds approximately to 50 mg of leaf material. For RWC, it corresponds to approximately 20 mg of leaf material.

Species (50-30% RWC)	Amount of leaves harvested for metabolite analysis	Amount of leaves harvested for RWC
Xerophyta viscosa	2-3 leaves	Half a leaf
Xerophyta elegans	1 leaf	Half a leaf
Xerophyta humilis	3 leaves	Half a leaf
Eragrostis nindensis	6 leaves	2 leaves
Craterostigma pumilum	2 leaves	1 small leaf
Myrothamnus flabellifolia	6 leaves	2 leaves

Table 4: Amount of leaves harvested per species at 50-30% RWC. For metabolites analysis, the amount corresponds approximately to 50 mg of leaf material. For RWC, it corresponds to approximately 20 mg of leaf material.

Species (fully dehydrated)	Amount of leaves harvested for metabolite analysis	Amount of leaves harvested for RWC
Selaginella	1 branch	Half a branch
Xerophyta viscosa	3 leaves	Half a leaf
Xerophyta elegans	2 leaves	Half a leaf
Xerophyta humilis	4 leaves	1 leaf
Eragrostis nindensis	8 leaves	2 leaves
Craterostigma pumilum	3 leaves	1 small leaf
Myrothamnus flabellifolia	8 leaves	2 leaves
Eragrostis tef	6 leaves	Half a leaf
Arabidopsis thaliana	6 leaves	1 leaf

Table 5: Amount of leaves harvested per species at fully dehydrated state. For metabolites analysis, the amount corresponds approximately to 50 mg of leaf material. For RWC, it corresponds to approximately 20 mg of leaf material.

Water content determination:

Before harvesting, Eppendorf tubes were labelled with species name, number of the replicate, date (and leaf type for E. nindensis: inner (i), outer (o)). A small amount of leaf (approximately 10 mg) was harvested and immediately weighed on a Metler analytical balance to the nearest five decimals to determine the fresh weight. Then, each sample was placed in an individual Eppendorf tube, filled with water and kept at 4°C in the dark overnight inside the cold room. The following day, after briefly drying the leaves on tissue, they were weighed again to determine the turgid weight. Lastly, the same samples were placed in a labelled foil packets, dried at 70°C for 48 hours and weighed to determine the dry weight. This procedure was applied to all the species to determine the RWC at the three different stages of fully hydrated, 50-30% and fully desiccated.

The relative water content at the fully hydrated stage was determined gravimetrically according to Barrs and Weatherley's (1962) formula:

$$RWC\ (\%)\ =\ ((Fresh\ Weight — Dry\ Weight)/(Turgid\ Weight — Dry\ Weight))\ x\ 100$$

The relative water content at 50-30% and at fully desiccated stages was determined by using the following formula:

$$AWC\ (\%)\ =\ AWC\ final/AWC\ initial\ *100$$

$$where\ AWC\ =\ (Fresh\ weight\ -\ Dry\ weight)/\ Dry\ weight$$

Harvesting for metabolite analysis:

Before harvesting, foil packets were labelled with species name, number of the replicate, date and leaf type. Leaf tissues (50 mg dry weight) were harvested from each plant and immediately snap-frozen into liquid nitrogen (this is done in order to avoid induction of metabolites due to the harvesting instead of water stress ones).

2.3 Freeze drying

After harvesting, samples were transferred in a freeze dryer and deep-frozen in a vacuum chamber in order to dry out and remove all the water by sublimation directly from the solid phase to the vapour phase and drawn off by the vacuum pump under very low pressure. In this way, samples won't have liquid water present in their cells ever again, avoiding the potential activation of enzymes.

2.4 Metabolites extraction

Samples were taken out of from the freeze-drier and conserved in Tupperware with silica gel inside a fridge at -20°C until the moment of extraction. From each sample, only 20 mg of leaf material was used and weighed in order to get the exact weight for each sample. Each of them was placed in a 1.5mg labelled Eppendorf.

Based on operational considerations at UCT and on the large amount of sample, the extraction was executed by following the 50% methanol at 70°C method (Roessner et al. 2000): Samples were ground, mixed with 500µl of analytical grade methanol, warmed up to 68°C for 15 minutes. Then

500µl of Milli-Q of water and 50µl of a 2mg/ml solution of Ribitol were added to each sample and afterwards sonicated for 15 minutes. In order to separate the solid phase from the liquid phase, samples were centrifuged for 15 minutes at 13000 x g/min at 4°C. Lastly, 160 µl of solution for each sample has been pipetted in a glass vial. Three synthetic averages samples were then prepared from each sample, as technical control.

2.5 Drying extracts *in vacuum*

After the extraction, the vials with the extracts were place in the Speed vac Savant SPD121P and dried *in vacuum* for 16 h using the Refrigerated Vapor Trap RVT4104. As last, the vials were sealed under Argon with ergonomic caps and then sealed all together in plastic bags under vacuum and conserved at -20°C.

2.6 Derivatisation

Immediately before GC-MS analysis, all samples were chemically derivatised, in order to render them volatile and heat-stable for gas chromatography. Dried extracts were derivatised by using the N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) and methoxamine hydrochloride (Roessner et al. 2000) injected inline. Retention index standards, in this case alkane series (from C9 to C36), were added at the same time. These were used during peak alignment and annotation by assigning each a retention index that is independent of any variation in the instrument and can be directly compared to retention characteristics of compounds in online databases.

2.7 GC-MS analysis

GC-MS analysis of derivatised extracts was performed on a GC-MS Thermo Scientific Trace 300 Triple Quadrupole mass spectrometer. It was fitted with a DB-5ms column or functional equivalent and the electron impact spectra was collected from m/z ratio (mass/charge) from 50 to 600. This technique permits the separation of complex mixtures of metabolites and distinguishes hundreds of different compounds in a single experiment, giving a fingerprint of the metabolism in many samples and allowing to detect the change in the metabolic composition within plants under desiccation stress.

2.8 Numerical analysis

2.8.1 Pre-processing

The Thermo raw files obtained by the GC-MS analysis were converted from a mzXML format using the Raw Converter version 1.1.0.19 from the Scripps Institute. Afterwards, the pre-processing was executed using the R libraries metaMS 1.16.0 and xcms 3.2.0, part of the Bioconductor project [1].

2.8.2 Principle component analysis (PCA)

Principle component analysis was executed by using the package ChemometricsWithR, version 0.1.11 [3]. Several plots were created for each species:

From each data set, a screeplot and a bar plot were created to visualize the contribution of the principal components to the total variability. This graph was used to choose the number of the principle components that would have been subsequently combined in different couples to plot the actual data set. Trials have been done only with the first few principle components explaining 80% of cumulative variance. PCA score plots were generated (Gabriel 1971) together with boxplots and loading plots. Score plots were used to represent the main couples of principal components that contributed to cluster the data per treatments. Boxplots were used to represent the variability on

the first principle component. Lastly, loading plots, were used to show the main metabolites contributing for a principle component. In order to further reduce the visual congestion of the plots, only the most prominent loading vectors were represented.

A heat-map was also produced in order to compare each species-treatment among each other and to visualize the abundance of the most important metabolites previously analysed.

2.8.3 Compound annotation

Compounds were annotated by using two different approaches: some of these compounds were found by spectra similarity search, by comparing their spectra with NIST database. Others were defined by spectral and retention index-based research comparing them with the Golm Metabolome Database [2].

3 Results and discussion

3.1 Drying process

Species	From hydrated to 50-30% RWC	From 50-30% RWC to desiccated	Total dehydration process
Selaginella	-	-	2 days
Xerophyta viscosa	20 days	7 days	≈ 30 days
Xerophyta elegans	≈20 days	≈10 days	≈ 25 days
Xerophyta humilis	≈ 8 days	≈ 6 days	≈ 14 days
Eragrostis nindensis	≈ 16 days	≈ 5 days	≈ 21 days
Craterostigma pumilum	≈ 15 days	≈ 7 days	≈ 21 days
Myrothamnus flabellifolia	≈ 8 days	≈ 7 days	≈ 14 days
Eragrostis tef	-	-	≈ 10 days
Arabidopsis thaliana	-	-	≈ 6 days

Table 6:Dehydration periods, from hydrated state to 50-30% RWC (late dehydration), from 50-30% RWC to desiccated state and total dehydration process.

The drying process can vary between species and it mainly depends on the complexity of the structure of the plant. *Selaginella*, which is a bryophyte, has a very simple structure and loses the water very rapidly (Oliver et al. 2000). Due to its rapidity of drying, it was not possible to record its medium water content. In higher plants, such as angiosperms, the dehydration process lasted longer and showed to be highly specific and related to morphological and physiological characteristics. All the angiosperm resurrection plants reached the desiccated state in a period that varies between 15 and 30 days. *Xerophyta viscosa*, for example, was the species that took longest to dry, almost a week more than the other two *Xerophyta spp*. This is because its leaves, are covered with patellar glands on the abaxial side that secrete viscous metabolites responsible in part for the regulation of the water loss rate (Farrant et al. 2015).

In general, all of them had similar dehydration patterns. Nevertheless, *Eragrostis nindensis* had a particular drying curve: it took 16 days to reach the 50-30% RWC, and after that point it dehydrated very rapidly, reaching the desiccated state in between 2 and 5 days, depending on the replicate. Another particularity is the different behaviour of its leaves during dehydration. Old senescent leaves dehydrated much faster than the young mature leaves, and when young leaves where at 50-30 % RWC, these old leaves where already at 20-10% RWC.

Within replicates of a same species, the drying rate appeared variable. Different reasons can have leaded to it: different soil structure and depth, different plants density and competition, harvesting during, before or after flowering, previous endured dehydration cycles.

Arabidopsis thaliana and Eragrostis tef dried faster (almost one week) than desiccation tolerant angiosperms and died at a water content of approximately 60-50% RWC.

3.2 Morphological changes upon dehydration

The clearest response that resurrection plants show to desiccation stress is their morphological changes. One of the most common responses among all resurrection plants is the curling or folding of their leaves upon dehydration, since it minimizes the light absorption on the adaxial surface, trying to expose only the abaxial side to light (Vicré et al. 2004). All the Xerophyta species under study showed this phenomenon, but to a much less extent in *Xerophyta viscosa*. This species has

elongated schlerophyllous leaves that allow only a minimal folding and the surface is protected by abaxial patellar glands that act as sunscreen, together with anthocyanin pigments (Farrant et al. 2015). *Eragrostis nindensis* also had a similar response: the young mature leaves curled upon dehydration, while the old ones didn't show any curling and simply lost their erect position and bended towards the ground, preparing to die. A very marked response appeared in *Myrothamnus flabellifolia*, where upon dehydration, the fan-like leaves curled towards the inside and folded towards the branch. In addition, the branches themselves folded towards the centre, in order to fully minimize the surface exposed to light. Another morphological change in the leaves was the turning of the abaxial colour into a reddish-brown colour. This change also appeared to happen in *Xerophyta elegans* and in *Craterostigma pumilum*, which are all homoiochlorophyllous and tend to accumulate a high concentration of anthocyanins (responsible for the colour change) for light protection by employing it as "sunscreen" for masking the chlorophyll (Christ et al. 2014; John P. Moore et al. 2007).

