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Sugar and oligosaccharide metabolism in *Eragrostis spp.* and *Xerophyta viscosa*

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Abstract

Desiccation tolerance (DT) is the ability of organs and organisms to survive the loss of more than 90% of its cellular water for extended periods and to recover full metabolic competence upon rehydration. In contrast to *Xerophyta viscosa* (a well-known DT species) not much is known about DT in *Eragrostis nindensis*, which is a DT species closely related to the desiccation sensitive species *Eragrostis tef*. Understanding how vegetative tissues of this resurrection grass (*Eragrostis nindensis*) survive without water will help inform crop improvement efforts and could also be used for biotechnological applications. Previous research suggests that some key desiccation-related sugars, like sucrose, help DT plants against several stresses due to water deficit. Because of this the main research question in this report is: 'How do desiccation and rehydration affect the carbohydrate metabolism of key desiccation-related sugars in the resurrection species *Eragrostis nindensis*?'. It is hypothesized that the metabolism of key desiccation-related sugars is affected differently in the younger (or 'inner') and older (or 'outer') leaves of *Eragrostis nindensis* during desiccation and rehydration. To test this hypothesis, both sugar metabolite pools and the activity of key enzymes involved with sucrose metabolism were measured in the inner and outer leaves of *Eragrostis nindensis* and compared to the leaves of *Xerophyta viscosa* and *Eragrostis tef* during dry-down and rehydration. Sugar quantitation was performed using a Dionex HPLC, while activities of invertase, sucrose synthase (SuSy) and hexokinase were measured using spectrophotometric techniques. Compared to desiccation sensitive tissue more sucrose, raffinose and stachyose is measured in DT tissue during desiccation and also enzyme activities support these observations. Additionally the inner leaves of *Eragrostis nindensis* and the leaves of *Xerophyta viscosa* show several similarities with particular reference to some key desiccation-related sugars. Although more repetitions are required for more statistical significance, this research suggests the metabolism of these sugars are affected differently in desiccation tolerant and desiccation sensitive tissues.

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Introduction

Water is essential for (plant) life on earth. It is involved in metabolism as both a reactant and a product of many processes and it is the medium in which the intracellular milieu is suspended (Farrant, Brandt, & Lindsey, 2007). Hydrophilic interactions are the driving force for the assembly of phospholipids into biological membranes and for the conformation of many proteins (Hoekstra, Golovina, & Buitink, 2001). Although the importance of water is known, water deficit is the most limiting environmental factor of plant productivity in the world (Bewley & Krochko, 1982). There is a large genetic potential for yield which is unrealized due to the need of better adaptation of plants to water deficit (Boyer, 1982; Shao, Chu, Jaleel, & Zhao, 2008). Most growing plants normally have relative water contents (RWC) around 85-100% and die whenever this value falls under 60-30% (Höfler, Migsch, & Rottenburg, 1941). There are three main categories of stress which cause plants to die due to water deficit (Vicré, Farrant, & Driouich, 2004): mechanical stress associated with loss of turgor (Iljin, 1957), destabilization or loss of membrane integrity (Vertucci & Farrant, 1995) and oxidative stress related to disruption of metabolism (Seel, Hendry, & Lee, 1992).

To avoid water stress plant species developed several abilities during evolution like a water loss resisting cuticle and curling or folding leaves. Due to these abilities a lot of species tolerate the loss of some water during (short) periods of limited water availability (Gaff & Oliver, 2013). However desiccation tolerance (DT) is the ability of organs and organisms to survive near complete water losses for a long period. The definition which is now commonly used for desiccation tolerance (DT) is: 'the ability of an organ or organism to survive the loss of more than 90% of its cellular water for extended periods and to recover full metabolic competence upon rehydration' (Bewley, 1979; Vertucci & Farrant, 1995; Walters, Farrant, Pammenter, & Berjak, 2002). Desiccation tolerance is common among prokaryotes, some algae and reproductive tissues from higher plants like spores, pollen and seeds but it is much rarer in vegetative tissues of higher plants (Gaff & Oliver, 2013). However there are approximately 135 species of angiosperms (mostly monocotyledonous) which are desiccation tolerant (also known as resurrection plants). They are able to survive the loss of up to 95% of total cellular water content and recover full metabolic activity within 72 hours of rehydration (Farrant et al., 2015). These desiccation tolerant angiosperms can manage several cycles of dehydration and rehydration in a year (Gaff & Oliver, 2013). It seems there is no limit on the number of cycles these plants can handle, if there are periodic opportunities for photosynthesis. Air dry leaves tolerate temperatures up to 60 ^{o}C and most species examined are able to survive this state for about 2 years (Gaff & Oliver, 2013).

Some of these DT species are resurrection grasses from the subfamily *Eragrostoideae* which are reported to grow in southern Africa and one of these resurrection grasses is *Eragrostis nindensis* (Gaff & Ellis, 1974) as shown in Figure 1 (Photo Guide to Plants of Southern Africa website, 2009), which is closely related to the desiccation sensitive agricultural species *Eragrostis tef* which is shown in Figure 2 (Wikipedia website, 2005). Tef is a cereal grown mainly in Ethiopia, which is used to make a variety of food products (Ingram & Doyle, 2003). At present cereals make up the bulk of current food supplies, but most species have poorly developed abilities to resist vegetative water loss (Farrant et al., 2015). *Eragrostis tef* for instance is a drought tolerant, but not a desiccation tolerant, crop. This means it can handle the loss of some water for short periods only. However, *Eragrostis nindensis* and cereals like *Eragrostis tef* are closely related, so understanding how vegetative tissues of the resurrection grass *Eragrostis nindensis* survive without water will help inform crop improvement efforts. This knowledge could also be used for biotechnological applications, which is a potentially exciting approach because the prevalence of desiccation tolerance in seeds and pollen suggests that most plant species do have the necessary genetic programmes for desiccation tolerance even though they are inactive in vegetative tissues (Bewley & Oliver, 1992).

Figure 1. **[*Eragrostis nindensis*].** Reprinted from *Photo Guide to Plants of Southern Africa* website, by N. Dreber, 2009, retrieved from www.southernafricanplants.net/plantdata_sub.php?Mspec_ID=2234 Copyright 2009 by N. Dreber

Figure 2. Eragrostis tef. Reprinted from *Wikipedia* website, by Wikipedia, 2005, retrieved from https://en.wikipedia.org/wiki/Eragrostis_tef Copyright 2005 by Wikipedia.

Another known resurrection plant is *Xerophyta viscosa*. This monocotyledonous plant, which lives in Africa on rocky terrain in exposed grasslands typically hanging off cliff edges like the those in Figure 3 (Farrant et al., 2015, p. 2), is a member of the family *Velloziaceae* (Porembski & Barthlott, 2000). Due to its growing location these plants have to suffer multiple abiotic stresses like intense solar radiation and intermittent periods of water deficit (Farrant, Cooper, & Nell, 2012). The extensive existing literature on *Xerophyta viscosa* makes it a valuable quality assessment species for this study.

Figure 3. Xerophyta viscosa **growing chasmophytically in the Cathedral Peak Area of the Drakensberg Mountains, KwaZulu Natal, South Africa.** Reprinted from "A molecular physiological review of vegetative desiccation tolerance in the resurrection plant *Xerophyta viscosa*," by J. M. Farrant et al., 2015, *Planta an International Journal of Plant Biology*, *242*, p. 408. Copyright 2015 by Springer.

To survive desiccation, resurrection plants have to overcome the three main categories of water stress (mechanical stress associated with loss of turgor (Iljin, 1957), destabilization or loss of membrane integrity (Vertucci & Farrant, 1995) and oxidative stress related to disruption of metabolism (Seel et al., 1992)) (Vicré et al., 2004). To protect themselves against mechanical stress due to water loss, almost all plants adjust their osmotic potential. However DT plants use two additional mechanisms to avoid mechanical stress (Farrant et al., 2007). The first phenomenon observed in several DT plants is (reversible) cell wall folding, which contributes to mechanical stabilization. Different DT species use different cell wall folding mechanisms based on various biochemical components. This is one of the important reasons which suggests that DT families evolved separately from each other (Gaff & Oliver, 2013). Wall folding also occurs in the main resurrection plant used in this research (*Eragrostis nindensis*), but for *Xerophyta viscosa* there is just little evidence for this phenomenon (Farrant et al., 2015). It seems there is no notable biochemical wall changes during drying. The leaf cell walls of

Eragrostis nindensis have constant high propositions of arabinose, present as arabinogalactan proteins (AGPs) in association with xyloglucans (Plancot et al., 2014). However its relative *Eragrostis tef* has significantly lower levels of xyloglucan-associated arabinose, while having similar chemical wall constituents (Farrant et al., 2012).

The second strategy which DT species use to avoid and reduce mechanical stress is the replacement of water with compatible solutes capable of substituting for the required hydrogen bonds of polar groups on the surface lost due to dehydration. Normally the loss of water results in increasingly viscous cytoplasm, denatured proteins and membrane fusions (Vertucci & Farrant, 1995). Substituting water molecules results, besides maintaining some turgor pressure, also in the prevention of some proteins to denature and it stabilizes the membrane integrity (Hoekstra et al., 2001). For this reason this second protective strategy is also used to overcome the second main category of stress which DT plants have to handle, the destabilization or loss of membrane integrity (Vicré et al., 2004). This phenomenon is used to form a so-called glassy state of the cytoplasm. In this state the cytoplasm vitrifies and it comes in a metastable state which is solid but it has the properties of a liquid. These glasses prevent the crystallization of embedded chemical compounds, fusion between membranes and conformational changes in proteins, due to their extremely high viscosity. This glassy state not only depends on the water content, but also on the temperature and its chemical composition (Hoekstra et al., 2001). This (air-dry) state of DT angiosperms is static and stable, growth stops and metabolism slows until it (nearly) ceases (Gaff & Oliver, 2013). Some carbohydrates are capable to substitute for these hydrogen bonds with water molecules (mainly sucrose and oligosaccharides). The higher the molecular weight of these sugars, the higher the needed temperature to form a glass (Hoekstra et al., 2001). There are also some proteins, like the late embryogenesis abundant LEA protein, and some amino acids which can form this glassy state (Farrant et al., 2007). The mechanism of substituting hydrogen bonds by sugars is shown in Figure 4 (Hoekstra, Golovina & Buitink, 2001, p. 434). Both cell wall folding and the replacement of water molecules by substitutes have some similarities with seed maturation (Mtwisha, Farrant, Brandt, & Lindsey, 2006; Vicré et al., 2004).

Generally DT plants can be divided into two groups, homoiochlorophyllous (HDT) and poikilochlorophyllous (PDT) types. HDT plants retain their photosynthetic apparatus and chlorophylls in a readily recoverable form by protecting them (Proctor & Tuba, 2002). PDT plants, like *Eragrostis nindensis* and *Xerophyta viscosa*, have a different strategy to survive periods of extreme water deficit with respect to their photosynthetic apparatus. PDT plants dismantle their photosynthetic apparatus, break down their chlorophyll during desiccation and resynthesize them after rehydration (Zoltan Tuba, Lichtenthaler, Csintalan, Nagy, & Szente, 1994). The advantage of this strategy, compared with the HDT strategy, is minimising photo-oxidative damage via the formation of reactive oxygen species (ROS) and not having to maintain a working photosynthetic system during a long inactive period of desiccation. However, disadvantages are slow recovery and high energy costs of reconstruction (Zoltán Tuba, 2008). This photo-oxidative stress is the third and last main category of stress which resurrection plants have to handle. It is the result of drying-induced disruption of the electron transport which causes oxygen free radical production and the major sites of such production are the mitochondria and chloroplasts (Vicré et al., 2004). Antioxidants are able to buffer this kind of stress and DT plants upregulate various of these protectants during drying (Kranner, Beckett, Wornik, Zorn, & Pfeifhofer, 2002).

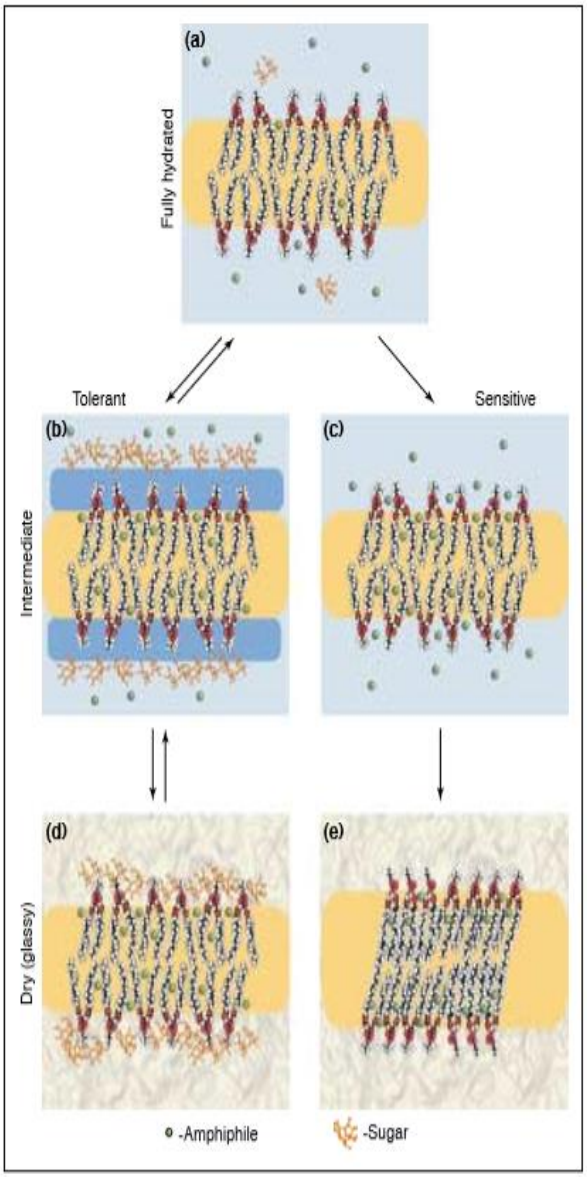

***Figure 4*. Membrane behaviour at different stages of water loss.** In fully hydrated cells (a), membrane lipids are in an undisturbed liquid-crystalline state. Upon water loss (intermediate water contents) …, this causes membrane disturbance in both tolerant (b) and sensitive (c) cells.… In the intermediate water range, the presence of preferentially excluded solutes (sugars) in tolerant cells (b) keeps the membrane surface preferentially hydrated (indicated by the blue band) and prevents membrane fusion. The absence of these solutes in the sensitive cells (c) might result in membrane fusion, as in model membrane systems. On further drying … the sugar molecules in tolerant cells replace water in the hydration shell of the membranes, thereby maintaining the spacing between phospholipid molecules.… In sensitive cells, the removal of water from the hydration shell in the absence of sugars results in packing of the phospholipid molecules, which leads to a phase transition into the gel phase.… Below 0,1 (g H_2O) (g dry weight)$^{-1}$, cytoplasmic components are immobilized in a glassy matrix, which might differ in properties between tolerant (d) and sensitive (e) cells.… The arrows indicate the reversibility of the processes during rehydration.… Reprinted and adapted from "Mechanisms of plant desiccation tolerance," by F. A. Hoekstra, E. A. Golovina and J. Buitink, 2001, *TRENDS in Plant Science, 6,* p. 434. Copyright 2001 by Elsevier.

In order to survive water deficit, several carbohydrates are involved in many ways. Previous research showed that *in vitro* the addition of sugars protect isolated chloroplasts and mitochondria (Heber & Santarius, 1964). However different conclusions are drawn in literature about the presence and role of carbohydrates in DT leaves. A major protectant sugar, especially in the late drying in angiosperm resurrection plants, seems to be sucrose. In experiments with different DT species a significant increase of the sucrose content was measured during drying and specially in fast dehydrated samples this increase was big (Crowe, 2014). In an experiment with *Sporobolus stapfianus*, another resurrection plant, there was an increase in sucrose, fructose and mainly glucose during the initial phase of drought stress (Ghasempour, Gaff, Williams, & Gianello, 1998). This increased glucose to fructose ratio could be an indication of amylolytic starch breakdown (Häusler, Schlieben, Schulz, & Flügge, 1998), but it could also be directly formed out of sucrose. In later stages of drying, in most DT species the glucose and fructose abundance in the leaves went down while the amount of sucrose went up, which proposed sucrose is accumulated at the expense of these sugars (Bianchi, Murelli, & Vazzana, 1991; Ghasempour et al., 1998). Although the degradation of glucose and fructose could also be the result from phosphorylation and metabolic processes (Yobi et al., 2012), it is proposed that sucrose is formed to replace water molecules.

Key enzymes which could be important during this accumulation and/or degradation of sucrose, are shown in Figure 5 (Sergeeva & Vreugdenhil, 2002, p. 363). First of all sucrose cleavage into glucose and/or fructose is catalysed by the enzymes invertase and sucrose synthase (SuSy) (Whittaker, Bochicchio, Vazzana, Lindsey, & Farrant, 2001). The existence of both enzymes provides alternative pathways for sucrose hydrolysis (Ap Rees, 1984). The reaction catalysed by invertase is irreversible. However the other reaction, catalysed by sucrose synthase (SuSy), is freely reversible and has a theoretical dynamic equilibrium (Geigenberger & Stitt, 1993). The reaction catalysed by hexokinase is a key step for the biosynthesis of sucrose out of glucose and with a lower efficiency also out of fructose. It catalyses the conversion from glucose into a monophosphate (using ATP) (Doehlert, 1989). Glucose-1-phosphate (G-1-P) is an intermediate step to form sucrose, but it can also form starch which is the main carbon source for the accumulation of glucose. To form sucrose it is converted into uridine diphosphate glucose (UDP-glucose), to form starch it will be converted into adenosine phosphate glucose (ADPGlc) (Sergeeva & Vreugdenhil, 2002).

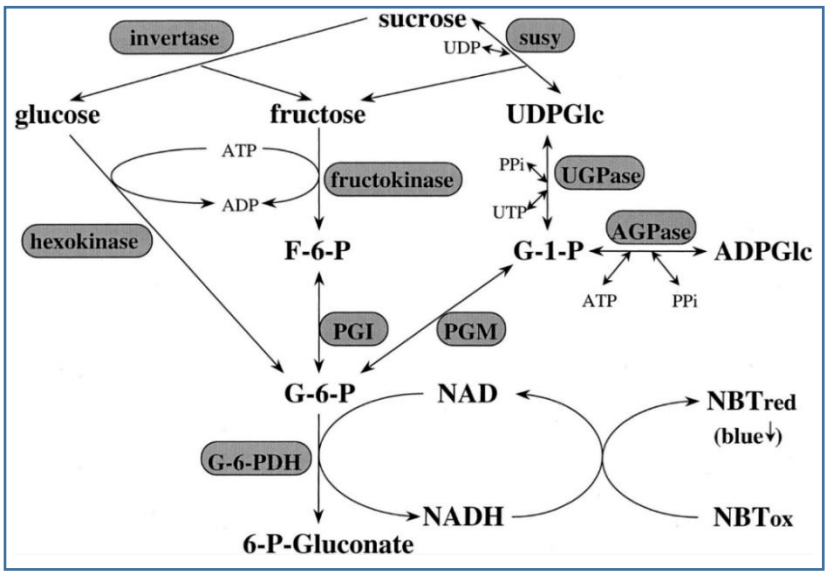

***Figure 5*. Scheme of reactions for histochemical assay of enzymes.** Reprinted from "*In situ* staining of activities of enzymes involved in carbo-hydrate metabolism in plant tissues," by L. I. Sergeeva & D. Vreugdenhil, 2002, *Journal of Experimental Botany, 53,* p. 363. Copyright 2002 by Society for Experimental Biology.

