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Research article

Vegetative desiccation tolerance in *Eragrostiella brachyphylla*: biochemical and physiological responses

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ABSTRACT

Eragrostiella brachyphylla is an angiosperm desiccation-tolerant resurrection plant, which can survive during desiccation in the air-dry state and recover completely on availability of water. The present study was conducted to understand the vegetative desiccation tolerance of *Eragrostiella brachyphylla* by evaluating its ability to recover the physiological, biochemical and morphological functions post desiccation. In order to understand the responses of *Eragrostiella brachyphylla* to desiccation and subsequent rehydration experiments were conducted in the hydrated state (HS), desiccated state (DS) and rehydrated state (RS). Scanning electron microscopy revealed significant changes between the three stages in the internal ultra-structures of leaves and stems. Compared to the other states, photosynthetic parameters such as chlorophyll a, chlorophyll b, total chlorophylland total carotenoid contents decreased significantly in the desiccated state. Superoxide radical ($O2^{\bullet-}$) content also increased, resulting in an oxidative burst during desiccation. Consequently, antioxidant enzymes such as catalase (CAT) superoxide dismutase (SOD) peroxidase (APX) and glutathione reductase (GR) activities were found to be significantly elevated in the desiccated state to avoid oxidative damage. Increased malondialdehyde (MDA) content and relative electrolyte leakage (REL) during desiccation provide evidence for membrane damage and loss of cell-wall integrity. During desiccation, the contents of osmolytes represented by sucrose and proline were found to increase to maintain cell structure integrity. After rehydration, all physiological, biochemical and morphological properties remain unchanged or slightly changed when compared to the hydrated state. Hence, we believe that these unique adaptations contribute to the remarkable desiccation-tolerance property of this plant.

1. Introduction

India's agriculture sector has to face a looming existential threat from climate change. About 50 % of the country is facing drought, with 16 % facing extreme or exceptional category of drought. The majority of the country's farmers still depend upon monsoon for their irrigation needs, 50–60% of farming in India is still rain-fed. Crop failure and drought havenow become a permanent scenario in the country. Parts of Karnataka, Maharashtra, Madhya Pradesh, Rajasthan, Chhattisgarh and Jharkhand reeling under drought are also extremely vulnerable to climate change. The present condition in India represents a severe economic constraint on agricultural production. Further, engineering of drought-tolerant crops with desiccation-tolerant (DT) traits identified from resurrection plants might be employed as a sustainable option. Elucidation of desiccation tolerance and its regulatory mechanisms in resurrection plant species may lead to the identification of unique features and processes of DT plants which can improve stress tolerance (Bartels, 2005; Blomstedt et al., 2018; Farrant and Moore, 2011; Alpert, 2005; Neeragunda et al., 2018).

Desiccation toleranceis present in the microbial, fungal, plant and animal kingdoms (Alpert, 2006). Vegetative desiccation tolerance is a very complex biological process that is present in less than 0.2% of the vascular plant species. Air-dryness or desiccation tolerance is present only in seed and in pollen of the plants, but comparatively few species have evolved desiccation-tolerance in their vegetative parts (Bartels, 2005; Farrant and Moore, 2011). Resurrection plants can recover their

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regular biological and physiological functions within several hours or a few days, depending on the species and size of the dried vegetative tissues (Tuba & Lichtenthaler, 2011; Aidar et al., 2017). In DT plants, water loss results in the disruption of membranes leading to prominent metabolic and mechanical damages (Levitt, 1980). Further, DT plants upon desiccation stress: (a) will limit the damage to repairable levels, (b) can maintain basal physiological integrity in the dried state, (c) mobilize their repair mechanisms very quickly (Bewley, 1995; Oliver et al., 1998; Farrant et al., 2003; Karbaschi et al., 2016).

Tolerance to desiccation in the plant kingdom is noted in most of the taxonomic groups ranging from pteridophytes to dicotyledons, but absent in gymnosperms (Gaff, 1986; Alpert, 2000). The habitats of DT species are ecological niches with unreliable rainfalls and seasonally inadequate water availability, DT species have been identified in all continents of the world. DT plants can live as outcrops of rock at moderate to low elevations in subtropical and tropical zones and also soils with minimum water retention capacity (Porembski and Barthlott, 2000). Most of the identified DT plants grow in the subtropics and tropics of Southern Africa and East-West Africa (incl. Madagascar), North America, Brazil, Australia, Eastern and Western Ghats of India (Kappen and Valladares, 1999).