Craterostigma pumilum also showed an inward curling of the edges of the leaves of the central rosette (it does not occur in the old outer senescent leaves)(Christ et al. 2014). In contrast, senescent leaves did not shrink and changed their colour from green to yellow.

These mentioned modifications are just some of a number of different ones associated with dehydration and can be considered adaptations of resurrection plants to minimize the damage from light and the consequent stress due to free radical production. The movement of leaves upon dehydration is also thought to reduce the effective transpiring surface during early stages of dehydration and/or to minimize the light radiation reaching the air-dry younger tissues (Vicré et al. 2004).

Species	Morphological changes at 50-30% RWC	Morphological changes at desiccated state
Selaginella	-	• Colour change from light green to grey • Consistency change from fluffy to stiff
Xerophyta viscosa	• Light green/yellow colour • Tougher consistency • Slight curling of leaves and erect position	• Intense yellow colour • Hard consistency
Xerophyta elegans	• Change in colour only in the leaves abaxial side to brown-purple • Folding of leaves edges inwards	• Complete change of abaxial side colour to dark-brown • Tough consistency • Complete folding of leaves
Xerophyta humilis	• Light-green/ yellow colour • Curled leaves	• Completely yellow colour • Tough consistency • Curled leaves • Presence of dry tips
Eragrostis nindensis	• Light-green/ yellow colour • Curled and erected young mature leaves • Bended downwards old leaves	• Yellow dry leaves
Craterostigma pumilum	• Turning of colour from bright green to darker green, with purple shade at the edges • Loss of consistency. From glossy and shiny to elastic and soft • Slight curling of leaves edges	• Completely brown-purple colour of the abaxial side • Very tough consistency • Leaves completely shrunk inwards
Myrothamnus flabellifolia	• Darker green colour of adaxial side, while brown on the abaxial side • Slight folding inward of leaves • Tougher consistency	• Dark-brown colour of abaxial side • Leaves completely folded inwards • Leaves folded towards the branch • Branches folded inwards

Table 7: Summary of the morphological changes happened during the dehydration process in resurrection plants.

3.3 Principal component analysis of GC-MS data

The purpose of this analysis was to detect which compounds are most strongly associated with desiccation tolerance in the studied species and which of them are in common among resurrection plants. A second step was to identify these compounds, when feasible. Since the dataset was massively multivariate, it has been challenging to reduce the large number of variables to a smaller number that could be easily analysed and visualised. Therefore, PCA analysis was performed on pre-processed mass spectral data from the different species from GC-MS datasets.

3.3.1 Quality of the results and experimental problems

While the GC-MS was running samples, its software together with the software of the computer controlling the machine had problems and failed in controlling the pressure. This caused a retention time shift, that leaded to the loss of some samples. Due to the restricted time of the project, the remaining samples didn't have time to be run. Therefore, the following species won't be present in the results: *Selaginella*, *Eragrostis nindensis*, *Eragrostis tef*, *Xerophyta elegans* dehydrated and desiccated.

Furthermore, due to an excessive injection of extracts, the certain chromatographic peaks were overloaded. This overload caused two problems, resulting in poor quantification of the largest peaks, such as sucrose and octulose.

At the same time the overloading gave the opportunity to analyse less abundant peaks, that would have been hidden by more abundant compounds in a normal analysis.

3.3.2 Xerophyta viscosa

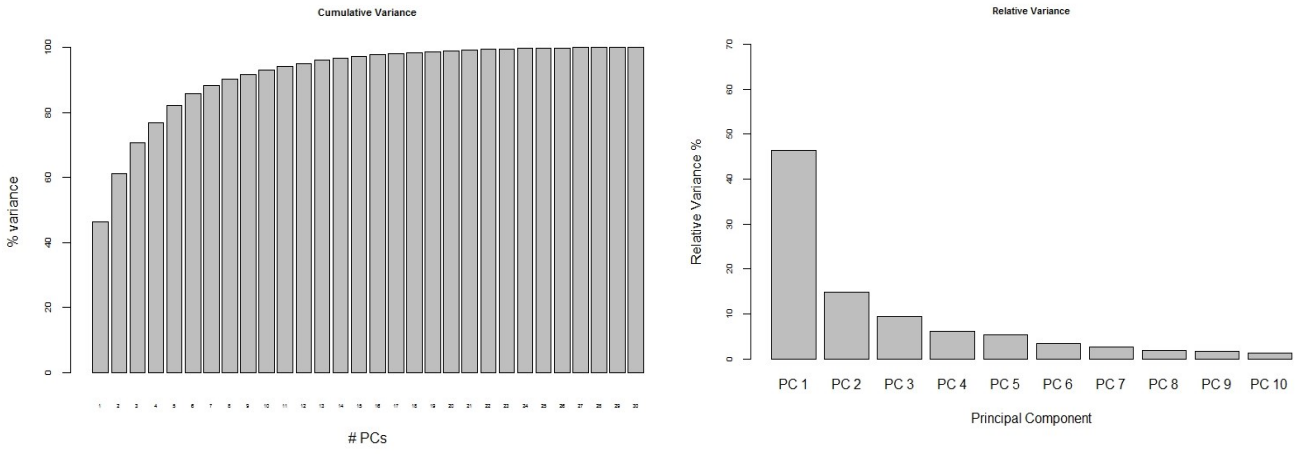


Figure 11: Cumulative and relative variance per Principal Component for GC-MS peaks of Xerophyta viscosa.

The variance within the data of Xerophyta viscosa is explained by the first five Principle Components, which together describe 80% of the variability of the samples. Among these five, only the first two principle components show a relevant clustering of the sample treatments (figure 13).

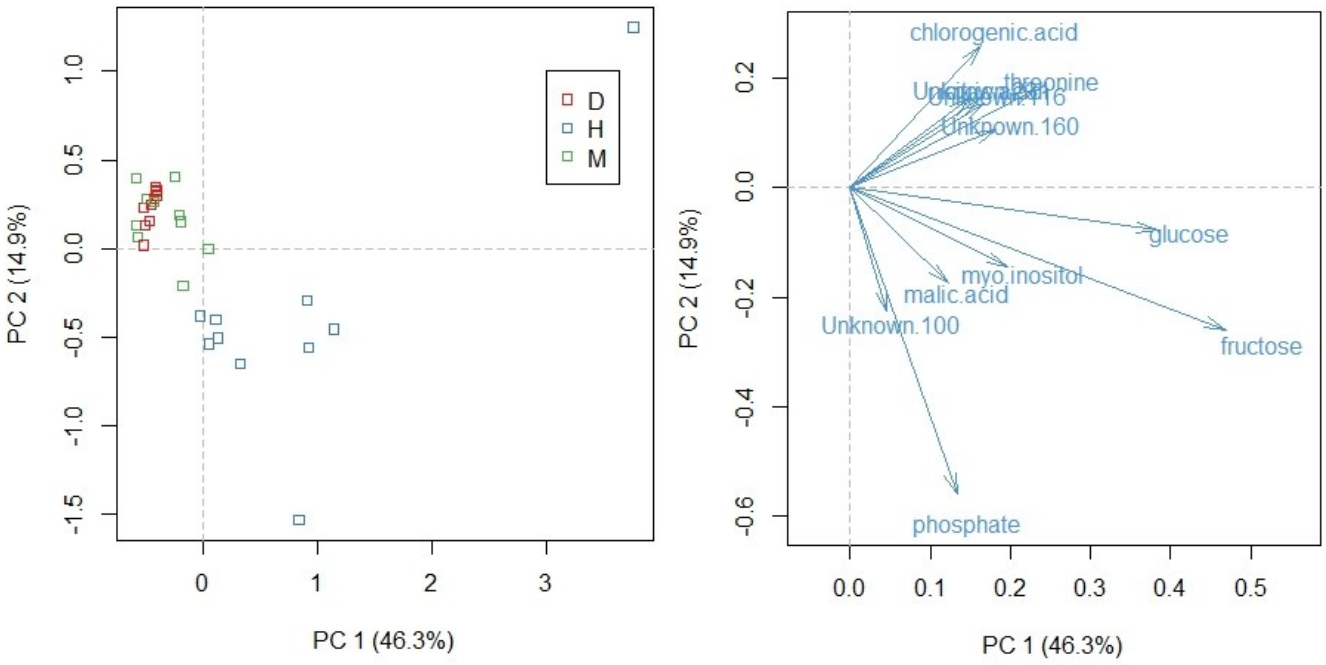
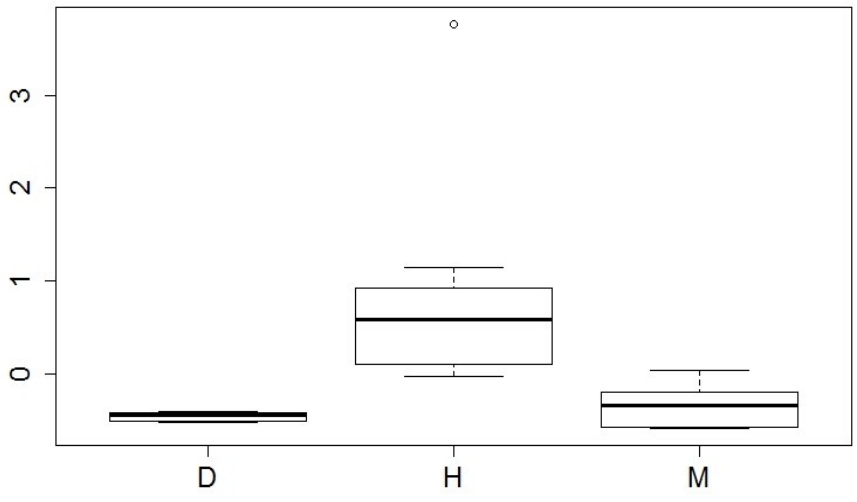


Figure 12: The image on the left represents a score-plot of the first and second principle components clustering for Xerophyta viscosa in the three treatments (D: desiccated, H: hydrated, M: medium dehydrated). The image on the right represents a loading plot with the main metabolites contributing to the first and second principle components.