Other sugars which are accumulated during drying in some DT species are raffinose and stachyose (Ghasempour et al., 1998). These sugars, both oligosaccharides, are α-galactosyl extensions of sucrose (dos Santos et al., 2011). In contrast to raffinose, which is found in several plant species, stachyose accumulates in the vacuole of only certain plant species (Janeček, Lanta, Klimešová, & Doležal, 2011). Previous research showed that these sugars function as osmolytes to maintain cell turgor, stabilise cell proteins, act as antioxidants to counteract the accumulation of reactive oxygen species (ROS) and/or help with the stabilisation of membrane phospholipids (Bartels & Sunkar, 2005; Farrant et al., 2007; Nishizawa et al., 2006), but it is proposed that these sugars mainly assist the role of sucrose by reducing the propensity of sucrose to crystallize at high contents (Caffrey, Fonseca, & Leopold, 1988). Another function that these oligosaccharides could have, is providing a carbon skeleton necessary for sucrose synthesis during dehydration (Illing, Denby, Collett, Shen, & Farrant, 2005).

Trehalose is present in most DT species, but is very rare in non-resurrection plants. There is some evidence that this sugar alone provided a level of DT in some human cells (Guo, Puhlev, Brown, Mansbridge, & Levine, 2000). Also in experiments with nematodes (Erkut et al., 2011) and yeast (Eleutheria, de Araujo, & Panek, 1993) mutants without this sugar did not survive even mild dehydration. Trehalose may supplement the effects of sucrose in drying tissue of these DT plants, especially for the protection of enzyme activity, despite the low contents (Ghasempour et al., 1998), but it is not detected in seeds or pollen (Gaff & Oliver, 2013) and it is also found in desiccation sensitive species when dried (Yobi et al., 2012).

Research questions and hypotheses

Previous research showed a higher total sugar content in air-dried leaves of resurrection plants than in air-dried leaves of non-resurrection plants (Ghasempour et al., 1998). For several carbohydrates there is evidence that they are important for desiccation tolerant (DT) plants (Crowe, 2014; Ghasempour et al., 1998; Martinelli, 2008). For this reason the main research question in this report is: 'How do desiccation and rehydration affect the carbohydrate metabolism of key desiccation-related sugars in the resurrection species *Eragrostis nindensis*?'. In general there are two types of plant growth strategies. Plant species of the first type have an efficient nutrient conversion, which means their maximum relative growth rate is low but their organs have a long life span. Type two plant species have a relatively high maximum growth rate, but they also have a high (leaf) tissue turnover rate (Johnson & Thornley, 1983; Schläpfer & Ryser, 1996). Because *Eragrostis nindensis*, like all grasses, has a high leaf tissue turnover rate, it is proposed it has another strategy to survive desiccation than *Xerophyta viscosa*. In *Xerophyta viscosa*, a plant species which has a relatively low maximum growth rate but whose organs have a long life span, senescence of all leaf tips was observed after desiccation and rehydration (Farrant et al., 2015). Previous research on *Eragrostis nindensis* showed that only the younger leaves rehydrate after desiccation and rehydration (Vander Willigen, Pammenter, Mundree, & Farrant, 2001). To survive desiccation this plant species senescence their older leaves in order to protect the younger ones, which means only the younger leaves will rehydrate when watered again and therefore function as DT tissue. This could be explained by their high leaf tissue turnover rate, which does not make it beneficial to rehydrate all leaves. Because of this the main hypothesis in this report is about the differences between the younger (or the 'inner') leaves and the older (or the 'outer') leaves of *Eragrostis nindensis*. It is hypothesized that the metabolism of key desiccation-related sugars is affected differently in the inner and outer leaves of *Eragrostis nindensis* during desiccation and rehydration. Additionally, it is proposed that the inner leaves of *Eragrostis nindensis* act similarly, with respect to the metabolism of key desiccation-related sugars, to the leaves of *Xerophyta viscosa* (both desiccation tolerant) and the outer leaves of *Eragrostis nindensis* act similarly to the leaves of *Eragrostis tef* (both desiccation sensitive).

Previous research suggests the main protectant in DT plants during desiccation is sucrose (Farrant et al., 2007; Scott, 2000). Therefor the first sub question in this report is: 'How do desiccation and rehydration influence the sucrose metabolism in *Eragrostis nindensis* and how does this compare to the well-known resurrection species *Xerophyta viscosa* and the desiccation sensitive species *Eragrostis tef*?'. Previous research suggests that during early drying response most plant species decrease their osmotic potential to extract more water out of their environment by accumulating more sugars like glucose and fructose (Morgan, 1984; Mullet & Whitsitt, 1996) and it also suggests that during late drying response those sugars are converted into sucrose (Farrant et al., 2007). Thus the hypothesis for this sub question is as follows: the mean sucrose content will increase and the mean glucose and fructose content will decrease during dehydration in DT leaves. The second hypothesis states that key enzymes involved with the accumulation of sucrose will be active during desiccation and key enzymes involved with the degradation of sucrose will be active during rehydration in DT leaves. It is also hypothesized that, although desiccation sensitive leaves have an early drying response by accumulating more sugars (Morgan, 1984), they do not have an increase or decrease in the carbohydrate contents after that period.

Other carbohydrates that are probably important in DT plants are raffinose, stachyose and trehalose (Ghasempour et al., 1998), although there is no consensus about their exact role yet. The second and third sub question in this report are therefore: 'How do desiccation and rehydration influence the quantities of the oligosaccharides raffinose and stachyose in *Eragrostis nindensis* and how do these quantities relate to the well-known resurrection species *Xerophyta viscosa* and the desiccation sensitive species *Eragrostis tef*?' and 'How do desiccation and rehydration influence the quantity of trehalose in *Eragrostis nindensis* and how do these quantities relate to the well-known resurrection species *Xerophyta viscosa* and the desiccation sensitive species *Eragrostis tef*?'. The hypotheses for these sub questions are that DT leaves will have an increase in the raffinose, stachyose and trehalose content when desiccated, where desiccation sensitive leaves will nog have an increase. In summary this gives the following research questions:

Main research question

How do desiccation and rehydration affect the carbohydrate metabolism of key desiccation-related sugars in the resurrection species *Eragrostis nindensis*?'

Sub research questions

How do desiccation and rehydration influence the sucrose metabolism in *Eragrostis nindensis* and how does this compare to the well-known resurrection species *Xerophyta viscosa* and the desiccation sensitive species *Eragrostis tef*?

How do desiccation and rehydration influence the quantities of the oligosaccharides raffinose and stachyose in *Eragrostis nindensis* and how do these quantities relate to the well-known resurrection species *Xerophyta viscosa* and the desiccation sensitive species *Eragrostis tef*?

How do desiccation and rehydration influence the quantity of trehalose in *Eragrostis nindensis* and how do these quantities relate to the well-known resurrection species *Xerophyta viscosa* and the desiccation sensitive species *Eragrostis tef*?

Materials and methods

To answer these questions, both sugar metabolite pools and the activity of key enzymes involved with the sucrose metabolism were measured. Sugar quantitation was performed using a Dionex HPLC, while activities of invertase, sucrose synthase (SuSy) and hexokinase were measured using spectrophotometric techniques. Because sucrose can only be degraded into glucose, fructose and uridine-diphosphoglucose (UDP-glucose) and accumulated out of UDP-glucose (Braun, Wang, & Ruan, 2013), quantitative measures of glucose, fructose and sucrose from the Dionex experiment in combination with the enzyme assays of three key enzymes involved in the sucrose metabolism (Winter & Huber, 2000), provide insights into the first sub question. The second and third sub-questions are addressed by measurements of raffinose, stachyose and trehalose in the Dionex experiment.

Plant material

For these experiments *Eragrostis nindensis* and *Eragrostis tef* plants were germinated on half-strength MS agar and then grown in commercial potting mix for approximately 4 months in a greenhouse. The *Xerophyta viscosa* plants used for these experiments had been grown in a greenhouse for 4 to 5 years in commercial potting soil. At the start of the experiment (day 0) the plants were transferred into a climate cabinet (16:8 hours & 15:25 ^{o}C light dark cycle) and fully hydrated leaf material was harvested from each individual plant. For the *Eragrostis nindensis* species inner (younger) and outer (older) leaves were harvested and analysed separately. In combination with the leaves of *Xerophyta viscosa* and *Eragrostis tef*, four different leaf groups were analysed. Watering was withheld until the plants had reached an air-dry state (<5% water content relative to the fully hydrated state). Leaf material was harvested from *Eragrostis nindensis* and *Eragrostis tef* plants at 2, 6, 8, 10 and 13 and from *Xerophyta viscosa* plants at 2, 6, 10, 15, 18, 20, 22 and 25 days after cessation of watering. After plants had equilibrated to an air-dry state, (day 13 and 25 for respectively the *Eragrostis* species and *Xerophyta viscosa*) all plants were watered again. When desiccation tolerant (DT) plants (*Eragrostis nindensis* and *Xerophyta viscosa*) regained full hydration, leaf material was harvested again from these plants. Half of the harvested leaves were immediately frozen into liquid nitrogen and stored at -80 ^{o}C. The rest was used, as comparable leaf tissue of the same plants, to determine the relative water content (RWC).

To calculate the relative water content (RWC), first the absolute water content (AWC) is calculated for each individual leaf.

AWC = (fresh weight – dry weight) / dry weight (1)

For each individual leaf group, an average of all AWC's of the 100% hydrated leaves (100% AWC) is used to calculate the relative water content (RWC) of each individual leaf.

RWC = AWC / 100% AWC (2)

Quantitative sugar measurements

Frozen leaf material was freeze-dried and approximately 10,5 mg from each sample was weighted for analysis. Tissue was homogenized using a bead mill and 1 mL 80% MeOH containing 400 mg/L melezitose was added to each sample. Samples were extracted for 15 minutes at 76 ^{o}C and dried in vacuo in a Speedvac vacuum centrifuge for approximately 2 hours. The dry extracts were redissolved in 1 mL mQ water and further diluted 10x in mQ water. The supernatants were analysed with a Dionex ICS5000 HPLC-system using a Carbopac PA1-column + Carbopac GA1 guard column (4 x 250 mm) at 30 ^{o}C. 10 µL injections were eluted with a 20-150 mM NaOH gradient over 30 min (+5 min 150 mM NaOH if required) at a flow rate of 1 mL/min. The column was rinsed between runs for 5 min with 500 mM

NaOH, followed by 10 min equilibration with 20 mM NaOH. Sugars were detected with a Dionex ED50 electrochemical detector and identified by comparison of their retention times with those of analytical. Quantitation was performed using separate standard calibration curves constructed for each sugar identified.

Extraction and measurements for enzyme activities

Frozen leaf material was freeze-dried and approximately 10,0 mg for each sample was weighed. Tissues were homogenized and 20 mg insoluble PVP was added to each tube. Samples were homogenized in 1,2 mL extraction buffer (50 mM HEPES (pH 7.4), 5 mM $MgCl_2.6H_2O$, 1 mM EGTA, 1mM EDTA, 10% glycerol, 0.1% BSA and 5 mM DDT) and centrifuged (for 10 minutes at 15000 RPM). Supernatants were aliquoted and stored at - 80 ^oC (extracts 1).

Insoluble invertase was extracted from the remaining pellets of the above samples. Pellets were washed twice with extraction buffer at 4 ^oC to remove soluble enzymes and were incubated overnight in 1 mL buffer (20 mM MES (pH 6.0) and 1.0 M NaCl). The extracts were centrifuged (for 15 minutes at 15000 RPM) and the supernatants were analysed for (insoluble) invertase activity (extracts 2). Invertase was measured in both sample supernatants, quantifying soluble (extracts 1) and insoluble (extracts 2) enzyme activities. Blanks for both extracts were obtained by boiling for 4 minutes. 25 µL of both extracts (soluble and insoluble enzyme fractions) and 25 µL of both corresponding blanks were transferred to four different tubes. To each tube 250 µL buffer (25 mM citrate/phosphate pH 5.2 and 25 mM sucrose) was added and after incubation at 30^oC for 45 minutes the reaction was stopped by boiling the contents of all tubes for 4 minutes. Reaction mixtures were transferred to a 96-well plate. Reaction products were analysed using a glucose kit (Diffchamp) according to manufacturer's instructions. Plates were read at 340 nm with a spectrophotometer (Molecular Devices Spectramax Plus) 3 minutes after initial sample introduction. Reaction substrate was added and plates were read again after 15 minutes reaction time.

Extracts 1 were used to measure the sucrose synthase (SuSy) activity. For each sample two wells on a 96-well plate were filled with 80 µL of these extracts. 125 µL of 2x buffer (50 mM HEPES pH 7.4, 1 mM EDTA, 1 mM EGTA, 5 mM $MgCl_2.6H_2O$ and 0.1 % BSA), 4.11 µL mQ water, 0,89 µL 400 U/mL PGM, 0,36 µL 1000 U/mL G6PDH, 1,79 µL 160 mM NAD, 1,79 µL 3 mM G-1,6-diP, 10,71 µL 150 mM PPi and 5,36 µL 2.5 mM sucrose were added to each well and the plates were read by a spectrophotometer (Molecular Devices Spectramax Plus) at 340 nm. For each sample 20 µL 150 mM UDP substrate was added to one well and 20 µL mQ water as blank to another and after 15 minutes of incubating the plates were read again by the spectrophotometer.

Extracts 1 were also used to measure the hexokinase activity. For each sample two wells on a 96-well plate were filled with 30 µL of extracts and to each well 125 µL 2x buffer (50 mM Bis-Tris pH 8.0 and 5 mM $MgCl_2.6H_2O$), 60.675 µL mQ water, 0.25 µL 1000 U/mL G6PDH, 1.575 µL 160 mM NAD and 12.5 µL 50 mM ATP was added. Plates were read at 340 nm by the same spectrophotometer. For each sample 20 µL 8 mM glucose substrate was added to one well and 20 µL mQ water to another as blank. After 15 minutes of incubation the plates were read once again using the spectrophotometer at 340 nm.

For all 4 enzymes (soluble and insoluble invertase, sucrose synthase (SuSy) and hexokinase) the enzyme activities is calculated the same way. First the absorbance of the 'normal' samples (formula 3) and blanks (formula 4) is calculated. The difference between those values is used to quantify the enzyme activities (formula 5).

absorbance 1 = absorbance after adding substrate - absorbance before adding substrate (3)

absorbance 2 = absorbance after adding mQ water - absorbance before adding mQ water (4)

relative absorbance = absorbance 2 - absorbance 1 (5)

Data analysis

Along with its relative water content (RWC), a lot of data points were obtained by the quantitative sugar and enzyme measurements for each sample. These data points represent the quantity of different sugars and enzyme activities at different moments during dry-down and rehydration for all 4 leaf groups. All raw data points are shown in appendix A.

To interpret these data, each data point was assigned to a category based on relative water content. The mean and standard deviation were calculated for each category and are presented in Figure 6 to 10. The X-axis represents the RWC and for *Xerophyta viscosa* and *Eragrostis nindensis* the last column represents a rehydrated category, which is not based on the RWC. Unfortunately, not all leaf groups have the same categories due to the difficulty of harvesting sufficient samples at intermediate water contents, given the number of plants available. For *Xerophyta viscosa* and *Eragrostis tef* all samples with more than 95% RWC and for *Eragrostis nindensis* (inner and outer leaves) all samples with more than 90% RWC were assigned to the fully hydrated category. All samples with less than 20% RWC were assigned to the desiccated category. Both DT species (*Xerophyta viscosa* and *Eragrostis nindensis*) have a rehydrated category containing samples harvested after rehydration.

Intermediate categories were assigned to make a distinction between early-drying and late-drying responses, based on the distribution of RWC in harvested leaf samples. For *Xerophyta viscosa* two extra categories (besides the fully hydrated, desiccated and rehydrated categories) were assigned. The first category contains all samples between 70% and 90% RWC and the second contains all samples between 45% and 50% RWC. Two extra categories were also formed for the outer (older) leaves of *Eragrostis nindensis* (70-80% RWC and 20-40% RWC) but for the inner (younger) leaves there were only enough samples for one extra category (40-55% RWC). For *Eragrostis tef* there were enough samples for a 70-90% RWC and a 30-70% RWC category.

Results

Glucose and fructose

Figure 6 shows the mean abundance of glucose and fructose at different stages during dry-down and rehydration for all four leaf groups (the inner and outer leaves of *Eragrostis nindensis* and the leaves of *Xerophyta viscosa* and *Eragrostis tef*) which are based on the raw data points shown in Figure A1-A8. For all leaf groups there are no significant differences between the absolute mean abundances of both monosaccharides in each relative water content (RWC) category respectively, which results in equal abundance patterns for both monosaccharides within each leaf group. The abundance patterns of all leaf groups are similar during early drying stages of dry-down for both monosaccharides. First the average quantities increase during early drying and at a certain moment during dry-down these quantities decrease again. While this inflection point appears to be at a different RWC for each species and leaf group, this is not totally clear due to the different RWC-categories. For the inner leaves of *Eragrostis nindensis* (Fig. 6a) and the leaves of *Xerophyta viscosa* (Fig. 6c) this decrease is much more profound than the decrease in the outer leaves of *Eragrostis nindensis* (Fig. 6b) and the leaves of *Eragrostis tef* (Fig. 6d). For the inner leaves of *Eragrostis nindensis* these quantities decrease till 27% and 24% (for glucose and fructose respectively) of its original, fully hydrated value. This steep decrease is also observed in the leaves of *Xerophyta viscosa*, which shows a decrease for both monosaccharides till 15% and 34% of its original value. The mean content of both glucose and fructose do not decrease below their original values at full hydration in both the outer leaves of *Eragrostis nindensis* and the leaves of *Eragrostis tef*. In absolute values there seems to be a difference between the inner and outer leaves of *Eragrostis nindensis* (Figures 6a and 6b) when desiccated. When desiccated the amount of glucose and fructose is 3,6 and 4,8 times higher in the outer leaves. However, this difference is not statistically significant at the 95% confidence interval. For most leaf groups the difference in mean monosaccharides abundances between the fully hydrated and the desiccated category are not statistically significant ($p > 0,05$), only the difference in the mean glucose abundance of *Eragrostis tef* between the fully hydrated and the desiccated category is ($p < 0,05$).

Sucrose

Figure 7 shows the mean abundance of sucrose at different moments during dry-down and rehydration for all four leaf groups which are based on the raw data points shown in Figure A9-A12. The abundance patterns of the inner leaves of *Eragrostis nindensis* (Fig. 7a) and the leaves of *Xerophyta viscosa* (Fig. 7c) show some similarities. Both graphs have an increase to a maximum value at the desiccated category and after this point the mean abundance decreases to between 97% and 116% of its original value when fully hydrated. For both leaf types of *Eragrostis nindensis* (Figures 7a and 7b) there is an increase in the mean sucrose content during early stages of dry-down (till 20% RWC), which is only statistically significant for the inner leaves of *Eragrostis nindensis* ($p < 0,05$). The mean abundance in the inner leaves of *Eragrostis nindensis* (Fig. 7a) is approximately 7-fold higher at the 40%-55% RWC-category and the mean abundance in the outer leaves of *Eragrostis nindensis* (Fig. 7b) is 3 times higher at the 20%-40% RWC category when compared to the corresponding fully hydrated category. This increase is also present in the leaves of *Xerophyta viscosa* (Fig. 7c) and *Eragrostis tef* (Fig. 7d) but is much lower (1,4 and 1,1 times higher than its original fully hydrated value) and is also not statistically significant ($p > 0,05$). However, the mean sucrose content for all four leaf groups is higher when desiccated than the mean sucrose content when fully hydrated. These abundances are approximately 2-fold higher the outer leaves of *Eragrostis nindensis* and in both the leaves of *Xerophyta viscosa* and *Eragrostis tef*. The abundance is approximately 7,5-fold higher in the inner leaves of *Eragrostis nindensis*. These differences are statistically significant ($p < 0,05$) except for the outer leaves of *Eragrostis nindensis*. In absolute values there seems to be a difference in the desiccated RWC-category (<20%) between the inner leaves of *Eragrostis nindensis* and the leaves of *Xerophyta viscosa* (Figures

7a and 7c) and the outer leaves of *Eragrostis nindensis* and the leaves of *Eragrostis tef* (Figures 7b and 7d). In the first case there is a mean sucrose content of 81,9 mg / g DW in the inner leaves of *Eragrostis nindensis* and 65,8 mg / g DW in the leaves of *Xerophyta viscosa*. The outer leaves of *Eragrostis nindensis* and the leaves of *Eragrostis tef* have a mean sucrose content of respectively 44,4 and 49,8 mg / g DW in the same category. However, these differences are not statistically significant ($p > 0,05$). When rehydrated the sucrose level in the inner leaves of *Eragrostis nindensis* (Fig. 7a) and the leaves of *Xerophyta viscosa* (Fig. 7c) decrease by 87% and 50%. This decrease is only statistically significant for the inner leaves of *Eragrostis nindensis* ($p < 0,05$). The sucrose content in the outer leaves of *Eragrostis nindensis* (Fig. 7b) does not have a strong decrease when rehydrated, it only decreases with 16%.

