# Metabolite profiling of *Eragrostis nindensis* during Desiccation and Recovery

By

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Declaration

I, **Erikan Baluku**, hereby declare that the work titled **"**Metabolomics of *Eragrostis nindensis* during Desiccation and Recovery" is my original research and that neither any part or whole work has been submitted for any degree or examination in any other university.

Signed by candidate

Signature: …… Date: 29/09/2023

Dedication

I dedicate this work to my parents, who opened the education journey for me, showed and taught me to love school and work hard for better results.

Acknowledgement

All thanks to the Almighty God for granting me the strength and grace to achieve my goals. As the culmination of this arduous journey comes into focus, I find myself profoundly grateful for the unwavering support and guidance that have propelled me forward. The completion of this dissertation would not have been possible without the contributions of numerous individuals who have shared their wisdom, encouragement, and expertise along the way.

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List of Abbreviations

| | |
|---|---|
| VDT | vegetative desiccation tolerance |
| ST | senescent tissue |
| NST | non-senescent tissue |
| FH | fully hydrated. |
| ED | early dehydration |
| LD | late dehydration |
| AD | air dry |
| GC-MS | gas chromatography-mass spectrophotometry |
| MSTFA | N-Methyl-N-(trimethylsilyl)trifluoroacetamide |
| PSL | Plant Stress Lab |
| NMR | nuclear magnetic resonance |
| rpm | revolution per minute |
| S/N | signal-to-noise |
| PCA | principal component analysis |
| PLSDA | partial least squares discriminant analysis |
| ANOVA | analysis of variance |
| FDR | false discovery rate |
| LSD | least significant difference |
| LC-MS | liquid chromatography-mass spectrophotometry |
| CE-MS | capillary electrophoresis mass spectroscopy |
| ROS | reactive oxygen species |
| BCAAs | branched-chain amino acids. |

GS/GOGAT - Glutamine synthetase/glutamine oxoglutarate aminotransferase

| | |
|---|---|
| CSV | comma-separated values. |

| | |
|---|---|
| RWC | relative water content |
| VDE | violaxanthin de-epoxidase |
| SPME | solid phase micro extraction |
| IMS | imaging mass spectrophotometry |
| GABA | gamma-aminobutyric acid |
| VOC | volatile organic compound |
| RFO | raffinose family oligosaccharides |
| VIP | variable importance projection |
| PCD | programmed cell death. |

Table of content

List of figures

List of tables

List of supplementary tables and Figures

Abstract

Resurrection plants are a unique group of angiosperms that can withstand cellular water loss of up to 95% and resume full metabolic activity upon rehydration. To withstand extreme water loss, they employ a plethora of molecular, physiological, and biochemical processes including accumulation of metabolites that shield the plant cells from photo-oxidative damage and reactive oxygen species. A global understanding of the whole plant using a multi-omics approach will provide more insights into how different parts of the plant deal with desiccation. This study aimed to identify the different metabolites that are differentially abundant in *Eragrostis nindensis* at different stages of dehydration and rehydration time points in both desiccation-sensitive senescent (ST) and desiccation-tolerant non-senescent (NST) leaf tissue using gas chromatography-mass spectrometry Furthermore, this study compared the shoot and root systems to unravel similarities and differences at the whole plant level in overcoming desiccation. The metabolomics data from the shoots between NST and ST showed that differentially abundant metabolites in NST act as major drivers for plant desiccation tolerance and also aid the plant post-recovery. The roots accumulated fewer metabolites than the shoots; however, some specific metabolites were shown to accumulate exclusively in the roots. These findings revealed that *E. nindensis* exhibits a metabolic shift with the abundance of sugars such as raffinose and sucrose, amino acids such as glycine and glutamic acid and organic acids such as alpha-ketoglutaric acid and citric acid during dehydration, resulting in accumulation of desiccation-responsive metabolites predominantly in NST compared to ST. The results demonstrated that the leaves have a different metabolic shift pattern that is more variable, and the roots' metabolome is less affected by desiccation. Post-rehydration, there is an accumulation of amino acids and organic acids to aid in the resumption of metabolism in NST compared to ST and roots. The accumulation of these metabolites may protect *E. nindensis* from the damage associated with rapid drying, as the accumulation of similar metabolites identified in this study has been reported to function as osmoprotectants, reactive oxygen species quenchers and compatible solutes that replace water during desiccation. The identified metabolites and metabolic process provide a great insight into the goal of improving drought tolerance in orphan and drought-sensitive crops.

# Chapter 1

## 1.1 Introduction

### 1.1.1 Desiccation tolerance and resurrection plants

Unfavourable weather conditions and changes in rainfall patterns have impacted crop productivity globally and drought poses a danger to food security in the future, as current trends indicate that Africa will not be able to fulfil Sustainable Development Goal (SDG) 2 (often known as "zero hunger") by 2030 (UNICEF, 2020). Water shortages and high temperatures are two significant interrelated stresses that have an impact on crop growth and productivity, which ultimately has an impact on food security (Cottrell et al., 2019; He et al., 2019). Due to the need to feed a growing population, expected to reach 8.6 billion by 2030 (https://www.un.org/en/desa/world-population-projected-reach-98-billion-2050-and-112-billion-2100), there is a need to devise means of producing drought-tolerant crops to increase agricultural productivity. To date, many methods have been used to solve the challenge of water deficit such as breeding of plants, planting of short-maturing and drought-tolerant types, timing modifications to cropping patterns, use of greenhouse technologies and the application of soil and water conservation techniques (Shiferaw et al., 2014), but low success rates have been reported. Most plants can withstand dehydration, but to varying degrees, and two forms of tolerance can be identified when water is continuously lost due to external factors (Hoekstra et al., 2001). Drought tolerance is considered the ability of plants to survive water loss while maintaining a high internal water content whereas desiccation tolerance generally refers to the ability of plants to tolerate dehydration up to 10% relative water content (RWC) equivalent to 0.1 $H_2Og^{-1}$ dry weight and restore full metabolism upon rehydration (Berjak, 2006; Oliver et al., 2020). This implies that some unique plants use desiccation tolerance as a mechanism to tolerate drought and the two types of tolerance are not synonymous (Alpert, 2005). Plants can also cope with drought stress in a variety of ways. They can be classified as physiological and morphological adaptations, and include processes such as developing longer roots, forming thicker cuticles, developing smaller and folded leaves, delaying leaf senescence, higher stomatal conductance, slower transpiration rates, and higher chlorophyll content (Aslam et al., 2015; Fadiji et al., 2022; Fang &

Xiong, 2015). Some plant species have shown to be drought tolerant but have vegetative tissues that are desiccation sensitive when exposed to extreme water loss (Ntuli, 2012). However, there is a unique group of plants termed “resurrection plants” that can survive extreme water loss up to 95% and subsequently resume their metabolic functions upon rehydration (Gaff, 1971). To date, this group encompasses over 600 terrestrial plants from 68 bryophytes and 10 ferns, with approximately 10 angiosperms families (Marks et al., 2021), and they display vegetative desiccation tolerance (VDT).

The majority of land plants with VDT are resurrection plants that evolved after VDT was first detected in the ancestors of early land plants. (Oliver et al., 2000). VDT is similarly seen in orthodox seeds as a basis for long-term seed viability (Smolikova et al., 2020). The vegetative tissue of most crops displays desiccation sensitivity during the early stages of plant growth making it difficult for crops to survive in harsh climatic conditions (Sherwin, 1995). *Arabidopsis thaliana* seeds, for instance, are desiccation-tolerant but lose that tolerance after germination (Maia et al., 2011). Unlike other plants, the vegetative tissues of resurrection plants are desiccation tolerant (Dinakar & Bartels, 2013; Farrant et al., 2009). Resurrection plants gradually suppress growth and reproduction during dehydration stages intending to achieve desiccation (Scott, 2000). Due to the ability of resurrection plants to resist extreme water losses, they have been positioned as unique models for understanding mechanisms used by plants to escape extreme drought thus providing strategies for the improvement of drought tolerance in crops (Tebele et al., 2021).

As plants try to adapt to the extreme water loss, they undergo several changes, the most notable of which are: i) cellular water efflux leading to cellular death due to plasmolysis; ii) altered metabolism, damage of biomolecules, and formation of free radicals due to oxidative stress; and iii) thylakoid membrane damage inhibiting photosynthesis (Li et al., 2009; Osakabe et al., 2014). Interestingly, resurrection plants can overcome all these changes to prevent cell death by developing unique molecular and metabolic mechanisms to protect themselves against damage caused by desiccation. These mechanisms include i) accumulation of compatible solutes that act as osmoprotectants to stabilize the proteins and cell membrane during water deficit stress (Georgieva et al., 2020; Moore et al., 2006), ii) production of stress-protective genes and proteins such as late embryogenesis proteins (LEA) and heat shock

proteins (HSPs) (Artur et al., 2019; Illing et al., 2005), iii ) production of reactive oxygen species (ROS) scavengers to reduce reactive free radicals and ROS (Farrant, 2007; Illing et al., 2005) and iv) production of protective metabolites to overcome desiccation (Challabathula et al., 2018; Keunen et al., 2013). (Ayub et al., 2021; Hossain et al., 2013; Y. Osakabe et al., 2014). Almost all these processes have been reported primarily in shoot systems. By contrast, roots have also demonstrated a distinct response to dehydration (Cao et al., 2017), and less research has provided insights into the underlying mechanisms. However, there is a root-shoot interaction during dehydration as evidenced by the fact that during dehydration, the root length and density restrict water intake and stimulate stomatal closure to reduce water loss in plants hence lowering the photosynthetic rate in plants (Cornic, 2000). Therefore, it is crucial to comprehend the intricate regulatory processes that coordinate the above- and below-ground plant component's response to desiccation.

Resurrection plants are classified as homoiochlorophyllous or poikilochlorophyllous species. The latter species degrade their chlorophyll and disassemble their thylakoid membrane during dehydration, and the photosynthetic apparatus is reconstituted upon rehydration. It is interesting to note that resurrection plants such as *Xerophyta humilis*, *X. viscosa* now *X. schlechteri* and *E. nindensis* have developed a unique mechanism to shut down photosynthesis to minimize the effects of photooxidative stress by protecting the photosynthetic apparatus (Beckett et al., 2012; Challabathula et al., 2015; Ginbot & Farrant, 2011). Poikilochlorophyllous resurrection species are mostly monocotyledons belonging to the velllozіaceae family. The homoiochlorophyllous species for example *Myrothamnus. flabellifolia* and *Craterostigma spp* retain their photosynthetic apparatus and use other mechanisms such production of ROS scavengers and reduction of light intensity from reaching the chloroplast by modification of leaf morphology through the accumulation of anthocyanins in their leaf surfaces, which serve as photon quenchers (Charuvi et al., 2015; Farrant et al., 2007; Morse et al., 2011).

The molecular and physiological basis of desiccation tolerance of several resurrection plants such as *M. falbellifolia, X. viscosa now X. schlechteri, Craterostigma plantagineum, C. wilmsii, X. humilis, E. nindensis,* and *Selaginella lepidophylla* (Cooper & Farrant, 2002; M.-C. D. Costa et al., 2017; Cushman & Oliver, 2011; Moore et al., 2004; Peters et al., 2007; Vander Willigen et al., 2001) have been well-studied

and have been shown to upregulate shared genes responsible for desiccation tolerance involved in protection of plant against abiotic stress. More so, the use of "omics" approaches to gain knowledge of biochemical mechanisms in most resurrection plants is increasing and as a result, the metabolomics analysis of *C. plantagineum, M. falbellifolia,* and *X. schlechteri* has revealed new information about key metabolites, notably those involved in antioxidant activity, photosynthetic apparatus shutdown, and the formation of suitable solutes (Bentley & Farrant, 2020; Radermacher et al., 2019). However, only a few studies have used a multi-omics approach on resurrection plants for example *Ramonda serbica Panc* (Vidović et al., 2022), *C. plantagineum* (Xu et al., 2021) and *Sporobolus stapfianus* (Yobi et al., 2017) to understand the complex network in the regulation of desiccation tolerance. All the multi-omics studies have been conducted on the shoot system and less data on the root omics.

### 1.1.2 “Omics” Approaches to Understanding Desiccation Tolerance

Advances in “omics” technologies have substantially changed approaches to understanding biological systems suggesting “top-down” and “bottom-up” approaches (Dai & Shen, 2022) and this offers an integrated tool for holistic investigation of biological systems. More so, multi-omics approaches have played a significant role in understanding plant biochemical processes that drive nutrient fixation and the response of plants to abiotic and biotic stress (Ramalingam et al., 2015; Raza et al., 2021; Yang et al., 2021). For instance, metabolomics has been substantially used in crop science to highlight the associations between plant genotype and phenotype under varying physiological and environmental conditions (Y. Osakabe et al., 2014; Paupière et al., 2017), and greatly improving the understanding of which transcripts, proteins and metabolites are produced during dehydration and subsequent desiccation (Gupta et al., 2021). Additionally, metabolomics assists in comprehending the intricate network as to how the synthesis of metabolites is regulated throughout a plant's response to abiotic stress during the adaptation process.

Multiple techniques have been used in the extraction and quantification of metabolites due to the different physical and chemical properties of biomolecules. The most commonly used approaches available for analysis include Gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), nuclear

magnetic resonance (NMR) spectroscopy, capillary electrophoresis mass spectrometry (CE-MS), and Fourier-Transform Infrared (FT-IR)spectroscopy (Fiehn, 2016; Hopfgartner et al., 2012; Kim et al., 2010; Maia et al., 2021; Zhang & Ramautar, 2021) detailed in Table 1-1. Using these techniques, metabolic changes in an array of metabolites from primary carbohydrates to secondary metabolites can be quantified and analysed. For instance, GC-MS was used to identify changes in primary and secondary metabolites during dehydration and rehydration in the resurrection plant *Haberlea rhodopensis* studied by (Moyankova et al., 2014). In a different study, Bianchi et al. (1991) used nuclear NMR spectroscopy to detect changes in primary and secondary metabolites in *C. plantagineum* during desiccation. However, some methods are limited depending on the type of analyte investigated. GC-MS has a major limitation because it analyses volatile compounds and samples are derivatized before being injected into the Column. Therefore, non-derivatized/non-volatile compounds can be analysed by LC-MS as an alternative method (Fiehn, 2016).

### 1.1.3 Gas Chromatography-Mass Spectrometer (GC-MS)

Gas chromatography (GC) is a separation technique that separates volatile compounds based on their affinity for a stationary phase within a column and a mobile phase (carrier gas). The compounds are introduced into the GC system, vaporized, and carried through the column. The different compounds interact with the stationary phase to varying degrees, leading to their separation. However, GC alone cannot provide detailed information about the chemical structure of the separated compounds. It is coupled with a mass spectrometer which is used to identify and quantify compounds based on their mass-to-charge ratio (m/z) and provides structural information through fragmentation patterns. The sample eluting from the GC column enters the MS, where the compounds are ionized and fragmented. The resulting ions are separated according to their mass-to-charge ratio, and their abundance is measured, generating a mass spectrum that represents the compound's molecular fingerprint (Turner et al., 2019).

The applications of GC-MS are vast and diverse. In environmental analysis, GC-MS is used for the detection and quantification of pollutants, such as volatile organic compounds (VOCs) (Ueta, 2022) and pesticides, in air, water, and soil samples (Santos & Galceran, 2002). In pharmaceutical analysis, it plays a critical role in drug

discovery, quality control, and metabolite identification (Togola & Budzinski, 2007). In forensics, GC-MS assists in the identification of controlled substances, determining accelerants in fire investigations, and toxicological analysis (Kabir & Furton, 2021). In food safety, it helps detect contaminants, such as pesticides, mycotoxins, and veterinary drug residues (Domínguez et al., 2020). Currently, it is beneficial to plant science in identifying and quantifying primary metabolites in plant tissues.

Over the years, advancements in GC-MS technology have improved sensitivity, resolution, and analysis speed. Fast GC techniques reduce analysis time, allowing for high-throughput analysis. The development of hyphenated techniques, such as GCxGC-MS, enhances the separation power by combining two GC columns with different selectivity (Winnike et al., 2015). Additionally, the coupling of GC with other techniques, like headspace sampling or solid phase microextraction (SPME) (Pieraccini et al., 2008), enables the analysis of volatile and semi-volatile compounds in complex matrices (Rao et al., 2016). In the current study, GC-MS was used due to its high sensitivity resolution as suggested by previous studies.

Table 1-1: Other metabolic profiling techniques and their functions

| Technique | Specific Function |
|---|---|
| Nuclear Magnetic Resonance (NMR) | Structural elucidation of metabolites.<br>Quantification of metabolites in mixtures.<br>Study of metabolic fluxes and interactions.<br>Investigation of protein-ligand interactions |
| Capillary Electrophoresis (CE) | Separation and analysis of charged metabolites.<br>Profiling of small, polar metabolites.<br>Analysis of nucleotides and organic acids. |
| Fourier Transform infrared Spectroscopy (FTIR) | Identification and characterization of metabolites based on functional groups.<br>Qualitative and quantitative analysis of metabolites. |
| Imaging Mass Spectrometry (IMS | Visualization and mapping of metabolites in tissue sections.<br>Spatial analysis of lipid distributions in cells and tissues.<br>Identification of biomarkers and metabolic pathways. |
| Liquid Chromatography-Mass Spectrometry (LC-MS) | Comprehensive analysis of metabolites in complex samples.<br>High-throughput profiling of small molecules.<br>Quantification of metabolites with high sensitivity.<br>Analysis of lipids, peptides, and drug metabolites |

### 1.1.4 Metabolic profiling of resurrection plants

Metabolites in plants are vital biomolecules which are necessary for basic metabolic functions like photosynthesis, respiration, and growth. They are found in all living plant tissues and comprise carbohydrates, lipids, proteins, and nucleic acids. Metabolic

profiling has been performed on several resurrection plants such as *C. plantagenium* (Xu et al., 2021), *S. stapfianus Selaginella spp* (Yobi et al., 2017; Yobi et al., 2012)*, X. schlechteri* (Radermacher et al., 2019), and *H. rhodopensis* (Moyankova et al., 2014*)* to study the changes in metabolism that occur during dehydration and rehydration to offer insight into the biochemical pathways that allow resurrection plants to survive and recover from significant water loss. Drought stress is the primary cause of many changes in metabolite abundance (Bashir et al., 2021), and changes are crucial for developing desiccation tolerance. A few metabolites which have been reported to accumulate in monocotyledonous resurrection plants that are closely related to the studied species are detailed in Figure 1-1, and they are anticipated to play a wide range of functions related to desiccation tolerance (Moyankova et al., 2014; Xu et al., 2021).

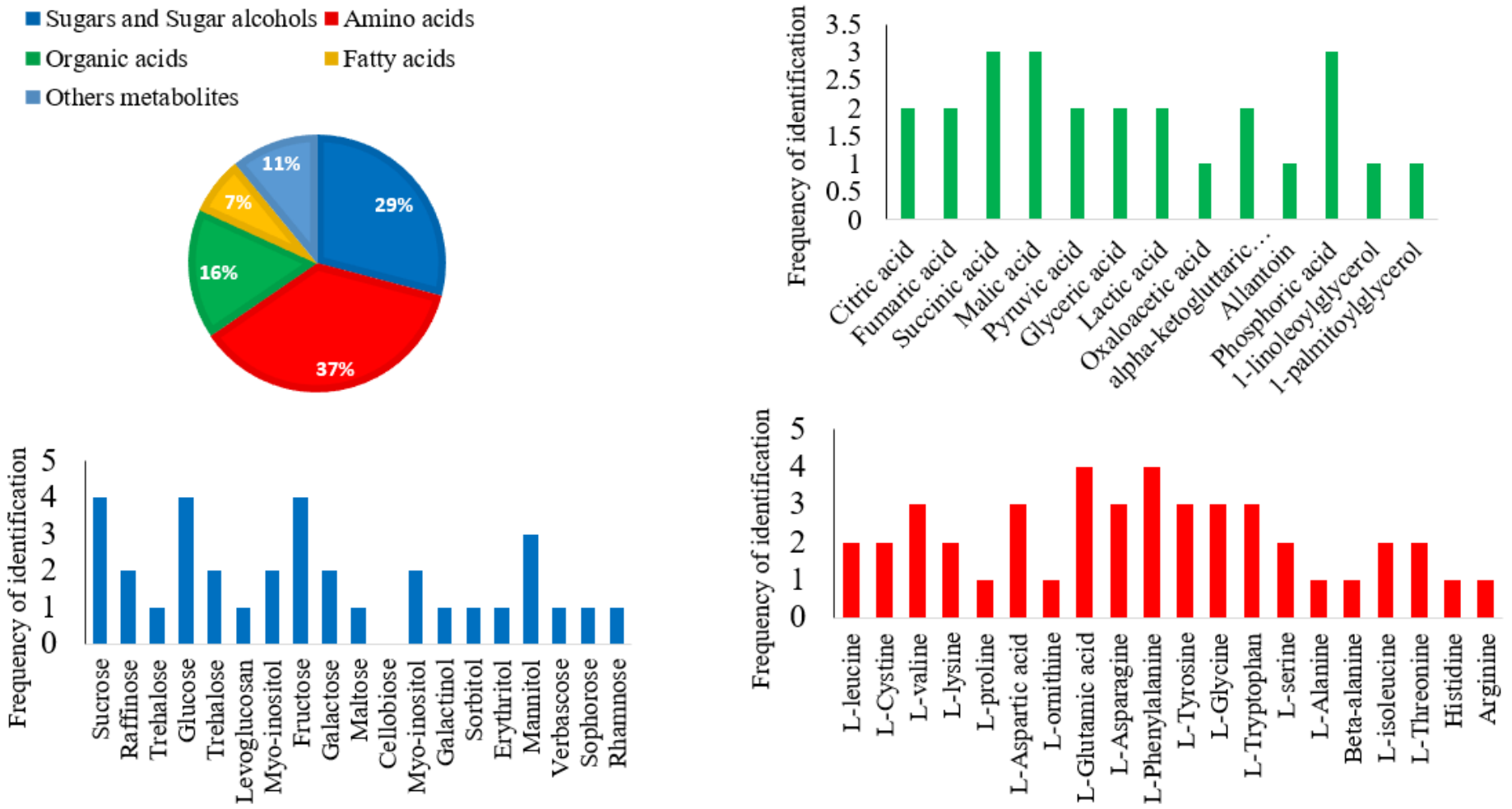



Figure 1-1: Summary of the most prominent metabolites identified in a selection of monocotyledonous VDT plants (Dace et al., 2023; Dinakar & Bartels, 2013; Gabier et al., 2021; Martinelli et al., 2007; Radermacher et al., 2019; Ricco et al., 2018; Whittaker et al., 2001)

#### 1.1.4.1 Metabolites involved in dehydration and rehydration.

Dehydration induces an array of physiological and biochemical changes in plants, including alterations to their primary metabolites including sugars, amino acids, and organic acids. Resurrection plants accumulate high levels of metabolites that act as ROS scavengers. These metabolites shield the plant from oxidative stress damage caused by an accumulation of ROS damage (Van den Ende & Valluru, 2008). Additionally, other metabolites that accumulate are involved in maintaining cell turgor, stabilizing cell membranes, and are involved in glass state formation (Farrant et al., 2007; Oliver et al., 1998). To survive, dehydrated plants must immediately regain their water balance and fix any damage to the photosynthetic apparatus. These processes require primary metabolites, which provide energy and the building blocks needed for growth and repair (Albini et al., 1999; Gechev et al., 2012). A study by Suguiyama et al. (2014) investigated the metabolite profile of the Brazilian resurrection plant *Barbacenia purpurea* Hook during desiccation and recovery and reported accumulations of sugars such as sucrose, amino acids such a glutamic acid and organic acids that are reported to aid the plant during desiccation and recovery, while a study by Peters et al. (2007) investigated the role of sucrose and raffinose as a protection mechanism in resurrection plant *X. viscosa* (Baker) now *X. schlechteri*.

#### 1.1.4.2 Trends of primary metabolites in response to desiccation tolerance

##### Sugars

Sugars are crucial for plants' ability to respond to dehydration and to withstand drought stress. Changes in levels of sugars have been extensively studied in a variety of resurrection plants as a universal response to desiccation tolerance recently reviewed in Dace et al. (2023). Other than acting as energy stores during dehydration, sugars play a role in maintaining a glassy state during desiccation (Buitink & Leprince, 2008) and preventing water loss through osmotic adjustment (Ahmad et al., 2020). When a plant is under drought stress, the concentration of solutes inside the cell decreases, causing water to move out of the cell and into the surrounding environment (Farrant, 2007; Oliver et al., 1998). This results in a loss of turgor pressure and can lead to dehydration and cell damage. Sugars such as sucrose and raffinose have been shown to accumulate within the cell to increase the osmotic potential and maintain turgor pressure, thereby protecting the plant from dehydration (Morse et al., 2011; Peters et al., 2007). Additionally, sugars have antioxidant properties that defend plants from

oxidative damage (Ende & Peshev, 2013). When cells are under drought stress, ROS can accumulate and cause deoxyribonucleic acid (DNA), protein, and lipid damage hindering plant growth. According to Van den Ende and Valluru (2009), sugars can function as ROS scavengers to lessen oxidative damage and shield cells from stress-related damage. Furthermore, sugars have the potential to function as signalling molecules (Sheen et al., 1999), causing adjustments in the metabolism and gene expression that assist the plant in overcoming dehydration stress. For instance, it has been demonstrated that trehalose increases plants' tolerance to drought stress by activating stress-responsive genes (Kosar et al., 2019). Finally, researchers have demonstrated that the accumulation of carbohydrates during dehydration results in shifts in gene expression, particularly those related to sugar metabolism (Ruan, 2014). During dehydration, genes linked to stress tolerance are increased, whilst genes linked to growth and development are downregulated. More so, during seed maturation, the accumulation of sugars in desiccation-tolerant embryos is postulated to cause a vitreous phase, a phase important in slowing down injury processes in embryos during germination (Williams & Leopold, 1989). Additionally, sugars such as raffinose and sucrose are found to prevent the chaotropic effects of high ion concentration in isolated chloroplasts hence having the ability to protect the photosynthetic apparatus. Additionally, raffinose increases the effectiveness of sucrose in the formation of a vitrified state (Caffrey et al., 1988), a state plants utilise in the protection of cellular components during desiccation and supports the hypothesis that the accumulation of these sugars remains a hallmark in desiccation tolerance and the fact that sucrose plays a key role both in vitro and in vivo metabolic processes (Hoekstra et al., 2001; Leslie et al., 1995).

Amino acids.

Amino acids are important molecules during desiccation, and they are involved in several metabolic processes. Similar to sugars, amino acids also contribute to osmotic adjustment and help maintain turgor pressure. Proline is a well-known amino acid that plays an important role during dehydration stress where its accumulation contributes to osmotic adjustments (Kavi Kishor & Sreenivasulu, 2014). Other amino acids such as histidine, asparagine, glutamic acid, aspartic acid, phenylalanine, and tyrosine have also been shown to increase during dehydration and most of them are involved in the formation of secondary metabolites that have a role in the acquisition of desiccation

tolerance (Martinelli et al., 2007; Yobi et al., 2019). Serine, threonine, and tyrosine stabilize protein structures by donating a hydroxyl group to shield proteins from denaturation caused by dehydration-induced damage. Furthermore, amino acids can neutralize ROS and guard against oxidative stress. The synthesis of amino acids such as proline, glutamine, asparagine, and arginine due to osmotic stress leads to the accumulation of compatible osmolytes to protect plants from abiotic stress (Hildebrandt, 2018). Amino acids can also function as signalling molecules. For instance, glutamate is known to trigger genes that respond to stress such as stomatal closure regulation genes and improve plants' tolerance to drought stress (Yoshida et al., 2016).

Fatty acids

Most of the fatty acids which have been studied have been demonstrated to play significant roles in responses to dehydration, particularly in maintaining the integrity of the cell membrane. Dehydration stress can cause damage to the cell membrane through ROS-mediated lipid peroxidation, resulting in the loss of membrane integrity and the leakage of cellular components. By decreasing membrane fluidity and increasing its packing density, fatty acids, especially those with unsaturated double bonds, can aid in stabilizing the membrane and preventing cell damage (Leekumjorn et al., 2009). Jasmonates, a product of linolenic metabolism and plant hormones, also serve as regulators for stress signalling (He & Ding, 2020). Additionally, fatty acids can also serve as an energy source for the plant during periods of dehydration stress, particularly in seeds and other storage organs. The breakdown of fatty acids through beta-oxidation predominantly in the peroxisome can produce ATP, which can be used to maintain cellular processes and support stress response mechanisms.

Phytohormones

Phytohormones play a critical role in improving the tolerance of plants against abiotic and biotic stress by facilitating the production of antioxidants to reduce the accumulation of ROS. Growth regulators such as salicylic acid, gamma-aminobutyric acid (GABA), and jasmonic acid enhance abiotic stress tolerance through the coordination of different signal transduction pathways to facilitate alteration in plant metabolism (Kohli et al., 2013; Verma et al., 2016).

As clearly stated above, metabolites play a significant role in the development of VDT. Despite an increase in metabolome investigations, there is significantly less information available when compared to transcriptomic studies. Understanding the biochemical processes that allow resurrection plants to endure and recover from severe water deprivation requires the use of metabolic profiling. To further understand the exact functions which various metabolites play in the dehydration and rehydration of resurrection plants, we investigate the resurrection grass *Eragrostis nindensis*, describe its metabolome modifications, and establish links between changes in the transcriptome (Madden, 2019) and proteome (van der Pas, 2023) previously studied in Plant Stress Lab (PSL). Most importantly, we are investigating both the shoot and root systems to understand how the whole plant overcomes desiccation.

### 1.1.5 Metabolic profiling of *Eragrostis nindensis*

*E. nindensis* (shown in Figure 1-2B ) is a desiccation-tolerant grass (Ginbot & Farrant, 2011; Pardo et al., 2020) which grows in clumps and has a short, oblique rhizome. Most of the species of *Eragrostis* are indigenous to most regions of Africa mostly southern Africa and may be found in the Northern Cape, Kalahari Sands, and forested short grassland. Other *Eragrostis* species, such as *E. tef*, are tolerant to drought but vulnerable to desiccation (Kamies et al., 2017), *E. nindensis* is a member of the poikilochlorophyllous group of resurrection plants, as it disassembles its thylakoid membrane upon dehydration and reassembles it upon rehydration. However, mature plant leaves have been shown to display senescence, a phenomenon referred to be the last stage of leaf development that is more commonly linked to age-related cell death, and this has led to the characterization of two different leaf tissues termed senescent tissue (ST) for old leaves which become desiccation sensitive and non-senescent (NST) for young leaves which are desiccation tolerant. Unlike the ST, the NST has been shown to recover full metabolic processes upon rehydration (Ginbot & Farrant, 2011). However, age-related senescence is an ongoing occurrence that is distinct from senescence that is untimely induced by environmental factors like extreme drought, salinity, and pathogen attack. This evidence shows that senescence is both a genetically controlled, age-dependent process as well as a process that is triggered by external factors like drought. According to several authors, plants can optimize resource use by relocating nutrients needed for growth from senescing tissue to developing tissues and organs that are not senescent (Durgud et al., 2018;

Guiboileau et al., 2010; Wingler et al., 2009; Yolcu et al., 2018). Through molecular methods, Madden (2019) identified 22 transcripts which were exclusively accumulated in ST. These transcripts had an overabundance of mitochondria-nucleus signalling and lacked transcripts related to the protection that was observed in NST. The findings of Madden's study show at the molecular level how exogenous factors may contribute to premature senescence, and more validation could be found in the identification of metabolites that are likely to contribute to senescence in plants, and this study seeks to answer this question.