Approximately 300 angiosperm resurrection plant species possess desiccation tolerance in their vegetative tissues. The resurrection grass *E. brachyphylla* belongs to one of the most abundant and most widespread angiosperms families: the Poaceae, *E. brachyphylla* is perennial, native Asiantropical plant. Inflorescence is composed of racemes and it hasfertile pikelets comprising 10–15 fertile florets; Glumes are lower persistent, upper deciduous; species in this family are usually sensitive to water loss and most individuals die when leaf water potentials fall below -4 MPa (Proctor and Smirnoff, 2000). However, some of the species in this family (e.g., *E. brachyphylla*) have evolved the ability to survive desiccation. The shoots and roots of these species canremain dried for considerable periodslike a seed. When rehydrated, these species can recover and continue to grow (Alpert, 2000).

When desiccation stress occurs quickly it results in lowered stomatal conductance and uptake of CO_2. These light-driven reactions will typically reset after rewatering by absorbing light energy with the interaction of chlorophyll-protein complexes (Dinakar et al., 2012). Mechanisms to prevent stress, includes increased pigment accumulation in vegetative tissues such as anthocyanins, rhodoxanthin, and betalanes, which protect photosynthetic tissue from photoinhibition and photobleaching by reducing the light intensity (Sherwin and Farrant, 1998). Imbalanced light reaction and downward biochemical pathways result in a lack of electron sinks and the system becoming over energised. Hence, it leads to reactive oxygen species (ROS) generation (Das & Roychoudhury, 2014; Dinakar et al., 2012). Antioxidants activated via stress-induced signaling is an essential process in the adaptation of oxidative stress during desiccation. Hence, the upregulated antioxidants are essential markers for drought stress. The control of scavenging and production of ROS are an essential role of plants to survive desiccation stress (Gill and Tuteja, 2010). The principal antioxidant defense system comprises catalase (CAT) superoxide dismutase (SOD) peroxidase (APX) and glutathione reductase (GR) enzymes (Hussain et al., 2016). Proline plays a significant role in osmotic adjustments and stress signaling, which leads to preserving cell turgor and removal of ROS by increasing the antioxidant enzymatic capacity, under stress conditions (Bartlett et al., 2014; de Campos et al., 2011). Desiccation stress reduces the starch content in leaf tissues (Whittaker et al., 2001; Norwood et al., 2000). Starch is converted into sugars such as sucrose and trehalose and these sugars are well known to contribute and maintain the turgor. Thus, they can prevent membrane fusions in the cell and protein denaturation during water stress (Pandey et al., 2010).

The present study aimed to evaluate the morphological, biochemical and physiological alterations that accompany the acquisition of desiccation tolerance in *E. brachyphylla.* The goal of this study is to quantify photosynthetic parameters, capture morphological changes, measure proline, sucrose, starch, lipid peroxidation, membrane stability, oxidative stress and antioxidant enzymes content during hydration, desiccation and rehydration in *E. brachyphylla*. To reduce the light absorption leaf blades of *E. brachyphylla* fold in half along the midrib and exposed abaxial surface as like *X. humilis, C. wilmsii* to minimisedamage from light (Farrant, 2000). The exciting factor in our study of desiccation tolerance in *E. brachyphylla* is how it protects itself against the damage caused by desiccation. Desiccation stress produces a morphological alteration in resurrection plants (Farrant et al., 2003). These findings enable us to understand better how plants withstand desiccation of their vegetative tissues and also to make inferences as to the processes biochemical, morphological and physiological adaptions to survive and recover from desiccation of vegetative tissues.