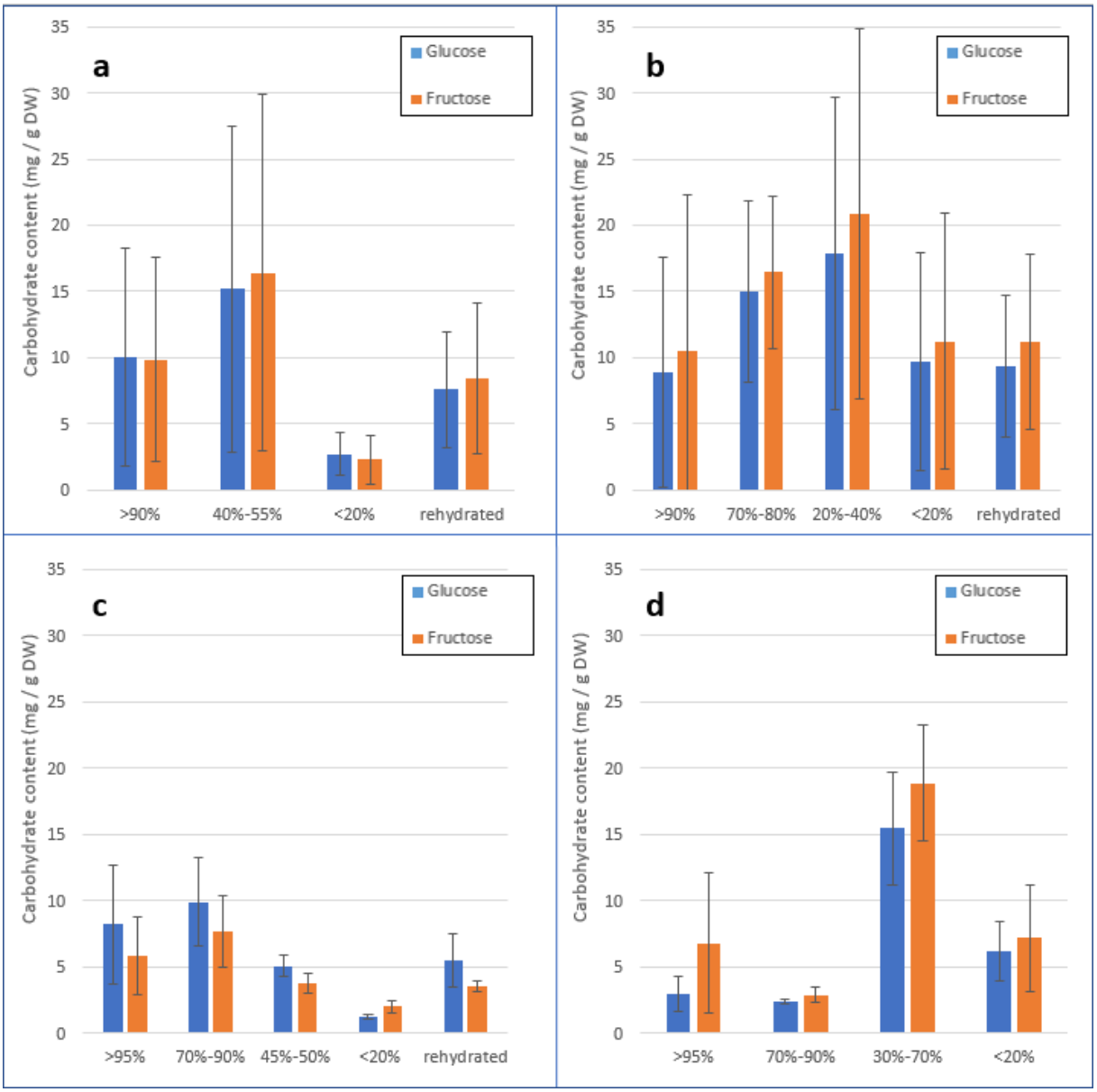

***Figure 6*. Mean monosaccharide contents (mg / g DW) in respectively the inner leaves of *Eragrostis nindensis* (Fig. 6a), the outer leaves of *Eragrostis nindensis* (Fig 6b), the leaves of *Xerophyta viscosa* (Fig. 6c) and the leaves of *Eragrostis tef* (Fig. 6d).** On the Y-axis, the mean carbohydrate contents (mg / g DW) of glucose (blue bar) and fructose (orange bar) is shown. The X-axis represents the various RWC-categories and if applicable a rehydrated category. Error bars represent the standard errors of the mean.

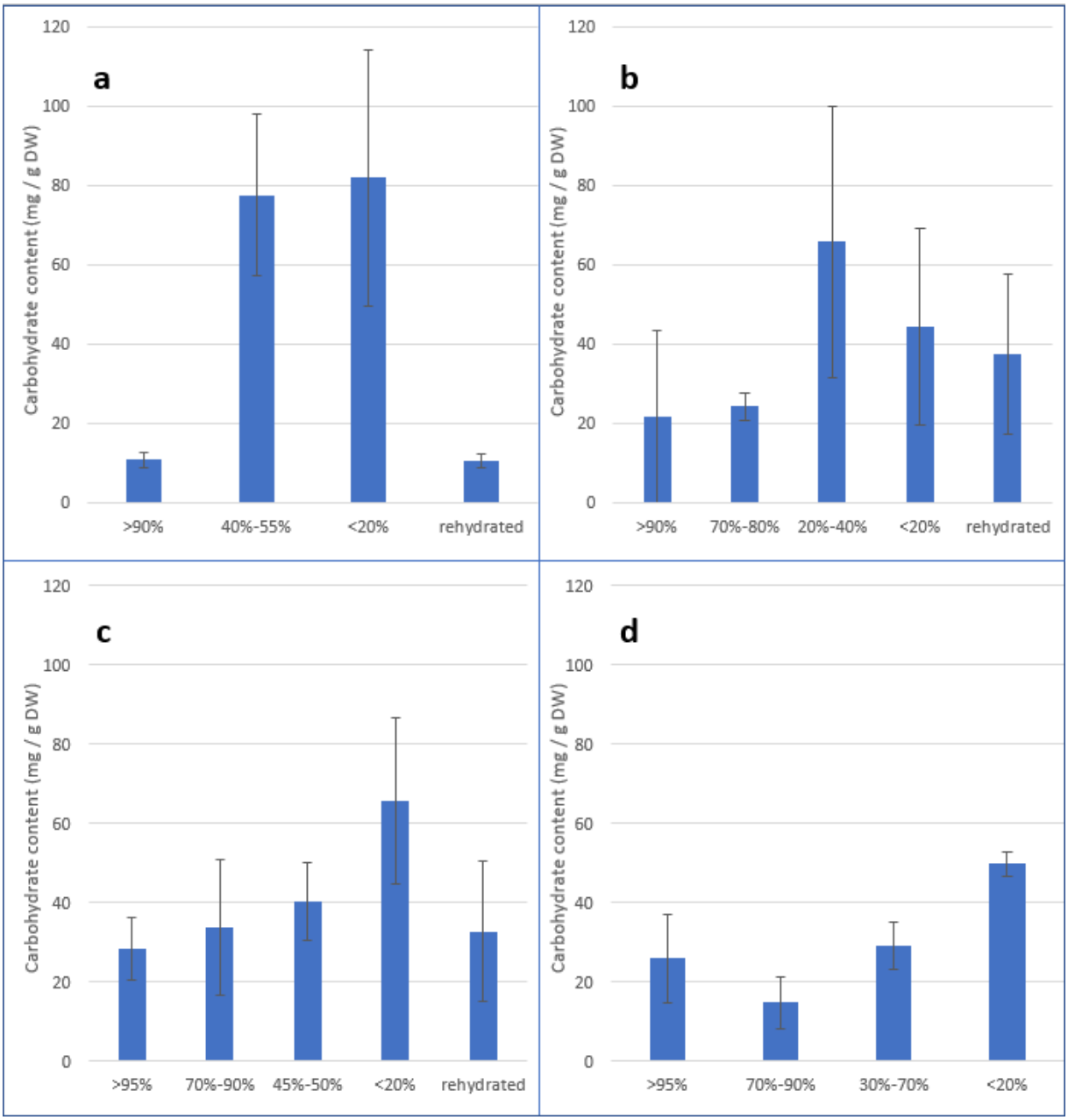

***Figure 7*. Mean sucrose content (mg / g DW) in respectively the inner leaves of *Eragrostis nindensis* (Fig. 7a), the outer leaves of *Eragrostis nindensis* (Fig 7b), the leaves of *Xerophyta viscosa* (Fig. 7c) and the leaves of *Eragrostis tef* (Fig. 7d).** On the Y-axis the mean carbohydrate content (mg / g DW) of sucrose is shown. The X-axis represents the various RWC-categories and if applicable a rehydrated category. Error bars represent the standard errors of the mean.

Enzymes

No insoluble invertase activity was detected in both leaf groups of *Eragrostis nindensis*. Figure 8 shows the relative activity of soluble invertase, sucrose synthase (SuSy) and hexokinase in the inner (Fig. 8a) and the outer (Fig. 8b) leaves of *Eragrostis nindensis*. After an increase in the relative activity of soluble invertase in the inner leaves of *Eragrostis nindensis* during early drying, the relative activity decreases till a minimum value of 0,008 at the desiccated (< 5% RWC) category. In the outer leaves of *Eragrostis nindensis* the relative activity of soluble invertase decreases during the entire dry-down till a minimum

value of 0,019 at the desiccated category. This activity increases after rehydration to 0,114 for the inner and 0,042 for the outer leaves. Differences in relative (soluble) invertase activity between the desiccated category (RWC < 5%) and the other RWC-categories of the inner leaves of *Eragrostis nindensis* are statistical significant ($p < 0,05$). The difference in relative activity between the inner and outer leaves of *Eragrostis nindensis* of the desiccated category (RWC < 5%) is also statistical significant ($p < 0,05$), other (soluble) invertase activities are not.

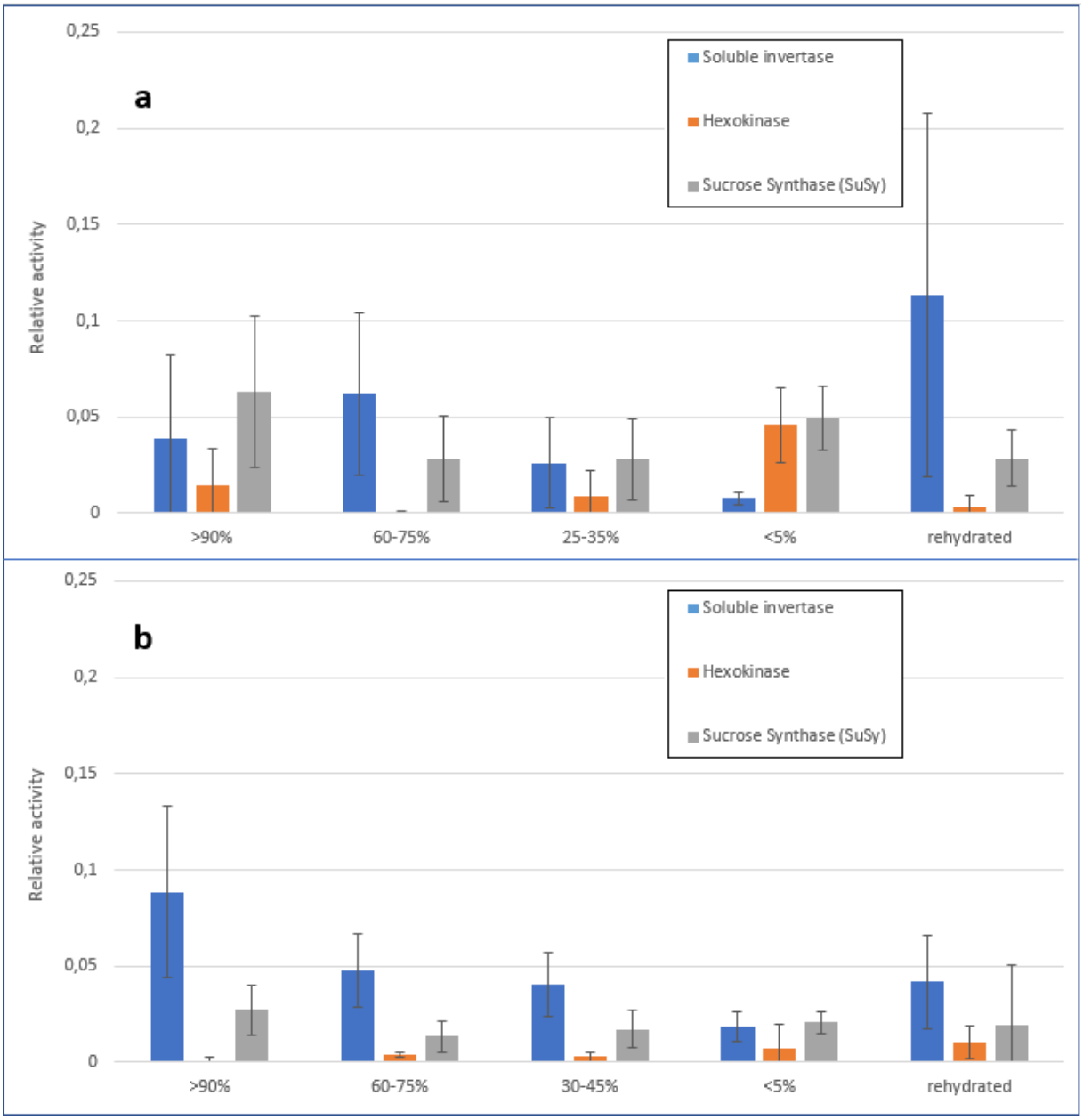

Figure 8. **Mean relative activities of soluble invertase, hexokinase and sucrose synthase (SuSy) in respectively the inner (Fig. 8a) and the outer (Fig. 8b) leaves of *Eragrostis nindensis*.** On the Y-axis the mean relative activity of soluble invertase (blue bar), hexokinase (orange bar) and sucrose synthase (grey bar) is shown. The X-axis represents the various RWC-categories and if applicable a rehydrated category. Error bars represent the standard errors of the mean.

The relative hexokinase activity of the outer leaves of *Eragrostis nindensis* (Fig. 8b) slightly increases during dry-down and rehydration till a value of 0,008 in the rehydrated category. The relative hexokinase activity in the inner leaves of *Eragrostis nindensis* (Fig. 8a) has a steep increase till a value of 0,046 at the desiccated category but decreases to 0,003 when rehydrated. All differences in relative hexokinase activity in the inner leaves of *Eragrostis nindensis* are statistical significant ($p < 0,05$) when compared to the desiccated category (RWC < 5%) and the same goes for the difference between the inner and the outer leaves of *Eragrostis nindensis* when desiccated. The relative sucrose synthase (SuSy) activity of the inner leaves of *Eragrostis nindensis* fluctuates during dry-down and rehydration between 0,063 and 0,028. For the outer leaves the relative activity is much lower and these fluctuations are more constant between 0,013 and 0,027. The differences between the inner and outer leaves is only statistical significant ($p < 0,05$) for the desiccated category (RWC < 5%).

Raffinose and stachyose

Figure 9 shows the mean abundance of raffinose and stachyose at different moments during dry-down and rehydration for all four leaf groups which are based on the raw data points shown in Figure A13-A20. For raffinose the abundance pattern is equal for all leaf groups, the raffinose content increases till the desiccated RWC-category and decreases when rehydrated. This increase (the difference between the fully hydrated and desiccated category) is the highest in the inner leaves of *Eragrostis nindensis* (Fig. 9a). The raffinose content in these leaves increases approximately 17-fold in the desiccated samples when compared to the fully hydrated ones. In the outer leaves of *Eragrostis nindensis* (Fig. 9b), the leaves of *Xerophyta viscosa* (Fig. 9c) and the leaves of *Eragrostis tef* (Fig. 9d) this increase is only nearly 3-fold, 2-fold and 4-fold of the original value when fully hydrated. These differences are statistically significant for the inner leaves of *Eragrostis nindensis*, the leaves of *Xerophyta viscosa* and the leaves of *Eragrostis tef* ($p < 0,05$). The raffinose content after desiccation is much higher in the inner leaves of *Eragrostis nindensis* (Fig. 9a) and the leaves of *Xerophyta viscosa* (Fig. 9c) when compared to the other leaf groups (Figures 9b and 9d). In the inner leaves of *Eragrostis nindensis* the abundance is 8,8 mg / g DW and in the leaves of *Xerophyta viscosa* it is 8,3 mg / g DW. In the other leaf groups it is only 5,7 and 3,5 mg / g DW for respectively the outer leaves of *Eragrostis nindensis* and the leaves of *Eragrostis tef*. However, these differences are not statistical significant ($p > 0,05$).

The abundances of the other measured oligosaccharide (stachyose) is totally different. When fully hydrated only a bit (0,2 mg / g DW) stachyose is measured in the leaves of *Xerophyta viscosa* (Fig. 9c). When desiccated in all leaf groups some stachyose is measured, however in the inner leaves of *Eragrostis nindensis* (Fig. 9a) and in the leaves of *Xerophyta viscosa* (Fig. 9c) these quantities are much higher. In the inner leaves of *Eragrostis nindensis* and the leaves of *Xerophyta viscosa* respectively 3,0 and 3,6 mg / g DW is measured at this RWC-category. The abundance for the outer leaves of *Eragrostis nindensis* (Fig. 9b) and *Eragrostis tef* (Fig. 9d) are only 0,3 and 0,2 mg / g DW at the same category. However these differences are only significant compared with the results of *Xerophyta viscosa* ($p < 0,05$). When rehydrated the abundance of the inner leaves of *Eragrostis nindensis* (Fig. 9a) decreases till the original value of the fully hydrated state of 0 mg / g DW. The stachyose quantity in the leaves of *Xerophyta viscosa* (Fig. 9c) also decreases till approximately 50% of its original value when fully hydrated. However, the stachyose abundances in the outer leaves of *Eragrostis nindensis* (Fig. 9b) keeps increasing till 0,7 mg / g DW when rehydrated (compared to 0 mg / g DW when fully hydrated), but its standard deviation is also very high. Only for *Xerophyta viscosa* these differences with the rehydrated samples are statistically significant ($p < 0,05$). For the leaves of *Xerophyta viscosa* and the leaves of *Eragrostis tef* the abundance differences for both oligosaccharides between the fully hydrated and the desiccated RWC-category are statistically significant ($p < 0,05$). For *Eragrostis*

nindensis only the difference in abundance between the fully hydrated and the desiccated RWC-category for raffinose in the inner leaves is statistically significant ($p < 0,05$).