Figure 1-2A demonstrates how the various tissues NST and ST can be phenotypically distinguished during sampling, with NST denoting young leaf and ST denoting old leaf.

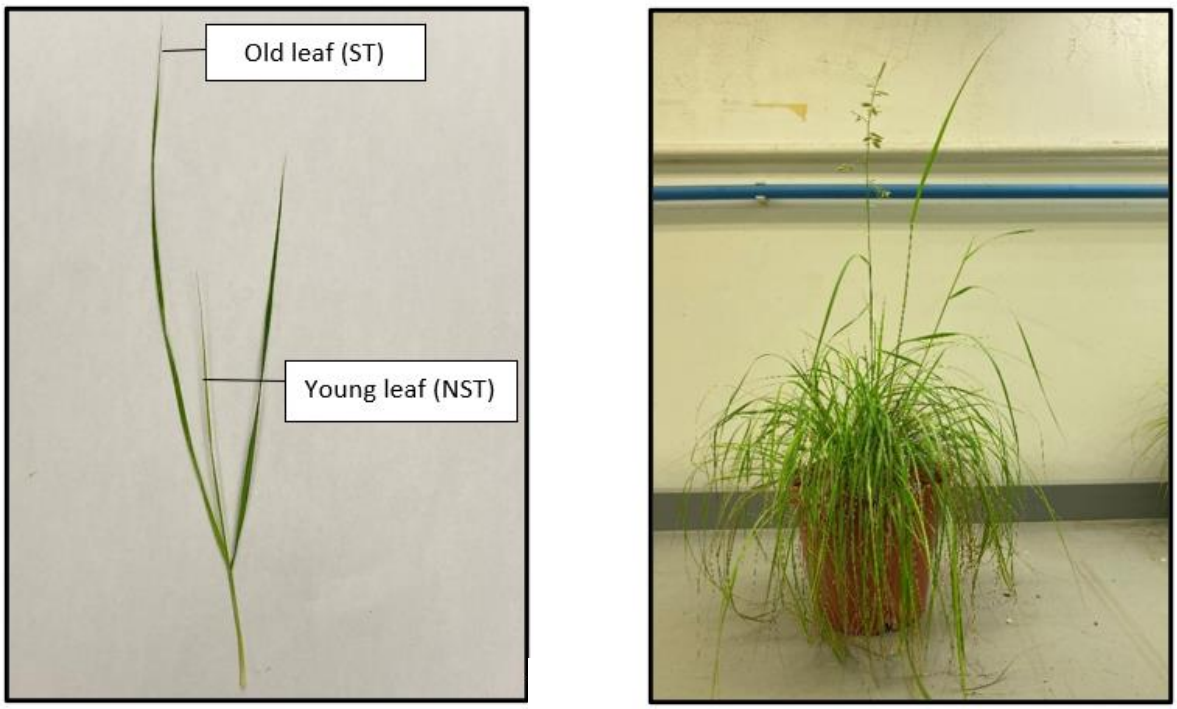



Figure 1-2: *Eragrostis nindensis* tillers and plant: (A) shows the tiller of E. nindensis showing young (non-senescent tissue ) and old desiccation sensitive (senescent tissue) and (B) is a fully hydrated and mature *E. nindensis.*

More research is required to fully comprehend the mechanism of desiccation tolerance and senescence regulation in *E. nindensis* to aid in the production of drought-tolerant crops, especially the orphan crop *E. tef,* which produces a gluten-free grain used in the food industry. *E. nindensis*'s genome was recently published (Pardo et al., 2020)

with unpublished dehydration and rehydration transcriptome (Madden, 2019) and proteomics (van der Pas, 2023) being available to myself. From these studies, the genome revealed a significant shift in gene expression during the different responses to desiccation especially during the late response (45-15% RWC) and rehydration. Abiotic stress response-related transcripts accumulated during dehydration and the downregulated genes were related to photosynthesis. Upon rehydration, the trend was reversed with upregulated genes significantly enriched in gene ontology terms related to photosynthesis (Pardo et al., 2020). Madden's (2019) work on the transcriptome revealed that biological reactions related to desiccation were enriched during the dehydration process, including raffinose metabolism, nitrogen fixation, amino acid transport, and photosynthetic shut-down that revealed regulation of chlorophyll metabolic reactions. Additionally, van Der Pas (2023) who worked on the same plant population used in transcriptomics, supported most of the findings from the transcriptome in the proteomic work by observing the upregulation of proteins enriched in gene ontology terms related to defence responses, protein transport, abscisic acid-activated signalling pathway, and response to biotic stimulus. While most species' omics have replicated similar findings from genome to proteome in several studies, resurrection plants' metabolomics studies have given researchers a clearer understanding of how the plants react to adaptation and defence mechanisms by producing a broad range of metabolites in response to abiotic stimuli (Xu et al., 2021; Yobi et al., 2017). The few studies that have been conducted on root systems have indicated similar responses in both leaves and roots e.g. a study by Norwood et al. (2003) conducted on *C. plantagineum* roots showed no visible signs of damage during dehydration, although it is unclear whether most of its root system can survive desiccation or whether to some extent the root system is important in desiccation. Research conducted on several crops indicates that shoots and roots respond differently to abiotic stress as they also play different roles in plant growth (Cao et al., 2017; Cattivelli et al., 2008). The latter being involved in nutrient and water uptake and the shoot is involved in the photosynthesis process for energy generation. Based on the different functions, we hypothesize a shoot-root relationship that could cause the translocation of some metabolites from the roots to the shoot and vice versa during the dehydration process. Since all plants recovering from desiccation utilise the roots as the primary site of water uptake, the root-shoot relationship is crucial in understanding the response to dehydration and rehydration. Therefore, this study

undoubtedly helps to uncover the essential metabolites involved in *E. nindensis's* desiccation tolerance, both leaves and roots in contrast to previous omics of the same plant done by Madden (2019) on transcriptomics and van Der Pas (2023) on proteomics (unpublished). By using metabolomics, there is optimism for future bioengineering processes and the development of various plant growth stimulants which could aid plants to withstand extreme environmental conditions.

### 1.1.6 Aim of the research

The study's major aim is to characterize the principal metabolites involved in the resurrection plant *E. nindensis's* tolerance to desiccation and to compare the metabolic responses in NST and ST of leaves, and roots.

Based on the findings by Madden (2019) and van Der Pas (2023) at the transcriptome and proteomic level respectively, we anticipate downstream production of metabolites caused by upregulated genes and proteins that are significantly involved in dehydration and rehydration. We demonstrate in the current work how omics analysis provides an enhanced grasp of a biological system's underlying reaction to additional scientific investigation.

The first step was to compare the changes in metabolites during the dehydration and rehydration processes between the desiccated and rehydrated states. Secondly, the study investigated the metabolic changes at various relative water contents: fully hydrated (85%–100%), early dehydrated (75%–65%), late dehydration (40%–55%), airdry (< 5%), and rehydrated time points at 24, 48 hours, and 1-week post-rehydration. Next, focus on the variations in metabolite response in ST and NST leaves during and after dehydration was done and lastly, the metabolic changes between the root and shoot system was investigated to gain a better insight into the whole plant metabolome.

### 1.1.7 Research questions to be answered.

The following questions will be answered in this study.

1. Are metabolites differently abundant among the fully hydrated, dehydrated, desiccated, and rehydrated tissues in *E. nindensis?*
2. Are metabolic responses similar or different in ST vs. NST?

3. What are the desiccation-induced metabolites in leaves and roots and how do these relate to desiccation and survival thereof?

# Chapter 2

## 2.1 Materials and Methods

### 2.1.1 Plant material and growth conditions

*Eragrostis nindensis* seeds were collected from plants grown in the field near Aggeneys, Northern Cape, South Africa, (29°16'41.1"S 19°00'25.4"E) with permission from the land manager Pieter Venter. These were germinated in soil mixture of potting soil, sand and vermiculite in ratios of 2:1:1 and were maintained in a growth room under the following environmental conditions: Day/night Cycle: variable between 16 hrs in light and 8 hrs in dark, light intensity: variable between 250 – 500 µmol $m^{-2}$ $s^{-1}$, temperature: Ambient temperature ranging from 25.0 $^{O}$C and 27.5 $^{O}$C depending on light intensity and relative humidity: 50-60%. The plants were watered twice a week and after 6 months of growth, the plants were ready for sampling. Sixty fully grown plants were pruned by removal of the dried senescent leaves and then acclimated for a further week before experimentation.

### 2.1.2 Experimental treatments and Sampling procedures.

During the sampling, young leaf tissue was regarded as non-senescent tissue (NST), and old leaf tissue as senescent tissue (ST). Fully hydrated samples were taken after the last day of acclimation where all the plants were fully watered in the dusk and sampling was done after 15 hours on the following day. Dehydration was induced by ceasing to water the soil and plants and different dehydration sampling points were estimated based on the physical appearance of leaves during the dehydration process especially the change of colour and folding of the leaves (see Figure 3-2Figure *2-1*). Plant tissue was then sampled at 3 hours after 'dawn' on the light part of the day and this was consistent across all the dehydration sampling points. Six plants were sampled for each treatment: Full Turgor (FT), Early dehydration (ED), late dehydration (LD), airdry (AD), rehydration at 24 hours (R24), 48 hours (R48) and 1 week. At each sampling point, the same plant was sampled for NST, ST, and root tissues. Approximately 20 entire leaves (ST and NST) were collected and flash frozen and several leaf blades per plant were pooled to increase biomass. Three leaves were utilised for relative water content (RWC) determination. For root RWC, 2-3 roots were

used depending on the size of the roots. For metabolite extraction, the harvested tissue was placed in a 15 mL conical centrifuge tube, quickly frozen in liquid nitrogen, and stored at -80°C until further analysis. The frozen tissue was further homogenized to a fine powder using a pestle and a mortar and then dried using SpeedVac **(**Savant SpeedVac SC100**)** operated in a cold room of 4 $^{O}$C before the extraction process. During root sampling, all the excess soil was rubbed off to avoid contamination of the root sample and the roots quickly snap frozen. Figure 2-1 illustrates the appearance of the plants at various stages of drying and recovery with varying plant tissues' appearance at the different sampling points of dehydration and rehydration.

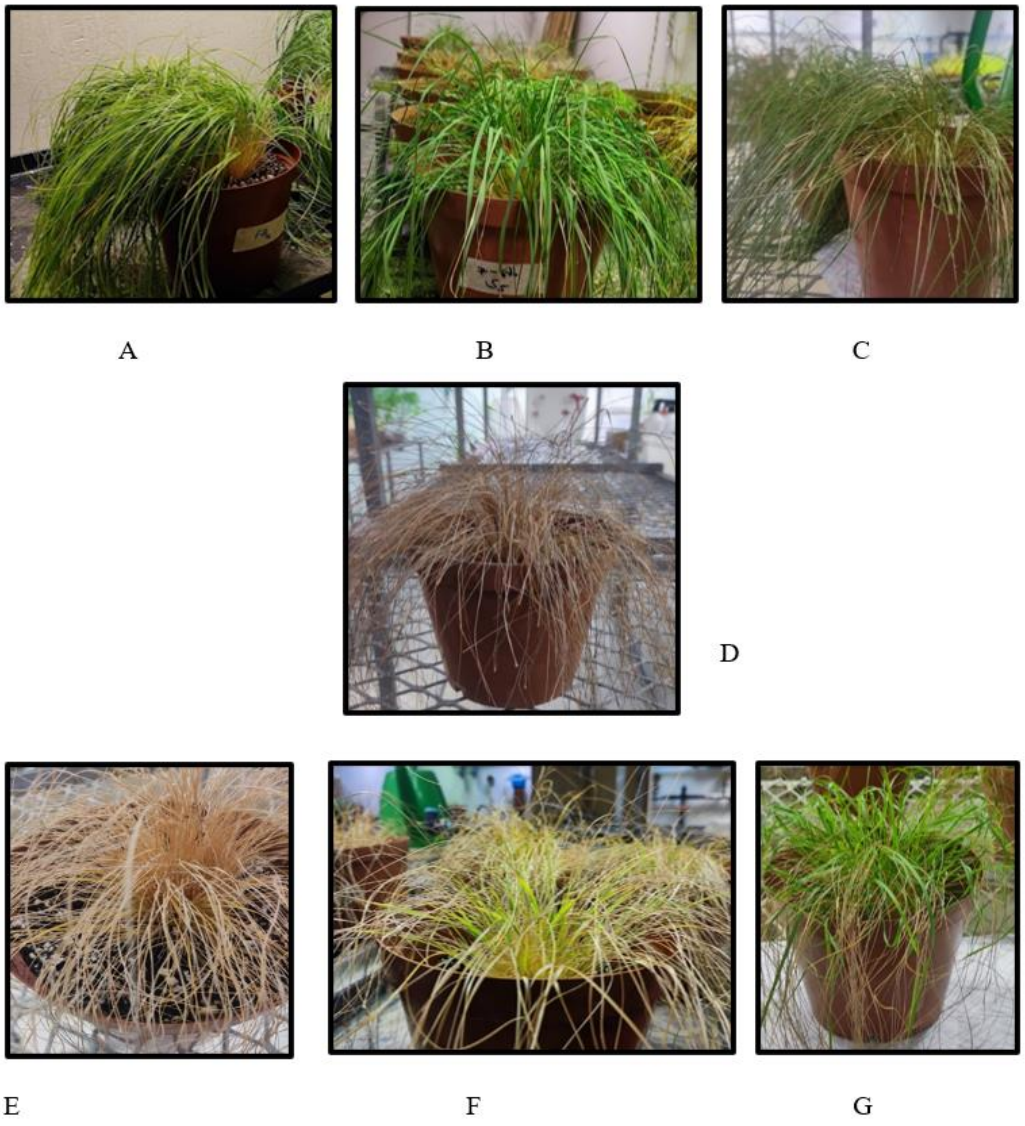



Figure 2-1: The appearance of the plants at various stages of drying and recovery. The same plant was sampled for NST, ST, and root at all sampling points with 6 biological replicates at each point. Approximately 20 leaves of each tissue type were collected, and 3 leaves were utilised for RWC. For root RWC, 2-3 roots were used depending on the size of the roots. Tissue appearance is shown before

and during dehydration: fully hydrated (A), early dehydration (B), late dehydration (C), air-dry (D), and rehydration 24 hours (E), 48hours (F), 1 week (G).

### 2.1.3 Sampling and Relative Water Content Determination

The relative water content (RWC) of the desiccated tissues was determined by using the gravimetric method outlined by Barr and Weatherley (1962) by using the following equation:

RWC (%) = Absolute water content final/ Absolute water content at full turgor x 100

### 2.1.4 Extraction and derivatisation

Extraction and derivatization were conducted according to Lisec et al. (2006) and Valledor et al. (2014) with minor modifications on the ratio of methanol to water. Ground samples were freeze-dried in SpeedVac and placed in a 4 $^{O}$C room until no more loss in weight acted as an indicator of completely dried samples. To preserve the integrity of the samples, 10 mg of finely ground leaf and root tissue were placed in 1.5 mL screw-capped tubes and placed in liquid nitrogen. Methanol-water extraction solvent was used and ribitol was added as an internal quantitative standard. Pre-cooled 1 ml of 50% methanol-water extraction solvent was added. The samples were vortexed for 30 seconds and then agitated on an orbital shaker for 45 minutes at 4 $^{O}$C and 1000 rpm. The samples were placed in a sonication bath for 15 mins and thereafter centrifuged for 15 minutes at 12000 rpm using a benchtop centrifuge. Aliquots of 100 µL were prepared from all samples and transferred to a fresh 1.5 mL tube. Samples were freeze-dried for 30 minutes before derivatization. Derivatization was performed on samples by the addition of Methoxyamination reagent and N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) to render them volatile for Gas chromatography-mass spectrophotometry (GC-MS) analysis. While MSTFA was prepared using the retention index standard mixture as stated by Lisec et al. (2006), the derivatization process started by adding 40 µL of methoxyamination reagent to all of the aliquots and samples were then shaken on an orbit shaker for 2 hours at 37 $^{O}$C and 1000 rpm. The aliquots were spun for 30 seconds before the addition of MSTFA. The derivation process was then completed by adding 70 µL of pre-warmed MSTFA to the aliquots and samples shaken for 30 minutes at 37 $^{O}$C and 1000 rpm.

### 2.1.5 Sample processing for GC-MS analysis

The derivatized samples were centrifuged for 2 mins and all aliquots were transferred to 10 µl glass vials suitable for GC-MS analysis. A library of standards used for analysis was previously prepared by Shandry Tsebele, a PhD student in the Plant Stress Lab (PSL), in the same manner using a mixture of sugars, organic acids, and amino acids. Amino acids (Alanine, Valine, L-tyrosine, L-histidine, Tryptophan, L-cystine, L-arginine, glycine, proline, serine, threonine, aspartic acid, methionine, L-phenylalanine, Isoleucine, Leucine, Glutamic acid, norleucine, and asparagine); sugar alcohols (mannitol, sorbitol, glycerol, erythritol, threitol, xylitol, ribitol, arabitol, allo-inositol, myo-inositol, lactitol, maltitol, and galactinol); sugars (Ribose, xylulose, fucose, allose, fructose, galactose, sucrose, glucose, raffinose, trehalose, maltose, cellobiose, mannose, sorbose, altrose, isomaltose, melibiose, and gentibiose); fatty acids (decanoic acid, stearic acid, and palmitic acid). All the peaks that spliced each other were identified based on the standards M/Z values and retention times to reduce ambiguity in the data set obtained from GC-MS for further analysis.

### 2.1.6 GC-MS/MS metabolite profiling of Sugars, amino acids, Sugar alcohols, Organic acids, and phytohormones

The derivatized samples were analysed on an Agilent Model 7890A gas chromatography system equipped with 7693 autosamplers and joined with a 7000C Triple Quadrupole mass spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). Using the autosampler setup in split-less mode, samples of 1 µL were injected at a pressure of 12.2 psi, a temperature of 240 $^{O}$C for the injection port, and a helium carrier gas flow rate of 1 ml per minute. The initial oven temperature was 80°C for 1 minute, followed by an 8°C per minute increase to 320°C, and the temperature was maintained for 1.5 minutes. The split ratio was changed to 1:29 (30 times dilution). The samples' analytes were separated using the Agilent column J&W 122-5532G DB-5ms+DG, which has an internal diameter of 0.25 mm and a length of 30 mm. The DB-5ms+DG is a low polarity GC column fitted with a non-polar phenyl arylene stationary phase satisfactory for acquisitive signal-to-noise ratio with better sensitivity and mass spectral integrity of analytes (Agilent, Germany). The real-time for the runs on the GC-MS system was preceded by a solvent delay of 6 minutes, making the chromatographic duration 35.5 minutes. The mass spectrophotometer was tuned for

an ion source of 230 °C  and an Electron-impact (EI) mode of 70 eV, documented with a mass range of 70 to m/z 500 at 20 scans per second and a gain factor of 1.

### 2.1.7 Peak identification

Using acquired spectrum data and the internal standard ribitol, the MassHunter B.05 GC MS/MS software (Agilent, Germany) was used to identify and quantify metabolites from the spectrum. The spectral data were deconvoluted to identify all the metabolites in the samples. The identification and quantification of specific metabolites were based on the NIST08 MS library (National Institute of Standards and Technology, Gaithersburg, MD, USA) and comparing retention indices based on the literature from previous authors (Babushok et al., 2007; Luedemann et al., 2008; Theofel et al., 2008). The sample that accumulated the majority of the metabolites was used to establish a master peak list, and the remaining samples were quickly aligned by analysing the batch to the peak list formed. This created an automatic peak alignment for all of the metabolites present in the different samples. The output was generated in Excel format, including columns for the various samples and rows for the detected metabolites. The raw data from the MassHunter B.05 GC/MS software contained the retention time, match factor and signal-to-noise ratio (S/N), and peak area for all the analytes in the samples. The analytes were quantified based on the comparison of their peak area with that of internal standard ribitol during the normalization process.

### 2.1.8 Data pre-processing

Raw data generated from the Mass Hunter B.05 GC/MS software was exported in Excel format. The dataset was interrogated, and all the contaminants were removed based on the blank sample comparison, and metabolites with incorrect ionization values were removed based on the retention times indicated by the standards. All metabolites with high accumulation in the blank, match factor less than 70%, and S/N less than 10 were eliminated from the final data to reduce ambiguity in the data. A total of 99 metabolites were identified based on the set parameter of which 7 of them were not annotated well and had no library match, these were termed as “unknown metabolites”. The polished raw data was then further pre-processed by normalization with internal standard ribitol and sample dry weight to reduce the bias in samples (Chen et al., 2014). The resulting data matrix was exported as comma separated values (CSV) file and uploaded to MetaboAnalyst 5.0 and R for statistical analysis.

### 2.1.9 Statistical analysis

#### 2.1.9.1 Normalisation and Principal Component Analysis

MetaboAnalyst version 5 (https://www.metaboanalyst.ca/ )(Pang et al., 2021) was used for comparative analysis using One-way Analysis of Variance (ANOVA) Fisher's LSD post hoc analysis method with a p-value of 0.05. Data statistical filters were applied based on the relative standard deviations and the suggested threshold was 30%, followed by further normalization using log transformation (base 10) that applies a mathematical transformation to individual samples to adjust systematic differences among samples, "mean-centered and divided by the square root of the standard deviation of each variable" denoted as Pareto scaling was applied to adjust each metabolite in the sample by scaling factor computed based on the dispersion of the variable. To test sample variability, principal component analysis (PCA) results were generated in a score plot displaying a 95% confidence region to distinguish variance in the different treatments. For datasets that did not show clear clustering, partial least squares discriminant analysis (PLS-DA) was performed, and successful clustering of the different sample treatments was achieved. To visualize the separations with the relative concentrations of the corresponding metabolites in each sample, the top 15 features were shown on a variable importance projection (VIP) scores plot calculated based on a weighted sum of absolute regression.

#### 2.1.9.2 Identification of Significant Metabolites

ANOVA Fishers's LSD post-hoc analysis method with $p < 0.05$ was performed on PCA clustered samples and metabolites identified with a False Discovery rate (FDR) of $q$ values < 0.05 were listed as significant. Heatmap hierarchical clusters based on a difference in the significant metabolites across drying and rehydration were performed to further validate the PCA clusters and ANOVA results (Zhao et al., 2014). The heatmap was generated from the normalized data and hierarchical clusters represented using Euclidean distance and all treatments were shown in groups.

#### 2.1.9.3 Fold change Analysis

The fold change analysis was used on targeted metabolites to compare the log2 transformed ratio between each metabolite in each group (Xia et al., 2009). Fold change analysis was performed to identify differentially abundant metabolites relative to the fully hydrated samples across the three tissues. Differentially abundant

metabolites were identified, and a Venn diagram was generated to show which specific metabolites were shared across the different groups. Additionally, the different metabolites that are uniquely differentially abundant in specific groups were identified.

2.1.9.4 Pathway analysis and KEGG

Metabolic profiling requires tools for the interpretation of metabolic data to clearly explain the functions of different metabolites. To interpret the results generated from fold change analysis and ANOVA, pathway analysis within MetaboAnalyst 5.0 was used for the visualization and functional interpretation of differentially abundant metabolites. Only well-annotated compounds based on pathway libraries were mapped, and statistically significant pathways were generated on a pathway impact graph and table Format.

Mapping was done with the KEGG database for *Arabidopsis thaliana* to find the significant pathways of the upregulated metabolites and associations between pathways to represent pathways shared across the groups. The metabolome view shows all matched pathways generated indicating the P-value calculated from enrichment analysis, FDR adjusted using FDR < 0.05, pathway impact value calculated from topology analysis, and the number of Hits from the uploaded data. A threshold of $p < 0.05$ was used, and significant pathways were generated based on ruling out all the non-significant ones (Wieder et al., 2021).

# Chapter 3 : Comparison of desiccation tolerance in non-senescent and senescent tissue

## 3.1 Results and Interpretation

### 3.1.1 Morphological and Relative Water Content analysis comparing non-senescent and senescent leaf tissues.

The RWC of NST and ST tissue was measured during dehydration and rehydration (Figure 3-1), with full turgor (> 85% RWC), early dehydration (ED) (75-60% RWC), late dehydration (LD) (55-40% RWC), air-dry (AD) (<5%) and rehydration at 24 hours, 48 hours and 1 week. These RWC ranges were selected based on defined RWCs that have been shown in previous studies on resurrection plants to be relevant to the dehydration process (Farrant et al., 2015; Farrant & Hilhorst, 2022; Gabier et al., 2021). This defined pattern correlates with specific changes in metabolic activity in the plant and it is believed that most resurrection plants exhibit this pattern of drying (Gabier et al., 2021). In the current study, all the studied plants showed an early response to dehydration at 75-60% RWC, before a drop to late response (55-40% RWC). The average time for late response to desiccated state was between 3-4 days. By day 16, the plants were < 5% RWC. Previous work on transcriptomics of *E. nindensis* indicated that the airdry state is reached by day 14 (Madden, 2019). The same rate of dehydration was seen in both the ST and NST, demonstrating that senescence is not decided by water loss but instead controlled while drying. On rehydration, NST showed total recovery accumulating over 70% RWC 1 week post-rehydration whereas most of the ST did not rehydrate (shown also in Figure 3-2) and the few ST that recovered showed a relatively low RWC of 20%, 1-week post-rehydration. This presumably justifies that ST cannot revive back to full metabolic response upon rehydration and similar observations have been reported in other resurrection plants that exhibit senescence *X. schlechteri* and *S. stapfianus* (T. Martinelli et al., 2007; Radermacher et al., 2019)

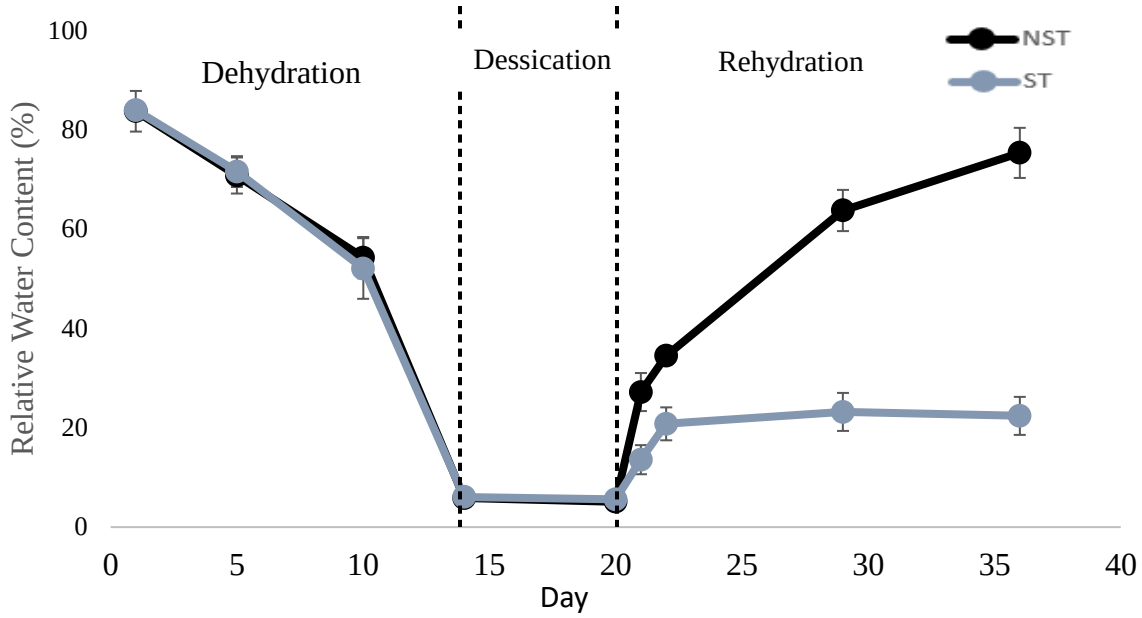



Figure 3-1: Relative water content of leaves of *E. nindensis* used for sampling. Five biological replicates were used for the experiments and error bars represent the standard errors across the biological replicates. RWC of NST and ST are shown in black and grey lines respectively.

### 3.1.2 Leaf morphology analysis in the regulation of photosynthesis

The morphology of the leaf tissues, as depicted in Figure 3-2, demonstrated the adaptation and regulation of photosynthesis. Both NST and ST experienced morphological changes in their leaves due to dehydration. Both leaf tissues appeared uncurled, green, and straight when they were at full turgor. While the NST demonstrated a coordinated pattern of rolling with some areas of the leaf left unrolled throughout the dehydration process, the ST displayed a disorganized pattern of rolling during early dehydration (see 75-60%, Figure 3-2). Upon reaching the airdry state, differentiation between the two cannot be made. However, upon rehydration only NST resumed accumulation of photosynthetic pigments at 48 hours, characterised by unrolled green leaves that survived desiccation whereas the ST leaves remained rolled and the colour remained unchanged post-rehydration (Figure 3-2). Unlike other resurrection plants, which can be distinguished between ST and NST tissue by leaf colour or leaf apex senescence (Radermacher et al., 2019), *E. nindensis* exhibits a differential drying pattern of NST and ST (Figure 3-2), and senescence mainly becomes apparent on rehydration.