The score-plot shows that both PC1 and PC2 are contributing to the clustering of the samples, mainly differentiating the hydrated samples from the dehydrated and desiccated ones. Looking at the loading plot it is clear that PC2 is positively correlated with chlorogenic acid and threonine, while negatively associated to phosphate. PC1 only shows positive association, mainly with fructose, glucose and myo-inositol. The clustering of hydrated samples in the first quadrant on the right represents a strong positive association with both the principle components and therefore suggests an association with the correlated compounds. Indeed, phosphate and the abovementioned sugars are highly present in the hydrated state of *Xerophyta viscosa*, while poorly present in the dehydration states (Suguiyama et al. 2014; Farrant et al. 2015; Berjak 2006; Moore et al. 2007).



In this last image (figure 14), the score distribution of desiccated, hydrated and dehydrated samples of Xerophyta viscosa for the first principle component is represented. From this picture it is possible to see that the variability is very small for dehydrated and desiccated samples, while higher in the hydrated samples.

Figure 13: Boxplot of score distribution of desiccated, hydrated and dehydrated samples of Xerophyta viscosa for the first principle component.

3.3.3 *Xerophyta humilis*

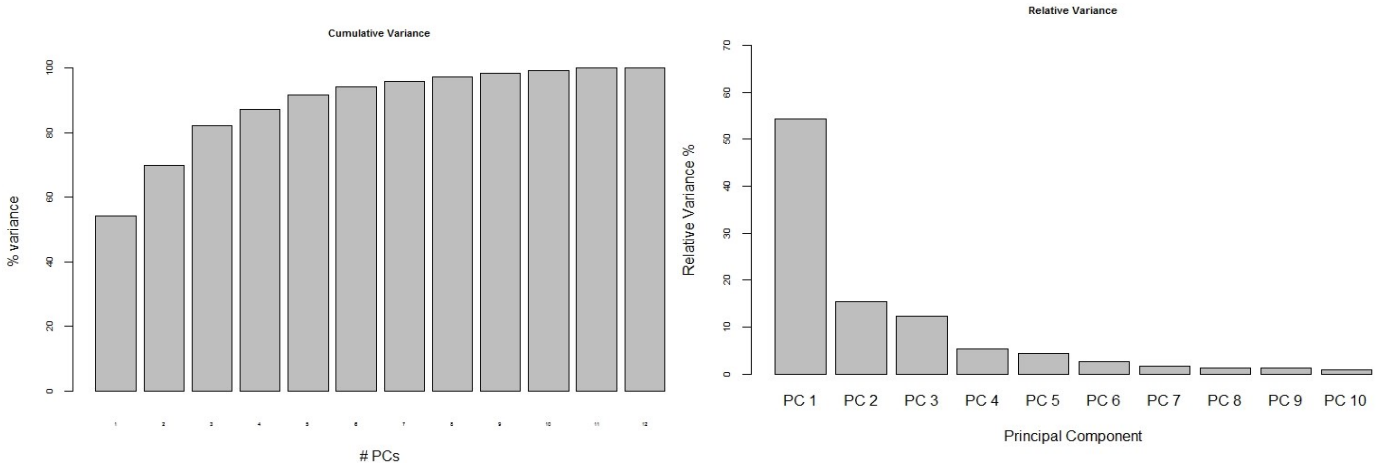


Figure 14:Cumulative and relative variance per Principal Component for GC-MS peaks of Xerophyta humilis.

The variance within the data of *Xerophyta humilis* is explained by the first three Principle Components, which together describe 80% of the variability of the samples. Among these three, only the first two principle components show a relevant clustering of the sample treatments (figure 15).

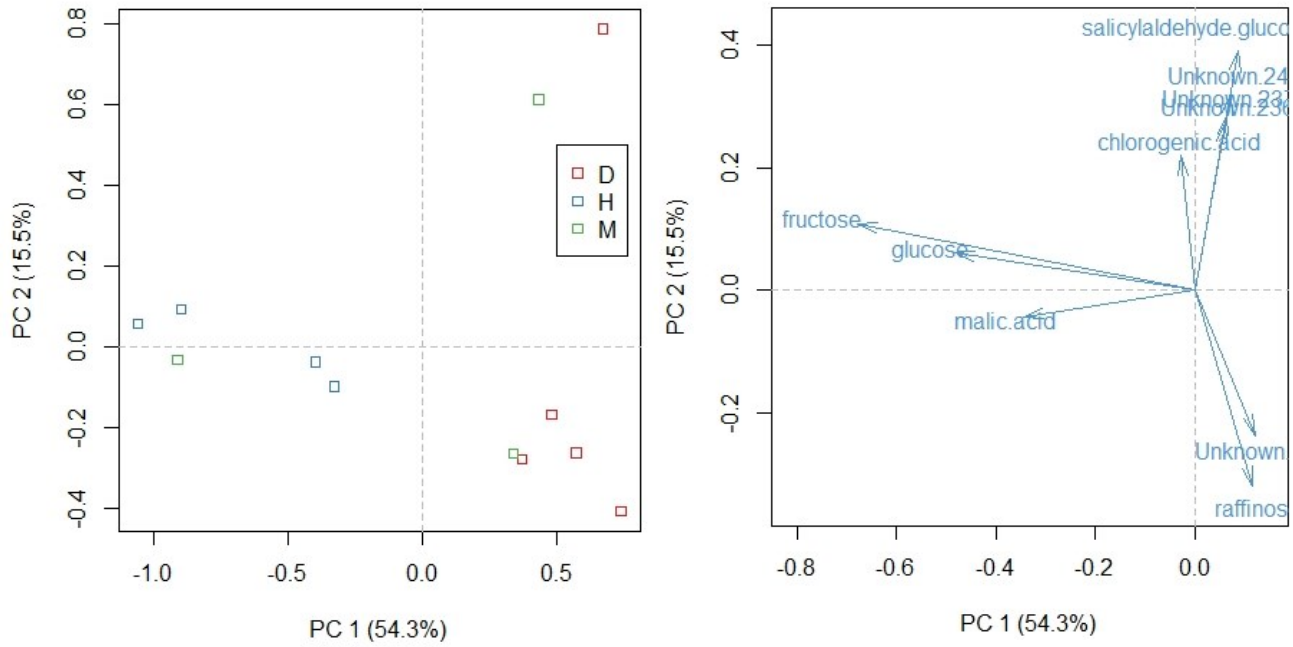
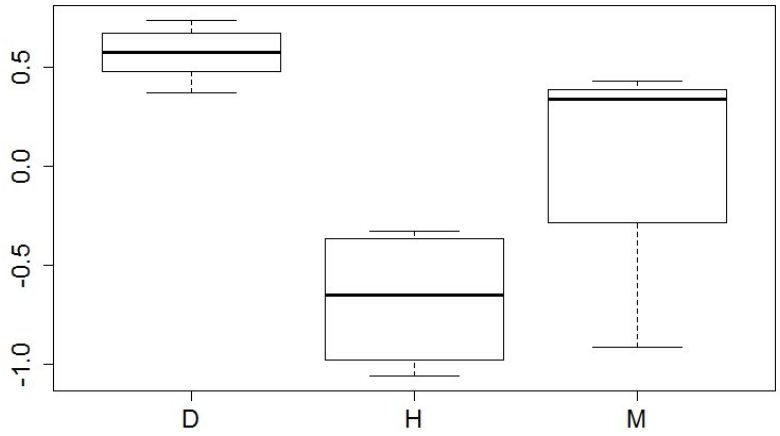


Figure 15: The image on the left represents a score-plot of the first and second principle components clustering for Xerophyta humilis in the three treatments (D: desiccated, H: hydrated, M: medium dehydrated). The image on the right represents a loading plot with the main metabolites contributing to the first and second principle components.

PC1 is mainly responsible for clustering the samples in different quadrants. Desiccated samples are clearly clustered on the right, while hydrated samples are slightly spread on the left quadrant. Medium dehydrated samples instead, don't have a pattern and are not associated with either of the two PCs. In the loading plots, fructose, glucose and malic acid are clearly negatively correlated with PC1 and are associated with hydrated samples. On the other hand, raffinose is mainly correlated to PC, negatively, and is associated with desiccated samples. There are other

compounds like salicylaldehyde-glucose and chlorogenic acid that are positively correlated with PC2 but don't seem to be clearly associated with a particular sample treatment.



In this last image (figure 16), the score distribution of desiccated, hydrated and dehydrated samples of *Xerophyta humilis* for the first principle component is represented.

PC1 explains quite accurately the desiccates samples while it shows a high variability for hydrated and mostly medium dehydrated samples.

Figure 16: Boxplot of score distribution of desiccated, hydrated and dehydrated samples of Xerophyta humilis for the first principle component.

3.3.4 *Xerophyta elegans*

Since for this species, only hydrated samples were available, it hasn't been possible to do a comparison among treatments. This species will be discussed later on in section 4.1 and compared with the hydrated samples of the other species.

3.3.5 *Myrothamnus flabellifolia*

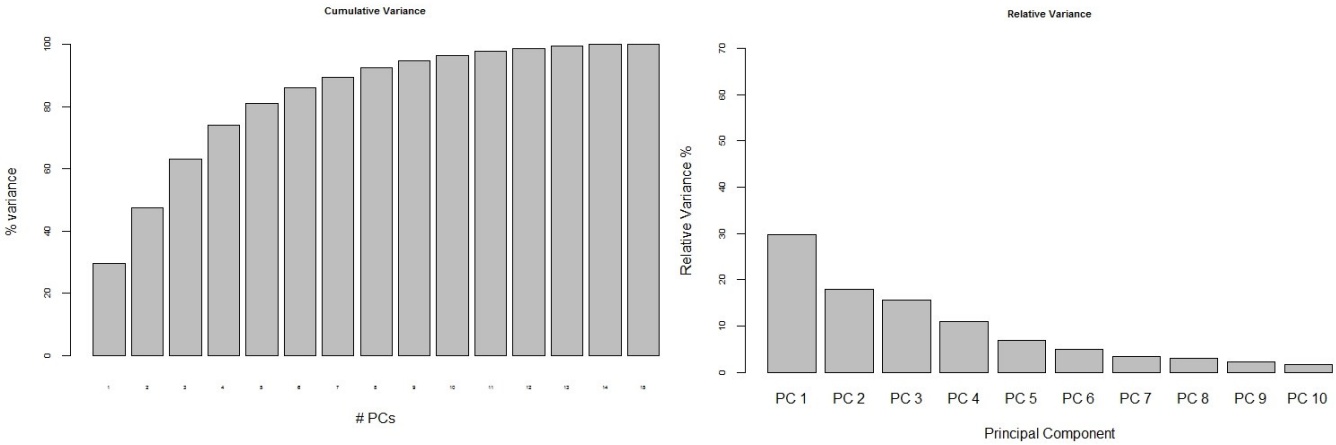


Figure 17: Cumulative and relative variance per Principal Component for GC-MS peaks of Myrothamnus flabellifolia.