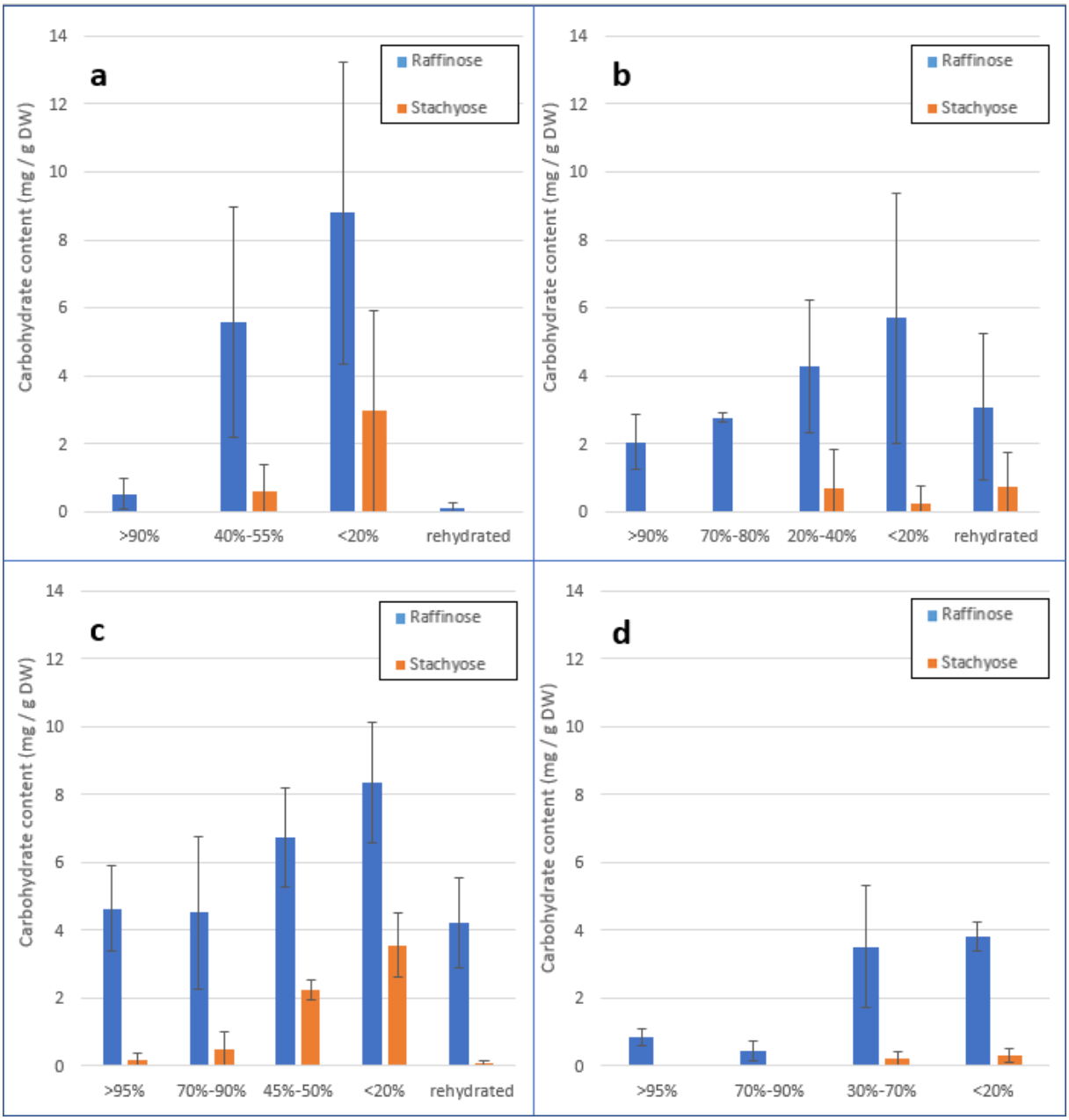

***Figure 9*. Mean oligosaccharide contents (mg / g DW) in respectively the inner leaves of *Eragrostis nindensis* (Fig. 9a), the outer leaves of *Eragrostis nindensis* (Fig 9b), the leaves of *Xerophyta viscosa* (Fig. 9c) and the leaves of *Eragrostis tef* (Fig. 9d).** On the Y-axis the mean carbohydrate contents (mg / g DW) of raffinose (blue bar) and stachyose (orange bar) is shown. The X-axis represents the various RWC-categories and if applicable a rehydrated category. Error bars represent the standard errors of the mean

Trehalose

Figure 10 shows the mean abundance of trehalose at different stages during dry-down and rehydration for all four leaf groups which are based on the raw data points shown in Figure A21-A24. The mean trehalose content stays at roughly the same level during dry-down and rehydration in three leaf groups, there are only some fluctuations in the outer leaves of *Eragrostis nindensis* (Fig. 10b). For

respectively the inner leaves of *Eragrostis nindensis* (Fig. 10a), the leaves of *Xerophyta viscosa* (Fig. 10c) and the leaves of *Eragrostis tef* (Fig. 10d), the mean abundance during the entire dry-down is 0,4; 0,02 and 0,2 mg / g DW. However, all differences in the trehalose abundance in the outer leaves of *Eragrostis nindensis* (Fig. 10b) between different RWC-categories are not statistically significant ($p > 0,05$). The minimum and maximum trehalose content in the outer leaves of *Eragrostis nindensis* (Fig. 10b) is 0,3 and 0,7 mg / g DW, which means both inner and outer leaves of *Eragrostis nindensis* have a higher trehalose content than the leaves of *Xerophyta viscosa* and *Eragrostis tef*. However, only the differences with *Xerophyta viscosa* are statistical significant ($p < 0,05$).

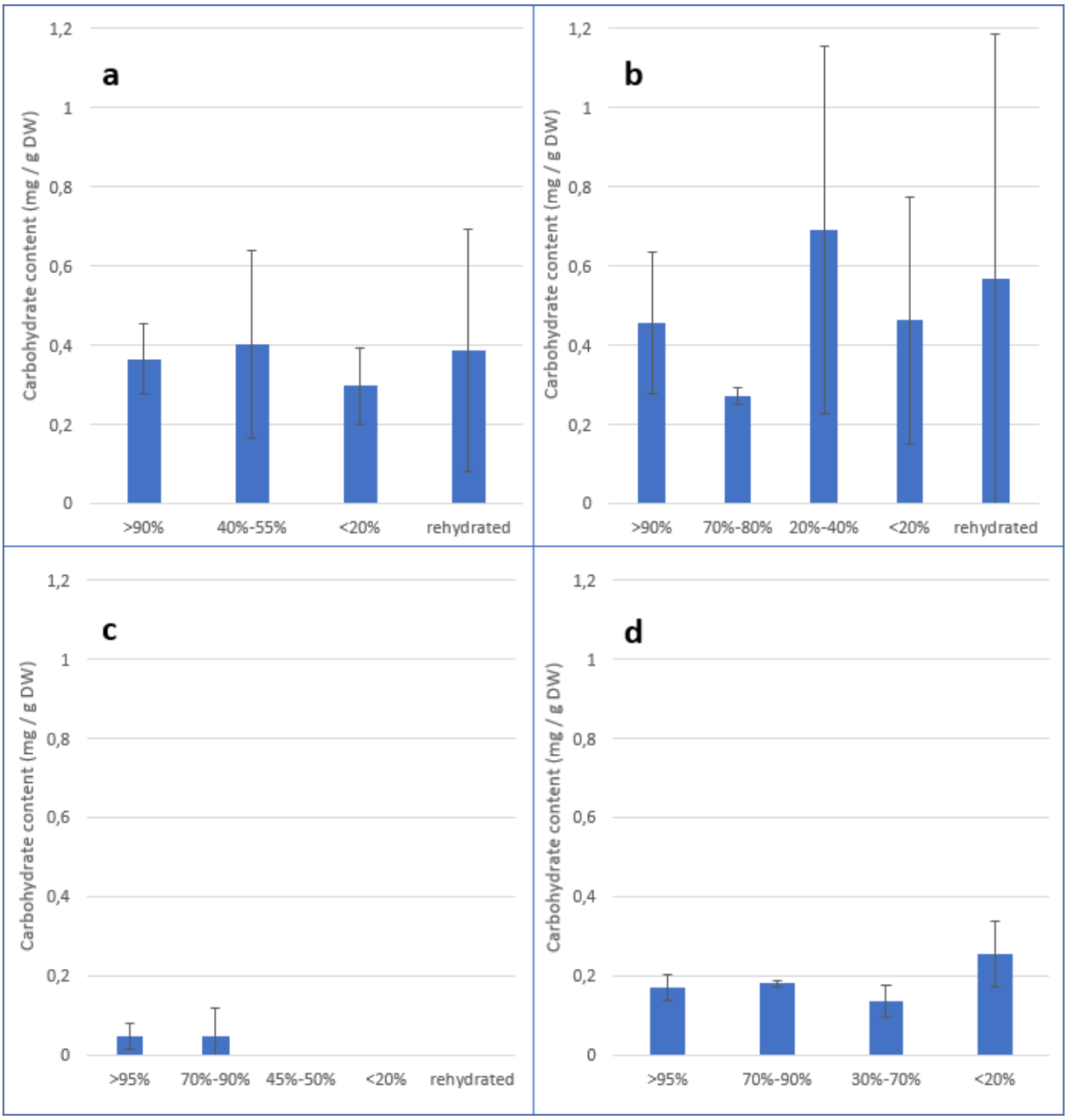

***Figure 10*. Mean trehalose content (mg / g DW) in respectively the inner leaves of *Eragrostis nindensis* (Fig. 10a), the outer leaves of *Eragrostis nindensis* (Fig 10b), the leaves of *Xerophyta viscosa* (Fig. 10c) and the leaves of *Eragrostis tef* (Fig. 10d).** On the Y-axis the mean carbohydrate content (mg / g DW) of trehalose. The X-axis represents the various RWC-categories and if applicable a rehydrated category. Error bars represent the standard errors of the mean.

Discussion

To answer the main question 'How do desiccation and rehydration affect the carbohydrate metabolism of key desiccation-related sugars in the resurrection species *Eragrostis nindensis*?' various quantitative measurements were done. Six different carbohydrates were quantified using a Dionex (HPLC), while activities of invertase, sucrose synthase (SuSy) and hexokinase were measured using spectrophotometric techniques. Previous research on *Eragrostis nindensis* showed that only the inner leaves rehydrate after desiccation and rehydration and therefor function as desiccation tolerant (DT) tissue (Vander Willigen et al., 2001). Because of that it is hypothesized that the metabolism of key desiccation-related sugars is affected differently in the inner and outer leaves of *Eragrostis nindensis* during desiccation and rehydration. Additionally, it is proposed that the inner leaves of *Eragrostis nindensis* act similarly, with respect to the metabolism of key desiccation related sugars, to the leaves of *Xerophyta viscosa* (both DT) and the outer leaves of *Eragrostis nindensis* act similarly to the leaves of *Eragrostis tef* (both desiccation sensitive).

To answer the first sub question 'How do desiccation and rehydration influence the sucrose metabolism in *Eragrostis nindensis* and how does this compare to the well-known resurrection species *Xerophyta viscosa* and the desiccation sensitive species *Eragrostis tef*?', both a sugar quantitation of glucose, fructose and sucrose and quantitative measurements of the invertase, sucrose synthase (SuSy) and hexokinase activities were performed using leaf samples with several relative water contents (RWC). It is proposed that during early drying response, most plant species lower their osmotic potential to extract more water out of their environment (Morgan, 1984; Mullet & Whitsitt, 1996). This is indeed observed in all leaf groups in this experiment. Prior to a medium RWC-category, the mean glucose and fructose abundance increases in all leaf groups. This means the glucose and fructose content is most likely constantly increasing until a certain RWC between the medium and the higher RWC-category. When desiccated there is a steep decrease in the mean glucose and fructose abundance in the inner leaves of *Eragrostis nindensis* and the leaves of *Xerophyta viscosa*. This decrease is also observed in the outer leaves of *Eragrostis nindensis* and the leaves of *Eragrostis tef*, but it is a lot less distinct. These results support the proposition that sucrose is formed at the expense of glucose and fructose during desiccation in DT leaves because it is the main protectant sugar (Farrant et al., 2007). The mean sucrose abundance patterns also support this hypothesis. In the inner leaves of *Eragrostis nindensis* a major increase of sucrose is measured when desiccated and a major decrease when rehydrated. To a lesser extent this pattern is also observed in the leaves of *Xerophyta viscosa* but it is not observed in the outer leaves of *Eragrostis nindensis* and the leaves of *Eragrostis tef*. Hexokinase catalyses a key step for the biosynthesis of sucrose out of glucose and fructose. Sucrose synthase (SuSy) is a key factor for sucrose accumulation and degradation (Doehlert, 1989; Whittaker et al., 2001). An increase in sucrose content was measured in the inner leaves of *Eragrostis nindensis* after desiccation and an increase in hexokinase and sucrose synthase (SuSy) activity were also detected after desiccation. After rehydration these activities decreased, which fits the measured decrease in sucrose. Invertase catalyses the cleavage of sucrose into glucose and/or fructose (Mohammadi, El Rhazi, Amine, Brett, & Brett, 2002). A major increase in invertase activity was measured in the inner leaves of *Eragrostis nindensis* after rehydration. This could be explained by the sucrose accumulation during the late stages of dry-down. A high sucrose content is not essential after rehydration and therefore it is degraded into glucose and/or fructose.

In previous experiments a significant increase in sucrose was also observed for *Xerophyta viscosa*, measuring nearly 5-fold increase in sucrose content between the fully hydrated and desiccated leaves (Peters, Mundree, Thomson, Farrant, & Keller, 2007). This is in contradiction to near 1,5-fold increase in this experiment and the absolute values are also very different between these experiments. The

highest sucrose content measured by Peters et al. (2007) (23,5 mg / g DW) is lower than the lowest sucrose content measured in this experiment (28,4 mg / g DW). Another difference between this experiment and previous experiments is the sucrose abundance pattern. In this experiment the highest increase in sucrose content is between the 45-50% RWC and <20% RWC categories. However, in previous experiments with the leaves of *Xerophyta viscosa* there is no increase in sucrose content in RWC-values lower than 55% RWC (Whittaker et al., 2001). A possible explanation for these differences could be the age of the *Xerophyta viscosa* plants and the amount of cycles (of dehydration and rehydration) they have undergone. Although Gaff and Oliver (2013) claim there is no limit on the number of cycles which DT plants can handle, provided there are periodic opportunities for photosynthesis, this could influence the exact results. It could be possible DT plants react slightly different to dehydration when they have already undergone several cycles of dehydration and rehydration, they still accumulate sucrose but the timing and quantity could be different. Most previous carbohydrate and relevant enzyme experiments with *Eragrostis nindensis* are not useful for comparison with this experiment. The insight that *Eragrostis nindensis* handles desiccation different than other DT plants like *Xerophyta viscosa* and the insight that only its younger (inner) leaves are functioning as its DT leaves is quite recent. Because of this, most available reports treat all leaves of *Eragrostis nindensis* equally and those results are based on an unknown combination of inner and outer leaves.

Most results in this experiment confirm the hypothesis that sucrose is the main protective carbohydrate during desiccation in *Eragrostis nindensis* and *Xerophyta viscosa* and it is formed out of glucose and/or fructose (Farrant et al., 2007). However, the results show that for both leaf groups approximately 10-15% of the required carbon is provided by fructose and glucose degradation, which means most sucrose is accumulated by starch mobilization (Smith, Zeeman, Thorneycroft, & Smith, 2003). Based on the measured differences between the inner and outer leaves of *Eragrostis nindensis*, there is indeed an important difference between those leaves with respect to the sucrose metabolism. Additionally the results of both DT leaf types and both desiccation sensitive leaf types show some similarities. This answers the first sub question 'How do desiccation and rehydration influence the sucrose metabolism in *Eragrostis nindensis* and how is this compared to the well-known resurrection species *Xerophyta viscosa* and the desiccation sensitive species *Eragrostis tef*?'.

To answer the second sub question 'How do desiccation and rehydration influence the quantities of the oligosaccharides raffinose and stachyose in *Eragrostis nindensis* and how do these quantities relate to the well-known resurrection species *Xerophyta viscosa* and the desiccation sensitive species *Eragrostis tef*?', a sugar quantitation of raffinose and stachyose with leaf samples of different RWC's was executed. It is proposed these sugars mainly assist the role of sucrose by helping to prevent the sucrose from crystallizing at high concentrations (Caffrey et al., 1988).

Raffinose accumulation was measured in all four leaf groups during dry-down, though in absolute values more raffinose is present in the inner leaves of *Eragrostis nindensis* and the leaves of *Xerophyta viscosa*. In both leaf groups (the inner leaves of *Eragrostis nindensis* and the leaves of *Xerophyta viscosa*) an average content of more than 8 mg / g DW was measured in the desiccated category and in the other two leaf groups (the outer leaves of *Eragrostis nindensis* and the leaves of *Eragrostis tef*) this value was lower than 6 mg / g DW. The stachyose abundance pattern is totally different from raffinose during dry-down and rehydration because no stachyose was measured in the fully hydrated leaf samples of *Eragrostis nindensis* and *Eragrostis tef* and only a small amount in the leaf samples of *Xerophyta viscosa*. All leaf groups accumulate stachyose during desiccation, but this accumulation is a lot more pronounce in the inner leaves of *Eragrostis nindensis* and the leaves of *Xerophyta viscosa*. When rehydrated a profound decrease in both raffinose and stachyose was measured in the inner

leaves of *Eragrostis nindensis* and the leaves of *Xerophyta viscosa*. However, for some samples it is possible stachyose was near the limit of detection for the HPLC (Dionex) method, which probably makes these results unreliable.

In previous experiments an increase in raffinose and stachyose was also measured during dry-down in the leaves of *Xerophyta viscosa*, though the pattern was slightly different. In this experiment both mean contents keep on increasing till the <20% RWC category. This means the mean content is increasing till a certain RWC between 5% and the highest value of the previous category, which is 50% for *Xerophyta viscosa*. In a similar experiment Peters et al. (2007) observed a linear increase till 50% RWC and after this point the raffinose and stachyose contents stayed constant in *Xerophyta viscosa*. There is also a difference in absolute values. The average raffinose content of the fully hydrated samples is quite similar in both experiments but when desiccated Peters et al. (2007) measured a raffinose content over twice as large compared to the results from this experiment. When rehydrated in both literature and this experiment a decrease in both oligosaccharides is observed, probably serving as an energy source for repair and recovery (Farrant et al., 2015).

Most results in this experiment confirm raffinose and stachyose play a role in DT plants. In absolute values both raffinose and stachyose mean contents are higher in the inner leaves of *Eragrostis nindensis* and the leaves *Xerophyta viscosa* when desiccated. Based on the measured differences between the inner and outer leaves of *Eragrostis nindensis*, there is indeed a difference between those leaves with respect to the raffinose and stachyose quantities. This answers the second sub question 'How do desiccation and rehydration influence the quantities of the oligosaccharides raffinose and stachyose in *Eragrostis nindensis* and how is this compared to the well-known resurrection species *Xerophyta viscosa* and the desiccation sensitive species *Eragrostis tef*?'.

To answer the third sub question 'How do desiccation and rehydration influence the quantity of trehalose in *Eragrostis nindensis* and how do these quantities relate to the well-known resurrection species *Xerophyta viscosa* and the desiccation sensitive species *Eragrostis tef*?' a sugar quantitation of trehalose with leaf samples of different RWC's was executed. Trehalose is present in most desiccation tolerant species, but is very rare in non-resurrection plants. Despite the low contents it is proposed trehalose supplements the effects of sucrose in DT plants (Ghasempour et al., 1998).