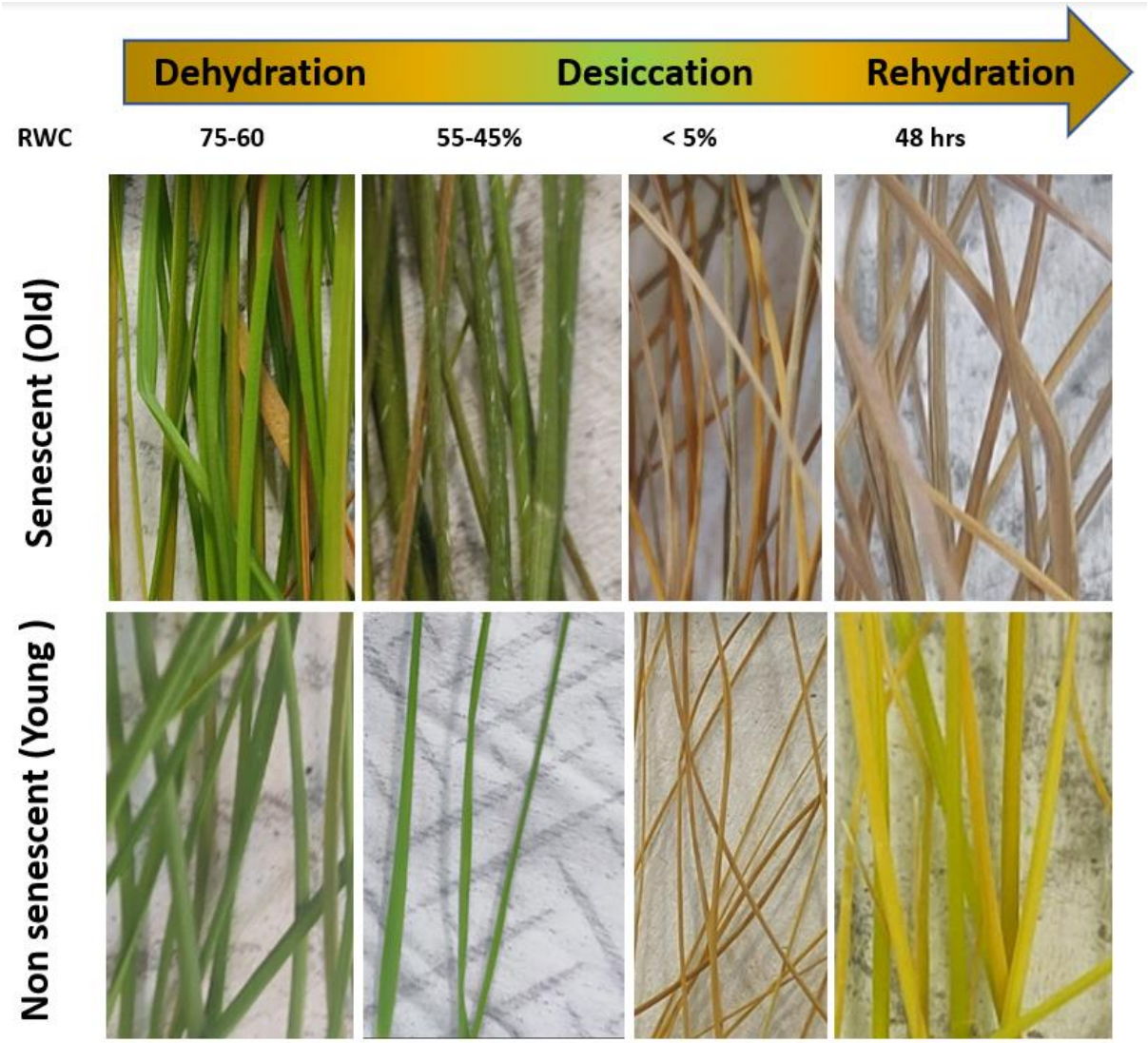



Figure 3-2: Leaf morphology of *E. nindensis*: Photos of ST (top panel) and NST (bottom panel) showing leaf morphology of E. nindensis during dehydration and rehydration.

### 3.1.3 Metabolite alteration during dehydration and rehydration in non-senescent and senescent leaf tissue

In leaves exposed to dehydration and rehydration, alterations in the accumulation of primary metabolites were examined. A total of 99 metabolites were identified of which 83 were differentially abundant ($P< 0.05$) between NST and ST, consisting of sugars, sugar alcohols, organic acids, amino acids, phytohormones, fatty acids and other metabolites (Appendix, Table S5-1). Additionally, individual tissues showed differences in the number of differentially abundant metabolites across the dehydration points. A total of 27 and 17 metabolites were differentially abundant in NST and ST respectively, with 9 metabolites shared by the two tissues and 18 identified exclusively in NST as depicted in Figure 3-3. More so, significant metabolites were exclusive to

NST including raffinose, myo-inositol, citric acid, ascorbic acid, glycine, quinic acid and erythritol that have been reported in desiccation-tolerant plants (Radermacher et al., 2019). Sucrose, phosphoric acid, and pyruvic acid were shared between both tissues. Upon rehydration, a total of 63 and 1 metabolites were significant in NST and ST respectively, with 1 metabolite shared by the two tissues and 62 identified exclusively in NST as depicted in Figure 3-4.

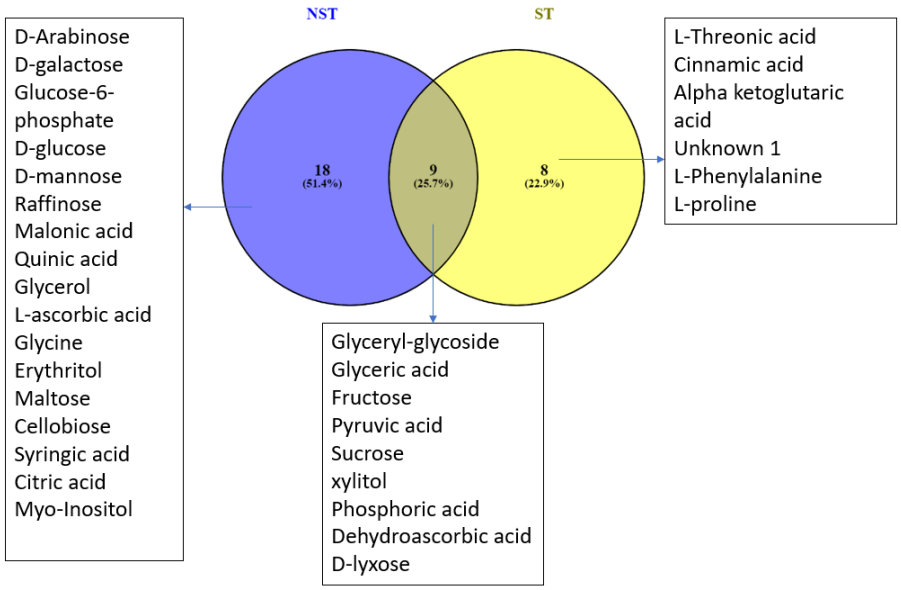



Figure 3-3: Total number of significant metabolites during dehydration in NST compared to ST. Colours indicate different tissue (blue = metabolites exclusive to NST, Yellow = metabolite exclusive to ST; green = metabolite shared between both NST and ST).

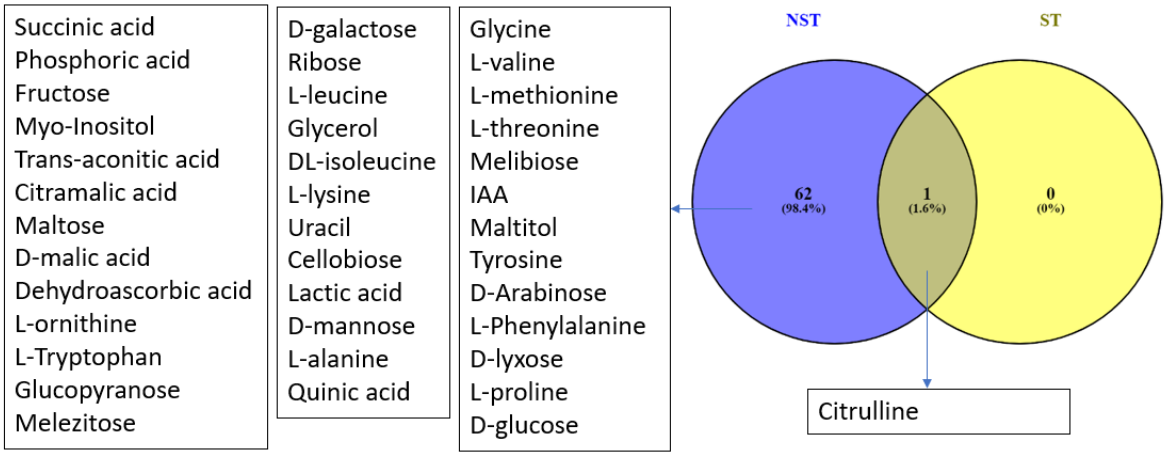
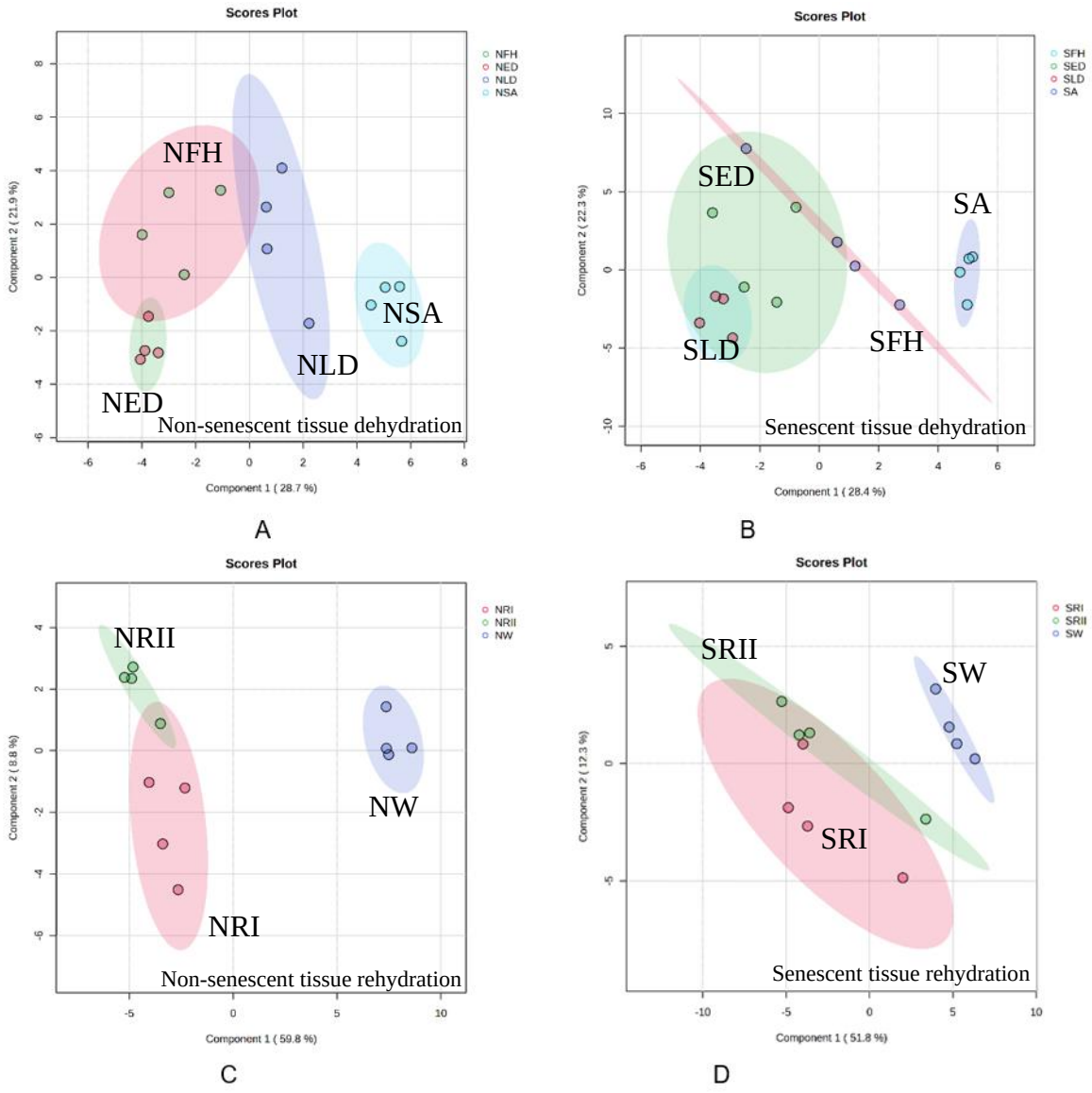



Figure 3-4: Total number of significant metabolites during rehydration in NST compared to ST. Colours indicate different tissue (blue = metabolites exclusive to NST, Yellow = metabolite exclusive to ST; green = metabolite shared between both NST and ST)

### 3.1.4 Variation of metabolites across dehydration in NST and ST

Partial Least Squares discriminant analysis (PLS-DA), a supervised analysis of a logarithm of principal component analysis (PCA) was performed to visualise the difference in the separation of metabolites across individual tissue types and RWC groups. The score plots showed clear separations clustered differently for the different tissue types and RWCs during dehydration and rehydration. For NST, the PLS-DA score plot (Figure 3-5A) showed a clear separation explained by a 50.6% variation with component 1 (28.7%) and component 2 (21.9%) during the dehydration. ST tissue showed a similar separation with a 50.7% variation (Figure 3-5B) across the dehydration stages. Variations in metabolites at the different time points were also evident upon rehydration. In NST, PLS-DA score plots explained 68.6% of the total variation (Figure 3-5C) in which the first and second PCs separately contributed 59.8% and 8.8%, respectively and 64.1% variation (Figure 3-5D) in ST. Based on the PCA results, VIP score plots (Figures 3.6-3.7) indicate a difference in the differentially abundant metabolites across the different stages of dehydration and rehydration time points.




NFH - Non-senescent fully hydrated (> 85% RWC) SFH - senescent tissue fully hydrated (> 85% RWC)

NED - Non-senescent early dehydration (75-60% RWC) SED - senescent tissue early dehydration 75-60% RWC)

NLD - Non-senescent late dehydration (55-40% RWC) SLD - senescent tissue late dehydration (55-40% RWC)

NSA - Non-senescent air-dry (<5% RWC) SA - senescent tissue air-dry (<5% RWC)

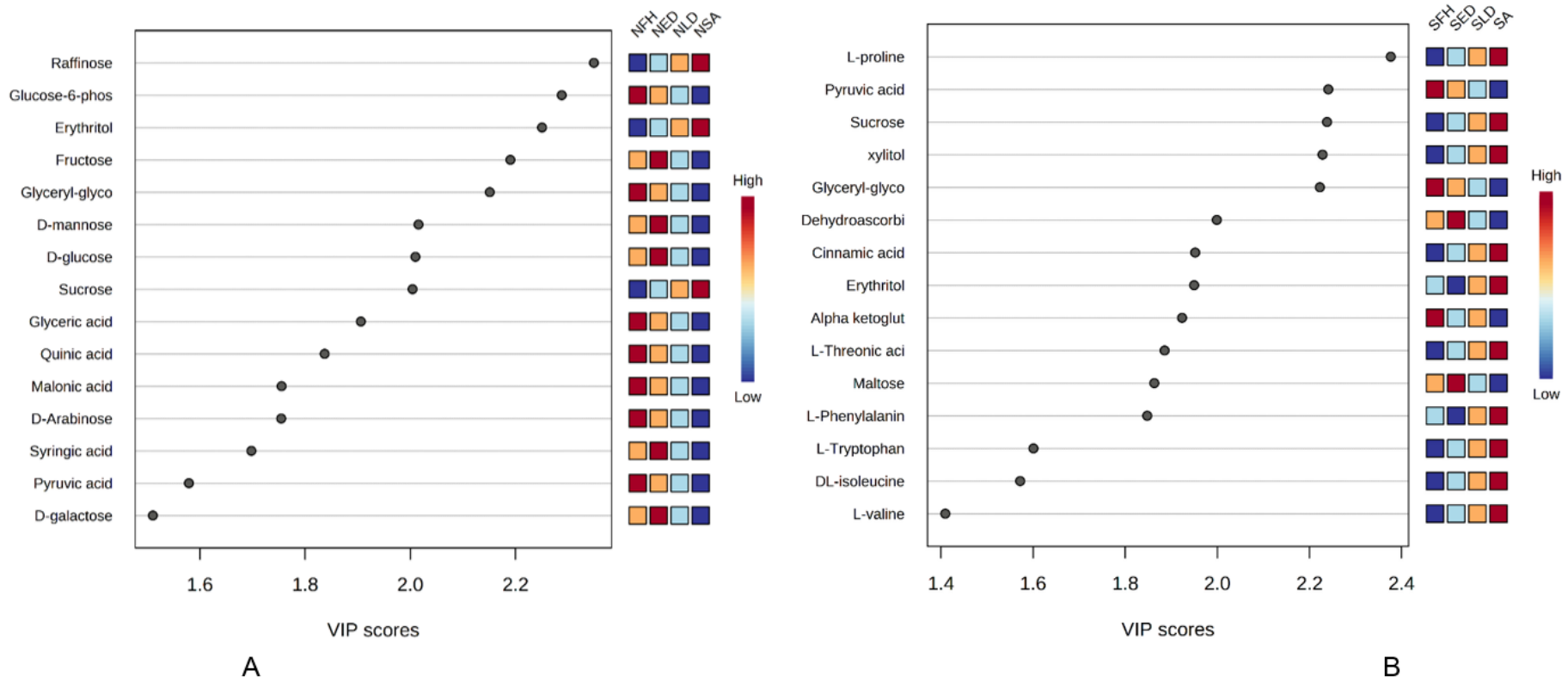
NRI - Non-senescent tissue 24 hours post rehydration SRI - senescent tissue 24 hours post rehydration

NRII - Non-senescent tissue 48 hours post rehydration SRII - senescent tissue 48 hours post rehydration

NW - Non-senescent tissue 1-week post rehydration SW - senescent tissue 1-week post rehydration

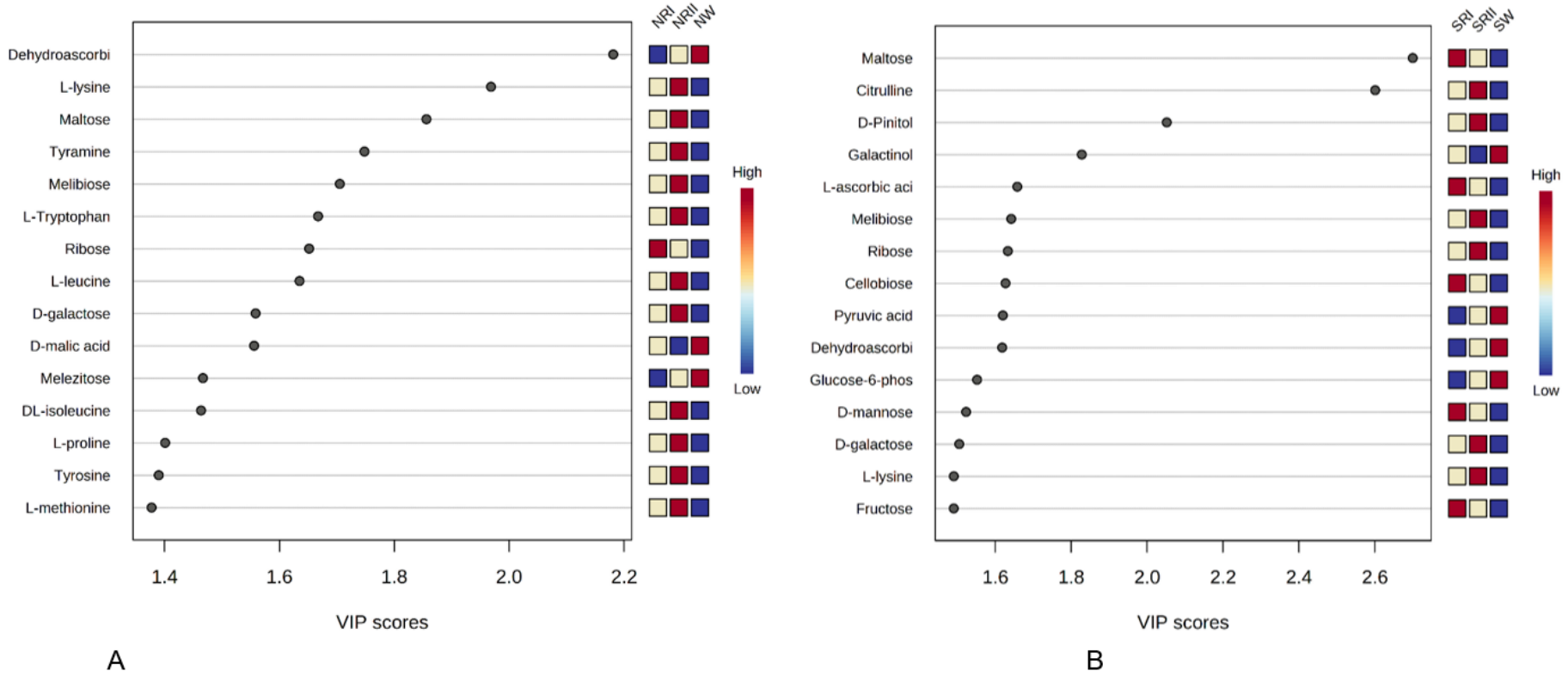
Figure 3-5: Score plots of *E. nindensis* dehydration and rehydration stages across the NST and ST based on principal component analysis. score plot A shows the four dehydration phases in the NST, Score plot B shows the dehydration stages of ST, score plot C shows the rehydration time points in the NST and score plot D shows ST rehydration time points. samples were classified based on RWCs, which are depicted in various colours.

The metabolites that affected the separation varied between the various tissue types. By using a VIP score plot (Figure 3-6), it was possible to see how the relative abundance of metabolites varied between the various tissue types and RWCs as previously shown by PCA (Figure 3.5) Raffinose had the largest abundance difference in the NST during the dehydration, accumulating at the dry stage (Figure 3-6A) unlike the ST which accumulated L-proline (Figure 3-6B) Differences in metabolites upon rehydration were also evident in PCA plots (Figure 3-5). Different metabolites varied by tissue type, with the NST's separation being affected by an accumulation of amino acids at 48 hours post-rehydration (Figure 3-7A) and the 24 and 48 rehydration periods for ST, the accumulation of more than 50% sugars had a significant impact on the separation (Figure 3-7B)




| | | | |
|---|---|---|---|
| NFH - Non-senescent fully hydrated | (> 85% RWC) | NED - Non-senescent early dehydration | (75-60% RWC) |
| SFH - Senescent fully hydrated | (> 85% RWC) | SED - Senescent early dehydration | (75-60% RWC) |
| NSA - Non-senescent air-dry | (<5% RWC) | NLD - Non-senescent early dehydration | (55-40% RWC) |
| SA - Senescent air-dry | (<5% RWC) | SLD - Senescent early dehydration | (55-40% RWC) |

Figure 3-6: Variable importance in projection (VIP) plots for *E. nindensis*: VIP score plot showing differences in metabolites across dehydration stages in non-senescent tissue (A) and senescent tissue (B) of E. nindensis. PLS-DA was used to distinguish between different metabolites across the different RWC groups. The respective metabolites' relative abundances (peak intensities) are shown in the coloured boxes on the right.



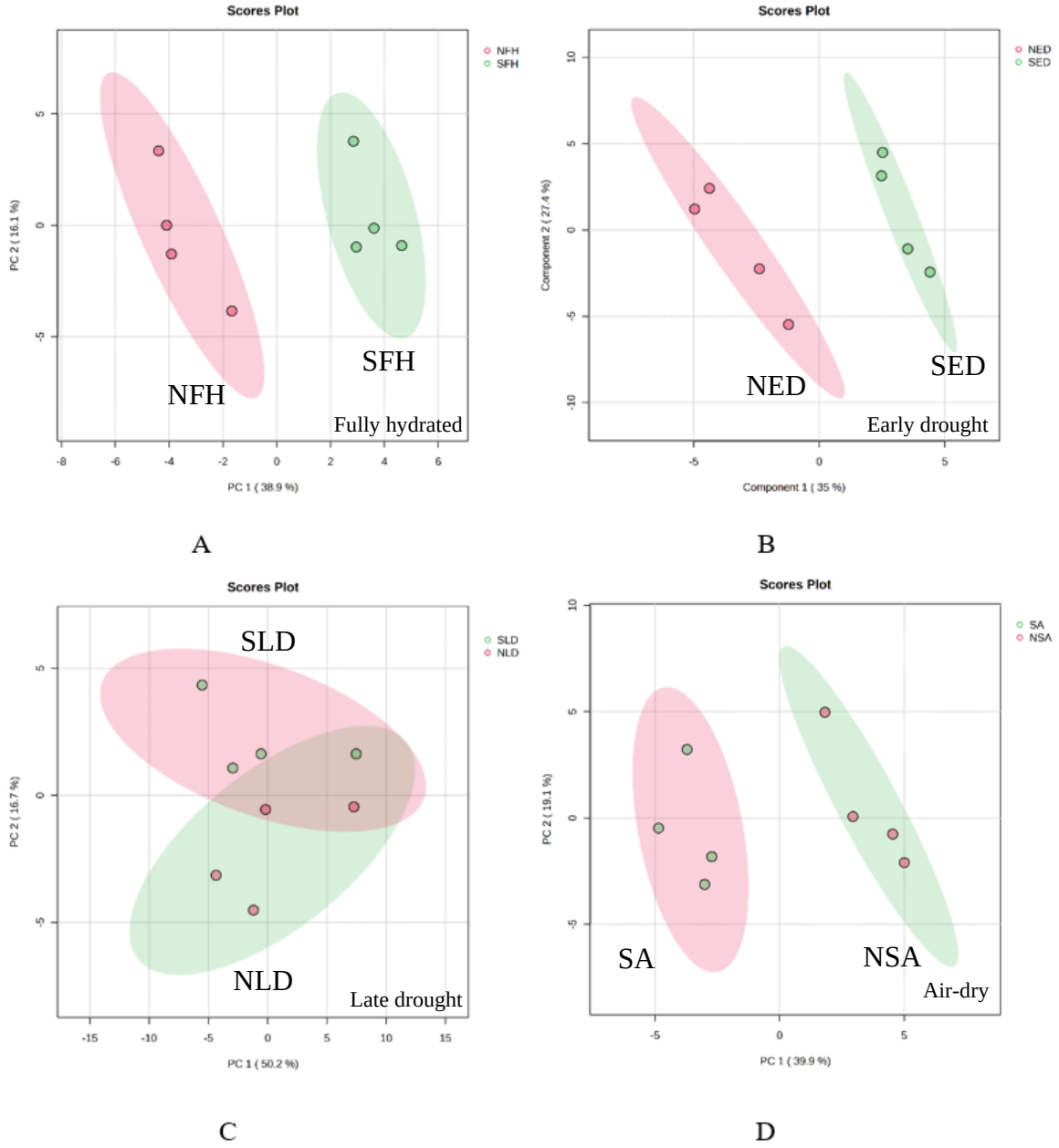

NRI - Non-senescent tissue 24 hours post-rehydration
NRII - Non-senescent tissue 48 hours post-rehydration
NW - Non-senescent tissue 1-week post-rehydration

SRI - Senescent tissue 24 hours post-rehydration
SRII -Senescent tissue 48 hours post-rehydration
SW - Senescent tissue 1-week post-rehydration.

Figure 3-7: Variable importance in projection (VIP) plots for *E. nindensis*: VIP score plot showing differences in metabolites upon rehydration in non-senescent tissue (A) and senescent tissue (B) of E. nindensis. PLS-DA was used to distinguish between different metabolites across the different RWC groups. The respective metabolites' relative abundances (peak intensities) are shown in the coloured boxes on the right.

### 3.1.5 Alteration in metabolite abundance during dehydration and rehydration between ST and NST

Independent differences across both tissues were observed, as previously depicted in Figure 3-5. To better understand the difference between NST and ST, further analysis was performed to investigate and identify metabolites that were differentially abundant based on the comparison of the two tissue types. Figure 3-8 illustrates the changes across dehydration that were determined by PCA at each relative water content group. Throughout dehydration, distinct separations with variances above 50% were seen, which either indicated an increase or reduction in metabolite abundance at each dehydration point. In contrast to early dehydrated tissue, which displayed 62.4% variation with PC1 (35.0%) and PC2 (27.4%) (Figure 3-8B), the PCA score plot at fully hydrated (Figure 3-8A) revealed clear separation clustered differently and explained by 55% variation with PC1 (38.9%) and PC2 (16.1%). Finally, the separation at the dry stage was described by 59% variance (Figure 3-8D) with PC1 (39.9%) and PC2 (19.1%) and late dehydration showing 66.9% variation explained by PC1 (50.2) and PC2 (16.7%) (Figure 3-8C). According to observations, PC1 accounted for the greatest difference amongst tissue types. Heatmap hierarchical clusters were utilized to demonstrate the abundance of the significant metabolites across the dehydration stages and rehydration time points in E. nindensis leaf tissue for both NST and ST to support PCA results. On identification of significant metabolites, ANOVA Fisher's LSD with $P< 0.05$ was used for comparison of two tissues. Out of 39 significant metabolites identified during dehydration, organic acids make up most metabolites (12) (Figure 3-11), sugar (11) (Figure 3-9B), sugar alcohol (7) (Figure 3-9B), amino acids (4), fatty acid (1) and other metabolites (4). More so, 54 significant metabolites were identified upon rehydration, with amino acids as the most abundant (17), followed by organic acids (14), sugar (11) (Figure 3-10B), sugar alcohol (8) (Figure 3-10A), phytohormones (2), fatty acids (1), and other metabolites (1).

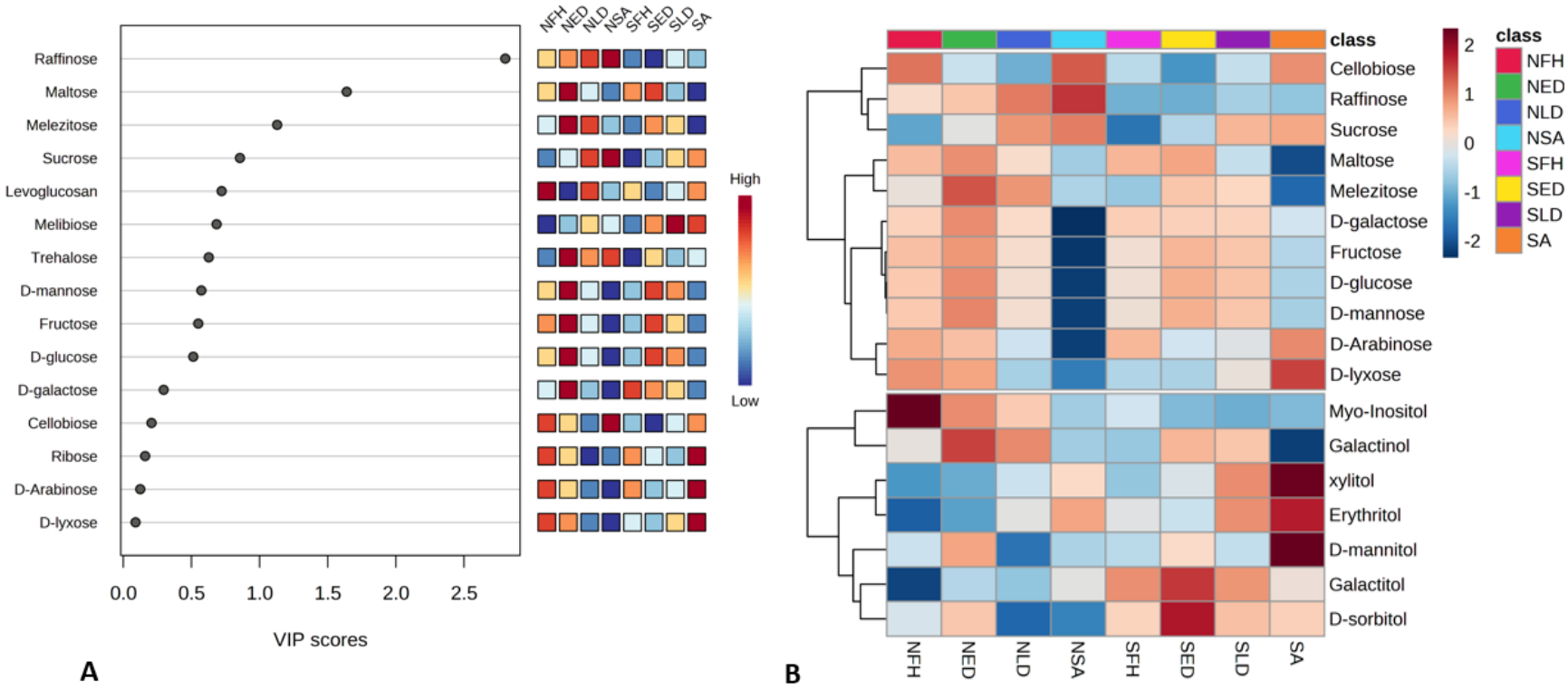



| | | | |
|---|---|---|---|
| NFH - Non-senescent fully hydrated | (> 85% RWC) | NED - Non-senescent early dehydration | (75-60% RWC) |
| SFH - Senescent fully hydrated | (> 85% RWC) | SED - Senescent early dehydration | (75-60% RWC) |
| NSA - Non-senescent air-dry | (<5% RWC) | NLD - Non-senescent early dehydration | (55-40% RWC) |
| SA - Senescent air-dry | (<5% RWC) | SLD - Senescent early dehydration | (55-40% RWC) |

Figure 3-8: Score plots of *E. nindensis* dehydration stages comparing the differences in NST and ST based on principal component analysis. (A) separation between fully hydrated ST and NST, (B) separation between early dehydrated ST and NST, (C) separation between late dehydrated ST and NST and (D) separation between air dry ST and NST. Samples were classified based on RWCs, which are depicted in various colours.