The variance within the data of *Myrothamnus flabellifolia* is explained by the first six Principle Components, which together describe 80% of the variability of the samples. Among these six, only the first two principle components show a relevant clustering of the sample treatments (figure 18).

PC1 is mainly responsible for clustering of the sample population. Not clear groups are shown, but itis still possible to see a pattern for each treatment. Hydrated samples are mainly on the first quadrant on the right, while desiccated samples, on the left. In the loading plot, fructose, glucose and glycerol results to be markedly positively correlated to PC1 and are indeed associated with hydrated samples. Quinic acid is negatively correlated with PC1 and seems associated with desiccated samples.

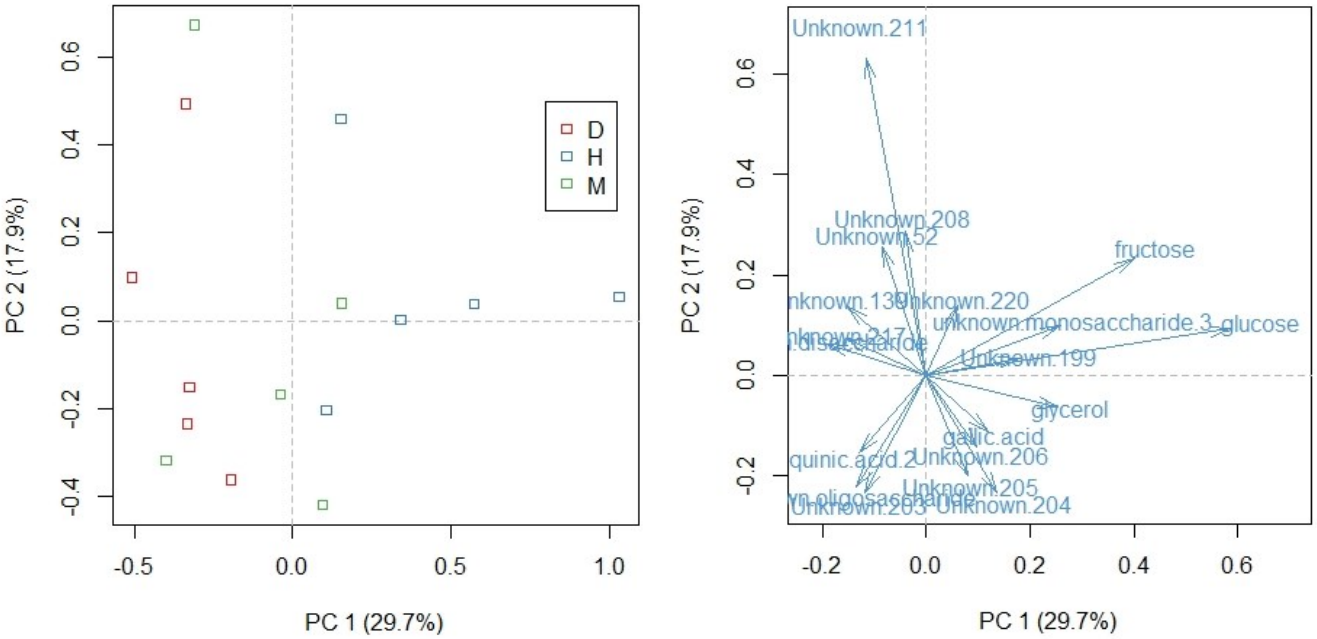
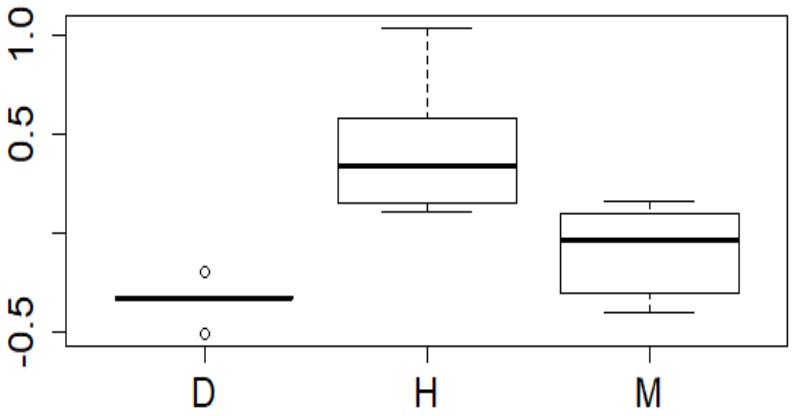


Figure 18:The image on the left represents a score-plot of the first and second principle components clustering for Myrothamnus flabellifolia in the three treatments (D: desiccated, H: hydrated, M: medium dehydrated). The image on the right represents a loading plot with the main metabolites contributing to the first and second principle components.



In this last image (figure 19), the score distribution of desiccated, hydrated and dehydrated samples of *Myrothamnus flabellifolia* for the first principle component is represented. PC1 explains quite accurately the desiccates samples while it shows variability for hydrated and mostly medium dehydrated samples.

Figure 19: Boxplot of score distribution of desiccated, hydrated and dehydrated samples of Myrothamnus flabellifolia for the first principle component.

3.3.6 *Craterostigma pumilum*

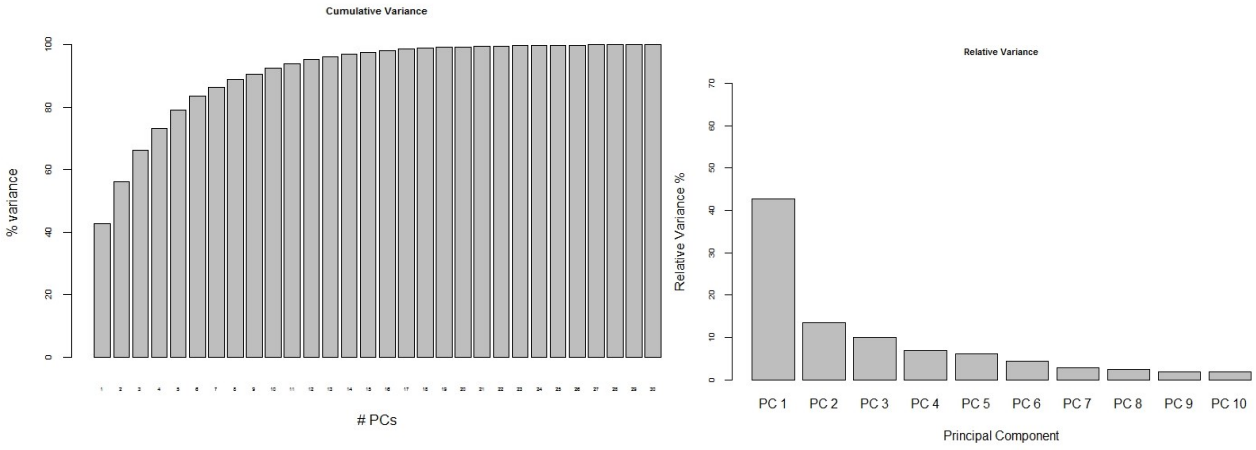


Figure 20: Cumulative and relative variance per Principal Component for GC-MS peaks of Craterostigma pumilum.

The variance within the data of *Craterostigma pumilum* is explained by the first six Principle Components, which together describe 80% of the variability of the samples. Among these six, both PC1 with PC3 and PC1 with PC5 show a relevant clustering of the sample treatments (figure 21 and 22).

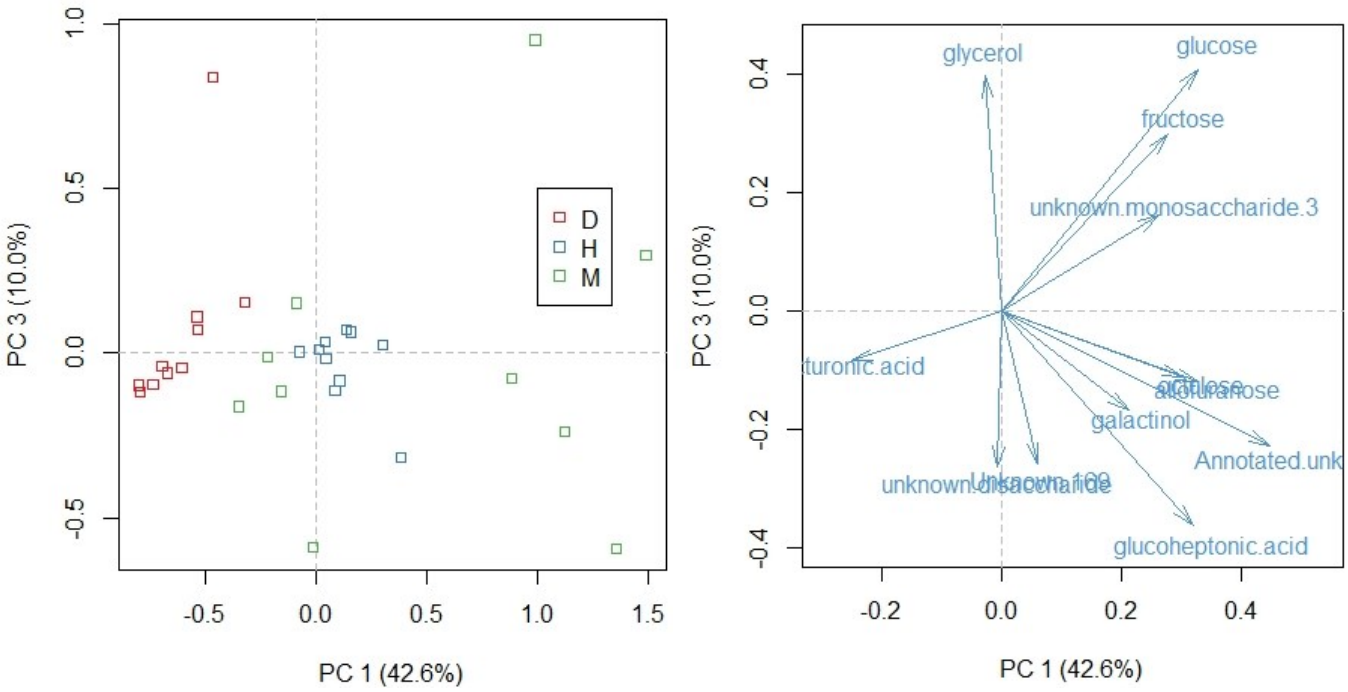


Figure 21: The image on the left represents a score-plot of the first and third principle components clustering for Craterostigma pumilum in the three treatments (D: desiccated, H: hydrated, M: medium dehydrated). The image on the right represents a loading plot with the main metabolites contributing to the first and second principle components.