During the whole dry-down there is no accumulation of trehalose measured in any of the four leaf groups in this experiment. The mean trehalose contents in the outer leaves of *Eragrostis nindensis* show some small fluctuations, but overall the trehalose contents remains on the same level in all leaf groups during the dry-down. In absolute numbers the mean trehalose content in both leaf types of *Eragrostis nindensis* are much higher than in the leaves of *Xerophyta viscosa* and *Eragrostis tef*. However, it is possible trehalose was near the limit of detection for the HPLC (Dionex) method, which probably makes these results unreliable. Previous research has not come to a consensus on the role of trehalose in DT plants. Ghasempour et al. (1998) did several comparable sugar quantitative measurements with eight different desiccation tolerant species. In some species no trehalose was measured, in some a constant level was measured and in some there was an accumulation of trehalose. Like in this experiment, no trehalose was measured by Ghasempour et al. (1998) in *Xerophyta viscosa*. Trehalose was measured in *Eragrostis nindensis*, but in an unknown mixture of inner and outer leaves.

Although the results of this experiment are comparable with previous research, no conclusive statements can be made about the role of trehalose in DT species. In some DT species, like *Xerophyta viscosa*, it is absent and although trehalose is rare in desiccation sensitive species it was measured in *Eragrostis tef* in this experiment. There is no clear difference in mean trehalose contents between the

inner and outer leaves of *Eragrostis nindensis* and there is also no accumulation measured during dry-down. This suggests desiccation and rehydration do not influence the trehalose content in all three investigated species and with that the third sub question ‘’How do desiccation and rehydration influence the quantity of trehalose in *Eragrostis nindensis* and how is this compared to the well-known resurrection species *Xerophyta viscosa* and the desiccation sensitive species *Eragrostis tef*?’ is answered.

With the help of all three sub questions, the main question ‘How is the carbohydrate metabolism of key desiccated-related sugars affected during desiccation and rehydration in the resurrection species *Eragrostis nindensis*?’ is answered. There are several results which support the hypothesis that the metabolism of key desiccation-related sugars is affected differently in the inner and outer leaves of *Eragrostis nindensis* during desiccation and rehydration. To draw any indisputable conclusions, future research is necessary with more repetitions. This results will have a higher statistical significance and more accurate graphs can be made without the use of RWC-categories. More repetitions potentially eliminate the current large dispersion as shown in Appendix A. This is especially notable during the mid-stages of dry-down. Extra repetitions could also help to eliminate potential dispersion caused by the differences in drying speed between plants of the same species. In this experiment the RWC content is used as a timescale for the dry-down, but in reality there were some major fluctuations in the rate of dehydration between plants which started a dry-down at the same moment. No research is done on the timing of sugar accumulation and degradation and it could be of high importance. In this experiment a distinction is made between inner and outer leaves of *Eragrostis nindensis*, but for all leaf groups the whole leaf is used to quantitate the carbohydrates. Since *Xerophyta viscosa* has a different DT strategy (senescence of all leaf tips) in comparison with *Eragrostis nindensis* (senescence of the older ‘outer’ leaves) it could be useful to research the exact position of all carbohydrates and carbohydrate accumulation within the leaves. This could be done using microscopy or by taking several samples from different locations on the same leaf performing quantitative carbohydrate analysis.

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Appendix A

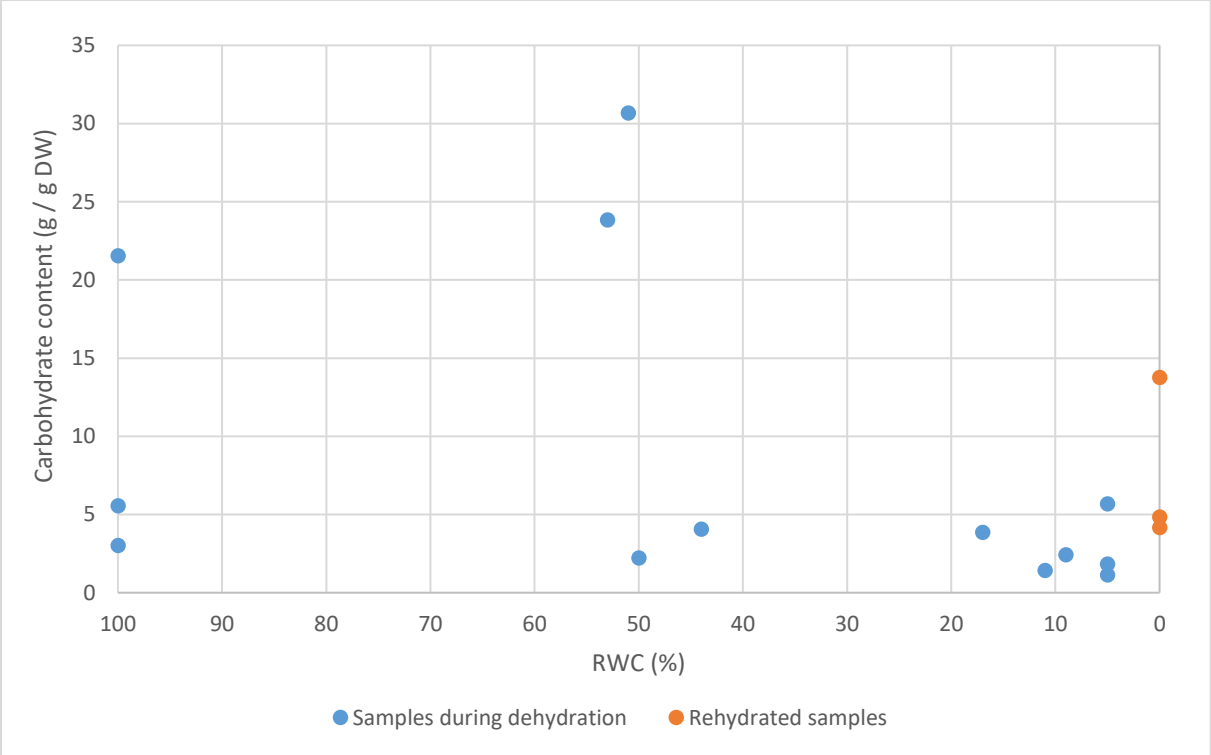

***Figure 1*. Raw data points for the glucose contents in the inner leaves of *Eragrostis nindensis*.** The Y-axis represents the carbohydrate contents (mg / g DW) of glucose and the X-axis represents the RWC. For a clear difference rehydrated samples are set at RWC 0%.

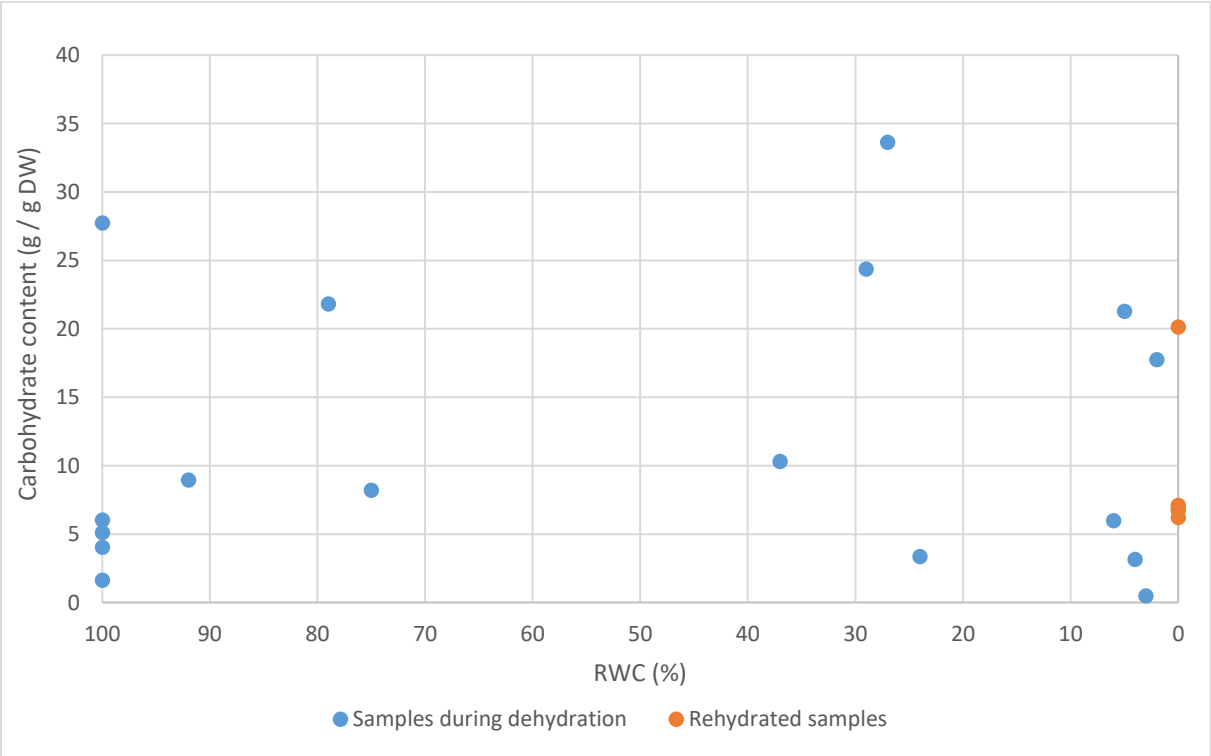

***Figure 2*. Raw data points for the glucose contents in the outer leaves of *Eragrostis nindensis*.** The Y-axis represents the carbohydrate contents (mg / g DW) of glucose and the X-axis represents the RWC. For a clear difference rehydrated samples are set at RWC 0%.

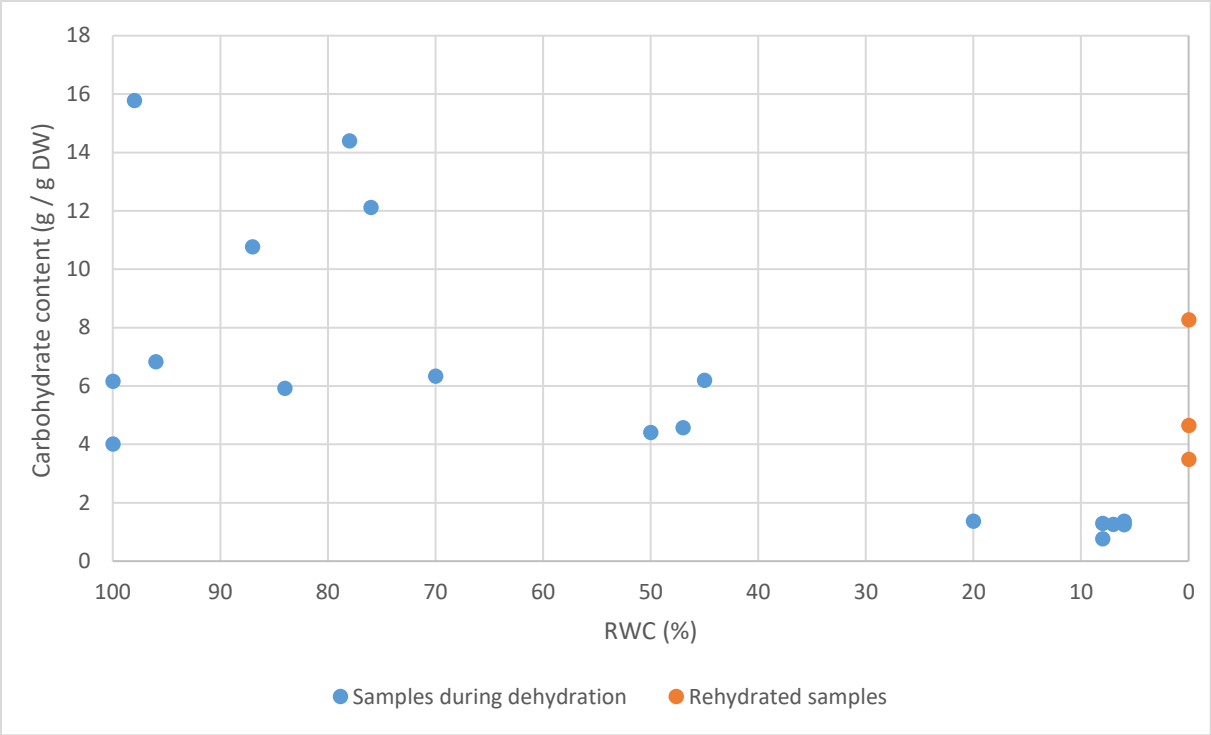

***Figure 3*. Raw data points for the glucose contents in the leaves of *Xerophyta viscosa*.** The Y-axis represents the carbohydrate contents (mg / g DW) of glucose and the X-axis represents the RWC. For a clear difference rehydrated samples are set at RWC 0%.

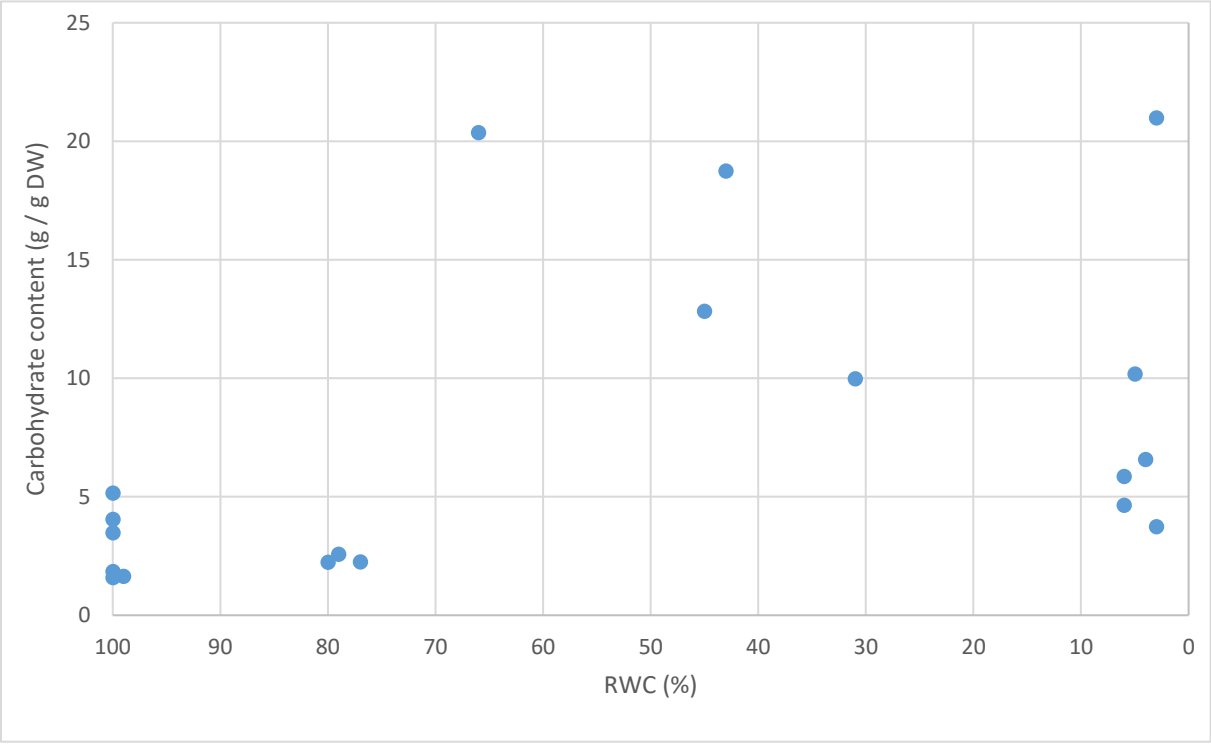

***Figure 4*. Raw data points for the glucose contents in the leaves of *Eragrostis tef*.** The Y-axis represents the carbohydrate contents (mg / g DW) of glucose and the X-axis represents the RWC.

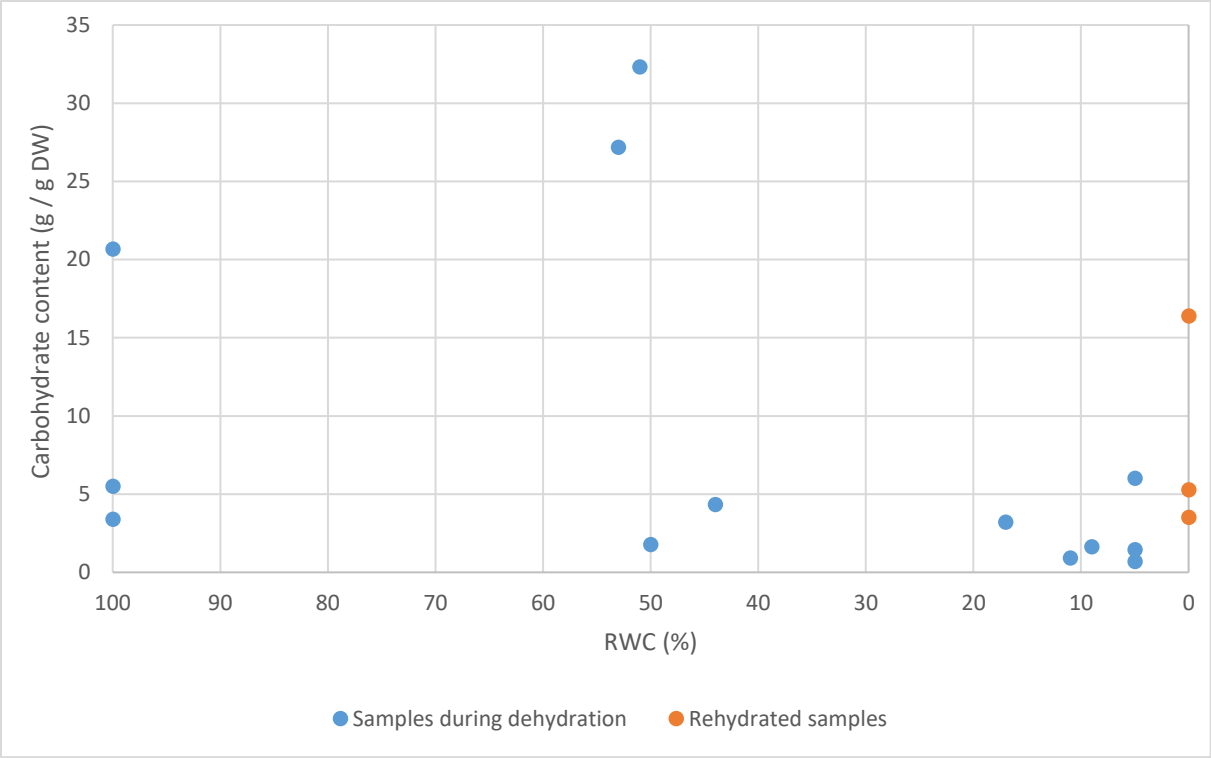

***Figure 5*. Raw data points for the fructose contents in the inner leaves of *Eragrostis nindensis*.** The Y-axis represents the carbohydrate contents (mg / g DW) of fructose and the X-axis represents the RWC. For a clear difference rehydrated samples are set at RWC 0%.