### 3.1.6 Metabolic changes in sugars, sugar alcohols, fatty acids and phytohormones during dehydration

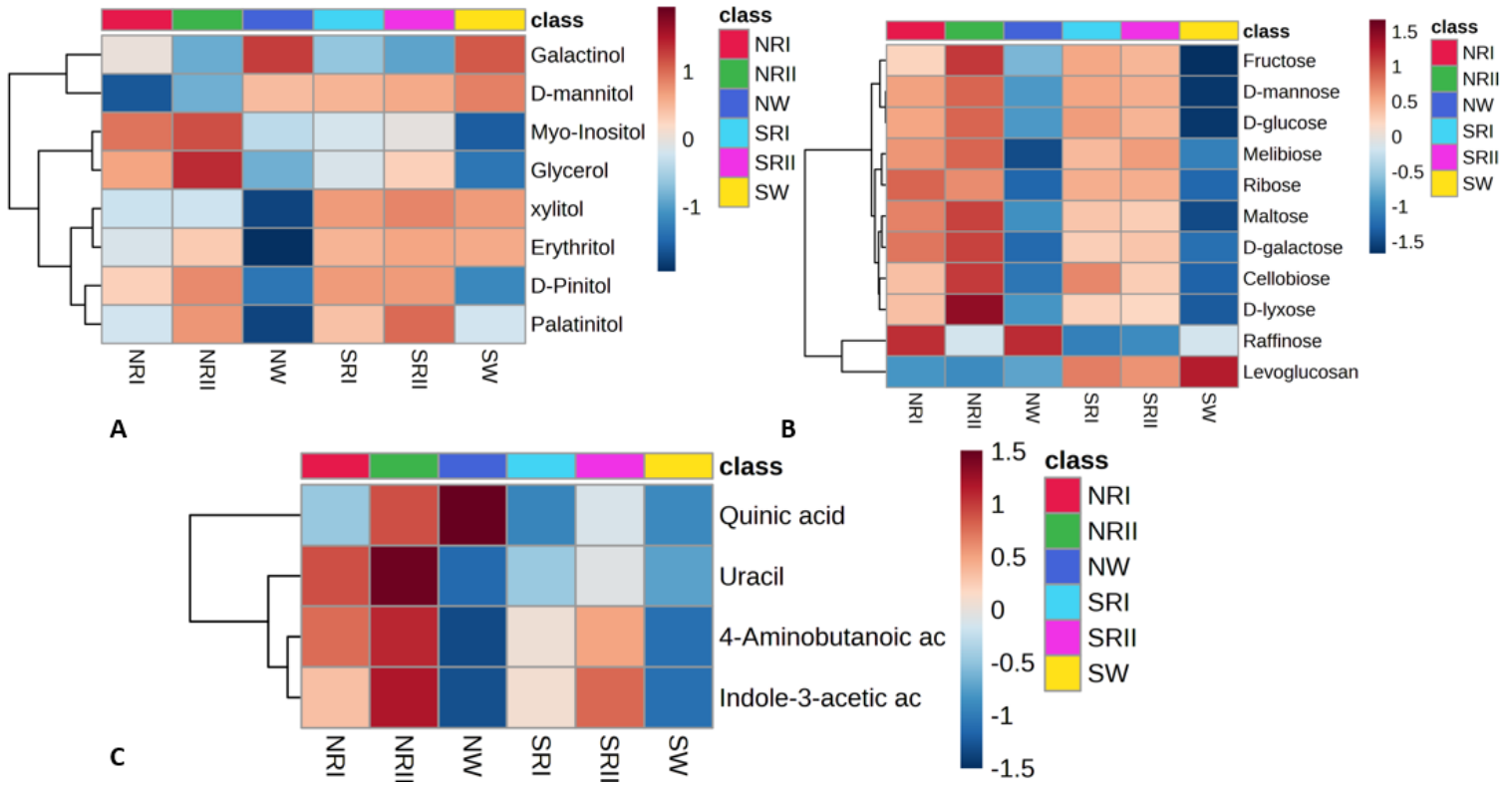
The accumulation and subsequent decrease of sugars during the early stages of dehydration and the transition to the desiccated state suggested the likelihood of different metabolic processes employed by individual tissues during desiccation tolerance, as previously hypothesized by van der Pas, (2023) and Madden (2019). The metabolic shifts associated with dehydration and rehydration are illustrated in Figure 3-9 and Figure 3-10 respectively. A differential abundance of 11 sugars was observed during the different dehydration stages as well as 7 sugar alcohols. Figure 3-9B reveals a notable accumulation of the sugars raffinose, and sucrose in the desiccated state in NST, unlike ST which accumulated sugar alcohols erythritol, D-mannitol and xylitol, accompanied by lesser quantities of other sugars. However, post-rehydration, raffinose levels remained high at 24 hours and decreased at 48 hours with increasing levels of glucose, fructose, maltose, cellobiose and galactose at 24 hours peaking up at 48 hours (Figure 3-10B). The metabolic shift was significant in NST unlike the counterpart ST which showed low levels of sugars post-rehydration (Figure 3-10B). The abundance of raffinose exhibited the most significant levels, with higher levels observed in NST at a dry state (5% RWC) compared to other tissue types and water contents, and levoglucosan displayed similarly high levels in ST, 1-week post-rehydration. Further analysis of various sugars and sugar alcohols revealed an increase in the levels of D-glucose, D-galactose, fructose, arabinose, D-lyxose, myo-inositol and galactinol during early dehydration, and later declined with low levels observed at dry state. The decline was significantly observed in NST than in ST as seen in Figure 3-9B. Differential accumulation of sugars, including D-lyxose and arabinose in the dry state in ST as well as raffinose and sucrose in NST (Figure 3-9A), was observed, mirroring the accumulation patterns of amino acids (Figure 3-13) to differentiate the metabolic processes in NST and ST. Notably, there was a significant decrease in the precursor metabolite myo-inositol for raffinose synthesis in NST at the airdry state as depicted in Figure 3-10B, and an increase in myo-inositol and glycerol in NST post-rehydration (Figure 3-10A). Fatty acids and phytohormones were also significant post-rehydration, Indole-3-acetic acid (IAA) and 4-aminobutanoic acid (GABA) accumulated post-rehydration and peaked at 48 hours (Figure 3-10C). Quinic acid levels decreased at 24 hours post-rehydration, then increased at 48 hours and peaked at 1 week post-rehydration (Figure 3-10C).




NFH - Non-senescent fully hydrated (> 85% RWC)
SFH - Senescent fully hydrated (> 85% RWC)
NSA - Non-senescent air-dry (<5% RWC)
SA - Senescent air-dry (<5% RWC)

NED - Non-senescent early dehydration (75-60% RWC)
SED - Senescent early dehydration (75-60% RWC)
NLD - Non-senescent early dehydration (55-40% RWC)
SLD - Senescent early dehydration (55-40% RWC)

Figure 3-9: VIP score plot and heatmap displaying differences in sugars and sugar alcohols that significantly accumulated across the dehydration stages for both NST and ST. (A) Differences were identified by PLS-DA and the coloured boxes indicate the relative abundances of metabolites of the corresponding tissue type and dehydration stages. Differences in significant sugars are also further represented by heatmap (B). Significance was based on ANOVA Fisher's LSD with $P< 0.05$.

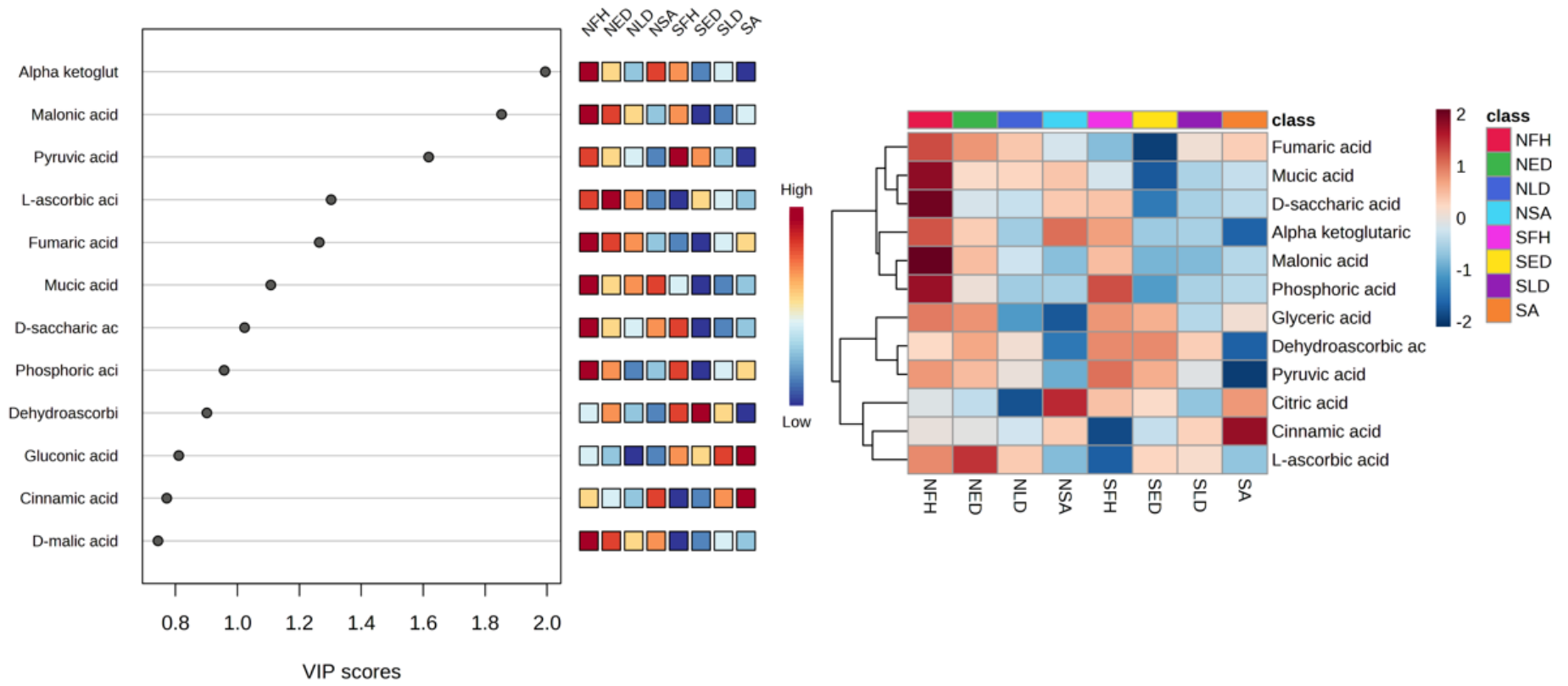



NRI - Non-senescent tissue 24 hours post-rehydration
NRII - Non-senescent tissue 48 hours post-rehydration
NW - Non-senescent tissue 1-week post-rehydration
SRI - Senescent tissue 24 hours post-rehydration
SRII -Senescent tissue 48 hours post-rehydration
SW - Senescent tissue 1-week post-rehydration.

Figure 3-10: Heatmaps of differentially abundant sugars, sugar alcohols, phytohormones and fatty acids in *E. nindensis* across the rehydration time points in NST and ST. A) heatmap of sugar alcohols B) heatmap showing sugars and c) heatmap showing phytohormones, fatty acids and nucleotides. Significance was based on ANOVA Fisher's LSD with P< 0.05.

### 3.1.7 Changes in organic acids abundance during dehydration and post-rehydration

In addition to sugars and sugar alcohols, 12 organic acids were found to be differentially abundant across the dehydration (Figure 3-11) and 14 post-rehydration (Figure 3-12) in E. nindensis. Levels of mucic acid were consistent throughout the dehydration process as observed in Figure 3-11. However, the accumulation of alpha-ketoglutaric acid and citric acid was more pronounced in the dry state in NST compared to other organic acids, while ST exhibited similar elevations in cinnamic acid (Appendix, Figure S5-3). The decline of all TCA cycle intermediates notably fumaric, pyruvic and malic acid during late dehydration (55-40%) (Figure 3-11) indicates a deceleration in respiration and a shift in metabolism towards the synthesis of metabolites crucial for protection during desiccation tolerance. The shift to defensive metabolism occurred when dehydration progressed beyond the late dehydration stage, leading to changes in metabolite levels. Additionally, the levels of phosphoric acid remained consistently low throughout the dehydration stages in both tissues and similarly low levels of malonic and D-saccharic acid were observed in ST (Figure 3-11). E. nindensis rehydrated upon watering after 16 days of desiccation (Figure 3-1) and re-initiated photosynthesis rapidly and the resumption of metabolism was further elucidated by an increase in TCA intermediates. High levels of succinic, lactic, pyruvic, mucic and phosphorous acid were observed at 48 hours post-rehydration and more pronounced in NST as shown in Figure 3-12. More so, alpha-ketoglutaric and oxalic acid peaked at 1 week post-rehydration. Contrastingly, the TCA intermediates were predominantly low in ST samples across rehydration time points (Figure 3-12). Most of the metabolites involved in the TCA cycle increased post-rehydration, an indicator of resumption of respiration and normal metabolism and the increase is predominantly seen in NST, unlike the ST. However, malic, Citramalic and dehydroascorbic acid decreased significantly post-rehydration up to 48 hours and later increased after 1-week post-rehydration.




NFH - Non-senescent fully hydrated (> 85% RWC)
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SFH - Senescent fully hydrated (> 85% RWC)
SED - Senescent early dehydration (75-60% RWC)
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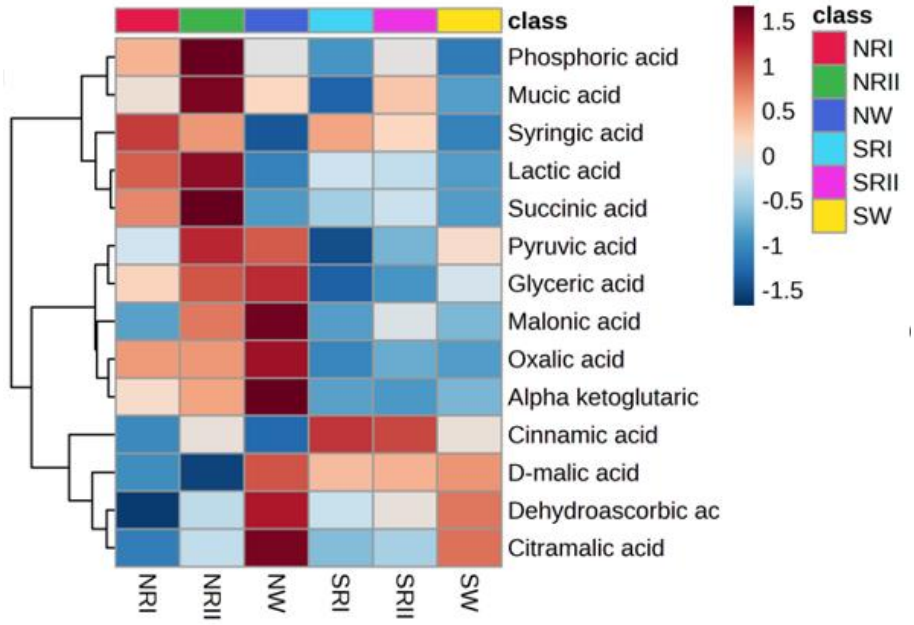
NLD - Non-senescent early dehydration (55-40% RWC)
SA - Senescent air-dry (<5% RWC)
SLD - Senescent early dehydration (55-40% RWC)

Figure 3-11: Heatmaps of differentially abundant organic acids in *E. nindensis* across the dehydration stages for both NST and ST. Heatmap of organic acids across dehydration stages. Differences were identified by PLS-DA and the coloured boxes indicate the relative abundances of metabolites of the corresponding tissue type and dehydration stages. Significance was based on ANOVA Fisher's LSD with P< 0.05.



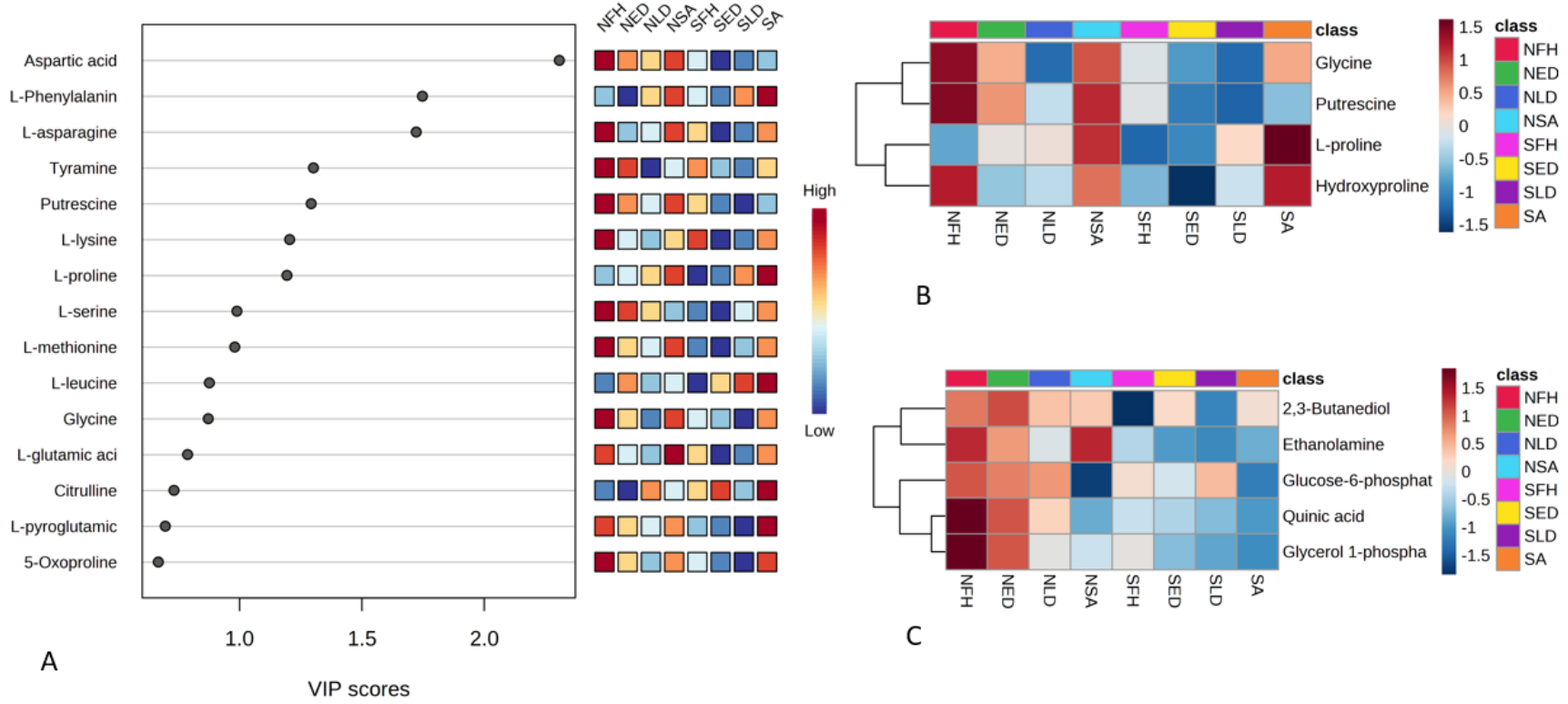

NRI - Non-senescent tissue 24 hours post-rehydration SRI - Senescent tissue 24 hours post-rehydration
NRII - Non-senescent tissue 48 hours post-rehydration SRII - Senescent tissue 48 hours post-rehydration
NW - Non-senescent tissue 1-week post-rehydration SW - Senescent tissue 1-week post-rehydration.

Figure 3-12: Heatmaps of differentially abundant organic acids in *E. nindensis* across the rehydration time points in NST and ST. A). Significance was based on ANOVA Fisher's LSD with P< 0.05.

### 3.1.8 Change in amino acids and other metabolites abundance during dehydration and post-rehydration

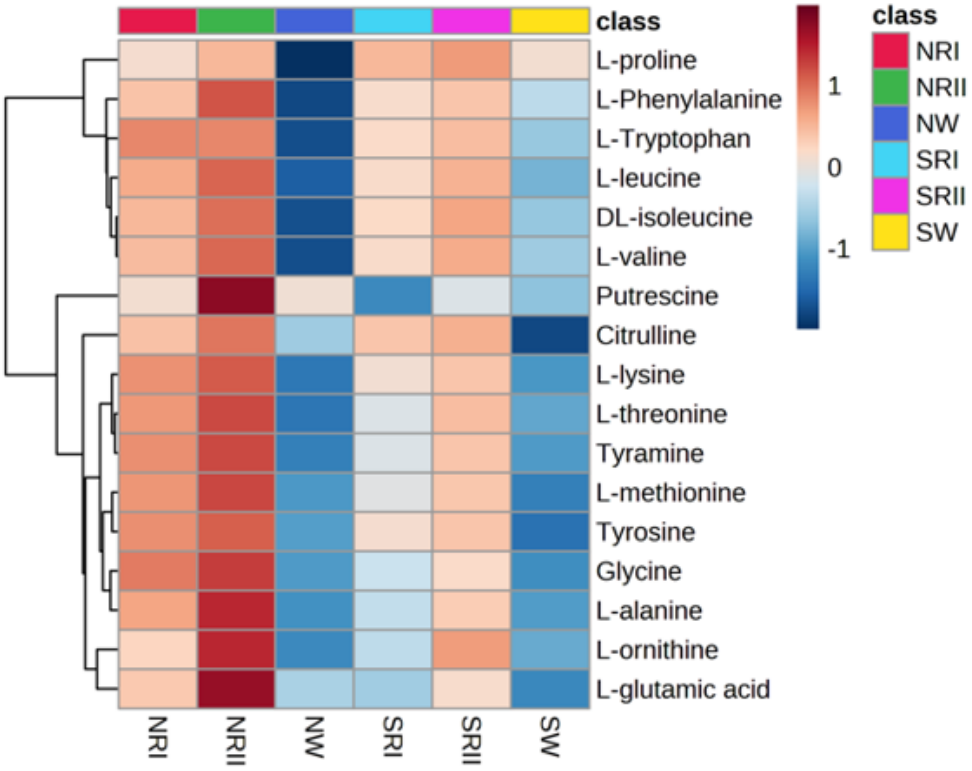
A total of 4 amino acids were differentially abundant upon dehydration in both tissues of E. nindensis (Figure 3-13B). The levels of the significant amino acids, including, glycine hydroxyproline and putrescine decreased significantly during dehydration, with an increase in abundance observed in the dry state. However, differences in amino acids that were not significant during dehydration are depicted in the VIP score plot (Figure 3-13A) and phenylalanine, tryptophan, proline, and glycine increased with higher accumulation seen in the dry state. Accumulation of aspartic acid, glutamic acid, methionine, asparagine, putrescine, and glycine was observed in NST. Similarly, the accumulation of leucine, citrulline, lysine, and proline during dehydration was more pronounced in ST, with slightly lower levels observed during both the early and late stages of dehydration (Figure 3-13A). The abundance of leucine, isoleucine, valine, and threonine in the dry state was predominantly higher in ST, as depicted in Figure 3-13A. Upon rehydration, there was a significant shift in metabolism, as most of the amino acid accumulated and peaked at 48 hours post-rehydration in NST, unlike the counterpart ST where low levels were observed as depicted in Figure 3-14. Glutamic

acid was the most abundant amino acid at 48 hours post-rehydration, and this is consistent with the role of glutamate as the primary nitrogen donor for the synthesis of other amino acids. Other amino acids that significantly increased post-rehydration included phenylalanine glycine, ornithine, methionine, and putrescine (Figure 3-14).




NFH - Non-senescent fully hydrated (> 85% RWC)
SFH - Senescent fully hydrated (> 85% RWC)
NSA - Non-senescent air-dry (<5% RWC)
SA - Senescent air-dry (<5% RWC)
NED - Non-senescent early dehydration (75-60% RWC)
SED - Senescent early dehydration (75-60% RWC)
NLD - Non-senescent early dehydration (55-40% RWC)
SLD - Senescent early dehydration (55-40% RWC)

Figure 3-13: VIP score plot displaying differences in amino acids and other metabolites across the dehydration stages for both NST and ST. Differences were identified by PLS-DA and the coloured boxes indicate the relative abundances of metabolites of the corresponding tissue type and dehydration stages. Differences in significant amino acids (B) and other metabolites (C) were also further represented by heat maps. Significance was based on ANOVA Fisher's LSD with $P < 0.05$.

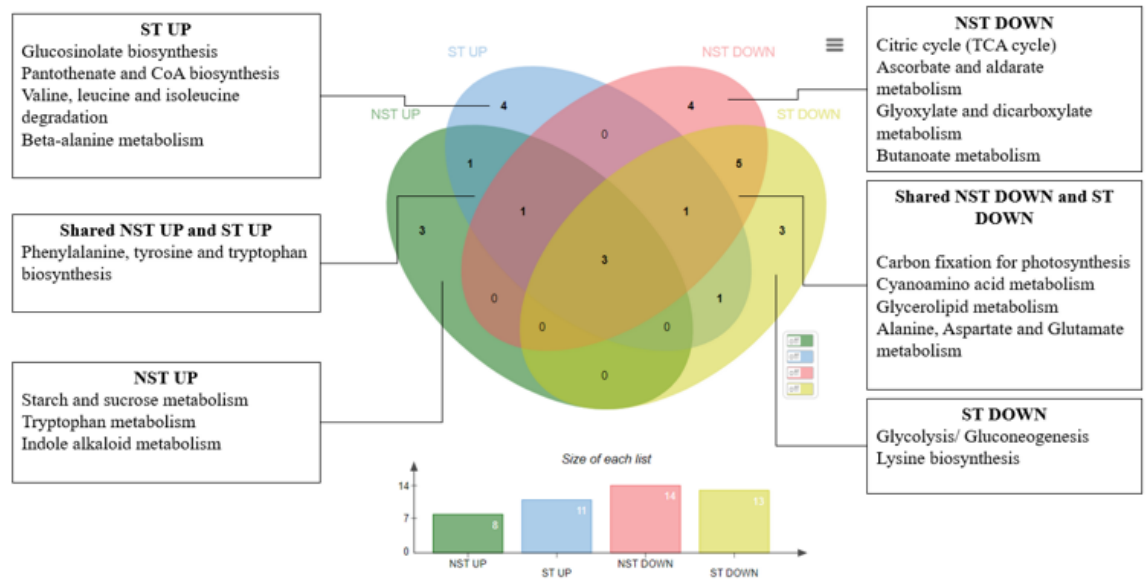



NRI - Non-senescent tissue 24 hours post-rehydration SRI - Senescent tissue 24 hours post-rehydration
NRII - Non-senescent tissue 48 hours post-rehydration SRII -Senescent tissue 48 hours post-rehydration
NW - Non-senescent tissue 1-week post-rehydration SW - Senescent tissue 1-week post-rehydration.

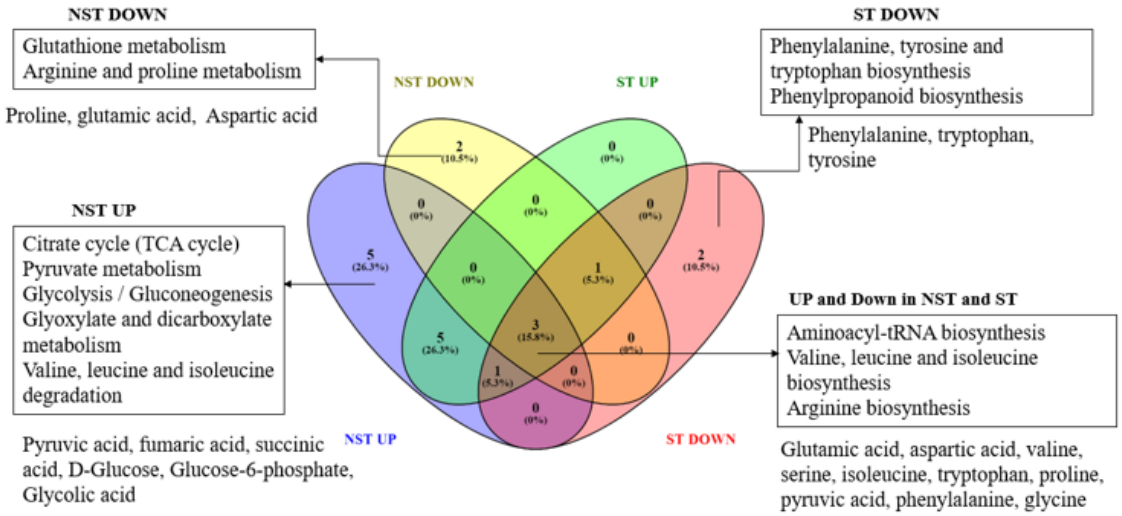
Figure 3-14: Heatmaps of differentially abundant amino acids in *E. nindensis* across the rehydration time points in NST and ST. Significance was based on ANOVA Fisher's LSD with P< 0.05.