PC1 is mainly clustering the sample population: desiccated samples on the left, hydrated samples in the centre and medium dehydrated samples between the centre and the right quadrant. PC1 is negatively correlated with turonic acid and seems associated with the desiccated samples. Glucose, fructose, octulose, allofuranose, galactinol, glucoheptonic acids and an annotated unknown compound are all positively correlated with PC1 and could be associated with both hydrated and medium dehydrated samples. The annotated unknown has been previously observed in other GC-MS metabolomic studies, but it hasn't been identified yet [2].

Also, in figure 22 PC1 is mainly clustering the sample population, but in a slightly different way: while desiccated and hydrated samples are still clustered in the same way, medium dehydrated samples seem to be less variable than in figure 21 and to cluster in between desiccated and hydrated samples. In this loading plot sucrose also appears and it is negatively correlated with PC1, being associated with desiccated samples. As before, glucose, fructose, octulose, allofuranose, galactinol and glucoheptonic acids are all positively correlated with PC1 and could be associated with both hydrated and medium dehydrated samples.

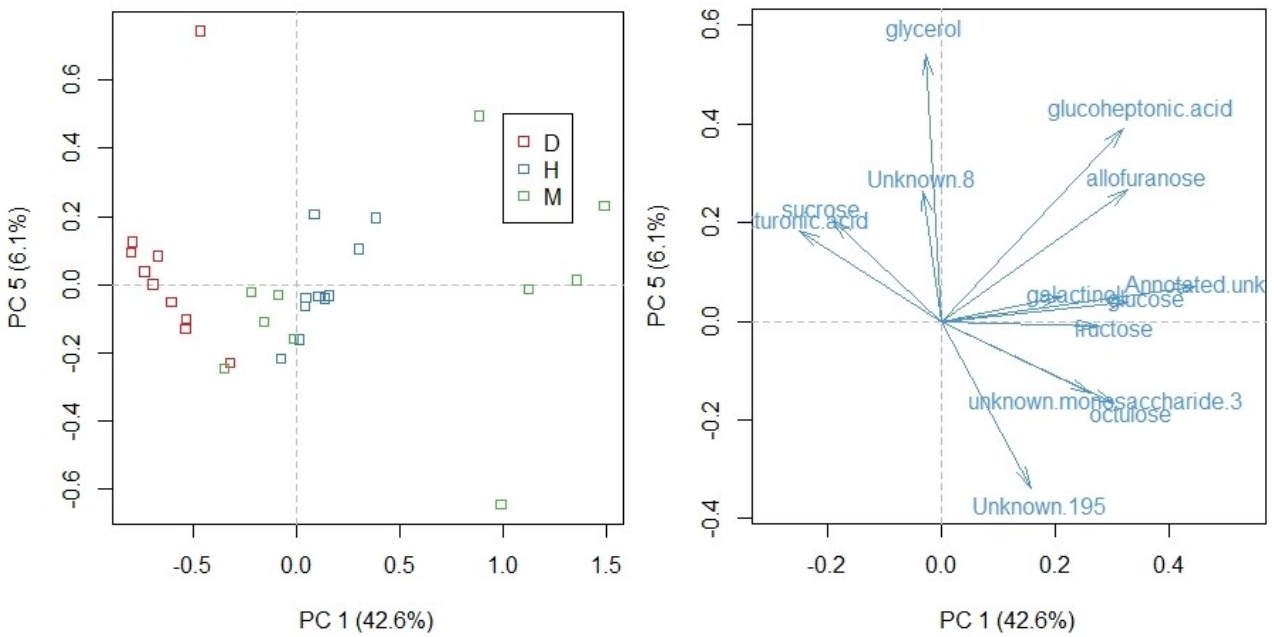
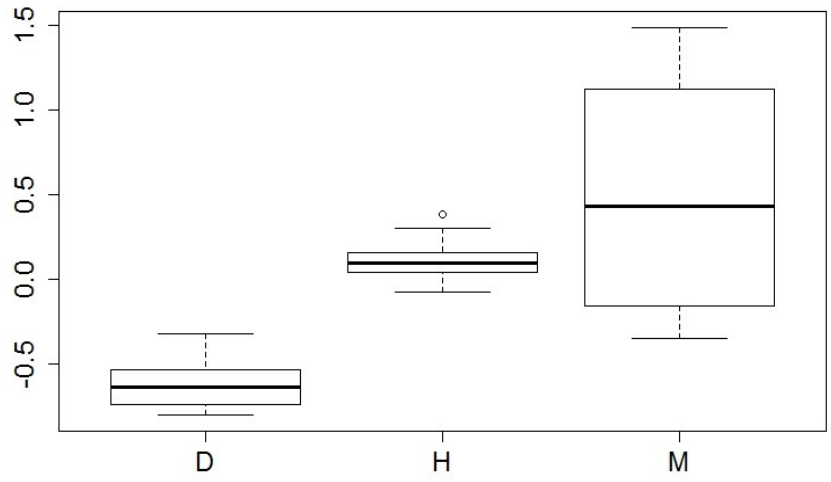


Figure 22: The image on the left represents a score-plot of the first and fifth principle components clustering for Craterostigma pumilum in the three treatments (D: desiccated, H: hydrated, M: medium dehydrated). The image on the right represents a loading plot with the main metabolites contributing to the first and second principle components.



In this last image (figure 23), the score distribution of desiccated, hydrated and dehydrated samples of *Craterostigma pumilum* for the first principle component is represented. PC1 explains quite accurately the desiccated and hydrated samples while it shows a high variability for hydrated samples.

Figure 23: Boxplot of score distribution of desiccated, hydrated and dehydrated samples of Craterostigma pumilum for the first principle component.

3.3.7 *Arabidopsis nindensis*

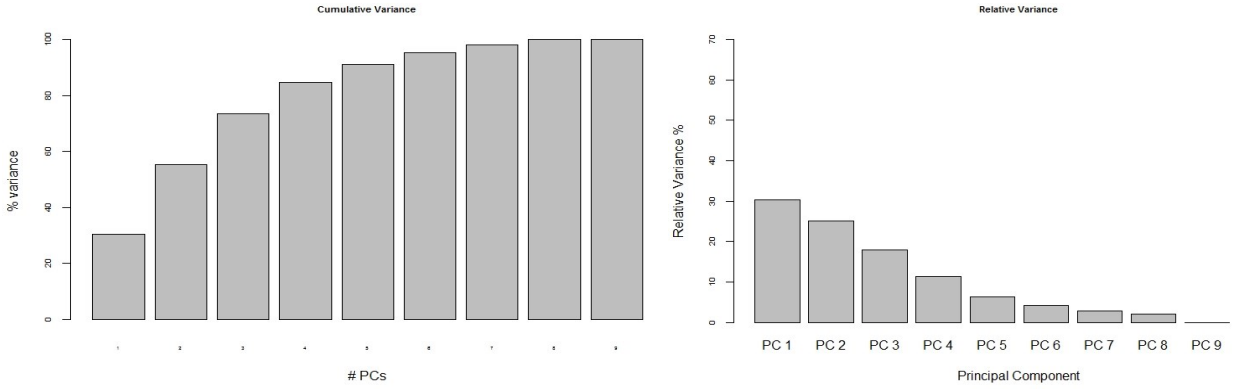


Figure 24: Cumulative and relative variance per Principal Component for GC-MS peaks of Arabidopsis thaliana.

The variance within the data of *Arabidopsis thaliana* is explained by the first four Principle Components, which together describe 80% of the variability of the samples. Among these four, the first and second principle components show a relevant clustering of the sample treatments (figure 25).

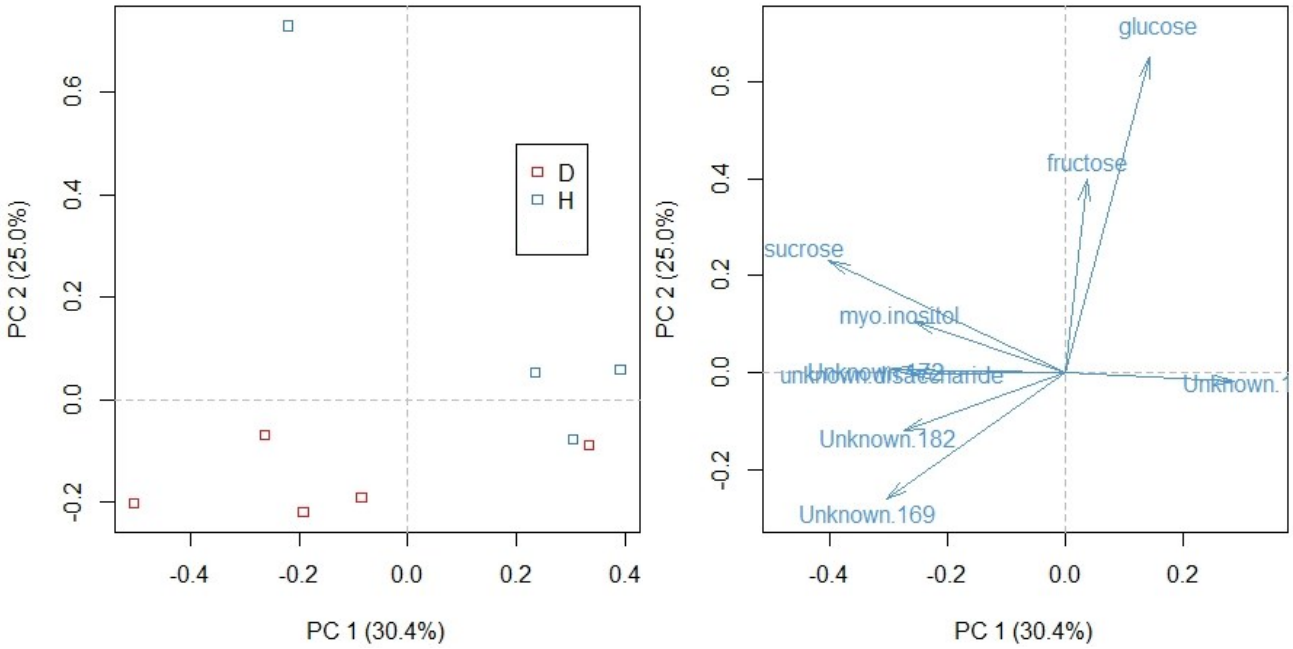


Figure 25: The image on the left represents a score-plot of the first and second principle components clustering for Arabidopsis thaliana in the three treatments (D: desiccated, H: hydrated, M: medium dehydrated). The image on the right represents a loading plot with the main metabolites contributing to the first and second principle components.

PC1 clusters desiccated samples on the left quadrant, while hydrated samples on the right quadrant. In the loading plot, various compounds such as sucrose and myo-inositol, are negatively correlated with PC1 and associated with the desiccated state. An unknown metabolite is positively correlated with PC1 and associated with the hydrated samples. Also, fructose and glucose are positively correlated, but with PC2 and might be present both in hydrated and desiccated, but in different abundances.