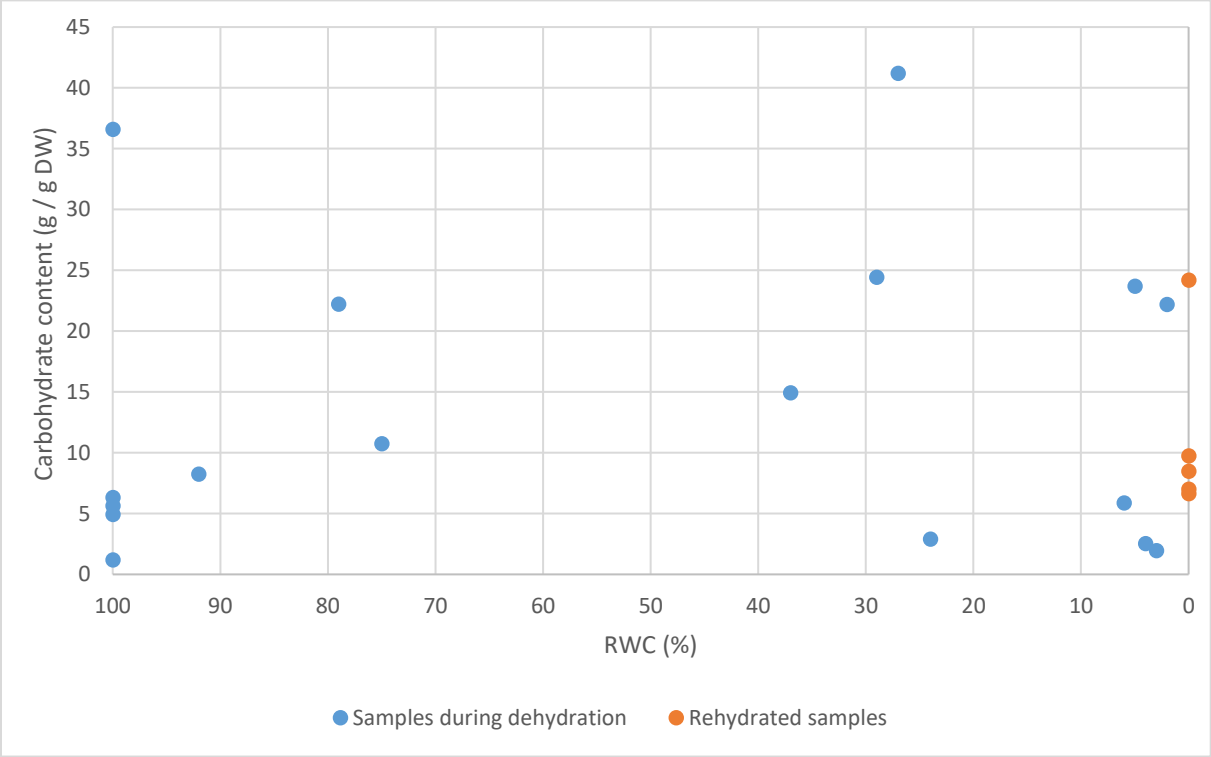

***Figure 6*. Raw data points for the fructose contents in the outer leaves of *Eragrostis nindensis*.** The Y-axis represents the carbohydrate contents (mg / g DW) of fructose and the X-axis represents the RWC. For a clear difference rehydrated samples are set at RWC 0%.

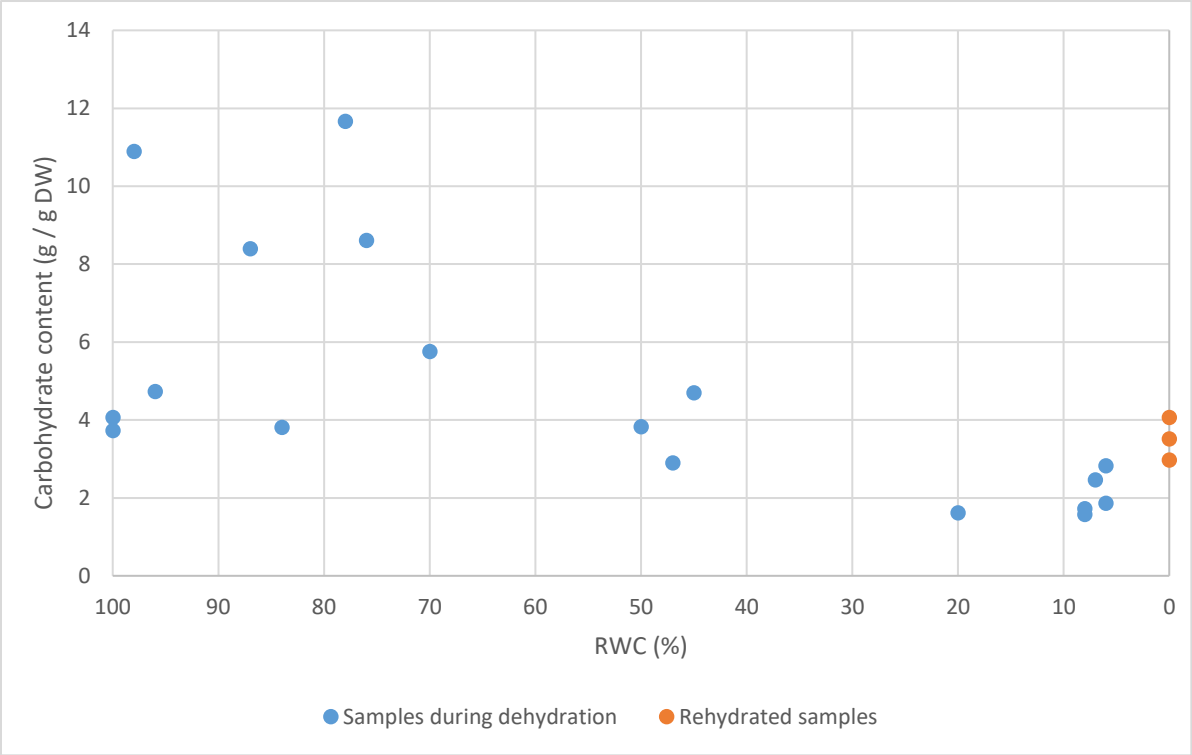

***Figure 7*. Raw data points for the fructose contents in the leaves of *Xerophyta viscosa*.** The Y-axis represents the carbohydrate contents (mg / g DW) of fructose and the X-axis represents the RWC. For a clear difference rehydrated samples are set at RWC 0%.

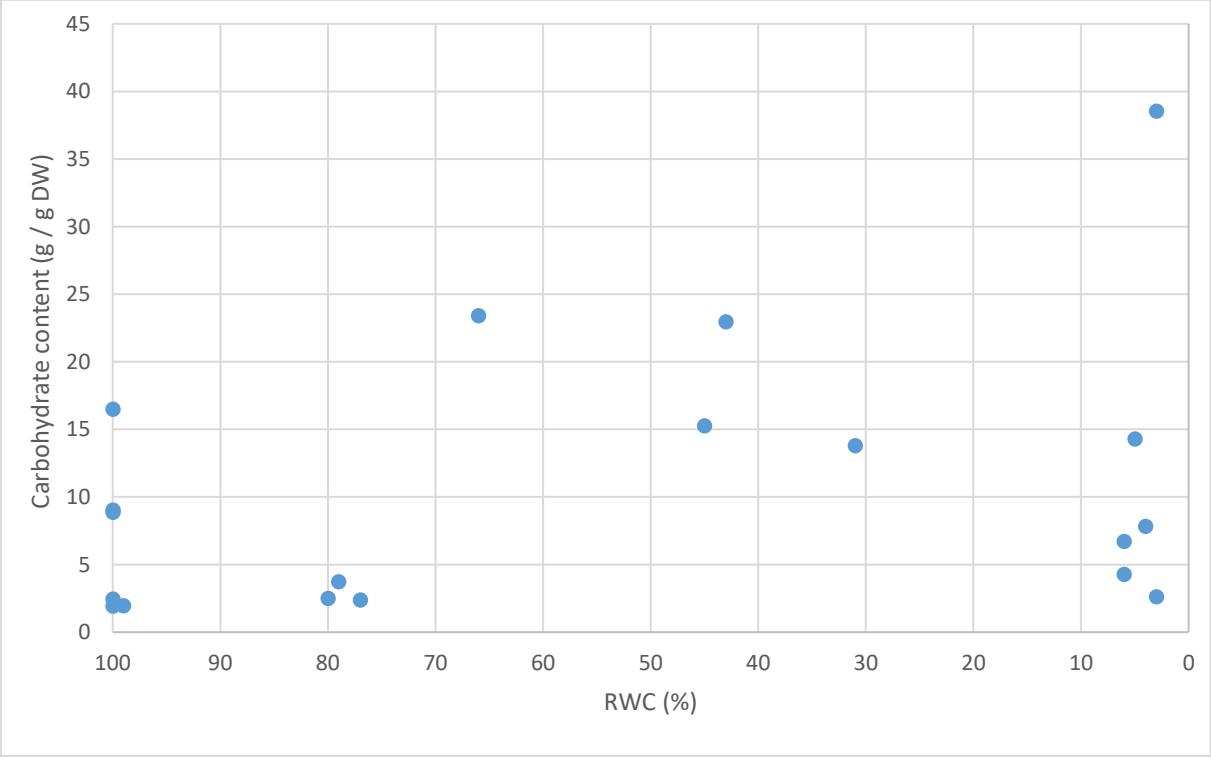

***Figure 8*. Raw data points for the fructose contents in the leaves of *Eragrostis tef*.** The Y-axis represents the carbohydrate contents (mg / g DW) of fructose and the X-axis represents the RWC.

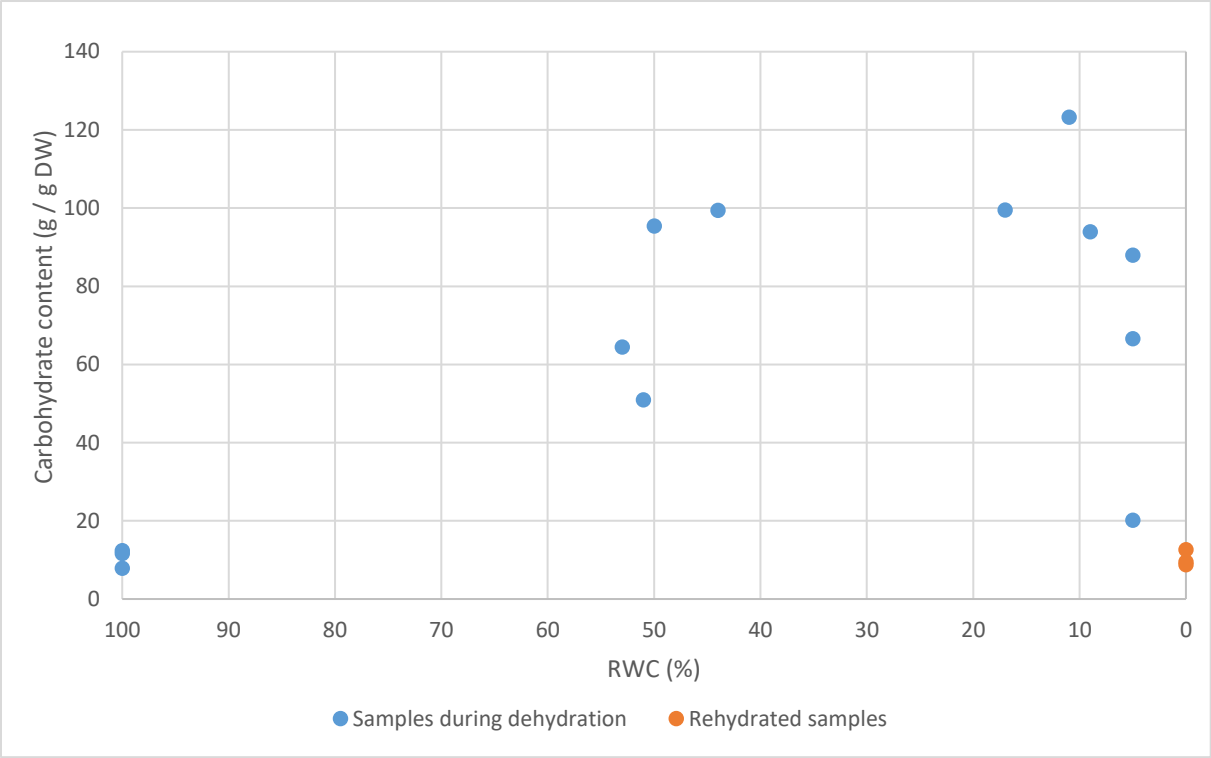

***Figure 9*. Raw data points for the sucrose contents in the inner leaves of *Eragrostis nindensis*.** The Y-axis represents the carbohydrate contents (mg / g DW) of sucrose and the X-axis represents the RWC. For a clear difference rehydrated samples are set at RWC 0%.

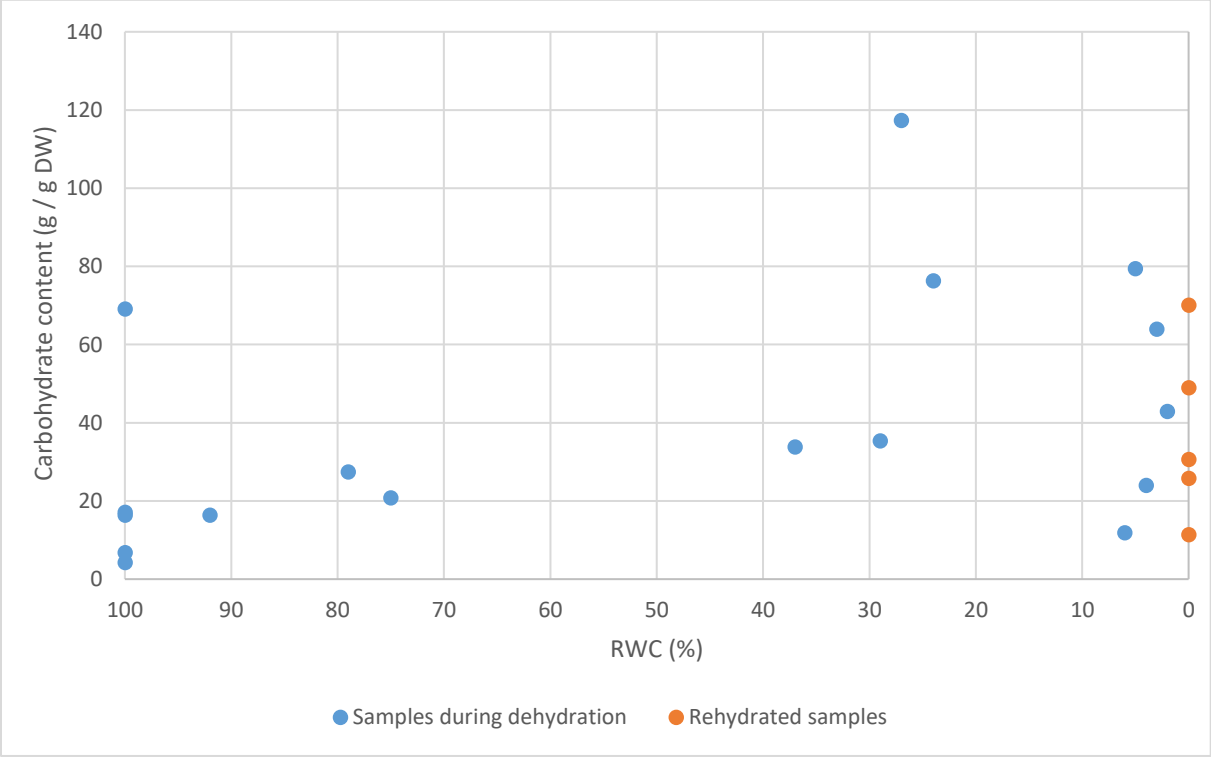

***Figure 10*. Raw data points for the sucrose contents in the outer leaves of *Eragrostis nindensis*.** The Y-axis represents the carbohydrate contents (mg / g DW) of sucrose and the X-axis represents the RWC. For a clear difference rehydrated samples are set at RWC 0%.

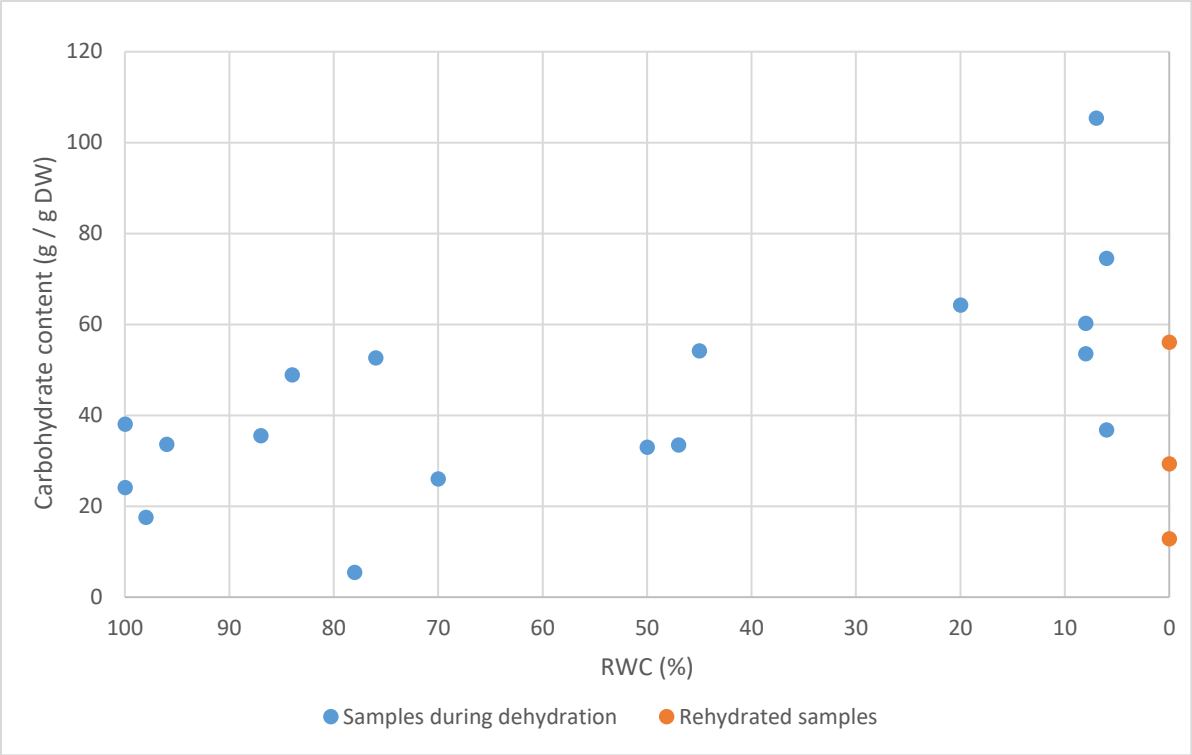

***Figure 11*. Raw data points for the sucrose contents in the leaves of *Xerophyta viscosa*.** The Y-axis represents the carbohydrate contents (mg / g DW) of sucrose and the X-axis represents the RWC. For a clear difference rehydrated samples are set at RWC 0%.

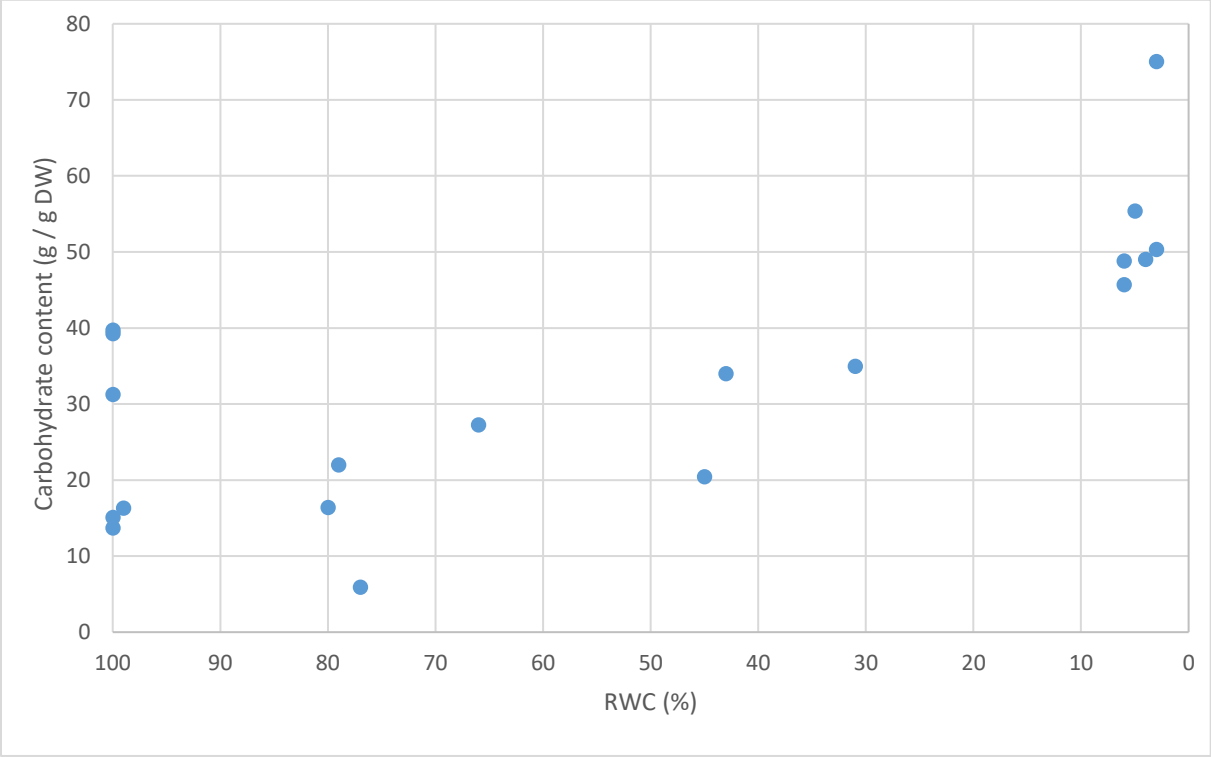

***Figure 12*. Raw data points for the sucrose contents in the leaves of *Eragrostis tef*.** The Y-axis represents the carbohydrate contents (mg / g DW) of sucrose and the X-axis represents the RWC.