### 3.1.9 Metabolic pathway analysis for differentially abundant metabolites.

Fold change analysis was done relative to the fully hydrated tissue control. This was to identify metabolites that were accumulating or decreasing during dehydration at different RWCs and post-rehydration. Pathway analysis was performed on all metabolites from fold change analysis using A. thaliana KEGG chassis as the pathway library to relate the biological functions of identified metabolites to different pathways (Figure 3-15). This revealed variations in metabolic pathways, suggesting different processes taking place during dehydration in NST and ST. Twenty-six and nineteen metabolic pathways were associated with dehydration and rehydration respectively. During dehydration, out of 29 metabolic pathways, 5 metabolic pathways including the TCA cycle, glyoxylate and dicarboxylate metabolism are downregulated exclusively in NST correlating with a decrease in TCA intermediates (Figure 3-15A) and the aforesaid metabolic pathways in addition to glycolysis and pyruvate metabolism were upregulated post-rehydration in NST (Figure 3-15B), an indication of resumption metabolic activity. Furthermore, phenylalanine, tyrosine, and tryptophan biosynthesis were upregulated in both NST and ST and exclusively downregulated in ST post-rehydration whereas 5 metabolic pathways were upregulated in both tissues during post-rehydration. Three metabolic pathways were associated with metabolites upregulated and downregulated in both ST and NST during dehydration and rehydration (Figure 3-15). Carbon fixation, cyan amino acid metabolism, glycerolipid metabolism and alanine, aspartate and glutamine metabolism were downregulated in both tissues whereas glycolysis/gluconeogenesis was exclusively downregulated in ST during dehydration (Figure 3-15A). Starch and sucrose metabolism was common to all NST dehydration stages, and this is consistent with high levels of raffinose, and sucrose. Glycolysis and TCA were not significant in ST correlating to low levels of sugars and TCA intermediates. None of the metabolic pathways was exclusively associated with metabolites upregulated in ST. Valine, leucine, and isoleucine degradation and beta-alanine metabolism were exclusively upregulated in ST.




A




B

Figure 3-15: Venn diagram of metabolic pathways associated with differentially abundant metabolites in NST and ST of *E. nindensis* during dehydration and rehydration relative to fully hydrated and air-dry tissue respectively. Venn diagram A indicates metabolic pathways associated with dehydration and Venn diagram B indicates metabolic pathways associated with rehydration. “UP” refers to metabolic pathways associated with accumulating metabolites and “DOWN” metabolic pathways associated with diminishing metabolites. All metabolic pathways analysis were performed with MetaboAnalyst 5.0 using A. thaliana as the pathway library with a P-value of 0.05 and false discovery rate (FDR > 0.2).

## 3.2 General discussion and conclusion

### 3.2.1 General Discussion

The purpose of this chapter was to compare NST and ST in *E. nindensis* during dehydration and rehydration. The protection of plant tissue during prolonged drought and subsequent desiccation requires a well-orchestrated set of processes to be put in place to ensure long-term survival and recovery. Additionally, resurrection plants regain full photosynthetic activity post-rehydration, indicating the presence of mechanisms to preserve the delicate photosynthetic apparatus during desiccation and post-rehydration. Prior research on the transcriptome of *E. nindensis* (Madden, 2019) had shown key changes in transcript abundance during the late dehydration stage, which is characterized by a decrease in water content below 40%RWC. During this stage, *E. nindensis* employs a strategy of disassembling the photosynthetic apparatus to effectively shut down photosynthesis, as observed in previous studies (Vander Willigen et al., 2001). The impact of dehydration on transcript and protein abundance is substantial, particularly when the tissues reach a nearly desiccated state at 25% relative water content (RWC), and this could induce significant changes in the tissues, ultimately leading them entering a state of quiescence below 10% RWC (Madden, 2019). The metabolome data exhibits comparable patterns, with shifts in metabolite concentrations observed during initial dehydration and further shifts occurring in the desiccated state (5%) (figures 3.7-3.12) and this elucidates that differences in transcripts and protein regulation have a distinct impact on the biosynthesis of metabolites. Therefore, the integration of multiple omics approaches offers a better understanding of *E. nindensis* during dehydration and rehydration. The metabolome to some degree validates several hypotheses postulated in the transcriptome (Madden, 2019) and proteome (van der Pas, 2023) studies. What is even more conclusive is that the population used for the metabolic study done herein is completely different to the population used for the transcriptomic and proteomic study, clearly demonstrating that changes observed are well conserved between populations. Although the exploration of the previous omics was beyond the scope of this project, they provided insightful direction to investigate the metabolic changes during dehydration and rehydration. The metabolomic profile of *E. nindensis* done herein thus adds to the multi-omics approach to understanding desiccation tolerance and the regulation of senescence in *E. nindensis* and illustrates the importance of

coupling a multi-disciplinary approach to understanding plant stress adaptation and tolerance.

#### 3.2.1.1 Physiological findings

With regards to physiological data, both NST and ST behave similarly in terms of dehydration (Figure 3-1) and differences were evident upon rehydration coupled with leaf morphology changes (Figure 3-2) suggesting differences in metabolic activity in these two tissue types. This corroborates that ST was not able to survive desiccation and ultimately underwent senescence whereas the NST was able to induce desiccation tolerance mechanisms during dehydration while suppressing senescence. However, senescence in leaves has been attributed to several factors and is still not clearly defined for most resurrection plants, including E. nindensis. During the course of experimentation, it was observed that E. nindensis recovered from desiccation at two weeks post-germination and was able to show total recovery of all leaves upon rehydration (data not shown). This suggests that senescence could be an age-dependent occurrence as has been hypothesized in the recently completed proteome on this species (van der Pas, 2023) but is perhaps accelerated by drought stress.

During dehydration, morphological differences were seen between the ST and NST (Figure 3-2) and it is interesting to note that the rolling of the NST was regular and uniform during the drying process. The NST showed controlled leaf rolling at the 55-45% RWC whereas the ST did not. Importantly, coordinated leaf folding is a typical response in resurrection plants, and it has been linked to lower rates of transpiration, which aids in temperature regulation and photoprotection (Farrant et al., 2007; Radermacher et al., 2019). Physiological research on *Eragrostis* species indicated that *E. nindensis's* stomatal conductance decreases transpiration and reduces carbon dioxide assimilation (Ginbot & Farrant, 2011) and this correlates with another study conducted by (Aidar et al., 2010) on a poikilochlorophyllous desiccation tolerant plant *Pleurostima purpurea* indicating a decrease in transpiration due to reduction in stomatal conductance. The regulation of stomatal conductance, which prevents water loss and gas exchange, has been attributed to metabolites including GABA and fumarate (Nunes-Nesi et al., 2007). Consistent with this, a rise in GABA predominantly in NST (see Appendix, Figure S5-6) and fumaric acids was observed in *E. nindensis* during ED (75%-60%) (Figure 3-11), suggesting that *E. nindensis* could be employing stomatal conductance as an initial mechanism of regulating water loss during

dehydration as also previously evidence a study by Vander Willigen et al. (2001) comparing *E. nindensis* with other *Eragrostis* species. Resurrection plants regain full photosynthetic activity after being rehydrated, indicating the presence of mechanisms to preserve the delicate photosynthetic apparatus during desiccation and upon rehydration. The recovery can be shown by leaf morphology and water uptake by the plants. Differences in the recovery of desiccation-tolerant plants have been observed in various studies. A study by Sherwin and Farrant (1996) highlighted a slow recovery in *X. viscosa* now *X. schlechteri*, a poikilochlorophyllous resurrection plant similar to *E. nindensis.* This slow recovery was attributed to the significant time required to restore the chloroplast and photosynthetic apparatus. In contrast, other species such as *Craterostigma wilmsii* exhibited faster recovery, while *M. flabellifolia's* slow recovery was due to dehydration-induced xylem embolism (Sherwin & Farrant, 1996). For *E. nindensis,* after 24 hours of rehydration, progressive unfolding of the NST leaves was observed and by 1 week post-rehydration, the NST leaves were back to their original pre-stressed green colour with RWC of about 70%. This suggests that *E. nindensis* could be exhibiting a slower recovery rate as it undergoes re-assembling of photosynthetic apparatus. Chlorophyll content on *E. nindensis* during dehydration and rehydration also showed that at 72hrs post-rehydration, which is roughly 40% RWC, re-greening occurred (Madden, 2019). No further rehydration was evident up to 1 week following rehydration as the ST leaves remained folded, indicating that the ST leaves could not recover post-rehydration. Commitment to senescence was thus evident in the ST owing to the lack of full recovery (see Figure 3-2, 48 hours). The proteome (van der Pas, 2023) and transcriptome (Madden, 2019) seem to suggest that beyond the 40% is when commitment to desiccation response and senescence is initiated but true senescence is only really evident upon rehydration. Notably, in a study by Vander Willigen et al. (2001) comparing *E. curvula* and *E. nindensis*, there was no recovery of outer leaves and the inner leaves appeared to preserve the integrity of the membrane under dehydration, which might have contributed to their recovery upon rehydration which is consistent with the current study's hypothesis indicating that exogenous factors such as water deficit could promote early senescence in older leaves. Several factors including high temperatures and heat stress have been reported to induce early senescence and this is consistent with a study conducted on sunflower leaves that showed early senescence characterised by increased $H_2O_2$ accumulation and a decline in antioxidant activity (Haba et al., 2014), which correlates with the current

study showing a low recovery rate due to lower levels of antioxidant-related metabolites observed in ST during dehydration as predetermined by the metabolic shift observed in Figure 3-13, and this seems to indicate the failure of ST to suppress senescence. Moreover, at 48 hours post-rehydration, NST had reached a RWC of roughly 40-50%, and akin to dehydration at the same RWC, a noticeable metabolic shift was seen. This is in agreement with a study by Vander Willigen et al. (2001), which found that after 40% RWC was achieved in inner leaves post-rehydration, the quantum efficiency of photosystem II gradually recovered, suggesting that photosynthesis had resumed, and this corroborates the high metabolic shift observed at 40% RWC post-rehydration. One might argue that 40% RWC at both ends of dehydration and rehydration serve as a tipping point as postulated by van der Pas, (2023). When *E. nindensis* reaches the 40% threshold during dehydration it commits to the desiccation response by increasing the breakdown of the thylakoids and shut down of photosynthesis (Madden, 2019). Metabolic shifts leading up to the 40% tipping point are required for drought response but not necessarily desiccation response but the 40% RWC commits the plant to desiccation. From an ecophysiological perspective, this makes sense since *E. nindensis* in its natural environment is likely to go through several rounds of short or prolonged periods of no water and as suggested thus far, the desiccation response requires a well-orchestrated set of processes which are fundamentally expensive and comes at the cost of sacrificing the ST. Conversely, when fully desiccated and rehydrated, *E. nindensis* might only commit to full recovery when cellular RWC reaches this 40% threshold. Thus, one might speculate that the 40% RWC serves as a key regulatory cellular water stage that commits *E. nindensis* to desiccation and full recovery as a significant metabolic shift was observed at this RWC.

#### 3.2.1.2 Sugar metabolism during dehydration and rehydration

During dehydration and post-rehydration, the PCA (Figure 3-5) indicated separations between both tissues which correlated with the metabolic shift seen in the results (Figure 3.3-3.15). Concerning sugars, it is commonly noted that during a water shortage, resurrection plants accumulate high levels of sugars such as sucrose and raffinose (Gabier et al., 2021; Illing et al., 2005; Peters et al., 2007). During the late to dry state in *E. nindensis*, an increase in raffinose levels along with sucrose was observed. The results are in agreement with the above data and the studies performed

as seen in the previous two omes (Madden, 2019, van der Pas, 2023). Raffinose appears to potentially have a role in osmoprotection in *E. nindensis* as it showed a defined pattern of accumulation upon dehydration and decreased at 48 hours post-rehydration. The accumulation of raffinose could be favoured by the simple sugars and sugar alcohols from the breakdown of starch, which decreased upon desiccation in both NST and ST, as also seen in *X. schlechteri* (Gabier et al., 2021). However, raffinose accumulation was exclusive to NST as seen in Figure 3-3 and Figure 3-9 and this further supports the predetermined desiccation tolerance mechanisms exclusively seen in the transcriptome and proteome of the same species, unlike ST which accumulated only sucrose in the air-dry state. Previous studies have also found that raffinose accumulation in *E. speciosa* seeds has a role in desiccation tolerance (Hell et al., 2019), particularly concentrating in subcellular compartments, which sheds light on the idea that resurrection plants' vegetative tissue can exhibit characteristics comparable to those seen in orthodox seeds (Matilla, 2021). Indeed, several resurrection plants have been shown to accumulate raffinose in the dry state following a period of late dehydration (Dace, 2014; Gabier et al., 2021; Oliver et al., 2011; Peters et al., 2007) indicating its particularity in how various resurrection plants can respond to abiotic stress by accumulating different metabolites, but also lends more credence to the rewiring of orthodox seeds theory (Costa et al., 2017). It is interesting to note that raffinose accumulation has been reported in mostly monocotyledonous and poikilochlorophyllous resurrection plants though more studies are needed to validate this theory. The overexpression of raffinose and galactinol synthase seen in the transcriptome (Madden, 2019) could potentially reflect the accumulation of raffinose in the current study during the late stages of dehydration. This is an illustration of how the integration of omics could aid in the comprehension of the metabolic process. Other studies have also shown raffinose accumulation and genes encoding raffinose synthase in desiccation-tolerant rice expressing the OsWRKY11 (Wu et al., 2009), and this seems to further support how elevated transcripts could to some extend regulate metabolic activity. Precursor metabolites such as galactinol and myo-inositol for raffinose biosynthesis decreased upon dehydration in the present study as they serve a critical role in raffinose family oligosaccharides (RFO) pathway. Studies have also reported accumulation of sugar alcohols, such as galactinol and myo-inositol, as osmoprotectants as well as antioxidants (Lah et al., 2023). However, their reduction in *E. nindensis* seems to suggest that their role in *E. nindensis* could be limited to RFO

synthesis. In the RFO pathway, myo-inositol combines with UDP-galactose via galactinol synthase activity to produce galactinol (ElSayed et al., 2014), and two enzymes, raffinose synthase and stachyose synthase, use galactinol as a galactosyl residue to generate raffinose and stachyose, respectively, from sucrose. This theory could serve as evidence that raffinose accumulation which is also concomitant to the increase in raffinose synthase (see Appendix, Figure S5-11) as demonstrated in the transcriptome of *E. nindensis* (Madden, 2019) and proteome (van der Pas, 2023), suggesting that these metabolites could be transformed into RFOs and that their role could be predominantly during the early stages of dehydration. Over ad above to the role of raffinose in acting as compatible solutes, previous studies have reported raffinose as an inhibitor of sucrose crystallization (Leinen & Labuza, 2006), which seems to suggest that accumulation of raffinose could potentially have a role in the stabilisation of sucrose, and the two sugars are important in glassy state formation for cellular protection in the desiccated state. Furthermore, LEAs have also been reported to aid stability to the glassy state, cross-linking with raffinose (Shimizu et al., 2010), suggesting that the LEA proteins that were reported by Madden (2019) to significantly increase in *E. nindensis* seem to contribute to the stability of glassy state.

Sucrose, along with raffinose, is thought to play a role in vitrification, a process employed by resurrection plants and seeds to preserve their cellular components in the latter stages of desiccation (Berjak, 2006; Farrant et al., 2007; Illing et al., 2005). According to a study by Brenac et al. (1997), the vitrification of sucrose across fluid membrane phospholipids maintained the membrane's integrity in maize while also contributing to its role in vitrification and a similar mechanism could be employed in *E. nindensis* as consistent accumulation of sucrose were observed in both tissues, though more pronounced in NST (Figure 3-9).

Sugars are also essential for osmo protection during desiccation (Ahmad et al., 2020). Osmolyte accumulation such as sucrose has been detected in *X. schlechteri* as a defence mechanism against oxidative stress brought on by ROS accumulation during dehydration (Gabier et al., 2021; Radermacher et al., 2019; Živanović et al., 2020). Due to its involvement in the synthesis of suitable solutes that replace the water in cells and preserve cell integrity during desiccation, sucrose accumulation in *E. nindensis* in the dry state could have a role in osmo-protection as levels are consistent with those that have been previously reported in other studies. Similarly, trehalose has

been shown to act as an osmolyte; however, levels of trehalose are reported to be low in most plants (Lunn, 2007) indicating that it could have a lesser role in osmo protection. An exception to this is the woody resurrection plant *M. flabelifollia* which accumulates large quantities of trehalose during dehydration (Drennan et al., 1993). In *E. nindensis*, trehalose accumulated primarily at early dehydration (75%-60%) with low levels at late dehydration in NST corroborating the underrepresentation of trehalose 6- phosphate synthase seen in the proteome data (van der Pas, 2013) therefore suggesting that trehalose could be playing a lesser role in osmoprotection for leaves of *E. nindensis* and that *E. nindensis* is primarily an RFO resurrection plant. Research has linked abscisic acid-mediated stomatal closure to trehalose metabolism (Wang et al., 2020). As a result, a slight increase in trehalose during the dehydration stages may not help in osmoprotection but could instead be likely to regulate responses leading to induction of slowing down transpiration as early strategies of drought avoidance, and the elevated levels of trehalose seen at early dehydration in the current study seems to reflect the role of trehalose at early stages of dehydration. The accumulation of D-arabinose and D-lyxose predominantly ST in the dry state could be attributed to cell wall leakage due to the likelihood of ST failure to maintain the cell wall integrity during desiccation as these metabolites form the components of the cell wall (Kotake et al., 2016). Therefore, the low levels observed in NST could indicate that the integrity of the cell membrane is maintained during desiccation though the available data might not be conclusive to justify this hypothesis. A study by Madden (2019) observed through ultrastructural studies that the ST had collapsed cell walls in the airdry state whereas the NST still had relatively intact cell walls.

During rehydration, sugars also play an important role as shifts in sugar metabolism were noted as seen in Figure 3-10. During early rehydration, Sucrose could potentially serve as a significant carbon source for the creation of cellular components and the regeneration of ATP through glycolysis in NST as the process was downregulated in ST. The declining levels of sucrose observed at 48 hours post-rehydration (see Appendix, Figure S5-7) correlates with its role, suggesting that NST could be utilising sucrose for growth and development during recovery as a review by Yaseen et al. (2013) which indicated how carbon plays a role in the *in vitro* plant growth and development. Additionally, raffinose levels were significantly reduced at 48 hours post-rehydration (Figure 3-10) which also seems to suggest that the catabolism of raffinose

could contribute to the formation of sucrose which is utilised as a carbon stock post-rehydration. This correlates with a previous study by Radermacher et al. (2019) on *X. schlechteri* which reported a decrease in levels of sucrose and raffinose post-rehydration in NST. Furthermore, the decrease in sucrose correlates with the accumulation of simple sugars such as fructose and glucose, where these molecules are fed into glycolysis. Thus, the decrease of both sugars at 48 hours post-rehydration is interesting, as this time point has been previously discussed (where the plant achieves about 40% RWC post-rehydration) as a tipping point, where resumption of glycolysis and TCA drive the synthesis of ATP. In addition, the accumulation of sugar alcohols was noted at 24 hours and peaked at 48 hours, indicating that they could play several roles post-rehydration. In particular, glycerol may slow down the rate of water loss during the anticipated water loss upon rehydration because it lowers the surface tension of water and exhibits strong water-binding activity via hydrogen bonding (Weng et al., 2011). These findings could imply that most resurrection species may require the production of polyols during rehydration as more water uptake is needed for cellular activities during recovery. The build-up of polyols may also aid in other metabolic processes, such as minimizing the formation of ROS in the cells (Shen et al., 1999)

#### 3.2.1.3 Regulation of photosynthesis processes and TCA during dehydration and rehydration

Cessation of photosynthesis and shutdown of metabolism is well reported as one of the strategies employed by poikilochlorophyllous resurrection plants to deal with desiccation (Farrant et al., 1999; Sherwin & Farrant, 1998). A similar mechanism has been reported in *Xerophyta* species (Gabier et al., 2021; Radermacher et al., 2019; Sherwin & Farrant, 1998). This is synchronous with the decrease in the TCA intermediates in the present study predominantly in NST, and the studies performed earlier on *E. nindensis* (Ginbot & Farrant, 2011). Thus, the significant metabolic shift in TCA intermediates could be attributed to changes in carbon influx at different points in the cycle to synthesize key metabolites and redirect ATP production from other sources to sustain the cells during desiccation. In the present study, A significant reduction in α-ketoglutaric, pyruvic, fumaric and citric acid during dehydration in both tissues was observed (Figure 3-11). The decrease in levels of α-ketoglutaric acid could have a role in the synthesis of glutamate via glutamate dehydrogenase (Wu et al.,

2016), a key nitrogen donor for the synthesis of nitrogen-containing molecules required during desiccation and upon rehydration. However, α-ketoglutaric acid increased in abundance in the air-dry state predominantly in NST and this could be due to the fact that it is required post-rehydration to synthesize glutamate. Thus, the decreasing levels of TCA intermediates suggest that *E. nindensis* does not differ much from other resurrection plants in desiccation tolerance by utilising a similar mechanism of shutting down the TCA cycle. Alternatively, the decline of TCA cycle intermediates notably fumaric acid during late dehydration in NST could indicate a decline in the respiration process and by contrast, the accumulation of α-ketoglutarate in the air-dry state could potentially indicate that low levels of respiration are maintained in *E. nindensis* during dehydration. Similar results have been reported by (Ginbot & Farrant, 2011), indicating that respiration was recovered on drying to below 30% RWC, thus supporting the hypothesis suggested by Farrant et al. (2007) that respiration is critical for resurrection plants to maintain their ability to provide ATP for the repair of stress-induced damage and facilitating plant protection mechanisms. Citric acid was shown to accumulate during the dry state and it has been reported to play a potential role in the formation of Natural Deep Eutectic Solvents (NaDES) which could be important in desiccation as proposed by Du Toit et al. (2020). Sucrose is another component in NaDES formation, and its accumulation in *E. nindensis* could indicate this role over and above other roles already discussed. NaDES formation has been proposed to form a third liquid phase which prevents protein denaturation as well as aids in the concentration of subcellular components by facilitating the formation of compatible solutes that replace water during desiccation (Choi et al., 2011). NaDES formation has been proposed to facilitate the continuation of respiration in the desiccated state in *X. schlechteri* (Radermacher et al., 2019)

The shift in metabolism was equally important during rehydration since poikilochlorophyllous plants are susceptible to photooxidative stress during the process of regaining photosynthetic competence (Sherwin & Farrant, 1996). The elevated level of pyruvic acid at 48 hours post-rehydration could imply that *E. nindensis* could be utilizing this molecule in the MEP pathway to synthesize carotenoids, which are likely to help in photoprotection. A study by Vander Willigen et al. (2001) on the same species reported an increase in carotenoids at 60% RWC which is relatively the same RWC seen at 48 hours in the current study, supporting the

hypothesis postulated above. Similarly, a study by (Cooper & Farrant, 2002) on *C. wilmsii* indicated an increase in the levels of carotenoids in the leaves upon rehydration at 48 hours which seems to correlate with these findings. This could indicate that both homoiochlorophyllous and poikilochlorophyllous plants utilise a similar mechanism to mask chlorophyll from excessive radiation during recovery. Notably, low levels of carotenoids have been seen during dehydration, indicating that their synthesis is predominantly essential during rehydration (Farrant et al., 1999). At 48 hours post-rehydration, most TCA intermediates peaked suggesting that energy metabolism via glycolysis has resumed. Notably, malic acid significantly decreased during rehydration, which correlates with an increase in the levels of pyruvic acid, suggesting that its decline is thought to contribute to pyruvic acid accumulation to maintain the TCA cycle during recovery. The decline of malic acid could also contribute to the production of $CO_2$ and NADPH (Sun et al., 2019), a reducing agent for anabolic activities and an enzyme in carbon metabolism, thus playing a role in photosynthesis.

3.2.1.4 Antioxidant activity mechanisms during dehydration and rehydration.

Quenching ROS is another mechanism commonly employed by resurrection plants, with the accumulation of ROS scavenging associated metabolites having been observed in other resurrection plants upon dehydration especially from 60% RWC to dry state (Gabier et al., 2021; Radermacher et al., 2019), with similar metabolite shifts seen in the current study. A review by Palma et al. (2002) indicated how the accumulation of ROS is likely to cause oxidation of essential proteins thus becoming a target for proteolytic digestion through ubiquitin-mediated protein degradation. To overcome the proteolysis of important proteins, *E. nindensis* showed a significant accumulation of antioxidant-associated metabolites at early stages of dehydration (75-60%) to late dehydration (55-40%). For instance, the accumulation of ascorbic acid (Figure 3-11) and its oxidised form dehydroascorbic acid was observed at early dehydration in both ST and NST. Ascorbate has been pronounced as the most abundant antioxidant that forms an antioxidant network in plants and most importantly its uniqueness of comprising a minimum of five distinct isoforms makes it a more efficient ROS scavenger in both the cytosol and chloroplast (Sharma & Dubey, 2005). Ascorbic acid is also used as a reducing agent to reduce hydrogen peroxide ($H_2O_2$) to $H_2O$, and notably, high levels of ascorbate have been repeatedly reported in the activation of ascorbate peroxidase (Caverzan et al., 2012), corroborating the

increased abundance of ascorbate peroxidase reported in the proteome data (van der Pas, 2023). The aforementioned enzyme elucidates the accumulation of ascorbic acid in the current study at early dehydration, indicating that ascorbic acid could have a role in detecting water deficit in *E. nindensis* as a ROS scavenger as previously reported in several studies (Awad et al., 2015; Gest et al., 2012; Osmond et al., 2000). Most of the metabolites involved in ROS quenching were more pronounced in the NST than in the ST. Alternatively, ascorbic acid could be acting as a co-factor for several enzymes, with special emphasis on violaxanthin de-epoxidase (VDE), an enzyme that is extremely important for preventing photo-damage to the photosynthetic machinery by releasing excess light energy received as heat through the conversion of violaxanthin (V) to intermediate product antheraxanthin. (Li et al., 2013). Ascorbic acid also reduces $Fe^{3+}$ to increase its uptake by plants (Grillet et al., 2014), which helps with a variety of cellular activities and reduces programmed cell death that is facilitated by ferroptosis (Distéfano et al., 2021) due to the accumulation of oxidised iron. This seems to support the hypothesis of downregulation of ferroptosis in the NST but promotion of it in the ST as speculated by van der Pas (2023).

3.2.1.5 Amino acid metabolism during dehydration and rehydration

Similar to other resurrection plants, the NST of *E. nindensis* accumulates amino acids that have been repeatedly reported to aid in abiotic stress response (Batista-Silva et al., 2019). Four amino acids were found to show significant differences and accumulated during late dehydration with maximum peaks observed at air-dry state. Most of these amino acids serve as antioxidants (e.g., glycine, putrescine, and proline), as well as nitrogen reservoirs (e.g., asparagine, glutamic acid, phenylalanine) to aid in nitrogen metabolism post-rehydration, thus facilitating recovery (Martinelli et al., 2007). The accumulation of precursor metabolites associated with antioxidant synthesis was observed in the dry state in the NST, suggesting that these metabolites could be utilised in metabolic pathways to produce antioxidants such as spermidine. Spermidine is synthesized through metabolic pathways that utilise putrescine as an intermediate (Graser & Hartmann, 2000) and the synthesis starts with ornithine aminotransferase catalysing the conversion of glutamate to proline, resulting in the intermediate Proline-5-carboxylate, which is then used in the synthesis of ornithine. Ornithine undergoes a decarboxylation reaction to form putrescine. Decarboxylated S-adenosylmethionine transfers a 3-aminopropyl moiety to putrescine via spermidine

synthase, resulting in spermidine (Majumdar et al., 2016), a key antioxidant in drought stress (Hussain et al., 2011). Thus, the decrease in levels of putrescine and proline depicted in the study during dehydration could be attributed to the production of antioxidants utilised by *E. nindensis* in scavenging ROS. Alternatively, proline and putrescine could be utilised in the GABA shunt (Bouché et al., 2004), a metabolic process involved in replenishing succinate into the TCA during desiccation in plants for cellular metabolic processes. In addition, accumulation of proline has been reported to accumulate under abiotic stress (Seki et al., 2007) and its accumulation has been observed to prevent ROS accumulation and preserve the cellular components to facilitate cell function during desiccation (Nanjo et al., 1999). Notably, varying levels of proline were observed in a study conducted by Tymms and Gaff (1979) on nine species of resurrection plants, where it was suggested that the proline levels were not related to drought tolerance in resurrection plants. However, the accumulation of proline in the present study is in agreement with already reported levels of proline accumulating in a sister species *Eragrostis brachyphylla* indicating a drastic increase in the desiccated state, and similarly in other resurrection plants*, X. humilis* (Dace, 2014)*, S. stapfianus* (Martinelli et al., 2007), *Selaginella bryopteris* (Pandey et al., 2010) and *Selaginella tamariscina* (Wang et al., 2010) elucidating that proline could have a role in desiccation tolerance. Under stress conditions in plants, proline has been reported to have several roles such as 1) cell membrane stability and stabilising sub-cellular components; 2) induction of stress protective protein and formation of compatible solutes; and 3) osmotic adjustments and stress signalling, resulting in the maintenance of cell turgor (Bartlett et al., 2014). Thus, proline accumulation in the current study suggests that proline could contributing to the desiccation mechanisms employed by *E. nindensis*.

Among the amino acids investigated, glutamic acid showed the highest abundance in the dry state (Figure 3-13) which is consistent with its role as a universal nitrogen donor for the synthesis of amino acids that are required and could be utilised by the plant in mitigating effects of desiccation (Singh, 1998). The accumulation of aspartic acid, along with glutamic acid in *E. nindensis* in the dry state in the NST could also be attributed to their role in the GOGAT pathway to synthesize amino acids that serve as antioxidants and osmolytes during dehydration amounting to the roles of proline and putrescine. Glutamate synthase/glutamine:2-oxoglutarate aminotransferase

(GOGAT), is the central enzyme in the de novo synthesis of glutamate from ammonia assimilation (Miflin & Lea, 1980), and the α-amino group can be transferred to numerous 2-oxo acid acceptors, yielding a wide range of amino acids. Thus, the accumulation of glutamic acid and aspartic acid predominantly in NST could potentially imply that the GOGAT pathway is switched on in the NST and this is supported with the proteome data on the same species (van der Pas, 2023), where the NST had 4 shared terms associated with glutamate and pyruvate processes during dehydration.

Glutamine can also be irreversibly converted to gamma-aminobutyric (GABA) through the action of glutamate decarboxylase. The overrepresentation of glutamate decarboxylase (see Appendix, Figure S-11) in the proteome data (van der Pas, 2023) could be contributing to the synthesis of GABA, which is seen to considerably accumulate at early dehydration (Appendix, Figure S-6) with low amounts of glutamate at the same stage (Figure 3-13A). It has been shown by several authors that plants that are subjected to abiotic stress generate high levels of GABA (Hasan et al., 2021; Roberts, 2007). GABA has been reported to increase plant resistance to abiotic stress via modulating cellular pH, ion transportation, antioxidant activity, and reactive oxygen species reduction (Gill & Tuteja, 2010; Hu et al., 2015). The abundance of GABA significantly increased in NST at the early dehydration stage (75%-60%) followed by a significant decline in the air-dry state, suggesting that GABA accumulation could be attributed to the decarboxylation of glutamate via the GABA shunt. Moreover, GABA as a signalling molecule (Roberts, 2007) plays a role in the production of secondary metabolites and is also reported as an intersection for most of the metabolic pathways that involve nitrogen and carbon metabolism (Michaeli & Fromm, 2015; Wang et al., 2023), suggesting that it could potentially have multiple roles in response to desiccation (Hasan et al., 2021).