2. Material and methods

2.1. Plant material and relative water content analysis

E. brachyphylla species were collected from Deverayanadurga Reserve Forest, located in Tumakuru district of Karnataka state, India, which is a Thorny Scrub and Dry Deciduous Forest (Coordinates-13.3737 N, 77.2075 E), Photographs of the fresh flower, twigs, were taken with Nikon D3200 DSLR Camera 32 MP (Figure 1). The collected specimen was identified with the aid of floras (Gaff, 1986; Clayton et al., 2008; Bhaskar and Kushalappa, 2013). Plants were maintained under greenhouse conditions (temperature: 21–24 °C, relative humidity 70–80 %, 11 h day and 13 h dark cycle) at Tumkur University with regular irrigation. A bunch of 28 days aged grass grown in the green house was taken for RWC analysis. RWC is a technique to measure the cellular water content of the plants. Three independent biological replicates, each consisting of three *E. brachyphylla* plants were used. Tissue samples (whole plant) were harvested before dehydration (100% RWC). All samples were collected at noon to avoid apparent differences in protein abundance caused by circadian or light-dark regulation. Healthy and young plants selected for RWC analysis, were hydrated in beaker (200 ml) containing double distilled water for a period of 4 h until there was no further increase in weight, such plants were considered to be in hydrated state (HS) and it served as a control. The hydrated plant tissues were dewatered for 48hours at room temperature until no further weight loss occurred, such plants were considered to be in desiccated state (DS). The desiccated plants were immersed in double distilled water to rehydrate for12 hours until no additional weight gain took place under similar light or dark cycle; these plants were considered to be in rehydrated state (RS) (Pandey et al., 2010). The dry weight of the rehydrated plant was obtained after baking at 105 °C for 30 min in a hot air oven (RT-150 RELITECH, India), followed by treatment at 80 °C until no further weight gain and stabilised. All plant material required for morphological and biochemical analysis were treated in the same way described above. The hydrated, desiccated and rehydrated plants (leaves for biochemical analysis, leaf + stem for ultrastructural analysis) were ground using liquid nitrogen with pestle and mortar and stored at -80 °C for further analysis. The water content in the samples was calculated as the difference between fresh weight and dry weight divided by the fresh weight (Yobi et al., 2017). RWC was expressed in percentage.

2.2. Ultrastructure

To obtain micro-morphological scanning electron microscope (m-mSEM) images of *E. brachyphylla* leaf and stems (hydrated, desiccated and rehydrated states), samples were immersed in liquid nitrogen to preserve structural features. It also facilitated the generation of cross sections of leaf and stem with a sharp blade. All samples were mounted onto stubs and coated with a small layer of gold by using sputter-coating equipment (Polaron SC7640, London, UK) to avoid compression of samples due to heat generated inside the SEM chamber. Then the samples were observed at 5 kV, and images in the analytical mode were acquired

Figure 1. Images of *E. brachyphylla*captured atDeverayanadurga Reserve Forest (Coordinates-13.3737° N, 77.2075° E), Tumakuru district of Karnataka state, India (A & B hydrated. C & D desiccated).

in different magnifications (200x, 500x and 1000x) by using the SEM-TESCAN Vega-3. Leaf and stem tissues were analysed for anatomical features:spongy mesophyll layer, epidermis and palisade mesophyll layer and also vascular cells (Berazaín et al., 2007).

2.3. Pigment content and photosynthesis analysis

HS, DS, and RS tissues of *E. brachyphylla* tissues were frozen with liquid nitrogen and homogenised using mortar and pestle. Chlorophyll and carotenoid contents were extracted with 80% acetone, centrifuged at 3000g using Centrifuge 5400 R (Eppendorf, CA, USA) at 4 °C. The absorbance of the supernatantwas measured at $\lambda = 470$, $\lambda = 644$ and $\lambda = 661$nm using BioSpectrometer Kinetic (Eppendorf, CA, USA). Total chlorophyll (a+b) and total carotenoid (x + c = xanthophylls and carotenes) were calculated according to Arnon (1949); Lichtenthaler (1987); Bulgari et al. (2019), and expressed on a fresh weight basis. Anthocyanin concentration was determined spectrophotometrically. Briefly, leaves were extracted in acidified methanol [methanol: water: HCl (79:20:1)] with continuous shaking at 0 °C for 72 h in the dark and centrifugedat 5000rpm for 10 min at 4°C. Absorbance of the supernatant was measured at $\lambda = 530$ and $\lambda = 657$ nm using the formula of Rayleigh: Corrected $\lambda_{530} = \lambda_{530} - 0.228\lambda_{657}$ (Murray and Hackett, 1991; Sims and Gamon, 2002). CO_2 exchange rate was measured with the flow-through Q-box CO650 Plant CO_2 Analysis Package andthe CO_2 exchange rate was calculated with the Logger Pro software (Qubit Systems, North America).