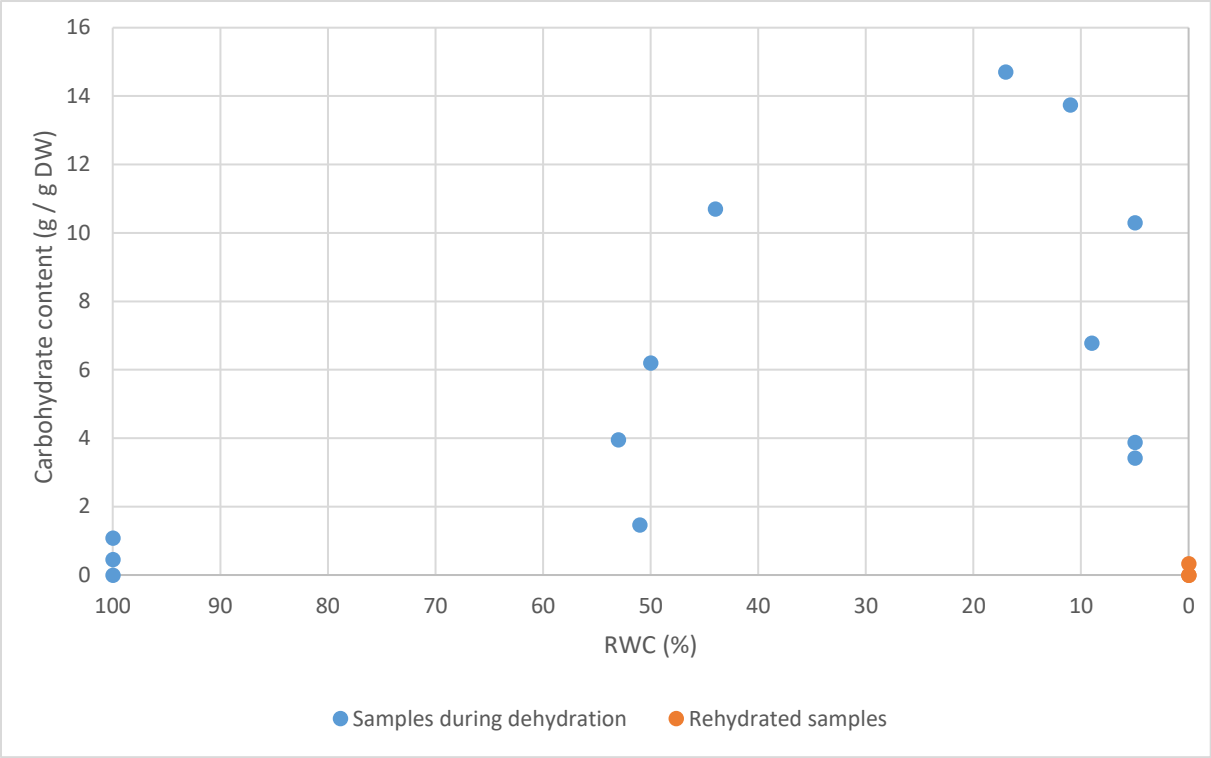

***Figure 13*. Raw data points for the raffinose contents in the inner leaves of *Eragrostis nindensis*.** The Y-axis represents the carbohydrate contents (mg / g DW) of raffinose and the X-axis represents the RWC. For a clear difference rehydrated samples are set at RWC 0%.

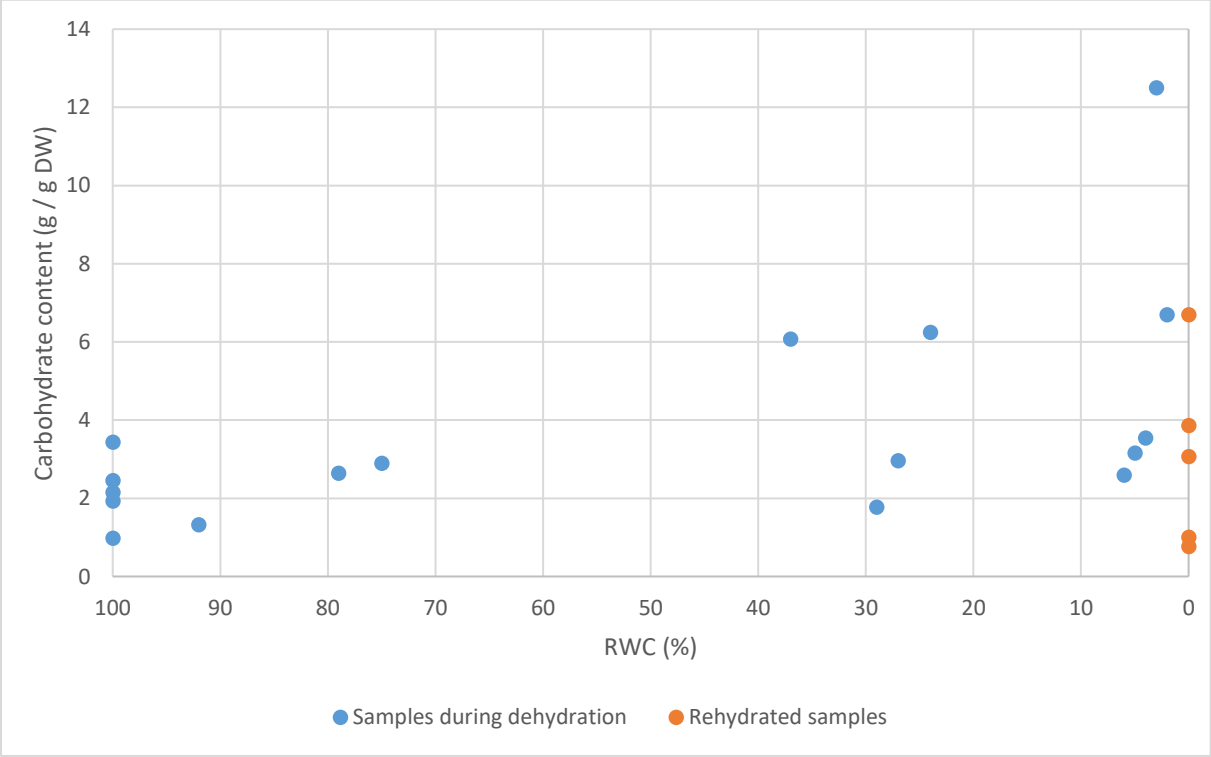

***Figure 14*. Raw data points for the raffinose contents in the outer leaves of *Eragrostis nindensis*.** The Y-axis represents the carbohydrate contents (mg / g DW) of raffinose and the X-axis represents the RWC. For a clear difference rehydrated samples are set at RWC 0%.

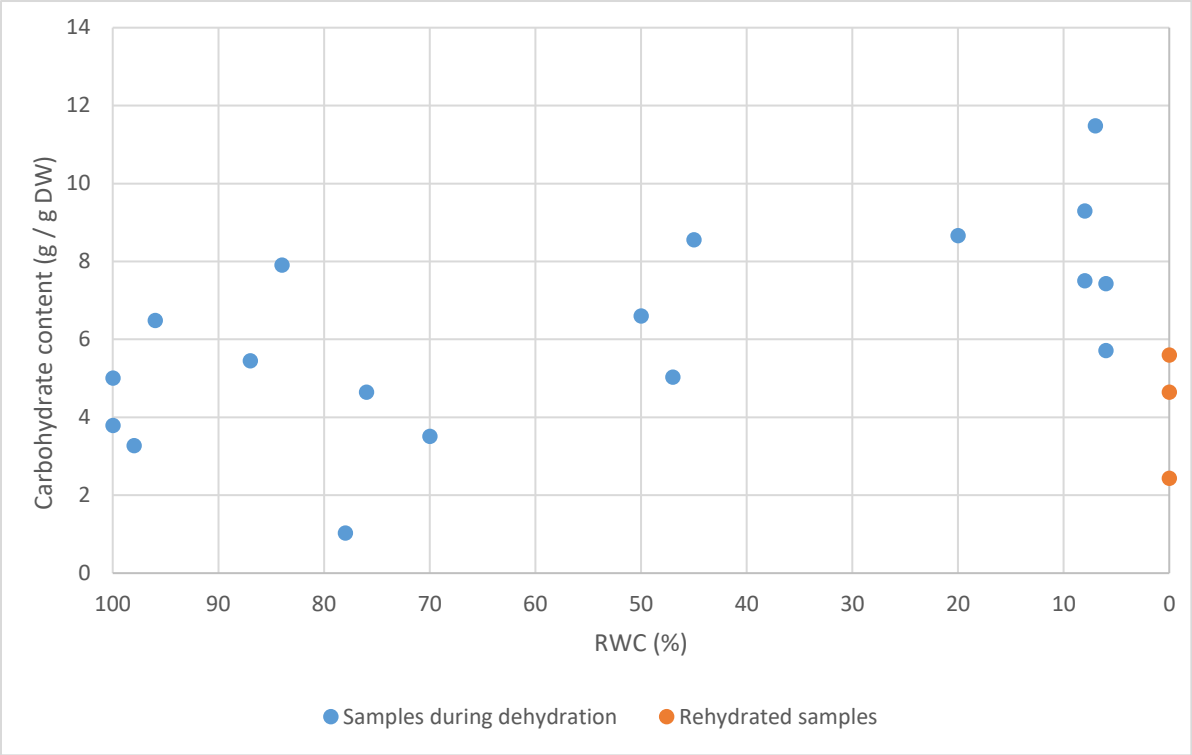

***Figure 15*. Raw data points for the raffinose contents in the leaves of *Xerophyta viscosa*.** The Y-axis represents the carbohydrate contents (mg / g DW) of raffinose and the X-axis represents the RWC. For a clear difference rehydrated samples are set at RWC 0%.

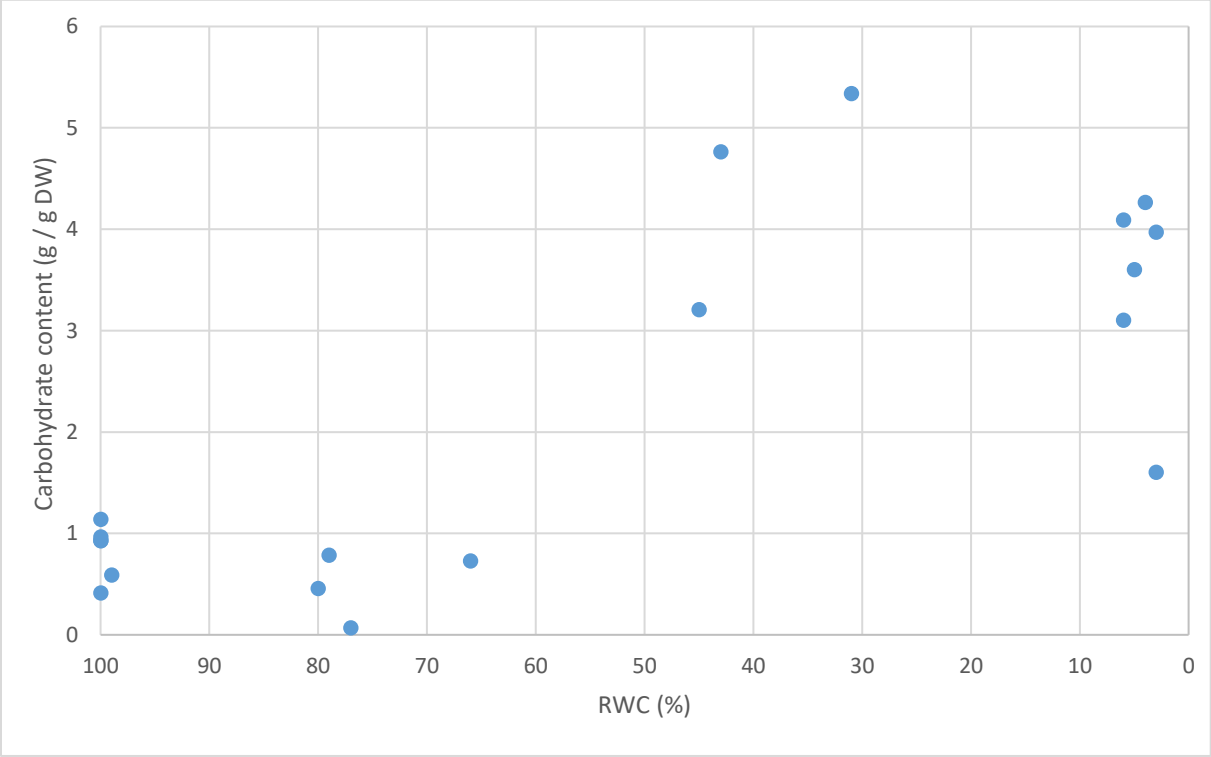

***Figure 16*. Raw data points for the raffinose contents in the leaves of *Eragrostis tef*.** The Y-axis represents the carbohydrate contents (mg / g DW) of raffinose and the X-axis represents the RWC.

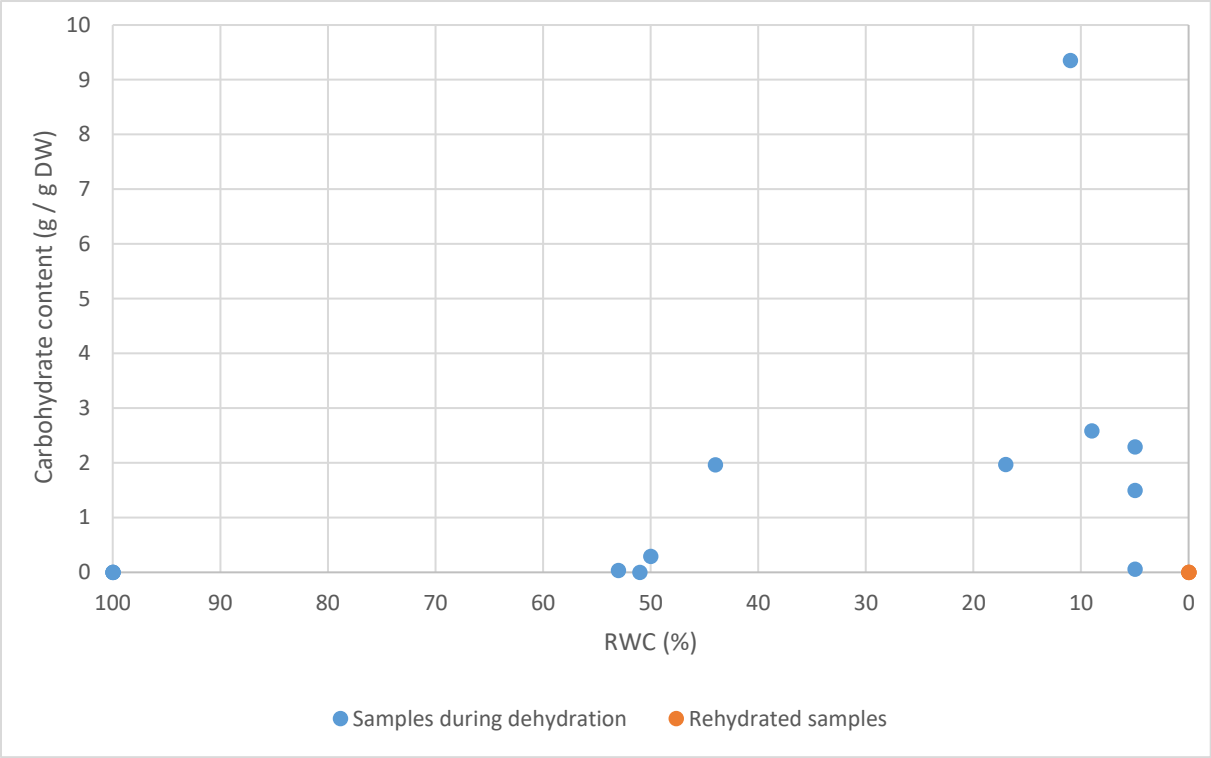

***Figure 17*. Raw data points for the stachyose contents in the inner leaves of *Eragrostis nindensis*.** The Y-axis represents the carbohydrate contents (mg / g DW) of stachyose and the X-axis represents the RWC. For a clear difference rehydrated samples are set at RWC 0%.

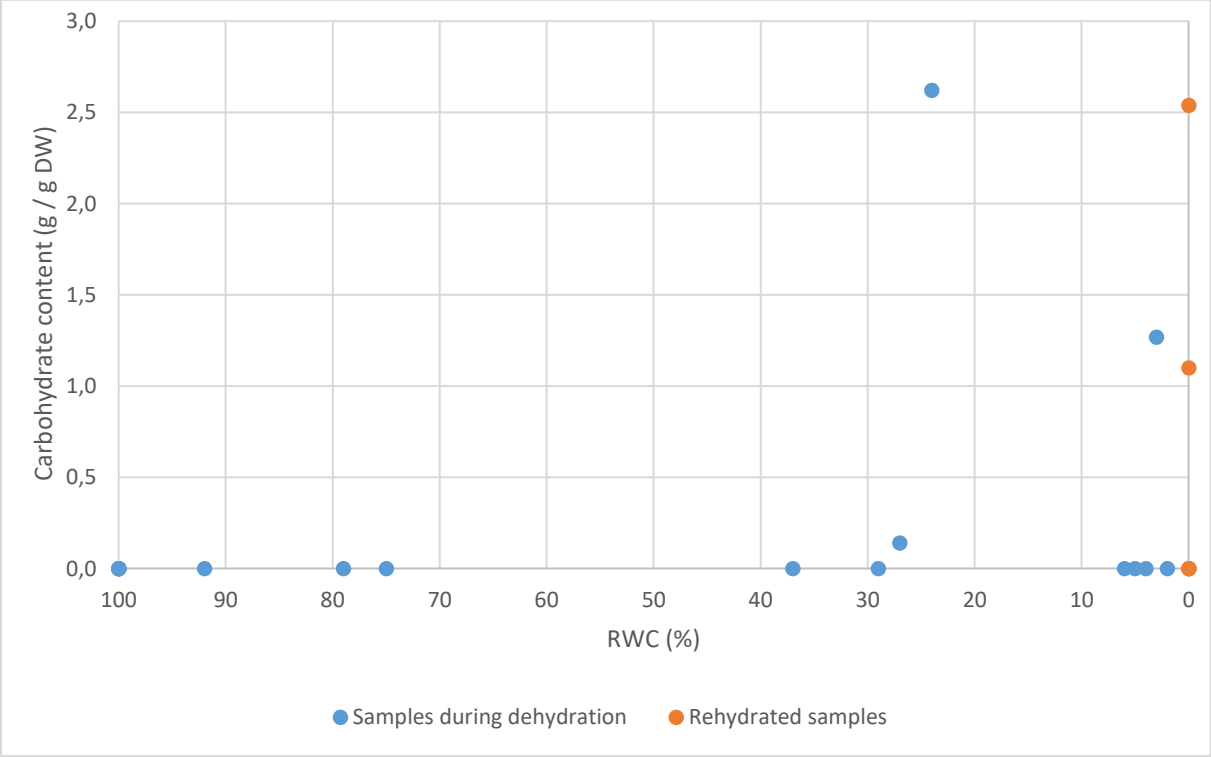

***Figure 18*. Raw data points for the stachyose contents in the outer leaves of *Eragrostis nindensis*.** The Y-axis represents the carbohydrate contents (mg / g DW) of stachyose and the X-axis represents the RWC. For a clear difference rehydrated samples are set at RWC 0%.