As most of the amino acids were shared between the NST and ST during dehydration, the accumulation of beta-alanine, leucine, and isoleucine remained high in ST while that of tryptophan, proline, glutamic acid, aspartic acid, and ornithine was elevated in NST, indicating that the NST could be geared toward quiescent state and the ST geared toward the onset of senescence. The accumulation of lysine and other branched-chain amino acids (BCAAs), such as leucine and valine, predominately in ST, could be related to fast protein turnover and the repurposing of some amino acids to create crucial amino acids needed to combat abiotic stress. Stress-induced protein

degradation could be another possible explanation for a surge in amino acids, but plants may also selectively produce certain amino acids for essential roles (Galili et al., 2016). Similarly, high levels of BCAAs accumulated in *S. stapfianus* (Yobi et al., 2017) and *X. schlechteri* (Gabier et al., 2021) under drought stress. More so, high levels of BCAAs were also detected in *A. thaliana* (Binder, 2010) and other plants (Kochevenko & Fernie, 2011), suggesting that these BCAAs could be produced during protein synthesis (Nie et al., 2018). Additional research on *A. thaliana* revealed that the accumulation of BCAAs is due to abscisic acid-regulated protein breakdown. By contrast, the accumulation of BCCAs in *E. nindensis* could be due to ubiquitin-mediated proteolysis triggered predominantly in ST. Similarly, in a study by Martinelli et al. (2007), BCCAs increased upon dehydration in older leaves of *Sporobolus stapfianus* which seems to align with the pattern observed in the *E. nindensis*. Conversely, decreased levels of BCCAs in NST suggest that they could act as alternative sources of carbon for the synthesis of sucrose at late stages of dehydration as outlined above. Moreover, oxidation of the (BCAAs) with lysine is thought to generate large amounts of ATP, which could be crucial for the survival of plants during desiccation (Hildebrandt, 2018). At a glance tryptophan, phenylalanine and hydroxyproline accumulated predominantly in the dry state in both tissues and these could have a role in desiccation by being involved in a series of biosynthetic pathways. The increased levels of aromantic acids phenylalanine, as well as tryptophan, were observed at late dehydration to dry state (40% to 5% RWC), and these abundances were more than two-fold greater in air-dry state and similar trends have been reported in *S. stapfianus, X. schlechteri* and *seleginella lepidophylla* (Oliver et al., 2011; Radermacher et al., 2019; Yobi et al., 2012) during dehydration. Tryptophan and phenylalanine serve as biosynthetic precursors for the synthesis of secondary metabolites via shikimate pathway which is key in desiccation tolerance. However, their accumulation in *E. nindensis* could be attributed to other factors as the proteome data (van der Pas) reported down regulation of enzymes involved in shikimate pathway.

Most amino acids significantly increased post-rehydration (Figure 3-14), which is consistent with their role as building blocks for protein synthesis, and particularly glutamine plays a role as a primary nitrogen donor for nitrogen metabolism, a process that is crucial for many cellular activities to promote growth and development of plants

(Häusler et al., 2014). The levels of glutamine decreased at 24 hours post-rehydration and significantly increased at 48 hours, which agrees with previous findings that reported a decrease in glutamine levels in *H. rhodopensis* (Moyankova et al., 2014). This could be attributed to the different times of rehydration. The decrease at 24 hours post-rehydration could reflect the synthesis of essential amino acids that play several roles in protein synthesis, including regulating root and shoot architecture, acting as signalling molecules and regulating flowering in plants (Hildebrandt et al., 2015). The presence of antioxidants such as ornithine reaching their highest levels at 48 hours, is expected to play a significant role in the prevention of photooxidative stress. The abundance of citrulline, a structural analogue of arginine, was observed to be elevated at the 48-hour time points following rehydration, and this could lead to the synthesis of arginine which is also a nitrogen storage metabolite and aids in the synthesis of polyamines during plant recovery (Yang & Gao, 2007). Moreover, quinic acid was found to be significant and accumulated during the initial stages of dehydration and post-rehydration in NST. The accumulation of quinic acid has been reported in *M. flabelifolia* to maintain the integrity of liposome membranes to reduce the effect of free radicles (Moore et al., 2005) and its accumulation in the current study could have a similar role in desiccation. The levels of quinic acid significantly reduced as the plant lost more water; this trend seems to indicate that quinic acid is being reduced in *E. nindensis* through the synthesis of its derivatives that could aid in maintaining cell membrane stability and antioxidant activity**.**

Concerning senescence that is hypothesized to be promoted in ST, the accumulation of cinnamic acid exclusively in ST during dehydration and post-rehydration seems to suggest that cinnamic acid could be involved in senescence. Cinnamic acid is a well-known allelochemical that causes plant cells to become lignified by redirecting the phenylpropanoid pathway (Mehmood et al., 2019). The higher accumulation of cinnamic acid in the ST could be indicative of programmed cell wall lignification which could further support the notion that the ST is committed to classical senescence whereas the NST is not. Additionally, metabolites identified in the ST correlated with those observed in a study by Watanabe et al. (2013) on *A. thaliana*, which indicated an increase in levels of BCCAs, maltose and galactinol as the leaves progressed to senescence, suggesting that the accumulation of similar metabolites in the current study could seem to contribute the senescence in ST. Other studies have also

reported a decrease in metabolites such as pyroglutamic acid, putrescine, raffinose and dehydroascorbic acid which play a key role in stress tolerance to decrease during leaf maturation (Kanojia et al., 2021). Therefore, the differences in the metabolite profile could potentially support the hypothesis that senescence in the ST is most likely age-dependent and that the ST might serve as a nutrient source for the recovering NST though not conclusive.

Metabolic pathway analysis was performed (Figure 3-15A) and revealed 14 different metabolic pathways in NST during dehydration, of which TCA cycle and glyoxylate and dicarboxylate metabolism were down exclusively in NST during dehydration and up upon rehydration. It corroborates the decline in respiration observed in a study conducted by Ginbot & Farrant (2011). This agrees with the over and above-discussed data on sugars and organic acids, validating the hypothesis of TCA cycle shutdown as a strategy employed by resurrection plants during desiccation as earlier discussed. The fact that this process was seen in NST could indicate that NST could be able to channel the ATP to essential biosynthetic processes that could help to mitigate abiotic stress which is not seen in ST. This is consistent with the transcriptome (Madden, 2019) and proteome data (van der Pas, 2023) indicating the shutdown of processes involved in the TCA cycle during the late stages of dehydration. The failure of ST to accumulate metabolites involved in TCA cycle and glycolysis during rehydration seems to support its failure to suppress senescence as these are vital metabolic pathways that generate ATP and NADH for different cellular activities and consist of precursors used in several biosynthetic pathways in plants. Therefore the accumulation of metabolites involved in the TCA cycle in NST could demonstrate the importance of their involvement and the results here point to a similar role observed in desiccation-tolerant grass in *S. lepidophylla* (Yobi et al., 2012) therefore corroborating the accumulation of metabolites as indicated previously in NST at 48 hours post-rehydration. Additionally, Sucrose and starch metabolism were exclusively up in NST, and this seems to correlate with sucrose and raffinose accumulation seen in NST as an ability to suppress senescence compared to ST that accumulated metabolites involved in valine, isoleucine, and leucine degradation. The reduction of metabolites involved in phenylalanine, tyrosine, and tryptophan biosynthesis in ST during rehydration could also indicate the failure to suppress senescence as the amino acids are involved in growth and development. Interestingly, tryptophan metabolism was

upregulated in NST during dehydration and this correlates to a study by (Kang et al., 2019) that reported an elevation in biosynthetic pathways of some amino acids in drought tolerant genotype of wheat grown under drought conditions. The biosynthetic pathways could be utilised for the biosynthesis of metabolites that are vital in desiccation in *E. nindensis.* However, some metabolic processes were shared between NST and ST such as carbo fixation for photosynthesis was down in both tissues during dehydration, implying that both tissues could sense desiccation though ST was not able to suppress the effects of desiccation.

### 3.2.2 Conclusions on the comparisons of NST and ST

The metabolome of *E nindensis* demonstrated key metabolites that are involved in desiccation and clear differences between NST and ST. NST showed a metabolic halt under acute water-deficit stress reflected by a decrease in metabolites involved in the TCA cycle, which is necessary to terminate energy-consuming processes and initiate a desiccation response. The current study is thought to indicate that more metabolic processes are focused on the NST in favour of promoting a quiescent state, whereas the ST is preparing for senescence. Concerning sugars, it was interesting to note that raffinose accumulation varied between the NST and ST, and the accumulation of raffinose exclusively in NST is likely to have played a key role in the osmoprotection and vitrification process leaving ST vulnerable to the effects of desiccation because a decrease in levels of raffinose was observed. Amino acids and organic acids also varied in the NST and ST during dehydration, where putrescine and α-ketoglutaric acid accumulated predominantly in NST, and this could be due to enhanced regulation in NST than ST in the synthesis of antioxidants and repurposing the energy for other key metabolic processes that can mitigate effects of abiotic stress. The difference in metabolite levels between the two tissues lends credence to the idea that senescence in ST could be age-related because some variation in metabolite abundance correlated to some metabolites that are implicated in senescence. The accumulation of most metabolites at 48 hours post-rehydration in NST is an indicator that most metabolic processes responsible for resumption of metabolism could be taking place at this time point and this has been reported by molecular, ultrastructure and physiological studies previously conducted on NST of *E. nindensis*. Interestingly, most of the results support the transcriptome (Madden, 2019) and proteome data (van Der Pas, 2023), providing more evidence that transcribed transcripts and translated

proteins could effectively regulate metabolic processes in the plant. Accumulation of senescence-related metabolites in the metabolome could be associated with the failure of ST to recover upon rehydration, providing further evidence that age-related senescence is accelerated by drought. This work builds on and adds to a multi-omics multi-disciplinary approach to understanding the mechanisms and processes involved in desiccation tolerance and senescence in *E. nindensis* which ultimately improves and narrows the selection of potential genetic targets for improved drought resistance in drought-sensitive crops through biotechnology.

# Chapter 4 : Comparison of desiccation tolerance in root and shoot system.

## 4.1 Results and interpretations

### 4.1.1 Comparative analysis of leaf and root Relative Water Content

Based on the conclusion between ST and NST as discussed in Chapter 3, NST was considered for comparison between the shoot and root (RT) system. Importantly, no visible signs of senescence and root death were evidenced during dehydration and desiccation. There was a significant difference in the RWC of NST and root during dehydration (Figure 4-1). During early dehydration, the RWC of the roots decreased to 30% after 5 days, and the NST still maintained at 70% RWC. From day 10, similar trends of decrease were observed in both NST and roots. Furthermore, the roots indicated a higher RWC (60%) at 24 hours post-rehydration, then decreased at 48 hours but increased to become similar to that of NST by 1 week post-rehydration. The rapid increase at 24 hours is thought to attributed to be the need for water in the shoot system as water is required for metabolic reactions such as the recovery of photosynthesis.

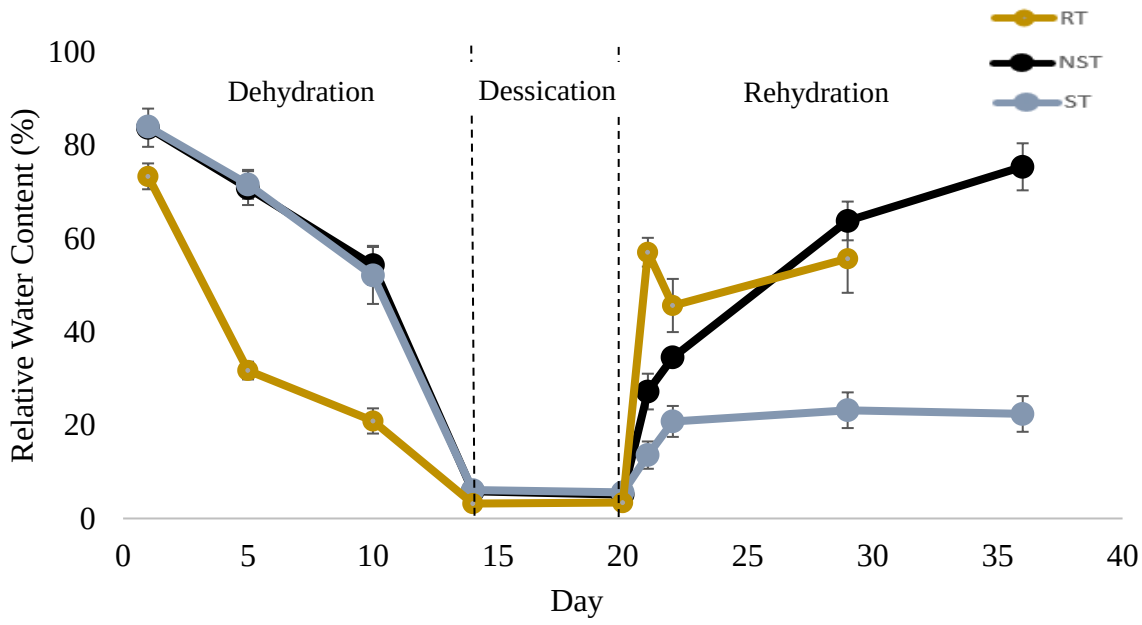



Figure 4-1: Changes in relative water content ( RWC) of *E. nindensis* tissues used for sampling. Five biological replicates were used for the experiments and error bars represent the standard errors across the biological replicates. The graph shows the relative water content of NST, ST and roots across all treatment points and on the same plants at each point.

### 4.1.2 Metabolic changes between NST and Roots during dehydration and rehydration

On analysis of separate tissues for significant metabolites across dehydration and rehydration, most of the metabolites were differentially abundant in the NST (24) compared to the roots (6) under dehydration and rehydration, and 44 were commonly present between leaf and root (Figure 4-2). Metabolites including raffinose, ornithine, phenylalanine, aspartic acid, galactitol, erythritol, quinic acid, and succinic acid were significant only in the leaf sample and trehalose, pyroglutamic, 5-oxo proline and glutamic acid in roots during dehydration and post-rehydration (Figure 4-2). The results show that during dehydration and rehydration, E. nindensis allocates more resources to leaves than roots.

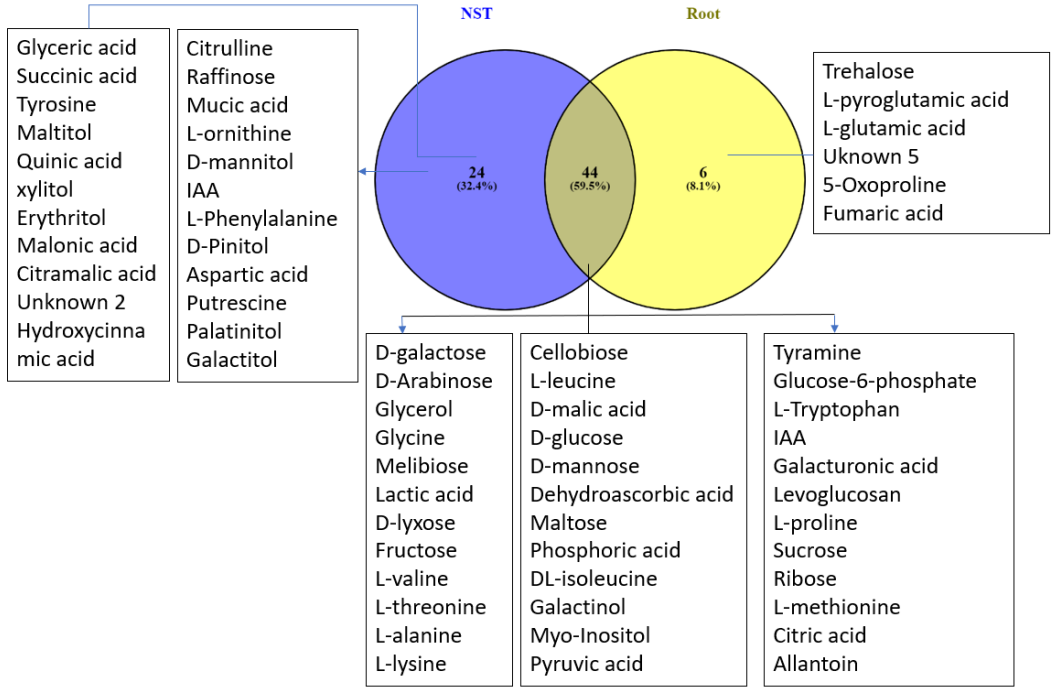
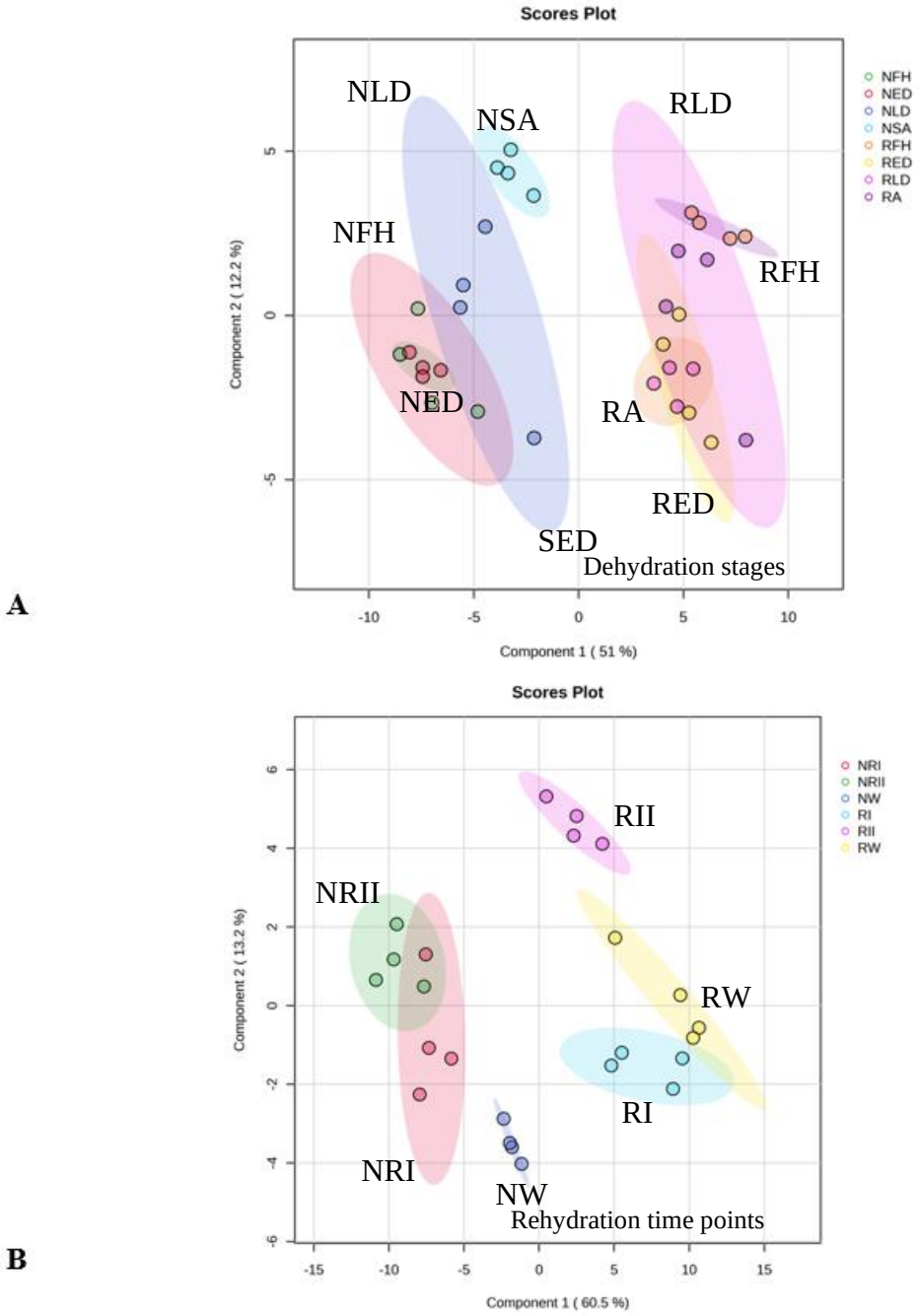



Figure 4-2: Venn diagrams of differential significant metabolites among non-senescent tissue and roots, metabolites exclusive to each tissue type and those shared among the two tissue types. Statistical significance was carried out with a p-value < 0.05.

The differences observed in the RWC data were further evidenced in the PCA results (Figure 4-3). The results explained 63.2% of the total variation during dehydration, in which the first and second PCs separately contributed 51% and 12.2%, respectively. Post-rehydration, there was an increase in total variation (73.7%), explained by component 1(60.5%) and component 2 (13.2%). Thus, a clear separation was observed between the shoot and root system.

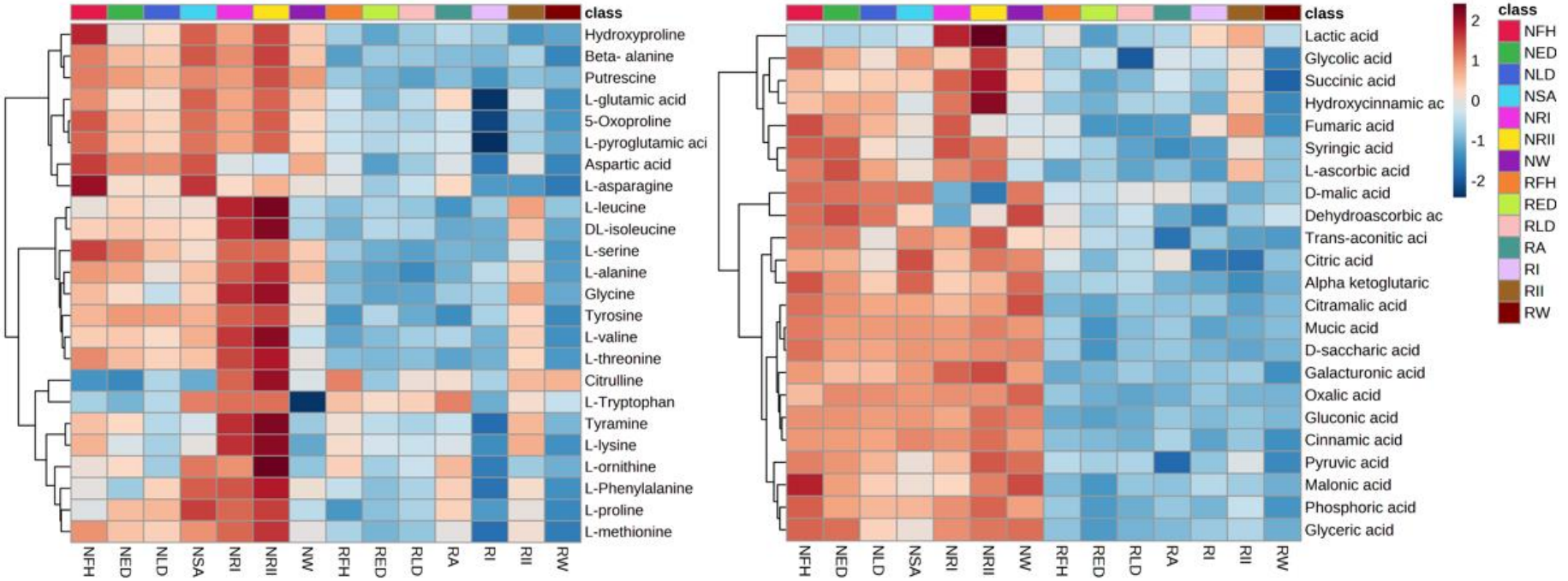



NFH - Non-senescent fully hydrated (> 85% RWC)
NED - Non-senescent early dehydration (75-60% RWC)
NLD - Non-senescent late dehydration (55-40% RWC)
NSA - Non-senescent air-dry (<5% RWC)
NRI - Non-senescent tissue 24 hours post rehydration
NRII - Non-senescent tissue 48 hours post rehydration
NW - Non-senescent tissue 1-week post rehydration

RFH - Root fully hydrated (> 85% RWC)
RED - Root early dehydration 75-60% RWC)
RLD - Root late dehydration (55-40% RWC)
RA - Root air-dry (<5% RWC)
RI - Root 24 hours post rehydration
RII - Root 48 hours post rehydration
RW - Root 1-week post rehydration

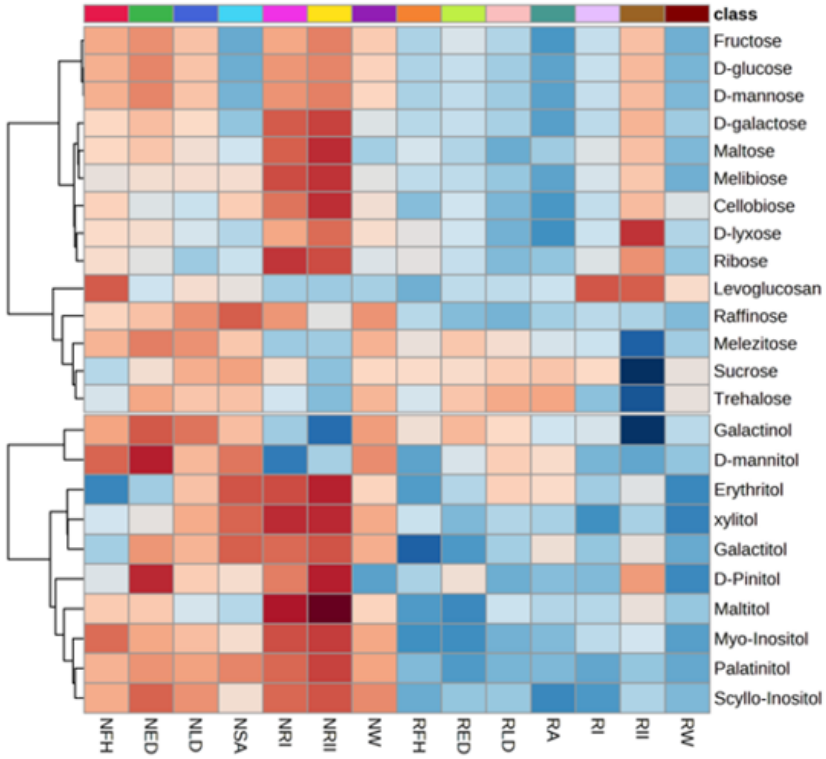
Figure 4-3: Score plots of *E. nindensis* during dehydration and rehydration stages comparing the differences in NST and roots based on principal component analysis. (A) separation across dehydration stages, (B) separation across the rehydration time points. Samples were classified based on RWCs, which are depicted in various colours of the small circles.

In addition, heat maps of differentially significant metabolites between the two tissues during the dehydration stages and post-rehydration were generated (Figure 4-4). Out of those 78 significant metabolites, amino acids make up most metabolites (24), followed by organic acids (23), sugar (14), sugar alcohol (10), phytohormones (2), fatty acids (1), and other metabolites (4). The heatmaps (Figure 4-4and **Error! Reference source not found.**) revealed a high accumulation of D-galactose, D-ribose, cellobiose, glucose, ornithine, threonine, glycine, valine, methionine, cellobiose, maltose, glucose, fructose, glycerol, galactinol, raffinose, mucic acid, mannitol, indole-acetic acid (IAA), phenylalanine, aspartic acid, putrescine and palatinitol in NST with decreased abundance in the roots. However, in the roots only 6 metabolites namely, allantoin, trehalose and tryptophan accumulated during dehydration whereas ribose, lyxose and levoglucosan accumulated during rehydration. In the NST most of the organic acids accumulated during dehydration and rehydration. Contrary, all organic acids decreased in the roots during dehydration whereas fumaric acid, succinic acid, L-ascorbic acid, and lactic acid increased at 48 hours post-rehydration, though the levels were still lower than those seen in NST (Figure 4-4B). The levels of trehalose during dehydration were higher in roots than those seen in NST. Most of the metabolites that were shown to be significant in the roots (amino acids oxo-proline, glutamic acid, pyroglutamic acid) reduced at 48 hours post-rehydration (Figure 4-4A) and similarly sucrose levels were predominantly low upon rehydration in roots (**Error! Reference source not found.**A).

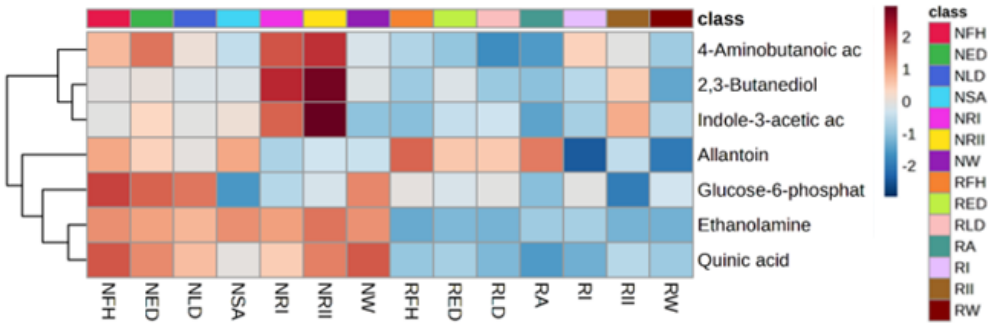



NFH - Non-senescent fully hydrated (> 85% RWC)
NED - Non-senescent early dehydration (75-60% RWC)
NLD - Non-senescent early dehydration (55-40% RWC)
NSA - Non-senescent air-dry (<5% RWC)
NRI - Non-senescent tissue 24 hours post rehydration
RFH - Root fully hydrated (> 85% RWC)
RED - Root early dehydration 75-60% RWC)
RLD - Root early dehydration (55-40% RWC)
NW - Non-senescent tissue 1-week post rehydration
NRII - Non-senescent tissue 48 hours post rehydration
RI - Root 24 hours post rehydration
RII - Root 48 hours post rehydration
RW - Root 1-week post rehydration.
RA - Root air-dry (<5% RWC)

Figure 4-4: Heatmaps of differentially abundant organic acids and amino acids in *E. nindensis* across the dehydration stages and rehydration time points in NST and roots. A) heatmap of amino acids and B) heatmap showing organic acids. Significance was based on ANOVA Fisher's LSD with P< 0.05.




A




B

NFH - Non-senescent fully hydrated (> 85% RWC)
NED - Non-senescent early dehydration (75-60% RWC)
NLD - Non-senescent early dehydration (55-40% RWC)
NSA - Non-senescent air-dry (<5% RWC)
NRI - Non-senescent tissue 24 hours post rehydration
NRII - Non-senescent tissue 48 hours post rehydration
NW - Non-senescent tissue 1-week post rehydration
RFH - Root fully hydrated (> 85% RWC)
RED - Root early dehydration 75-60% RWC)
RLD - Root early dehydration (55-40% RWC)
RA - Root air-dry (<5% RWC)
RI - Root 24 hours post rehydration
RII - Root 48 hours post rehydration
RW - Root 1-week post rehydration.