In this last image (figure 26), the score distribution of desiccated, hydrated and dehydrated samples of *Arabidopsis thaliana* for the first principle component is represented. PC1 substantially explains desiccated samples, while hydrated samples show more variability.

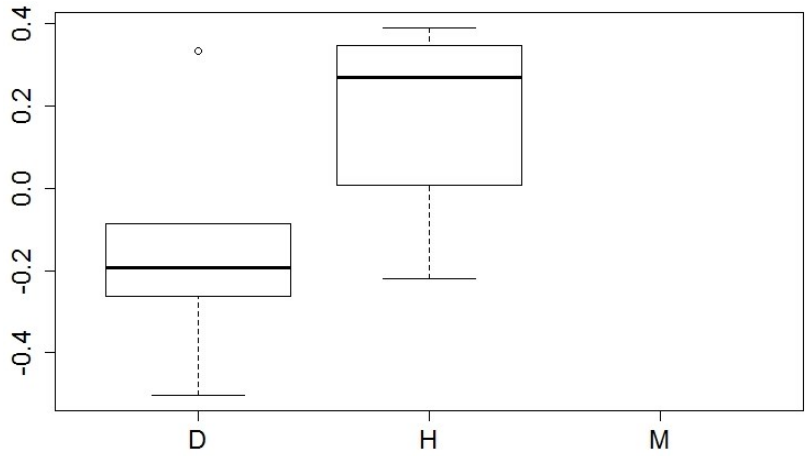


Figure 26: Boxplot of score distribution of desiccated and hydrated samples of Arabidopsis thaliana for the first principle component.

3.3.8 Total PCA

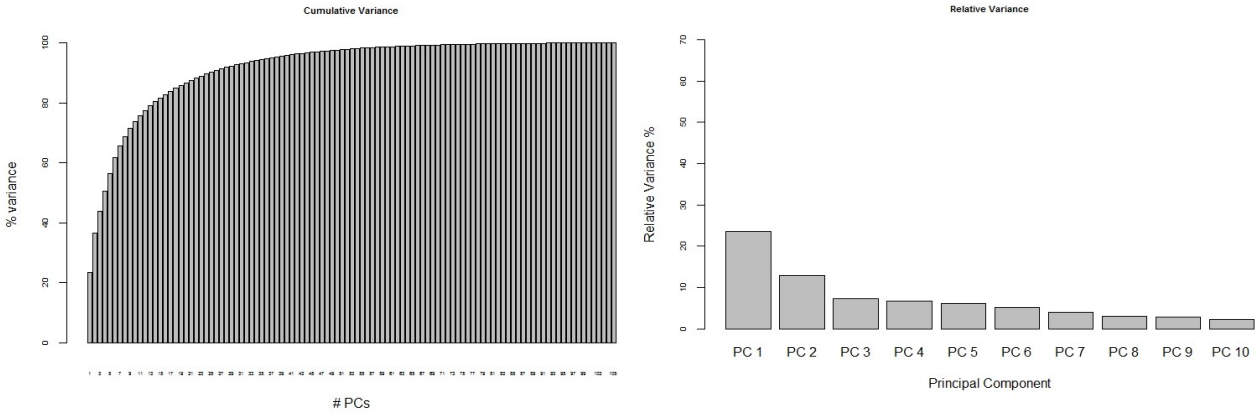


Figure 27: Cumulative and relative variance per Principal Component for GC-MS peaks of all species together.

The variance within the data of *all species together* is explained by the first fifteen Principle Components, which together describe 80% of the variability of the samples. Among them, the first and second principle components show a relevant clustering of the sample per species (figure 28), while the first and the third cluster the samples per treatment (figure 29).

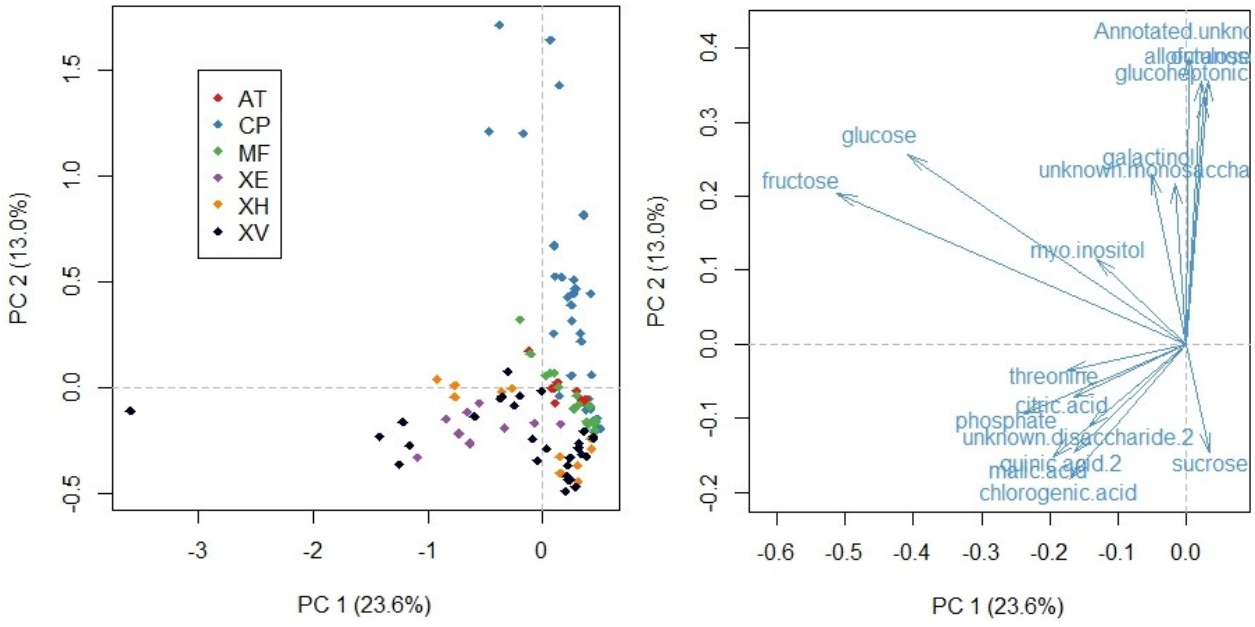


Figure 28: The image on the left represents a score-plot of the first and second principle components clustering the samples per species (AT: Arabidopsis thaliana, CP: Craterostigma pumilum, MF: Myrothamnus flabellifolia, XE: Xerophyta elegans, XH: Xerophyta humilis, XV: Xerophyta viscosa). The image on the right represents a loading plot with the main metabolites contributing to the first and second principle components.

From the score plot it is possible to individualize some similar or different clustering, that are mainly influenced by PC2. *Xerophyta viscosa*, *Xerophyta humilis* and *Xerophyta elegans* are all clustered mainly in the lower quadrant on the right. *Myrothamnus flabellifolia* and *Arabidopsis* are centred, while *Craterostigma pumilum* is located on the higher quadrants. PC2 is highly positively correlated with allofuranose, octulose, glucoheptonic acid and galactinol and it is indeed very much associated with Craterostigma pumilum. PC2 is then negatively correlated with sucrose, threonine, citric acid, phosphate and chlorogenic acid, which are all known to be present in *Xerophyta* species (Farrant et al. 2015; Lehner et al. 2008), and it is indeed what the score plot shows. Glucose and fructose are negatively correlated with PC1 and their orientation in the middle suggest that are present in all species.

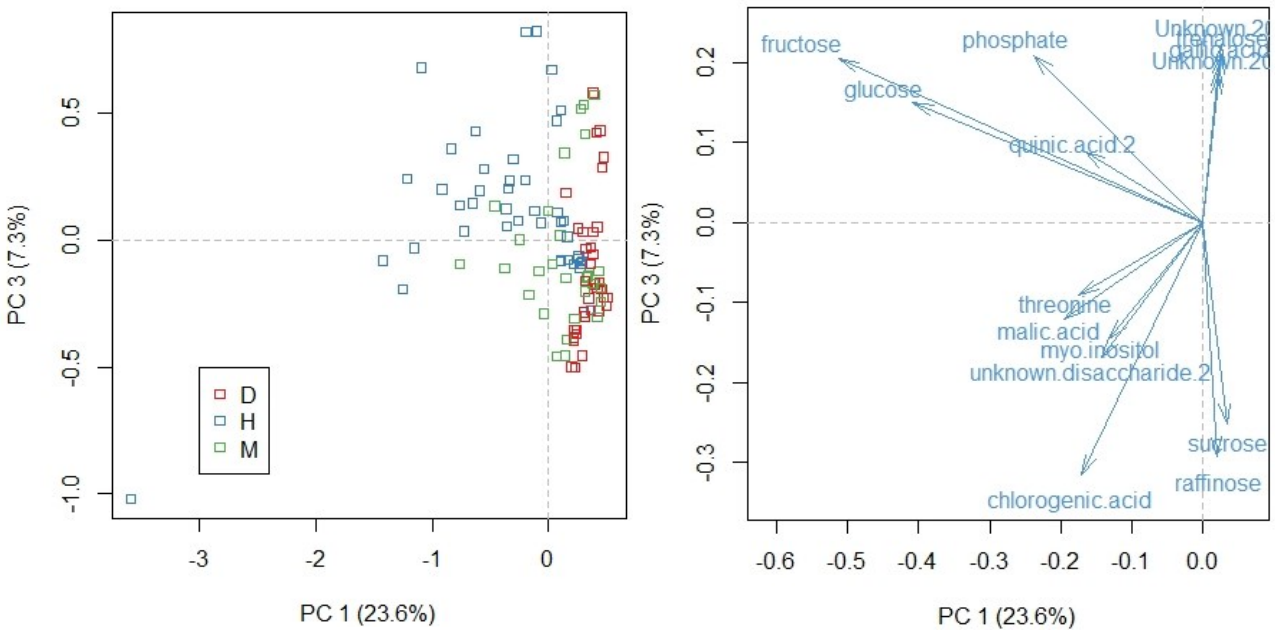


Figure 29: The image on the left represents a score-plot of the first and third principle components clustering all the samples per treatment. The image on the right represents a loading plot with the main metabolites contributing to the first and second principle components.

PC1 clearly cluster the desiccated samples of all the species from the hydrated samples. While the desiccated response seems similar to all the species, the hydrated samples show a variable composition. PC2 is negatively correlated with sucrose and raffinose mainly and is associated with desiccated samples. PC1 is negatively associated with several compounds such as phosphate, fructose, quinic acid, glucose, threonine, malic acid, myo-inositol and chlorogenic acid. All together they indeed explain the variability of the hydrated samples.