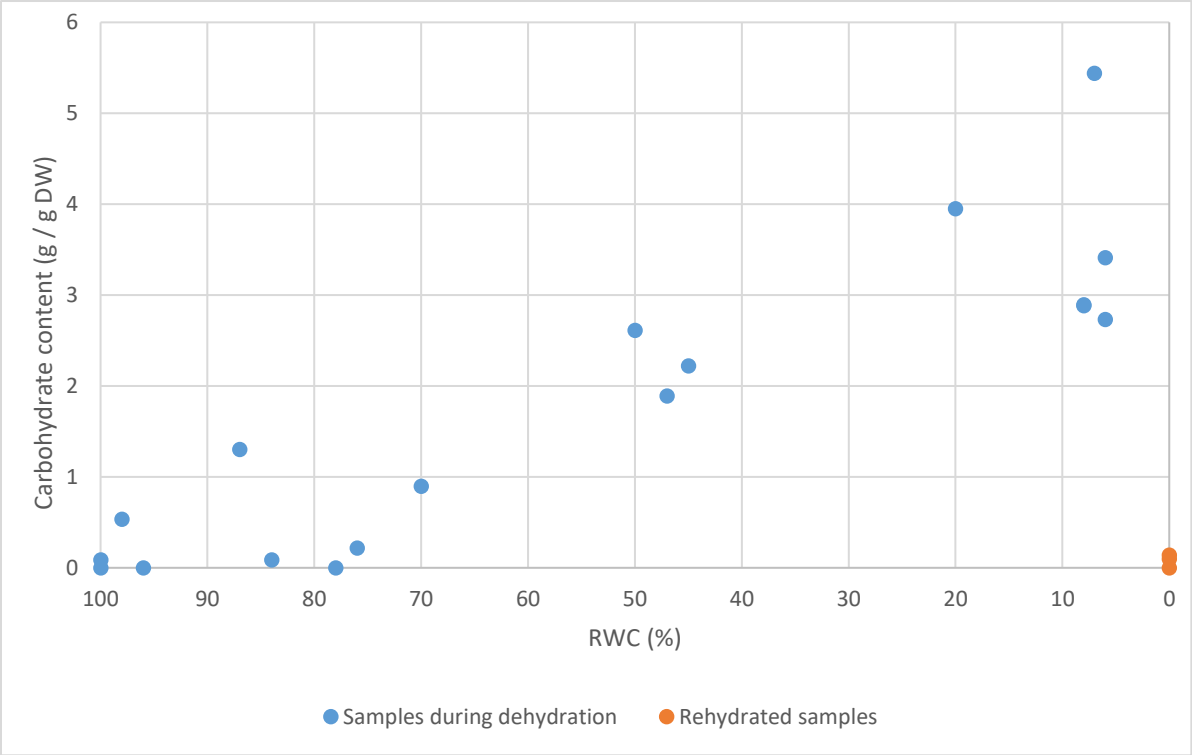

***Figure 19*. Raw data points for the stachyose contents in the leaves of *Xerophyta viscosa*.** The Y-axis represents the carbohydrate contents (mg / g DW) of stachyose and the X-axis represents the RWC. For a clear difference rehydrated samples are set at RWC 0%.

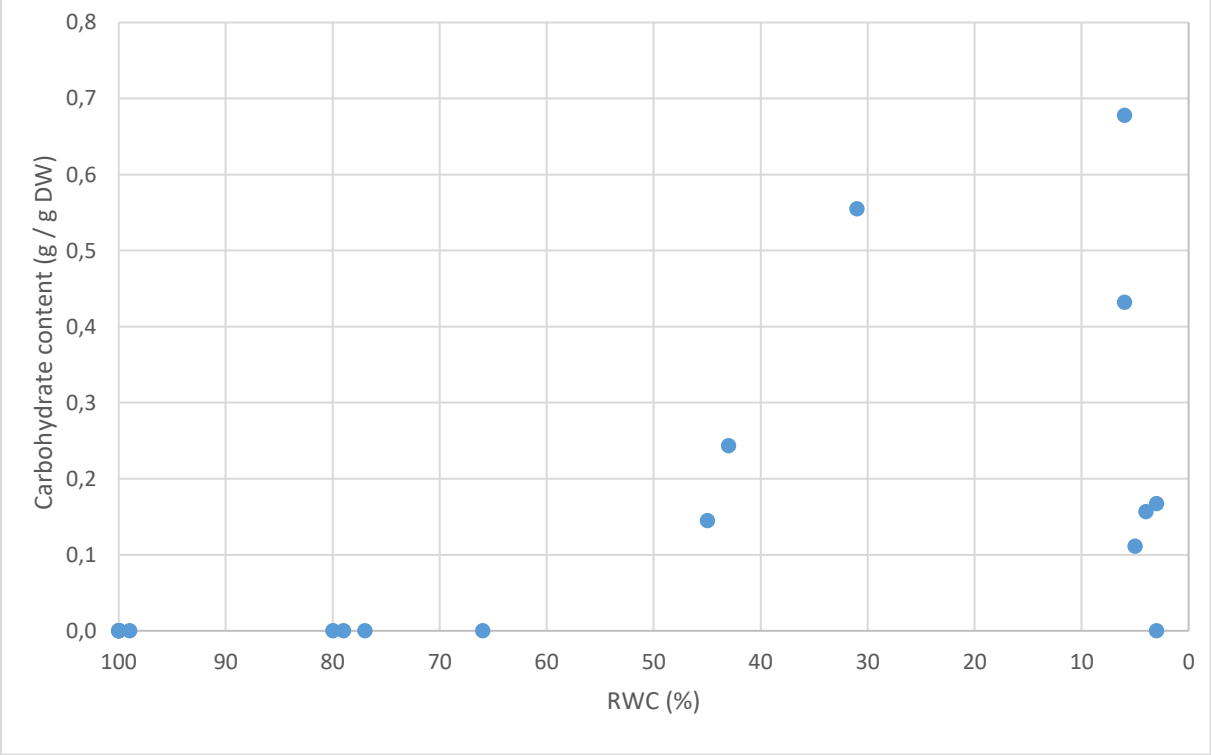

***Figure 20*. Raw data points for the stachyose contents in the leaves of *Eragrostis tef*.** The Y-axis represents the carbohydrate contents (mg / g DW) of stachyose and the X-axis represents the RWC.

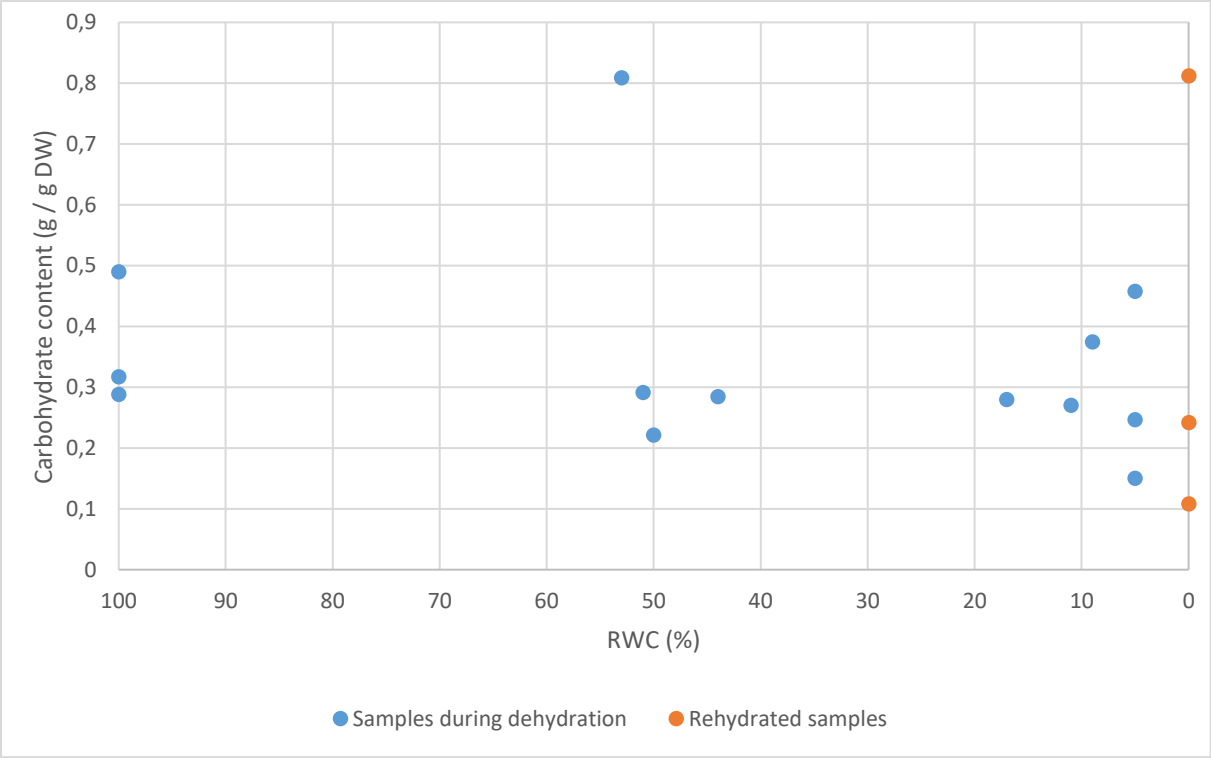

***Figure 21*. Raw data points for the trehalose contents in the inner leaves of *Eragrostis nindensis*.** The Y-axis represents the carbohydrate contents (mg / g DW) of trehalose and the X-axis represents the RWC. For a clear difference rehydrated samples are set at RWC 0%.

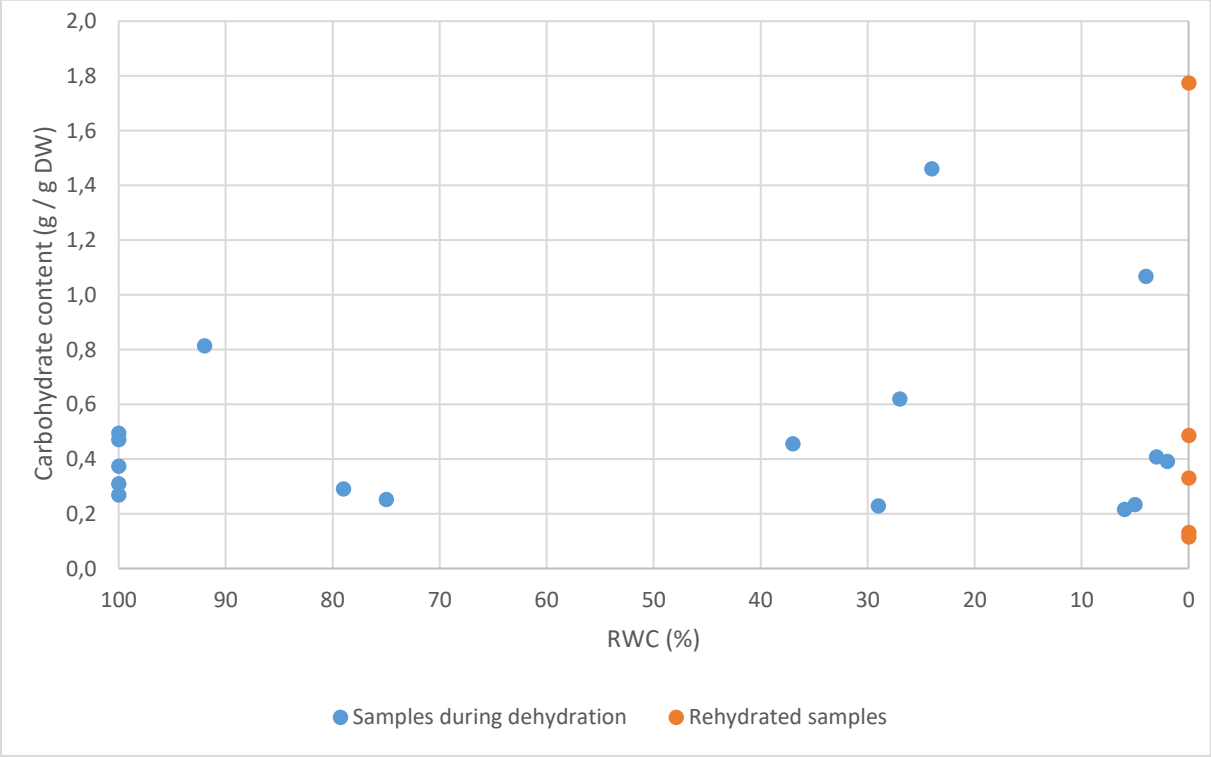

***Figure 22*. Raw data points for the trehalose contents in the outer leaves of *Eragrostis nindensis*.** The Y-axis represents the carbohydrate contents (mg / g DW) of trehalose and the X-axis represents the RWC. For a clear difference rehydrated samples are set at RWC 0%.

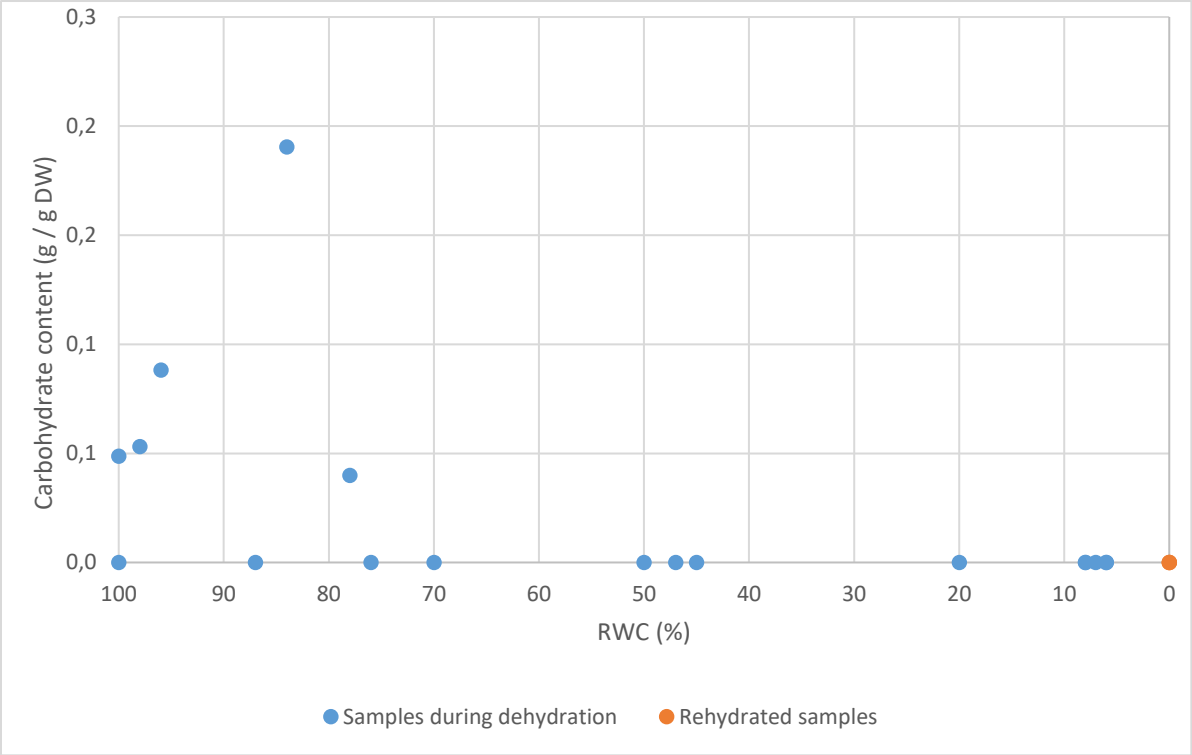

***Figure 23*. Raw data points for the trehalose contents in the leaves of *Xerophyta viscosa*.** The Y-axis represents the carbohydrate contents (mg / g DW) of trehalose and the X-axis represents the RWC. For a clear difference rehydrated samples are set at RWC 0%.

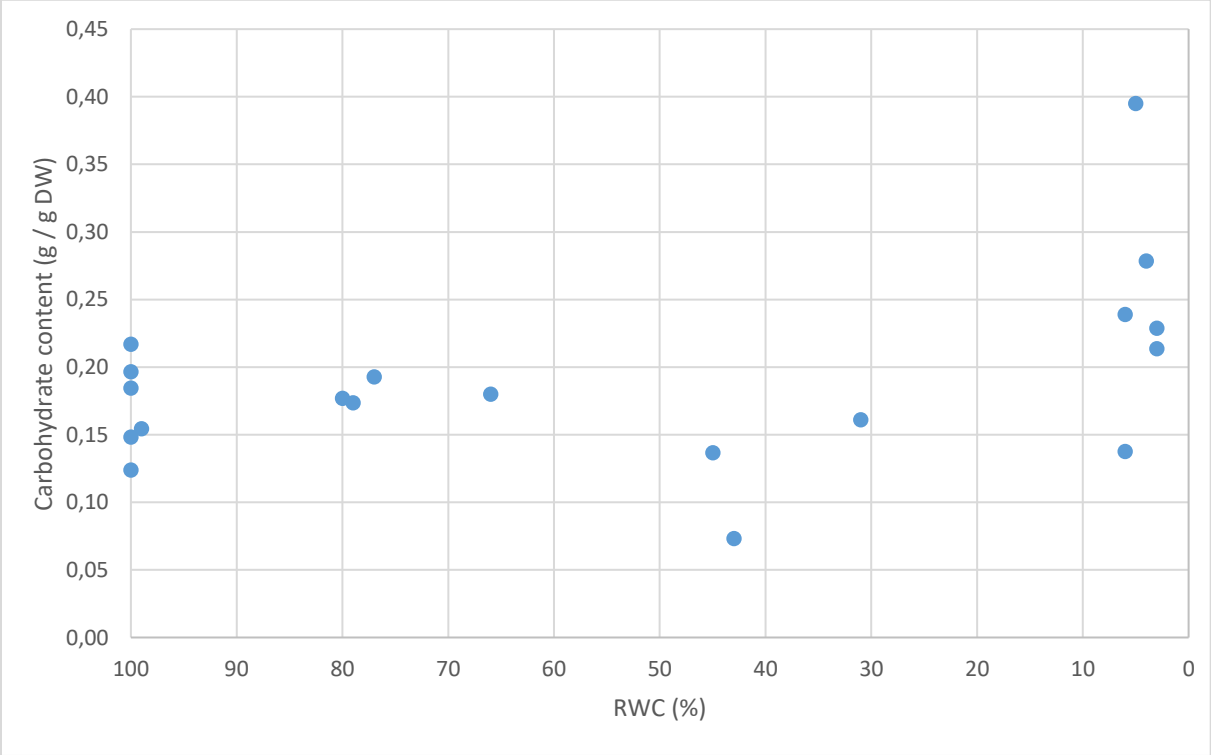

***Figure 24*. Raw data points for the trehalose contents in the leaves of *Eragrostis tef*.** The Y-axis represents the carbohydrate contents (mg / g DW) of trehalose and the X-axis represents the RWC.