Figure 4-5: Heatmaps of differentially abundant sugars, sugar alcohols, phytohormones and other metabolites in *E. nindensis* across the rehydration time points in NST and roots. Significance was based on ANOVA Fisher's LSD with P< 0.05.

## 4.2 General Discussion and conclusion

### 4.2.1 General Discussion

Multiple studies have linked several methods used to deal with desiccation tolerance with the accumulation of several metabolites in leaves for quenching ROS, acting as compatible solutes and signalling molecules (Moyankova et al., 2014; Xu et al., 2021; Yobi et al., 2012). Most, if not all, have primarily focused on the aerial parts of the plant resulting in a general lack of understanding of the changes occurring belowground. The current study explored the metabolic alterations in the shoot and root to understand which metabolic processes are used by the whole plant to mitigate the associated damage from drought and desiccation stress. NST was used to compare the shoot with the root, and this was because it showed over 80% recovery at 1 week upon rehydration. The whole plant was analysed and assessed for allocation of metabolites for different metabolic functions in roots and leaves, thereby assessing the contrasting responses of shoot and root to desiccation. From the physiological data, the roots' response to dehydration is different as roots illustrated a rapid decrease in water content in the first 5 days of water deficit (Figure 4-1). The hasty loss of water by roots could be due to the ‘cohesion-tension’ hypothesis which is one of the ways water movement across plants from roots to leaves caused by transpiration to facilitate stomata opening allowing gaseous exchange for photosynthesis (Steudle, 2001; Vesala et al., 2017). However, a physiological study conducted on *E. nindensis* by Ginbot and Farrant (2011) showed a clear decline in transpiration rate after 3 days of dehydration which suggests that this could contribute to the gradual decrease in roots RWC during late dehydration to air-dry state as less water is pushed upwards striking a balance between the shoot and root water content. Importantly, the decline in transpiration correlates with leaf folding as stated in Chapter 3 and it is reported to be an initial mechanism to deal with drought stress correlating with the accumulation of GABA at early dehydration and could likely have a role in the regulation of stomatal conductance as discussed in Chapter 3.

Concerning metabolic alterations, the accumulation of sugars is likely to enhance the plant's response to abiotic stress as earlier discussed in Chapter 3 and supported by other studies (Ahmad et al., 2020; Caffrey et al., 1988). The current study indicated an

accumulation of sugars, but their relative accumulation was higher in the NST than in the roots. NST highly accumulated raffinose and sucrose during dehydration, whereas roots accumulated trehalose during dehydration and then levoglucosan, d-lyxose and ribose upon rehydration. Interestingly, raffinose was not significant in comparison of ST with roots and low levels of trehalose were observed in air dry state in ST (see Appendix Figure S-10), and this adds up to the previous processes discussed in Chapter 3 speculating the failure of ST to recover as most of the metabolic shifts could not favour the survival of ST post-rehydration. The fact that sugars are important energy stores during desiccation as postulated in Chapter 3, leaves and roots might be utilising different energy stores due to their differences in sugar accumulation. Additionally, sugars play a role in plant growth and development and are involved in several metabolic processes in plants (Mishra et al., 2022). However, low levels of sugars were indicated in the roots during dehydration, and this could imply that the leaves could be utilising more sugars in protecting the photosynthetic apparatus of the plant as it is needed for the resumption of metabolism upon rehydration. Thus, the roots could be translocating most of the sugars to the shoot as more metabolic processes take place in the shoot than root. The low levels of trehalose in the leaves also seem to indicate that trehalose could be retained in the roots during translocation and likely to have a role in abiotic stress response in roots. Additionally, the higher accumulation of sugar alcohols in NST rather than roots might play a vital role in oxidative damage protection as previously discussed in Chapter 3. Since the leaves are directly exposed and affected by drought and the fact that they contain photosynthetic apparatus, they require a series of metabolic processes to adapt to abiotic stress and this could be the reason for the accumulation of more sugars and sugar alcohols seen in *E. nindensis* NST than the roots. Low levels of sucrose and sugar alcohols, mannitol and erythritol were detected in the roots, which is contrary to a study by Norwood et al. (2003) that indicated accumulation of sucrose in roots of *C. plantagineum*. Therefore, the low levels of sucrose in the current study seem to suggest that *E. nindensis* drains its carbohydrate reserves in the roots to the shoot system to aid in the survival of the NST during desiccation and post-rehydration. This is further shown by low to no traces of sucrose observed in roots at 48 hours post-rehydration, indicating that sucrose could be remobilised for the synthesis of simple sugar required for plant growth and development. Furthermore, previous studies have suggested that a plant may regard a series of sources as creating a priority among the

sinks, and the young leaves and roots are the major sinks during early growth stages (Wardlaw, 1990), and this could suggest that most of the sugars could alternatively be mobilised and translocated via the phloem to the NST hence the low levels indicated in the roots. Interestingly, levoglucosan, a cell wall sugar (Szechyńska-Hebda et al., 2016) peaks upon rehydration in roots suggesting metabolic activity within the cell wall post-rehydration. This rapid accumulation of levoglucosan has not been reported in previous studies, thus unclear but could reflect the repair of plant cell walls during plant recovery as levoglucosan is formed from pyrolysis of cellulose. The accumulation of ribose at 48 hours followed by decrease at one week upon rehydration could also be attributed to its role as a building block for (Deoxyribonucleic acid (DNA) and Ribonucleic acid (RNA) synthesis that is required for expression and synthesis of metabolites during development of the plant and also as source of ATP generation (Riggs et al., 2016).

As earlier indicated, the roots accumulated high levels of trehalose during dehydration. Trehalose has been proposed to play a role in the acquisition of desiccation tolerance in bacteria, fungi, yeast and plants (Elbein et al., 2003). Accumulation of trehalose has been reported in several resurrection plants mostly in the lower order *Selaginella* species as part of their mechanism to survive desiccation (Dinakar & Bartels, 2013). In addition to what is discussed in Chapter 3**,** trehalose can help prevent protein denaturation by decreasing cellular osmotic potential and acting as a chemical chaperone (Benaroudj et al., 2001). Similarly, trehalose accumulation in *S. lepidophylla* is associated with the protection of protein and membrane structures during desiccation (Goddijn & van Dun, 1999) and this is thought to comprehend the role indicated in the present study. Previous studies conducted on plant rhizobia also indicated that trehalose is vital for the survival of rhizobium under symbiotic relationships and alleviates abiotic and biotic stress (Bharti et al., 2022). Thus, the accumulation of trehalose in roots and NST is thought to play a role, in conjunction with sucrose in coping with abiotic stress though not conclusive whether it was produced from roots or soil microbiome. Furthermore, Garcia et al. (1997) reported trehalose accumulation in rice roots 3 days following salt stress, and a study by Asami et al. (2019) showed how the accumulation of sucrose and trehalose-6-phosphate in the roots of *Tripogon loliiformis* helped the plant survive desiccation. This correlates with the current study that indicated high levels of trehalose in roots during dehydration

suggesting that it could play a vital role in the survival of roots that showed a high level compared to NST and ST. A previous study by Sarkar et al. (2007) reported that trehalose could also regulate autophagy in mammals via the M-TOR-independent autophagy pathway. Autophagy is a biological process that is linked to increased lifespan in both mammals and plants. This association can be attributed to its crucial function in maintaining cellular homeostasis by eliminating damaged or unnecessary cellular components. A study conducted by Williams et al. (2015) identified a novel role of trehalose in the regulation of autophagy to prevent programmed cell death (PCD) and suppress senescence in the leaves of *T. loliiformis*. This seems to suggest that trehalose could play a similar role in autophagy induction and senescence suppression in the roots of *E. nindensis*. Therefore, the accumulation of trehalose in the roots of *E. nindensis* could imply that it is being utilised to activate autophagy corroborating a unique survival strategy in roots to survive desiccation. The root system of *E. nindensis* demonstrated survival of desiccation without induction of senescence. ST showed reduced levels of trehalose during dehydration (see Appendix Figure S-10), and this could contribute to ST failure to overcome. This further supports the hypothesis that the metabolic shift between the roots and ST does not support survival of the latter. It is interesting to note that there was no regeneration of new roots post-rehydration, and all the old roots were able to recover and survive desiccation unlike the ST that showed no recovery.

Alternatively, the accumulation of trehalose in the roots of *E. nindensis* during dehydration and peaking at the dry state could be associated with its analogous role to sucrose in stabilizing dry membranes and the vitrification process during desiccation (Crowe & Crowe, 1990; Leslie et al., 1995). What seems to be happening is that there could be tissue-specific expression of genes related to raffinose (in leaves) and trehalose (in roots); suggesting that as a whole plant, it cannot be exclusively classified as a pure RFO accumulator as indicated in Chapter 3. This further strengthens the argument that roots need to be included in further studies to gain a much better understanding of the global changes in the plant and that focusing only on aerial tissue inevitably leads to misinformation. The hypothesized mechanisms above seem to indicate the ability of the root to inhibit senescence could contribute to the survival of the whole plant because of continuous metabolic activity during desiccation to facilitate water and nutrient uptake during dehydration and post-recovery.

As discussed in Chapter 3, the accumulation of amino acids upon dehydration could enhance the ability of plants to quench ROS and act as intermediates in various biosynthetic pathways that are vital during desiccation. All amino acids accumulated to high levels in NST compared to the roots. However glutamic acid, pyroglutamic acid and oxo-proline showed a significant alteration upon dehydration and rehydration in roots, unlike the NST that showed alteration in levels of aspartic acid, phenylalanine, citrulline and putrescine. The alteration in roots indicated a decline in levels of glutamic acid and oxo proline upon rehydration at 48 hours post-rehydration (Figure 4-4A), contrary to NST which showed an increase in levels of these amino acids. This seems to support the hypothesis that amino acids could be translocated from source to sink when there is a need for utilisation. Accumulation of glutamic acid has been associated with desiccation tolerance in many resurrection plants and high levels have been noticed in the leaves of resurrection plants as indicated in Figure 1-1. However, less is known for roots. The decline in glutamic acid in roots is consistent with its accumulation in leaves at the same rehydration point, suggesting that glutamic acid could be translocated to the leaves for nitrogen metabolism. Proline and phenylalanine accumulated in both roots and NST in the dry state, but significant levels were seen in roots and a previous study indicated that similar levels of proline enhanced root hair formation and increased root biomass leading to plant growth even under harsh conditions (Meena et al., 2019). A previous study by Ghorbanzadeh et al. (2023) indicated an accumulation of phenylalanine and isoleucine in roots of drought-tolerant rice under drought stress conditions which correlates with the current finding which illustrated an accumulation of phenylalanine, a vital precursor for the synthesis of secondary metabolites that act as antioxidants during desiccation. In the roots, there was a significant increase observed in the levels of tryptophan compared to amino acids during desiccation. L-tryptophan serves as a physiological precursor to auxin and plays a critical role in mitigating the effects of drought (Rao et al., 2012). The synthesis of auxin occurs through the shikimic acid pathway, starting with tryptophan and previous studies have demonstrated that an elevation in auxin concentrations facilitates the growth of shoots and roots in a nitrogen-rich environment (Zhang et al., 2023). Furthermore, a study by Bowne et al. (2012) reported an increase in tryptophan exclusively in drought-tolerant wheat varieties. Considering the involvement of tryptophan in the metabolic pathways of glycine, serine, and threonine , it is plausible

to suggest that tryptophan may enhance roots of *E. nindensis* to overcome desiccation by contributing to the process of root elongation.

The abundance of the organic acids was higher in the NST than in the roots. The leaves significantly accumulated citric acid and alpha-ketoglutaric acid in the dry state, however, most of the organic acids apart from malic acid and citric acid decreased in the roots. This seems to suggest that the organic acids could be playing a role in the TCA cycle and other metabolic reactions in the NST rather than the roots. A previous study on maize roots showed reduced activity of TCA cycle enzymes in root mitochondria hence reduced generation of ATP and NADH (Jiang et al., 2006). Therefore, the low accumulation of organic acids observed in roots could be attributed to the mobilisation and translocation to the shoot system to generate the required ATP for metabolic processes during desiccation. Conversely, fumaric acid, ascorbic acid and succinic acid accumulated at 48 hours upon rehydration in roots. Their accumulation seems to reflect the resumption of the TCA cycle to generate ATP-dependent metabolic processes indicating a similar role observed in NST. Besides organic acid, fatty acids are also vital during desiccation, especially in maintaining cell wall integrity, carbon source reserve and synthesis of signalling molecules (He & Ding, 2020). However, a decrease in fatty acids abundance was observed in the roots and ST, and the explanation is unclear unlike the NST that accumulated significant levels of quinic acid, a key metabolite in protecting the membranes of cells, and high levels have been reported in leaves of *M. flabelifolia* (Bentley et al., 2019*)*. The higher levels of quinic acid in the NST could be due to chloroplast lipid liberation following the disassembly of thylakoids whereas the low levels in the roots could be due to the fact that roots do not have organelles that could be broken down to increase levels of quinic acid.

In the current study, allantoin, a purine metabolite in the nitrogen cycle accumulated during dehydration in both NST and roots. It was highly abundant in the roots in the dry state and similar levels were reported in roots of a drought-tolerant genotype of rice (Ghorbanzadeh et al., 2023) grown under drought-stress conditions. More so, a study on wheat reported an accumulation of allantoin in wheat exposed to water and nitrogen limitation. Additionally, rice treated with exogenous allantoin at different concentrations under drought stress conditions accumulated significant metabolites involved in desiccation including soluble sugars and up-regulation of genes related to

scavenging ROS (Lu et al., 2022). The high abundance of allantoin in the dry state evaluated here suggests that it could play a comparable function in the roots of *E. nindensis* by increasing antioxidant activity during desiccation. These outcomes are additionally comparable to those seen in the DT grass *S. stapfianus*, in which allantoin accumulation increased when RWC dropped to 40% or less (Oliver et al., 2011). From the differences in the metabolic shifts between shoot and root, the majority of metabolites that accumulated in ST during desiccation were not key in driving processes that overcame desiccation, and this could lead to failure of tissue to recover post-rehydration.

### 4.2.2 General conclusions and prospects.

The metabolic study presents key information to understanding the drivers of desiccation tolerance of *E. nindensis* at the omics level. Despite using a different population of *E. nindensis* from that used in previous omics studies, there were several similarities, suggesting a high degree of conserved mechanisms between populations. Most of the metabolites were more differentially abundant in the NST compared to ST and roots. The accumulation of desiccation-responsive metabolites in predominantly NST represents the survival capability of NST unlike the ST that had few differentially abundant metabolites, clearly indicating the differences in the two tissues and this is also seen in the physiological data. The metabolites that accumulated in NST have been previously reported in desiccation tolerance and this seems to suggest that similar processes that drive desiccation tolerance are seen in *E. nindensis*. The metabolism in NST indicated a shift towards the production of osmolytes and antioxidants with accumulation of raffinose, sucrose, aspartic and glutamic acid with the latter being involved in biosynthetic pathways for synthesis of antioxidants. Considering the fact that putrescine, a precursor for the biosynthesis of secondary metabolites accumulated exclusively in NST, it would be interesting to do targeted metabolomics and secondary metabolite analysis for example in all dehydration levels and see if some of these precursors could be involved in the synthesis of key secondary metabolites such as spermidine that is involved in formation of alkaloids that can aid in mitigation of abiotic stress in *E. nindensis*, though the previous omes did not show any relationship between secondary metabolites and desiccation even when it's known in some other resurrection plants such *M. flabellifolia* where secondary metabolism is essential. One of the interesting observations was the

possibility of channelling of TCA intermediates to other biosynthetic pathways observed in NST for instance α-ketoglutaric acid decreases during dehydration and this seems to support the possibility of being utilised in GS/GOGAT pathways for the synthesis of glutamine, an amino acid that could be involved in nitrogen metabolism during dehydration and recovery. The GS/GOGAT pathway seems to be shut down in ST because there was less abundance of all TCA intermediates. However, the accumulation of BCAAs in ST raises some speculation as to whether these amino acids could be utilised in nitrogen recycling to aid in the survival of NST, but this might not be conclusive and further investigations are needed to validate this hypothesis. Most of the metabolic processes were downregulated in ST as observed in the metabolic pathways analysis, indicating the failure of ST to suppress senescence.

While there was evidence of differences in metabolite accumulation across the NST and the ST, there is no evidence as to whether the metabolites are translocated from the ST to the NST during the process of senescence in the ST and whether the ST sacrifices itself for the survival of NST. It would be interesting to use other techniques such as isotope labelling and NMR to further investigate which metabolites are translocated or recycled from one tissue as the current study speculates that there could be translocation of metabolites from the ST to the NST, the roots to the NST and the roots to the ST. Furthermore, the accumulation of glutamic and aspartic acid in NST compared to ST and roots could be attributed to the nitrogen remobilisation process, suggesting that NST and roots could be utilising glutamine as a major nitrogen donor upon rehydration. The low abundance of metabolites such as raffinose, glutamic acid, sucrose and aspartic acid in the ST could also suggest that at least the tissue could be failing to mitigate the effects of desiccation and therefore committing to senescence. Depending on the data generated, there was less evidence to validate the senescence observed in the ST, however, it could still be age-dependent as hypothesized by van der Pas (2023). There is also an argument that cinnamic acid accumulation can cause plant cell wall lignification, which is one of the senescence indicators. However, there is no evidence to validate whether the accumulation of cinnamic acid in ST could be an indicator of senescence. The ultrastructural studies by Madden (2019) did not indicate any instance of lignification and despite the lack of confirmatory analysis, it remains a hypothesis as less data is available to validate the relationship of cinnamic acid to age-dependent senescence. KEGG pathway analysis

demonstrated metabolic processes that are involved in desiccation such as starch and sucrose metabolism which might be responsible for the accumulation of raffinose exclusively in NST compared to ST that demonstrated upregulation of metabolic processes that could be involved in senescence such as valine, isoleucine and leucine degradation reflecting of accumulation BCAAs in ST. The shoot-root interaction was an interesting one; the two tissues showed an exclusive accumulation of raffinose and trehalose in NST and root respectively during desiccation. This is the first study to show such a difference in resurrection plant tissues and this is presumably an indication that different parts of the plant could be employing different metabolic processes to mitigate the effects of desiccation. The decrease in sucrose and glutamic acid at 48 hours in the roots also indicated the possibility of translocation from the ground to the aerial parts of the plant though the current study could not validate this hypothesis. Due to differences in the metabolite composition between NST, ST and roots, with NST showing greater variability and roots showing fewer alterations, could indicate that more metabolites are likely to be allocated to the NST than the roots and ST, and hence resource remobilization and translocation could differ across different tissues. There was less data to understand the processes that are involved in desiccation tolerance in the roots as fewer metabolites varied across dehydration and post-rehydration. Therefore, it would be interesting to further investigate to what extent the roots are able to mitigate desiccation by using other omics data sets and ultrastructure studies. Most importantly, it would be important to understand whether there could be a root-associated microbiome because a recent study by Tebele et al. (2023) reported how roots and soil microbiome have contributed to the accumulation of metabolites involved in the desiccation tolerance of plants . However, the information in the results may, with more research, aid in understanding the desiccation tolerance of the entire resurrection plant. Recent progress in grass seed grafting (Reeves et al., 2022) holds promise to elucidate the compartmentalised contribution of desiccation tolerance between the roots and shoots. *E. tef* shoots can be grafted onto *E. nindensis* roots and vice versa to explore to what extent each compartment contributed to desiccation tolerance.

To conclude, we hypothesize that there could be translocation between the roots and NST or ST and roots. However, the differential abundance of metabolites observed in NST seems to contribute to all the metabolic processes that aid in the survival of *E.*

*nindensis*. The phenomenon of vegetative desiccation tolerance remains a fascinating one and will continuously draw the attention of scientists to explore new processes involved in it. This study was able to unravel the key metabolites involved in the desiccation tolerance mechanism employed by the whole plant to mitigate the effects of desiccation. However, much of such metabolomic work especially on roots to understand which metabolites drive vegetative desiccation tolerance is needed. This research however provides more supporting information to support some of the findings from the transcriptome and proteome data. This could therefore help in the selection of potential candidate genes for the genetic transformation of sister species *E. tef* and other crops such as sorghum and maize.

# Chapter 5 : Appendix

## 5.0 Supplementary data

Table S5-1: Table showing differentially abundant metabolites in both senescent and non-senescent tissue during dehydration and rehydration. ($P < 0.05$)

| Metabolites | f.value | p.value | | f.value | p.value |
|---|---|---|---|---|---|
| 2,3-Butanediol | 19.24 | 1.29E-13 | D-mannose | 9.4276 | 1.08E-08 |
| Glyceryl-glycoside | 18.938 | 1.69E-13 | DL-isoleucine | 9.1292 | 1.70E-08 |
| Dehydroascorbic acid | 16.43 | 1.88E-12 | Maltose | 8.9725 | 2.17E-08 |
| xylitol | 16.139 | 2.53E-12 | Myo-Inositol | 8.5238 | 4.44E-08 |
| D-galactose | 15.542 | 4.73E-12 | Erythritol | 8.4978 | 4.63E-08 |
| Lactic acid | 14.277 | 1.89E-11 | Phosphoric acid | 8.086 | 9.13E-08 |
| Melibiose | 13.948 | 2.76E-11 | L-valine | 7.9591 | 1.13E-07 |
| D-Arabinose | 13.728 | 3.56E-11 | Unknown 7 | 7.5592 | 2.25E-07 |
| L-leucine | 13.577 | 4.25E-11 | L-Threonic acid | 7.4433 | 2.75E-07 |
| Fructose | 12.839 | 1.03E-10 | Raffinose | 7.4075 | 2.93E-07 |
| Pyruvic acid | 12.746 | 1.15E-10 | Succinic acid | 7.3563 | 3.21E-07 |
| D-lyxose | 12.729 | 1.18E-10 | Cinnamic acid | 6.7692 | 9.30E-07 |
| Quinic acid | 12.606 | 1.37E-10 | Tyramine | 6.7556 | 9.54E-07 |
| Cellobiose | 12.438 | 1.69E-10 | Galactinol | 6.6665 | 1.13E-06 |
| D-malic acid | 11.731 | 4.19E-10 | Malonic acid | 6.0554 | 3.64E-06 |
| Glyceric acid | 11.589 | 5.05E-10 | Glucopyranose | 6.0388 | 3.76E-06 |
| Glycine | 11.393 | 6.54E-10 | Mucic acid | 5.9075 | 4.88E-06 |
| Uracil | 10.986 | 1.14E-09 | Glucose-6-phosphate | 5.8704 | 5.26E-06 |
| Glycerol | 10.717 | 1.65E-09 | L-proline | 5.8245 | 5.77E-06 |
| Unknown 1 | 10.383 | 2.64E-09 | Melezitose | 5.6703 | 7.87E-06 |
| D-glucose | 10.045 | 4.29E-09 | Tyrosine | 5.5995 | 9.10E-06 |
| Putrescine | 5.0967 | 2.60E-05 | Syringic acid | 3.2911 | 0.001681 |
| Galacturonic acid | 5.0068 | 3.16E-05 | Unknown 3 | 3.2501 | 0.001861 |

| | | | | | |
|---|---|---|---|---|---|
| L-alanine | 4.9179 | 3.83E-05 | L-methionine | 3.2077 | 0.002068 |
| Citrulline | 4.7434 | 5.60E-05 | Unknown 2 | 3.1477 | 0.002402 |
| Glycerol 1-phosphate | 4.48 | 0.000101 | D-sorbitol | 3.1378 | 0.002463 |
| D-mannitol | 4.448 | 0.000108 | Oxalic acid | 3.1243 | 0.002547 |
| L-Phenylalanine | 4.4382 | 0.000111 | Fumaric acid | 2.9608 | 0.003844 |
| L-lysine | 4.3312 | 0.000141 | Allantoin | 2.8219 | 0.005468 |
| Ribose | 4.2337 | 0.000177 | Citramalic acid | 2.8096 | 0.005641 |
| L-Tryptophan | 4.0432 | 0.000275 | Indole-3-acetic acid | 2.6784 | 0.007889 |
| Sucrose | 3.899 | 0.000386 | Hydroxycinnamic acid | 2.6477 | 0.008535 |
| L-threonine | 3.8629 | 0.00042 | Gluconic acid | 2.5823 | 0.010096 |
| Maltitol | 3.855 | 0.000428 | L-ornithine | 2.5793 | 0.010174 |
| D-Pinitol | 3.8213 | 0.000464 | Unknown 6 | 2.5619 | 0.010641 |
| Ethanolamine | 3.6578 | 0.000686 | L-glutamic acid | 2.5032 | 0.012376 |
| Levoglucosan | 3.6411 | 0.000714 | Aspartic acid | 2.3588 | 0.017963 |
| 4-Aminobutanoic acid | 3.5898 | 0.000809 | L-ascorbic acid | 2.2068 | 0.026595 |
| Galactitol | 3.5469 | 0.000897 | Glycolic acid | 2.1566 | 0.030268 |
| Alpha ketoglutaric acid | 3.4181 | 0.001229 | Unknown 4 | 2.0356 | 0.041326 |
| Hydroxyproline | 3.418 | 0.00123 | Citramalic acid | 2.8096 | 0.005641 |
| Scyllo-Inositol | 3.3429 | 0.001479 | Indole-3-acetic acid | 2.6784 | 0.007889 |
| Palatinitol | 3.3327 | 0.001517 | Hydroxycinnamic acid | 2.6477 | 0.008535 |
| Syringic acid | 3.2911 | 0.001681 | Gluconic acid | 2.5823 | 0.010096 |
| Unknown 3 | 3.2501 | 0.001861 | L-ornithine | 2.5793 | 0.010174 |
| L-methionine | 3.2077 | 0.002068 | Unknown 6 | 2.5619 | 0.010641 |
| Unknown 2 | 3.1477 | 0.002402 | L-glutamic acid | 2.5032 | 0.012376 |
| D-sorbitol | 3.1378 | 0.002463 | Aspartic acid | 2.3588 | 0.017963 |
| Oxalic acid | 3.1243 | 0.002547 | L-ascorbic acid | 2.2068 | 0.026595 |
| Fumaric acid | 2.9608 | 0.003844 | Glycolic acid | 2.1566 | 0.030268 |
| Allantoin | 2.8219 | 0.005468 | Unknown 4 | 2.0356 | 0.041326 |

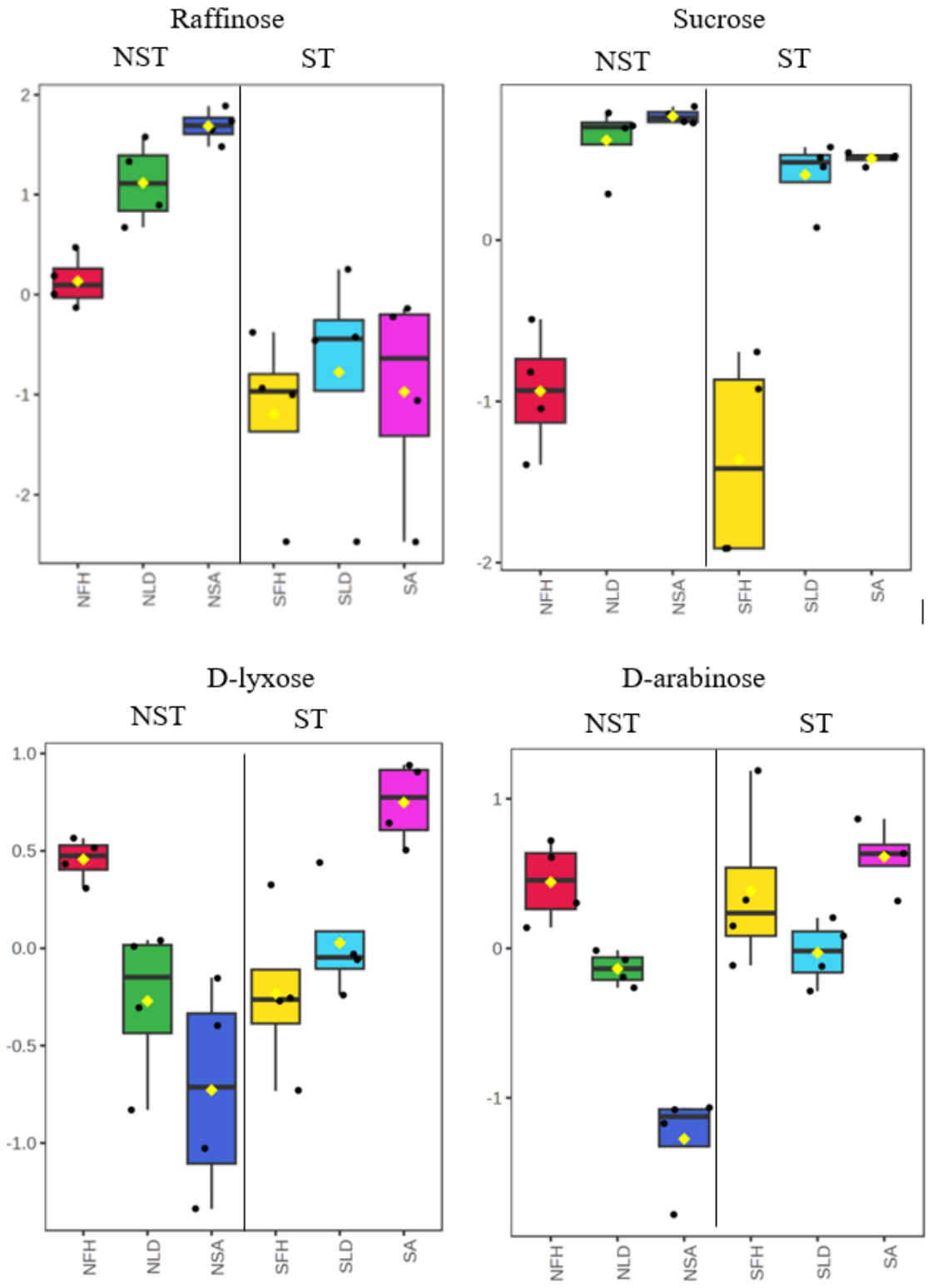



Figure S5-1: Differences in the abundance of sugars in *E. nindensis* dehydration conditions between non-senescent tissue (NST), in red, and senescent tissue (ST), Statistically significant changes are represented as box plots where a log2 fold change of >1 represents significant higher abundances in the NST compared to the ST.