3.4 Heat map

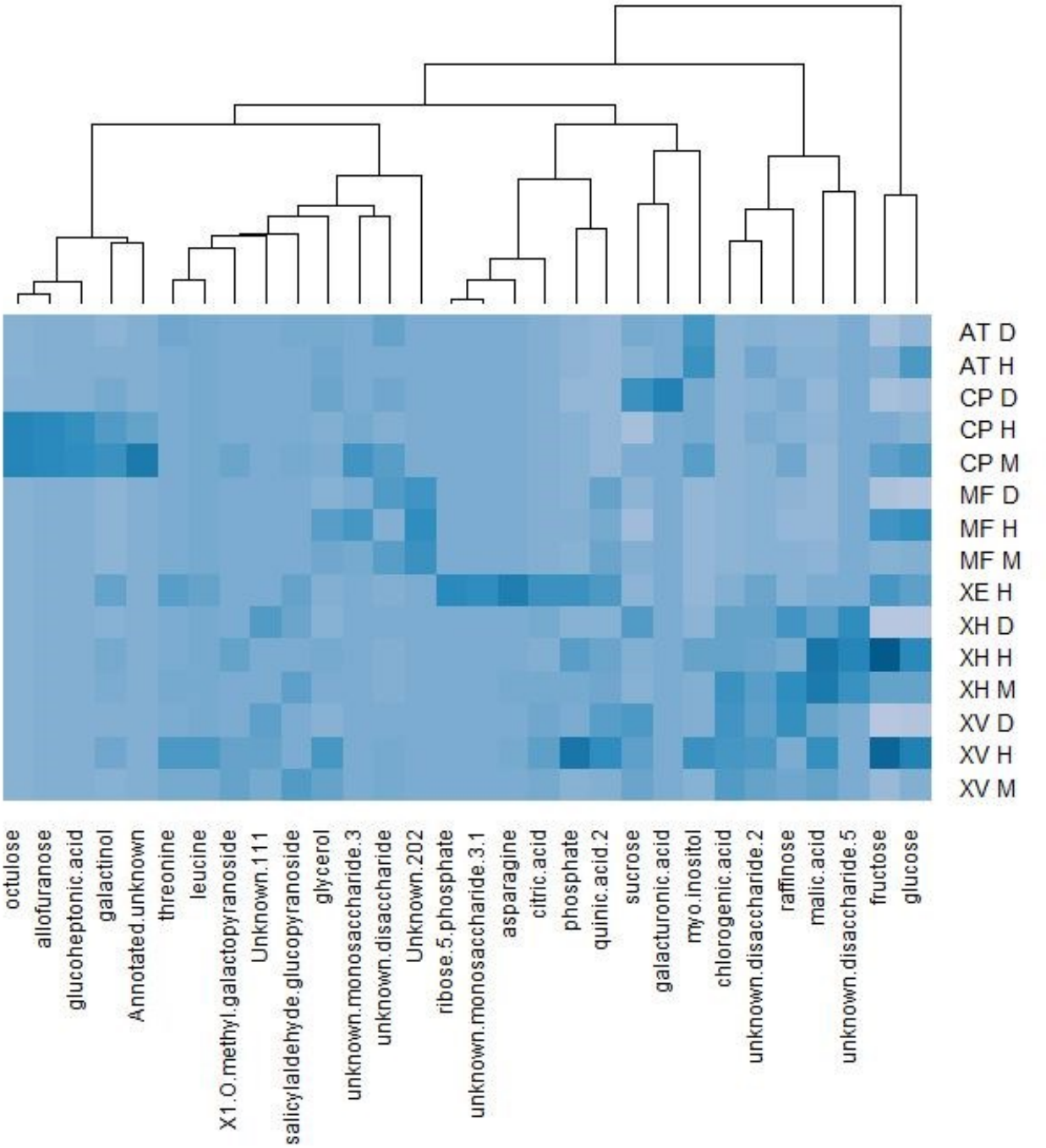


Figure 30: Heat map representing mean, scaled and normalized scores for each experimental group (species + treatment) of the 30 most variable metabolites in the total database. The labels on the right of the map represents the combination of species and treatments. The first two letters represent the species. AT: Arabidopsis thaliana, CP: Craterostigma pumilum, MF: Myrothamnus flabellifolia, XE: Xerophyta elegans, XH: Xerophyta humilis, XV: Xerophyta viscosa. The last letter represents the treatment. H: hydrated, M: late dehydration, D: desiccated.

The analysed species have been compared across the three different dehydration treatments in a heat-map (figure 11) that shows the most variable compounds of the total data set and their abundance in each of the experimental groups.

In general, *Xerophyta* species have a very similar metabolite composition and response during dehydration. They indeed have a very similar abundance of the same compounds, even though

Xerophyta elegans (hydrated only) differentiate itself for a few different compounds. Differently, *Craterostigma pumilum* and *Myrothamnus flabellifolia* are quite different from the *Xerophyta spp* and have other kind of compounds that are more abundant and that seems species specific. Lastly, *Arabidopsis thaliana* distinguishes itself from resurrection plants because all its compounds are much less abundant and even if it has some sugars in common, like glucose and fructose, their response to dehydration is much less marked. The desiccated samples reflect the metabolites composition of its dead state, since under 60-50% RWC *Arabidopsis* can't survive.

Comparing metabolites among species, some evident similarities are encountered among all of them. Firstly, sugars are highly represented in this subset of highly-variable compounds in all resurrection plants. Mono and disaccharides like glucose and fructose are most abundant in the hydrated state, mainly in *Xerophyta viscosa* and *X. humilis,* and they appear markedly decreased in the desiccated state. Also, raffinose and sucrose, are abundant in most of the species, mainly in the desiccated state. Sucrose would be expected to be highly abundant in the desiccated state (Suguiyama et al. 2014; Farrant et al. 2015; Berjak 2006). Nevertheless, due to peak overloading, sucrose peaks areas were underestimated.

As reported in literature (Suguiyama et al. 2014; Farrant et al. 2015; Berjak 2006; Moore et al. 2007), large amount of sugars like glucose and fructose are expected in the hydrated state, while during desiccation they decrease drastically. They are potentially dangerous at high concentrations because they can participate in Maillard reactions, and their down-regulation could help in limiting damaging reactions for the plant. Their decrease might also limit respiration and associated ROS production (John P. Moore et al. 2007). In contrast, raffinose and sucrose are highly abundant in desiccated state because of their multiple protective functions, such as vitrification of the cytoplasm, stabilization of membranes and their compatible solutes role in vacuoles (Farrant et al. 2015; Farrant et al. 2009; Farrant 2000).

There are other compounds that are present in similar amounts in almost all species. Myo-inositol, a sugar alcohol, is abundant in the hydrated state of all *Xerophyta* species, in *Craterostigma* and in *Arabidopsis*. It is also reported to be abundant in the desiccation tolerant *Selaginella lepidophylla*, which is very similar to the *Selaginella* species that should have been analysed in this project (Pampurova and Van Dijck 2014). Myo-inositol is known to be associated to hydrated tissues, while it decreases upon dehydration as it is a precursor (Oliver et al. 2011) for important metabolites (ex. raffinose and stachyose) synthesis pathways (Farrant et al. 2015; Lehner et al. 2008; Suguiyama et al. 2014). In addition, it possesses the ability to scavenge hydroxyl radicals as a way of reducing oxidative damage in the early stages of dehydration (Oliver et al. 2011; Yobi et al. 2013). As well, quinic acid is observed as a GC-MS peak in the hydrated samples of all *Xerophyta* species and in particular of *Myrothamnus.* This compound is never found in its free form and might appear in the data because of the breakdown of products of quinic acid complexes during sample processing and derivatization. But it is indeed representative of a number of other acids such as chlorogenic acid in *Xerophyta* species and 3,4,5-tri-O-galloylquinic acid in *Myrothamnus*. These two acids are both involved in protection of membranes against desiccation and play a role against oxidative damages (Moore et al. 2007; Suguiyama et al. 2014). Lastly, glycerol is also abundant in the hydrated state, mostly of *Xerophyta viscosa* and *Myrothamnus*, and it is also present in *Selaginella lepidophylla* (Pampurova and Van Dijck 2014). It is reported to cover some functions during dehydration, such as

to reduce the surface tension of water and to strongly bind water via hydrogen bonding. It therefore might limit the rate of water loss during the drying process (Yobi et al. 2013).

From the heat map it is visible that some metabolites are mainly common *in Xerophyta* species. Amongst them, there are malic acid, chlorogenic acid, phosphate, leucine and threonine. All of them are highly abundant on the hydrated state only, whereas the chlorogenic acid seems to be maintained over dehydration. Malic acid is mainly involved in primary energy metabolism and it decreases upon dehydration. Chlorogenic acid might play a role against a potential oxidative damage and it is indeed maintained in the dehydrated state (Suguiyama et al. 2014). Phosphate is highly involved in a lot of different metabolic processes but one hypothesis could also be that it is related to the myo-inositol 1-phosphate synthase, which has been reported (Lehner et al. 2008) to be associated to abiotic stresses and to be responsible for decreased abundance of sugars in the drying process (Farrant et al. 2015). Lastly, the amino acids leucine and threonine have been reported to be associated with early dehydration responses and to be highly abundant in this phase (Zhang and Bartels 2018). Furthermore, these amino acids, derivates of oxaloacetate and pyruvate, have been observed to be also associated with heat stress responses in Arabidopsis and their accumulation increased during initial dehydration (Guy et al. 2008).

A final analysis can be done on species specific metabolites, that appear to be highly abundant only in one species. In Xerophyta elegans hydrated samples, citric acid and asparagine seem to be much more abundant than in any other analysed species. Citric acid is an organic acid and is primarily a cycle intermediate of the tricarboxylic acid cycle. It is known to cover various functions during desiccation: it is believed to be accumulated in numerous small vacuoles to facilitate mechanical stabilization in the dry state. Also, it might be implicated in stabilization of the subcellular milieu by vitrification (Farrant et al. 2015).

The amino acid asparagine might instead be important for metabolic mobility of nitrogen reserves for metabolic recover during dehydration (Gaff and Oliver 2013).

Craterostigma pumilum reports a very particular metabolite composition. In the hydrated state and in the dehydrated state (50-30%) it is shown a very high abundance of sugars such as octulose, allofuranose, galactinol and glucoheptonic acid. Octulose might be a reserve substance, like starch, used for the redirection of carbon flow for sucrose accumulation (Moore et al. 2009; Oliver et al. 2000; Pampurova and Van Dijck 2014). At the same way, galactinol is proposed to donate galactosyl for synthesis of raffinose under water restriction (Suguiyama et al. 2014). Both galactinol and the previously mentioned myo-inositol are involved in the biosynthesis pathways of raffinose. In particular, L-myo-inositol is a precursor of galactinol and together with UDP-Galactose is used for the synthesis of galactinol by the enzyme galactinol synthase. In the end, galactinol is addressed for the synthesis of raffinose by the enzyme raffinose synthase (Sengupta et al. 2015) [4].