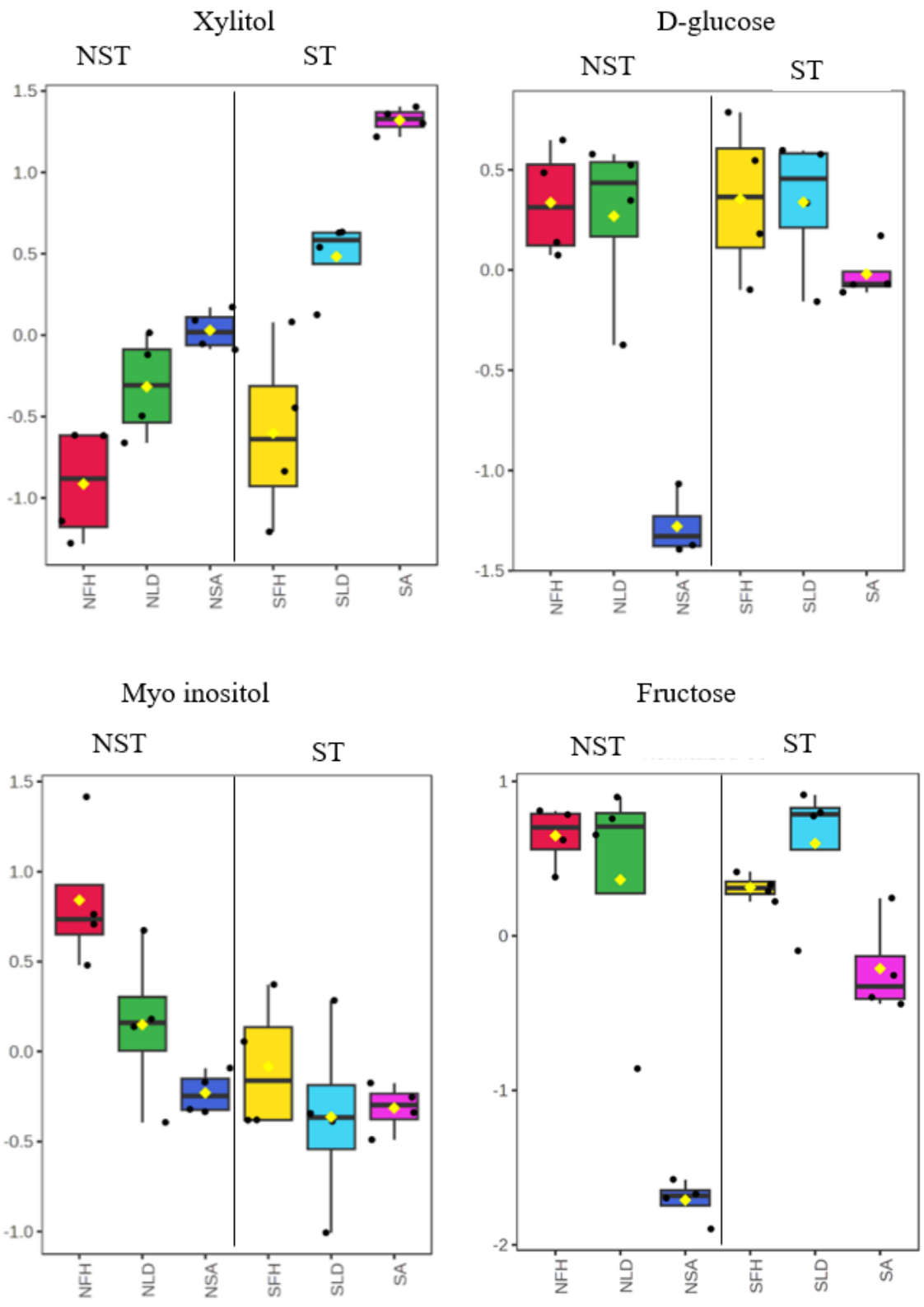



Figure S5-2: Differences in the abundance of sugars and sugar alcohols in *E. nindensis* dehydration conditions between non-senescent tissue (NST), in red, and senescent tissue (ST), Statistically significant changes are represented as box plots where a log2 fold change of >1 represents a significantly higher abundance in the NST compared to the ST.

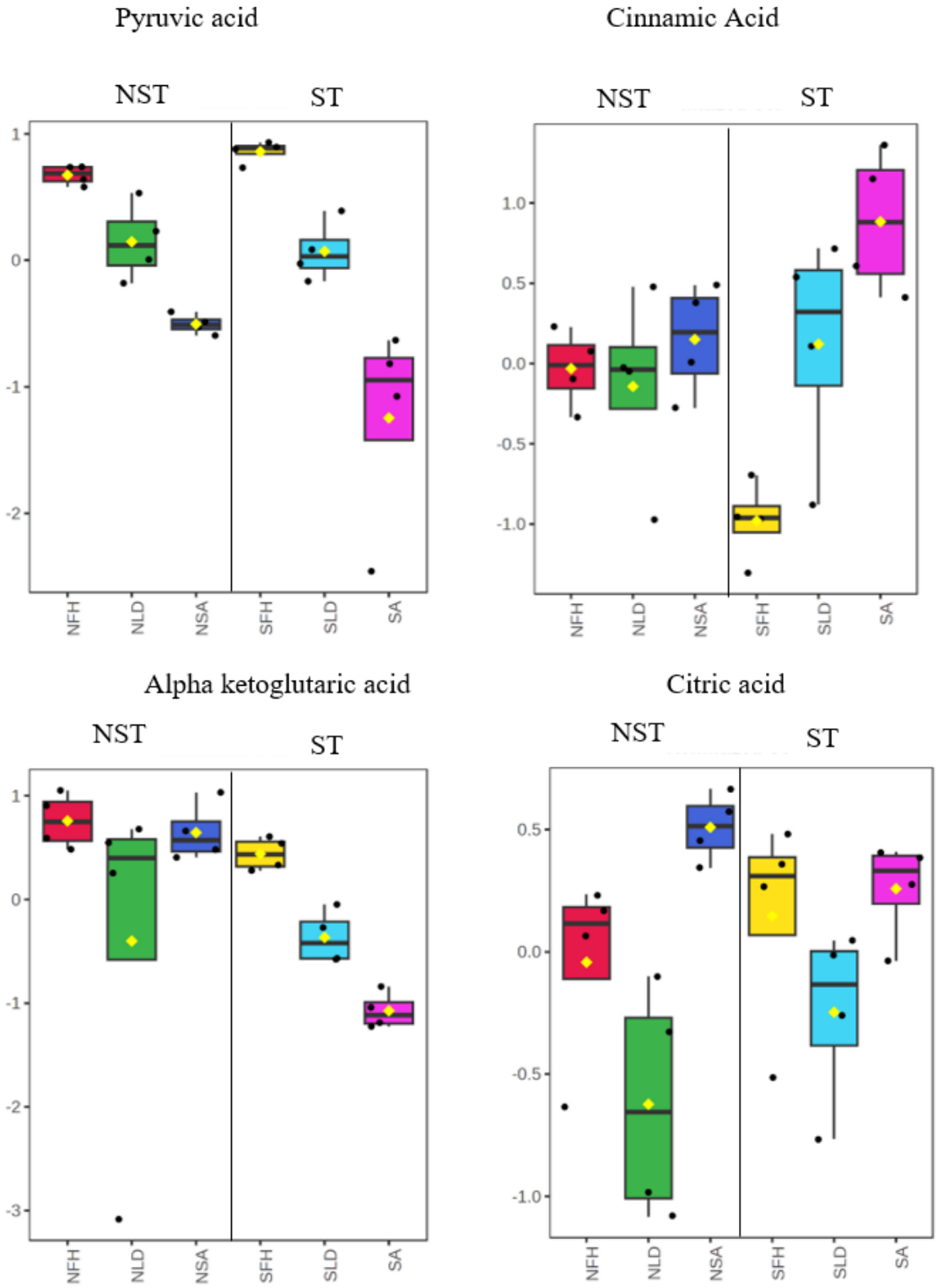



Figure S5-3: Differences in the abundance of sugars alcohols in *Eragrostis nindensis* dehydration conditions between non-senescent tissue (NST), in red, and senescent tissue (ST), Statistically significant changes in fold change are represented as box plots where a log2 fold change of >1 represents significantly higher abundances in the NST compared to the ST.

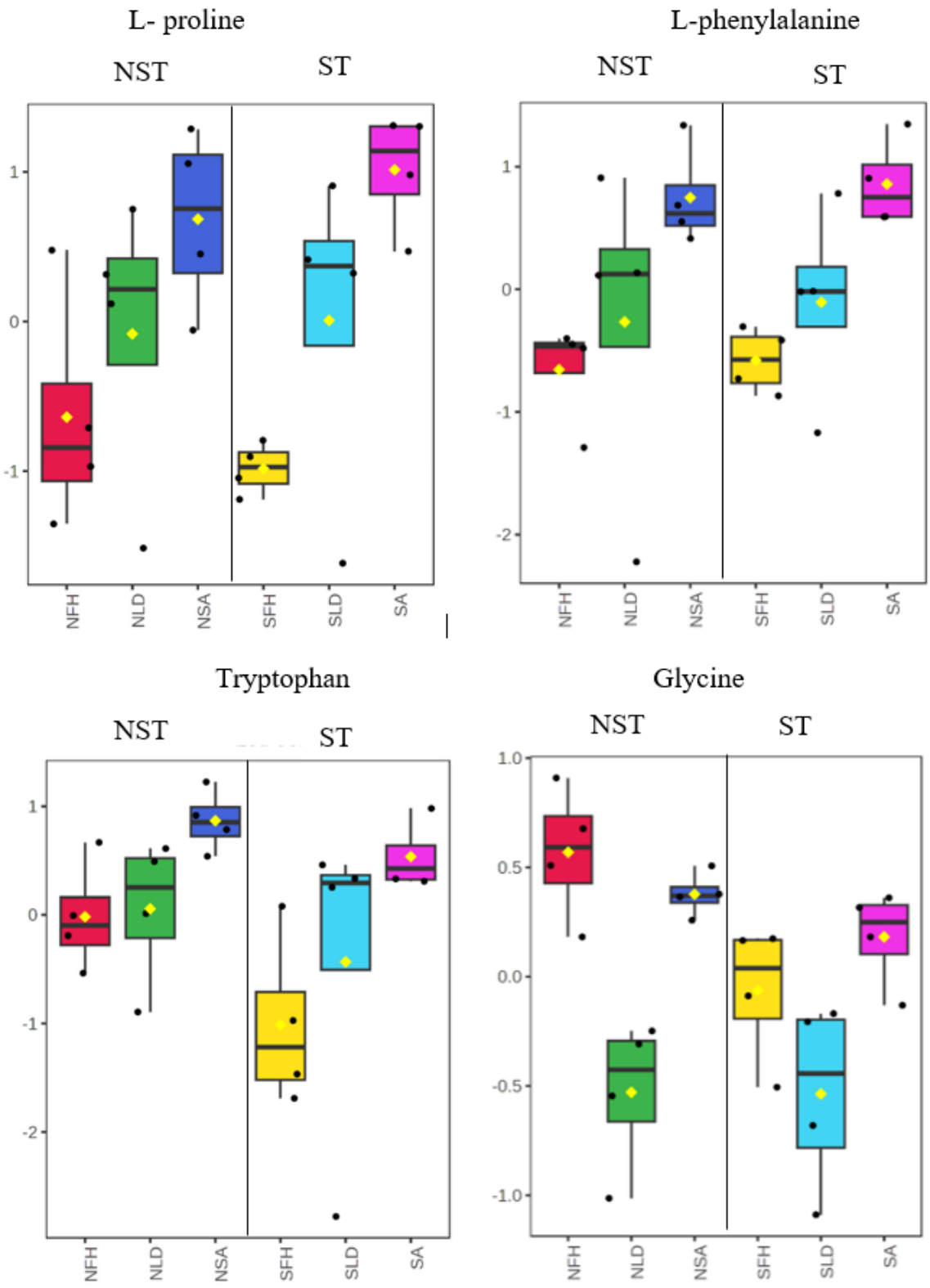



Figure S5-4: Differences in the abundance of amino acids in Eragrostis nindensis dehydration conditions between non-senescent tissue (NST), in red, and senescent tissue (ST), Statistically significant changes are represented as box plots where a log2 fold change of >1 represents significantly higher abundances in the NST compared to the ST.

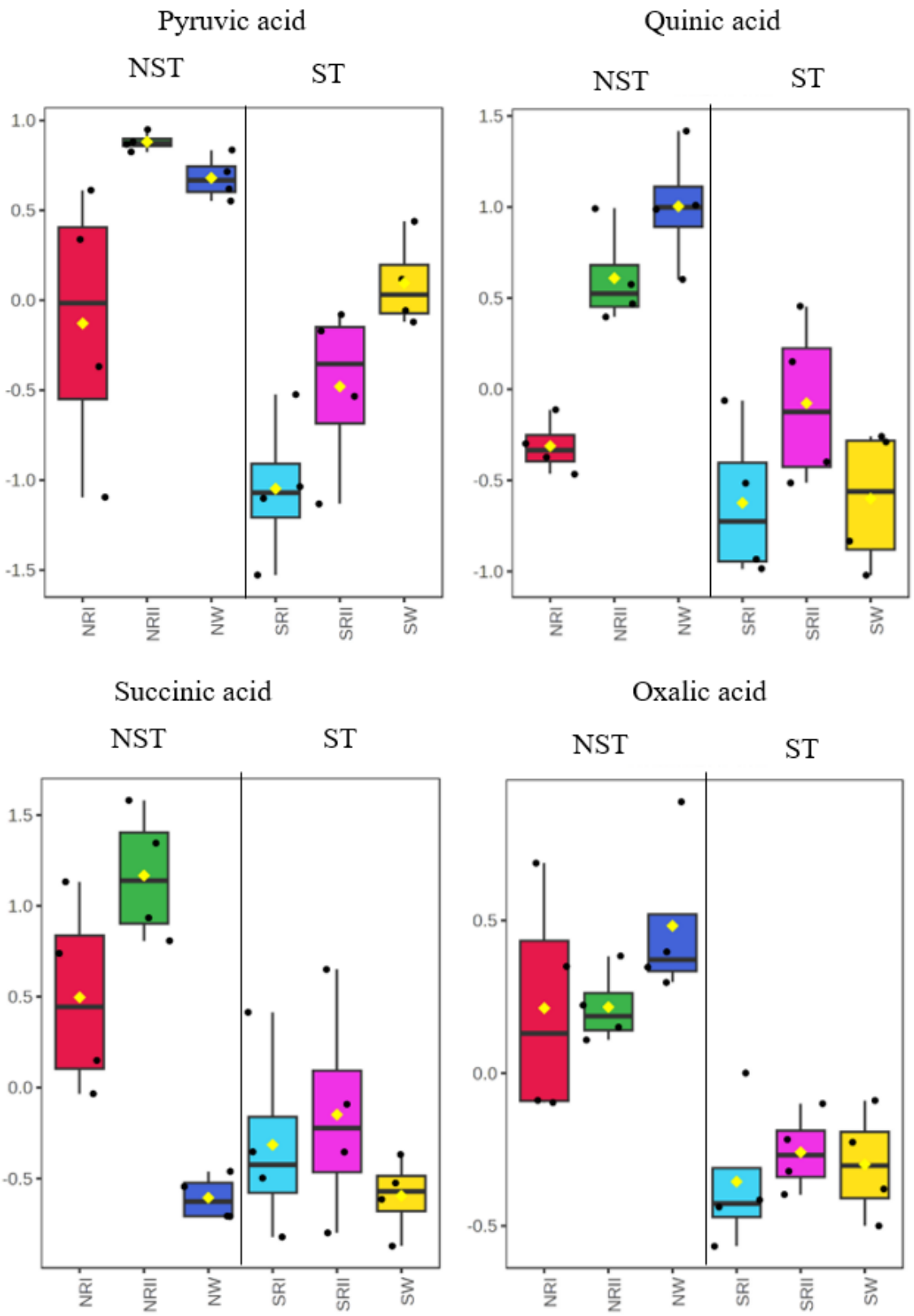



Figure S5-5: Differences in the abundance of TCA intermediates in *E. nindensis* rehydration time points between non-senescent tissue (NST), in red, and senescent tissue (ST), Statistically significant changes are represented as box plots where a log2 fold change of >1 represents significantly higher abundances in the NST compared to the ST.

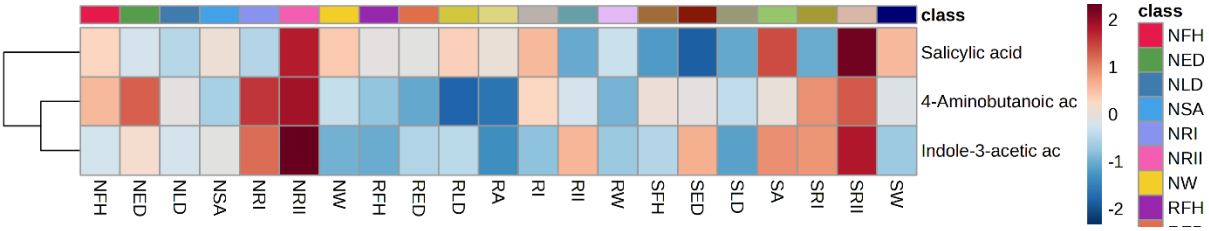



Figure S5-6: Heatmaps of differentially abundant phytohormones and other metabolites in *E. nindensis* across the rehydration time points in NST and ST and roots. Significance was based on ANOVA Fisher's LSD with P< 0.05.

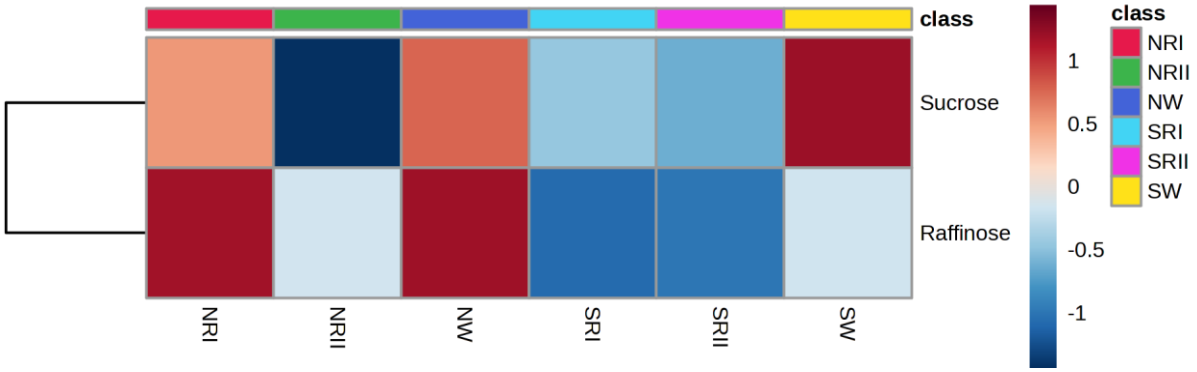



Figure S-7: Heatmaps of differentially abundant sugars in *E. nindensis* across the rehydration time points in NST and ST. Significance was based on ANOVA Fisher's LSD with P< 0.05.

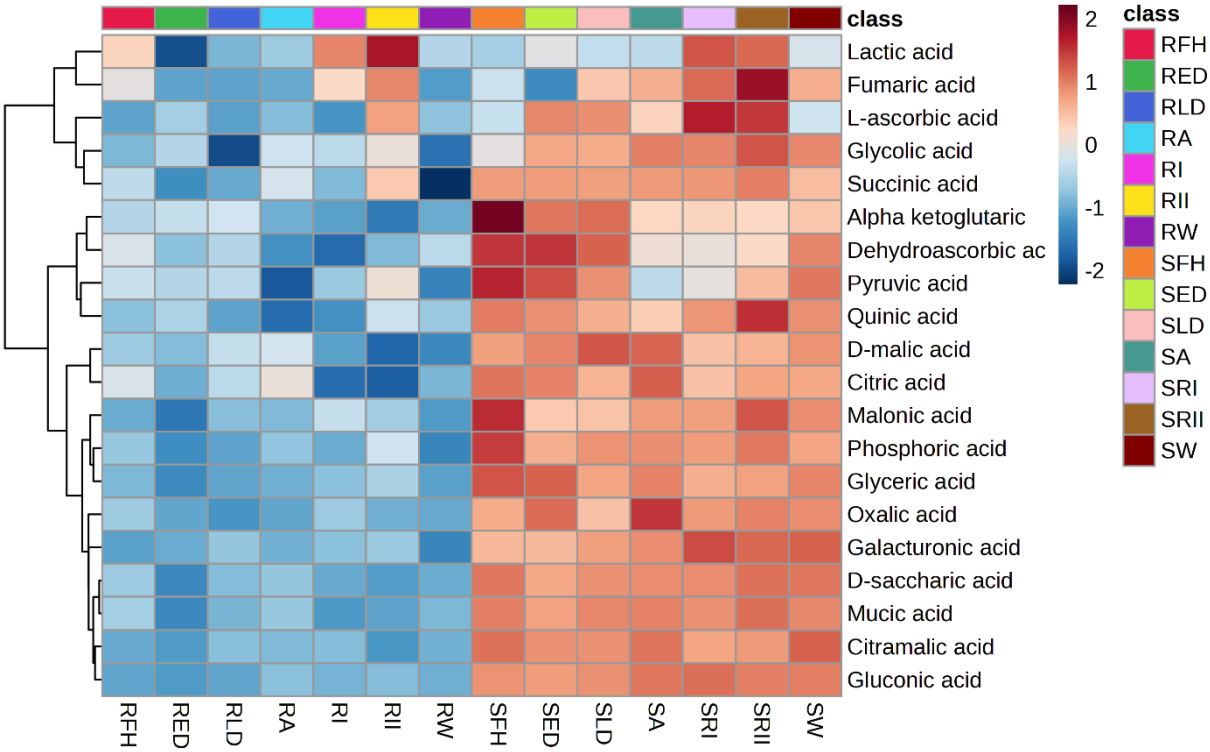



Figure S5-8: Heatmap of differentially abundant organic acids in *E. nindensis* across dehydration and rehydration time points in roots and ST. Significance was based on ANOVA Fisher's LSD with P< 0.05.

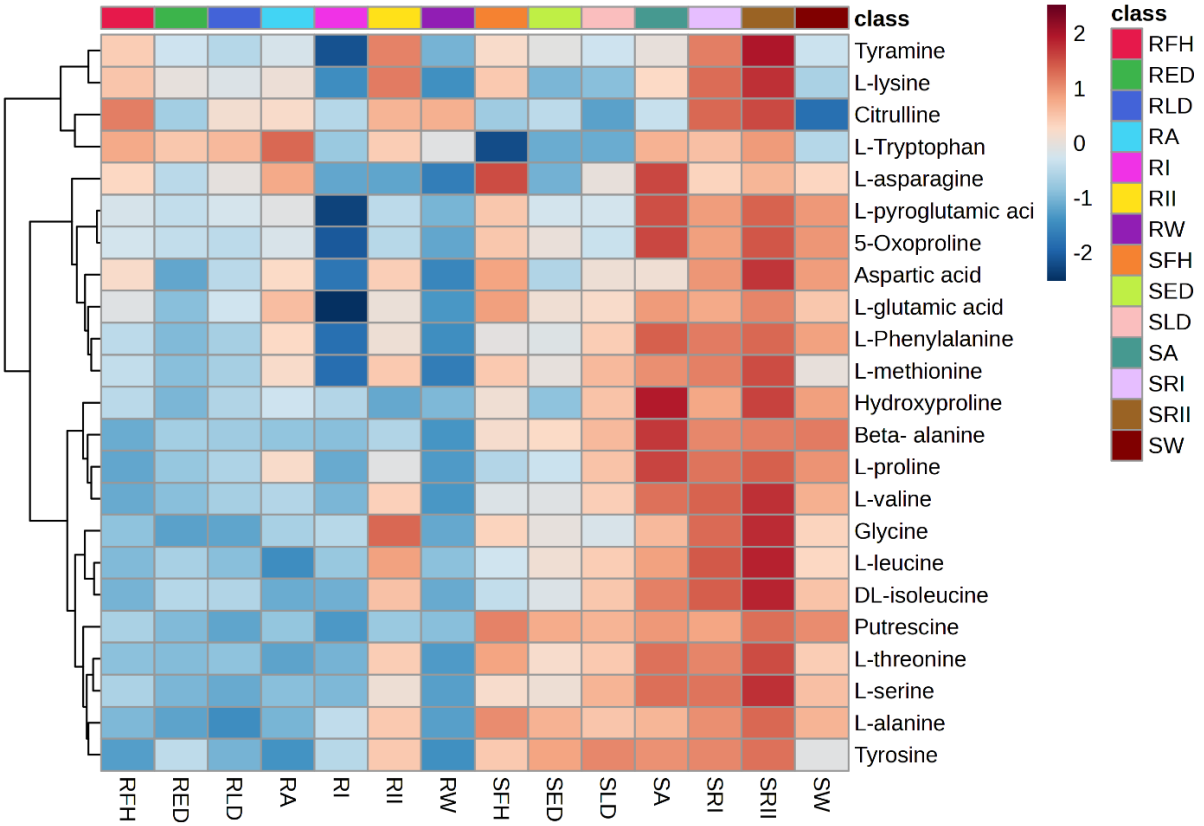



Figure S5-9: Heatmap of differentially abundant amino acids in *E. nindensis* across dehydration and rehydration time points in roots and ST. Significance was based on ANOVA Fisher's LSD with P< 0.05.

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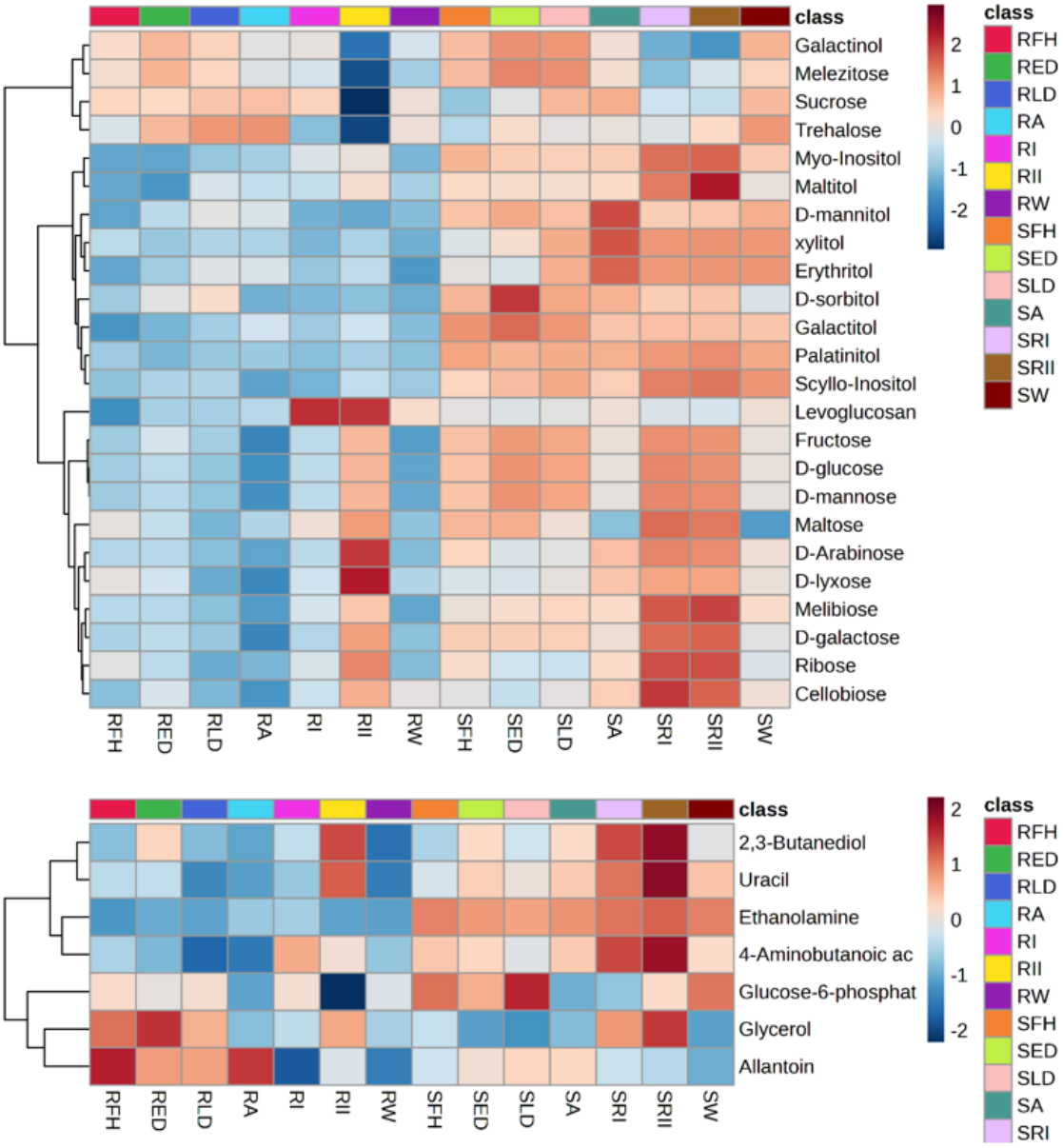


Figure S5-10: Heatmap of differentially abundant sugars, sugar alcohols and other metabolites in *E. nindensis* across dehydration and rehydration time points in roots and ST. Significance was based on ANOVA Fisher's LSD with P< 0.05.

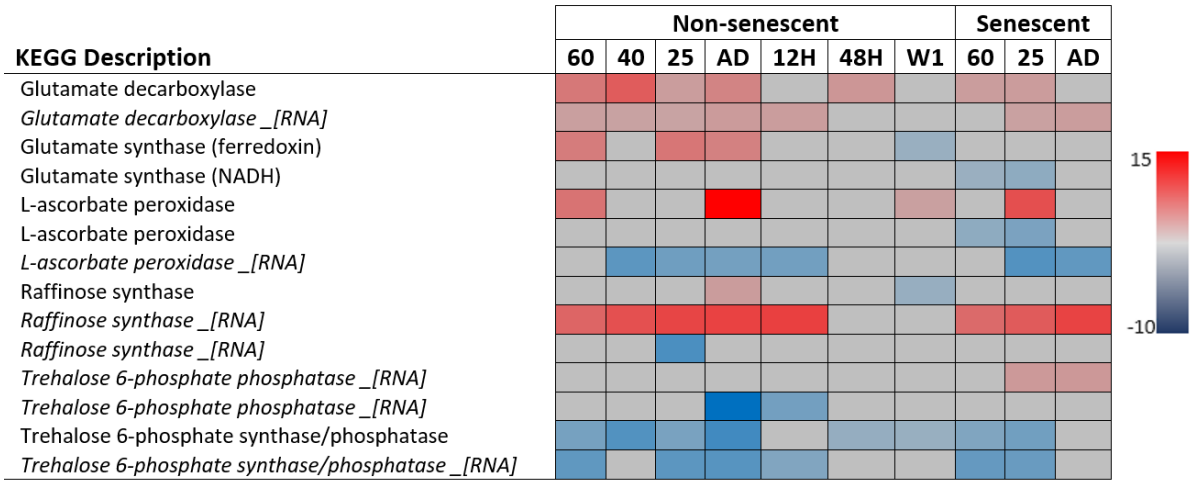


Figure S5-11: Heatmap showing a selection of proteins from the proteome and transcripts from the transcriptome as log2 fold values. Note that 12h NST and 18 ST are exclusive to the transcriptome whereas 48h NST and W1 NST are exclusive to the proteome. Fold changes displayed here represent the greatest/lowest for the respective description.

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