In the desiccated state *Craterostigma* reports a very high abundance of galacturonic acid, which is a sugar acid associated to the desiccated state (Vicré et al. 2004)

3.5 Biochemistry and adaptations

SPECIES	WATER CONTENT	ERENGY METABOLISM AND CARBON SOURCES	MECHANICAL STABLIZATION	MEMBRANE PROTECTION	ANTIOXIDANT FUNCTION	REPAIR MECHANISMS
Xerophyta viscosa	H	glucose, fructose, myo-inositol, galactinol, malic acid, phosphate	threonine, leucine, glycerol			threonine
	M					
	D		sucrose, raffinose	sucrose, raffinose	sucrose, raffinose, myo-inositol, chlorogenic acid	sucrose, raffinose
Xerophyta humilis	H	glucose, fructose, myo-inositol, galactinol, malic acid, phosphate	threonine, leucine, glycerol			threonine
	M					
	D		sucrose, raffinose	sucrose, raffinose	sucrose, raffinose, myo-inositol, chlorogenic acid	sucrose, raffinose
Xerophyta elegans	H	glucose, fructose, phosphate, citric acid, galactinol	citric acid			asparagine
Craterostigma pumilum	H	glucose, fructose, octulose, allofuranose, galactinol, annotated unknown				
	M	octulose, allofuranose, galactinol, annotated unknown				
	D		sucrose, raffinose	sucrose, raffinose		
Myrothamnus flabellifolia	H	glucose, fructose				
	M					
	D		sucrose	sucrose	3,4,5-tri-o-galloylquinic acid (quinic acid)	
Arabidopsis thaliana	H	glucose, fructose, myo-inositol				
	D (dead)					

Table 8: Summary of the most abundant metabolites, grouped per functionality among each species and the water content at which they are present (H: hydrated, M: late dehydration 50-30% RWC, D: desiccated). Metabolite's functions have been hypothesised by consulting literature.

In table 8, metabolites have been grouped by function per species and treatment.

Metabolites involved in energy metabolism are highly abundant in the hydrated state and some of them, like glucose, fructose and galactinol, are in common to all species. In *Xerophyta viscosa* and in *Xerophyta humilis* there are a few compounds, such as myo-inositol and malic acid, which are much more abundant than in the other species. These compounds might be specific to poikilochlorophyllous plants, since in homoiochlorophyllous species they are evidently much less abundant. Myo-inositol is particularly interesting, because related to the biosynthesis of raffinose (Farrant et al. 2015; Oliver et al. 2011). As resulted in the heat map, both in the desiccated state of *Xerophyta viscosa* and *Xerophyta humilis*, myo-inositol abundance is much lower than in the hydrated state, while raffinose is higher. This indeed suggests the employment of myo-inositol for raffinose accumulation.

Myrothamnus differentiates itself from all the other species because of the very low abundance of any sugars involved in energy metabolism, apart from glucose and fructose. This is indeed reflected in its raffinose abundance, which is the lowest of all DT species, together with galactinol and myo-inositol.

Craterostigma pumilum shows a much wider range of sugars, which include octulose, allofuranose, glucoheptonic acid and an annotated unknown (which is the most abundant one in the dehydration state M). This high abundance of sugars in the hydrated and dehydration states reflects in the abundance of sucrose, which is highly present in the desiccated state. As already known, sucrose is accumulated from the mobilization of carbon sources, like octulose (Moore et al. 2009; Oliver et al. 2000; Pampurova and Van Dijck 2014). Galacturonic acid is also very abundant in the desiccated state and might be related to the very high abundance of the annotated unknown in the hydrated and dehydration states.

Comparing all DT species with *Arabidopsis*, not very marked differences are present in the abundance of sugars at the hydrated state. Indeed, fructose, glucose and myo-inositol, which are also important for energy metabolism in desiccation sensitive plants, are abundant in the hydrated state.

Looking at DT adaptive mechanisms, metabolites can play a role in three different ways: contribute to mechanical stabilization, minimize membrane degradation and limit oxidative damages caused by ROS production. Metabolites can cover one or multiple functions depending on the species.

The main metabolites involved in mechanical and membrane stresses are sugars like sucrose and raffinose. Both of them are present in almost all resurrection plants and function as protection metabolites in several ways: they contribute to the vitrification of the cytoplasm to improve the stabilization of the subcellular milieu, they accumulate in vacuoles for mechanical stabilization and they replace H bonds for membrane stabilization (Farrant et al. 2015; Farrant et al. 2009; Farrant 2000). Vitrification of the cytoplasm might also be operated by other compounds. Farrant et al. (2015), suggest that threonine and glycine might be actors in vitrification and they resulted to be abundant mainly in the desiccated state. This result is incongruent with the results reported in this study, where threonine, and leucine, another amino acid, are mainly present in the hydrated state while much less in the desiccated state.

Farrant et al. 2015 also proposed that citric acid might be involved in vitrification and in accumulation in vacuoles and they reported it to be mainly abundant in the desiccated state of *Xerophyta viscosa.* Differently, in this study, citric acid resulted more abundant in the hydrated state than in the desiccated state of Xerophyta viscosa. An interesting result is that in *Xerophyta elegans*, citric acid is much more abundant than in any other resurrection plant, and it might be interesting to analyse if it would increase upon dehydration.

Lastly, glycerol is suggested to protect cells from water loss by reducing the surface tension of water and to strongly bind water via hydrogen bonding (Yobi et al. 2013). It is present in the hydrated state of both *Xerophyta viscosa* and *Myrothamnus flabellifolia*.

The metabolites involved in minimizing oxidative stress are quite variable among species and seem to be very much related to the type of response of the photosynthetic apparatus. In poikilochlorophyllous plants, such as *Xerophyta viscosa* and *Xerophyta humilis*, chlorogenic acid, a polyphenol composed by quinic acid, is highly abundant in both dehydrated and desiccated states and it might act as antioxidant (Farrant et al. 2015; Suguiyama et al. 2014).

For the same species, Farrant el al. (2015) suggest that also sucrose, raffinose and myo-inositol have antioxidant functions, by acting as sensors that stimulate the activation of antioxidant genes

expression under oxidative stress. Another metabolite, which hasn't been individualized in this study, but that is an important antioxidant in Xerophyta viscosa is 1-cys peroxiredoxin, which is also present in desiccation tolerant seeds (Farrant et al. 2015). The very low abundance of both chlorogenic acid and in minor part of quinic acid in *Xerophyta elegans*, might suggest that this species employs different kind of metabolites for oxidative stress. The very high abundance of citric acid might suggest that some organic acids could be some of the antioxidant actors.

In *Myrothamnus flabellifolia* another polyphenol named 3,4,5-tri-O-galloylquinic acid, also derivate of the quinic acid, is an important antioxidant metabolite (John P. Moore et al. 2007).

Furthermore, even though not shown in the PCA, high amount of pigments such as anthocyanins are accumulated during dehydration, mainly in homoiochlorophyllous species, in order to mask their photosynthetic apparatus from the light. Indeed, as reported in chapter 3.2, *Xerophyta elegans*, *Craterostigma* and *Myrothamnus*, reported a clear change of their abaxial colour upon dehydration, from green to dark purple.

In resurrection plants, metabolites are also very important during recovery and in literature a few samples have been reported. In *Xerophyta viscosa and Xerophyta humilis*, sucrose and raffinose might be accumulated in the desiccated state as energy source and might be useful during rehydration for rapid carbon mobilization (Farrant et al. 2015). Indeed, during rehydration, poikilochlorophyllous plants need a lot of energy to re-build their photosynthetic apparatus and to re-store their chlorophyll content. This might explain why in these species, sucrose and mainly raffinose, are more abundant than in other homoiochlorophyllous species. Farrant et al. (2015), also suggest that amino acids like threonine and glycine, might be highly present in the desiccated state as consequence of protein degradation and might be later used as a pool of free amino acids for recovery upon rehydration.

Lastly, in hydrated *Xerophyta elegans*, very high amounts of asparagine are found and it might be used as nitrogen source for metabolite recovery upon rehydration (Gaff and Oliver 2013).

3.6 Suggestion for future research

Since some differences in metabolites composition have appeared when comparing poikilochlorophyllous plants with homoiochlorophyllous plants, it would be interesting to further investigate if there is an actual difference in their desiccation induced protective mechanisms.

First of all, it would be interesting to analyse *Xerophyta elegans* in all its dehydration levels and see if in the desiccated state raffinose would be abundant or not and if it would reflect the low levels of myo-inositol in the hydrated state. Secondly, it would be interesting to observe which metabolites with antioxidant functions would be accumulated by *Xerophyta elegans* in the desiccated state in order to discover if these antioxidants would be related or not to citric acid.

Another interesting research would be to investigate which metabolites are involved in recovery mechanisms, since it would give a more complete idea of desiccation tolerant mechanisms and it would explain better the accumulation of certain metabolites in the desiccated state.

Another plant that would be very interesting to study is *Mohria caffrorum.* This plant is indeed very particular, since it is seasonally desiccation tolerant (Farrant et al. 2009). The analysis of the change of its metabolite composition from desiccation sensitive to desiccation tolerant would provide additional information on important metabolites involved in DT mechanisms.

4 Conclusions

After having analysed each species' metabolites composition at the three stages of the dehydration process, it is possible to conclude that in all resurrection plants there is a clear distinction in metabolites composition between the hydrated state and the desiccated state. No evident differences are present between the dehydrated (50-30%) and desiccated state. This result might have been influenced by the high variability of water contents among the replicates of the dehydrated samples. The use of replicates with a narrower range or a specific water content (40%), might have given a different result.

The desiccation sensitive species Arabidopsis thaliana has reported a very small difference between hydrated and dehydrated states, which confirms the more limited metabolic response of desiccation sensitive species to drought conditions.

After having compared the composition and abundance of metabolites in the plants under study, it is possible to outline that some clear differences in desiccation-induced metabolites are present among resurrection plants. Some of these metabolites appeared to be species specific or in other cases specific to a particular group. However, also evident similarities in desiccation-induced metabolites are found among resurrection plants. One example is sucrose, which resulted in

In conclusion, desiccation tolerance is a very complex phenomenon and it involves a wide variety of processes and metabolite changes. This study gives a first hint on which are some of the key protection mechanisms that are induced during desiccation, but further researches on species specific metabolites would help in gaining a greater understanding of the full spectrum of protection metabolites involved in desiccation tolerance. In addition, studying the rehydration process and the repair mechanisms involved, would give a completer and broader idea of the whole complex mechanism of desiccation tolerance.

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