2.4. Proline determination

Extraction and spectrophotometric estimation of proline was carried out in leaf samples of *E. brachyphylla*. Approximately 600mg of hydrated, desiccated and rehydrated leaf samples were homogenised in mortar and pestle with liquid nitrogen and 10 ml of 3% aqueous sulfosalicylic acid. Equal volumes of filtered homogenate, glacial acetic acid and acid-ninhydrin, were boiled for an hour in a water bath. 4ml of toluene was added and tubes were placed on ice to terminate the reaction. The chromophore-containing layer was used to measure the absorbance $\lambda = 520$nm after room temperature achieved. Proline concentration was expressed on a fresh weight basis (μmol g^{-1} FW) (Gururani et al., 2015).

2.5. Activity analysis of antioxidant enzymes

The activities of catalase (CAT), superoxide dismutase (SOD), peroxidase (POD) and glutathione reductase (GR) were determined in HS, DS and RS tissues of *E. brachyphylla*. They were ground separately at 4 °C in 6 mL of extraction buffer-1 (50 mM PBS, pH 7.8) to assay SOD and CAT and in 6 mL extraction buffer-2 (100 mM PBS, pH 7.0 for POD and GR assays). The homogenates were collected and centrifuged at 15000 g at 4 °C for 20 min. The activity of the catalase was assayed by the measurement of the initial rate of disappearance of H_2O_2 by the technique delineated by Chance and Maehly (1995). CAT activity assay solution (3 ml) contained 50 mM phosphate buffer (pH 7.0), 5.9 mM H_2O_2 and 0.1 ml enzyme extract. The decreased rate of absorbance at $\lambda = 240$ nm was recorded in BioSpectrometer Kinetic (Eppendorf, CA, USA), and the activity was expressed in μmol min^{-1} g^{-1} Protein. The activity of peroxidase was assayed by a modified method of Rao et al. (1995). The reaction solution contained 0.1 mL of enzyme extract, 50 mM O-methoxyphenyl, 100 mM PBS (pH 7.0) and 40 mM H_2O_2(30%) and the activity was expressed in μmol min^{-1} g^{-1} Protein. Glutathione reductase activity was assayed using the method of Halliwell and Foyer (1978). The reaction mixture consisted of 0.5 mM GSSG, 50 mMTris-HCl, 0.2 mM NADPH and 5 mM $MgCl_2$. GR activity was determined by measuring the disappearance of NADPH at $\lambda = 340$ nm, within 3 min and expressed as the number of μmol of NADPH oxidisation Units mg^{-1} protein (Wang et al., 2010). Superoxide dismutase activity in *E. brachyphylla* was assayed by its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT). 6 mL reaction solution consisted of 50 mM PBS (pH 7.8), 130 mM methionine, 750 μM nitro blue tetrazolium chloride (NBT), and 100μM $EDTA^{-}Na^{2+}$, 20 μM riboflavin and 0.1 mL of enzyme extract. The reaction solution was incubated for 10 min under fluorescent light with an intensity of 50 μmol m^{-2} s^{-1} at 20 min. The absorbance was determined at $\lambda = 560$ nm. The amount of enzyme required to inhibit the photochemical reduction of NBT by 50% was expressed as Units mg^{-1} protein (Wang et al., 2010), known as one unit of SOD activity.

2.6. Measurement of lipid peroxidation

Lipid peroxidation in *E. brachyphylla* tissues were quantified spectrometrically by following the protocol of Heath and Packer (1968).0.2 g of HS, DS and RS leaves were ground with pestle and mortar in 2 mL of

0.25% thiobarbituric acid prepared in 10 % trichloroacetic acid. The ground tissues were heated for 30 min at 95 °C and centrifuged for 10 min at 10,000 g in centrifuge5400 R (Eppendorf, CA, USA). Later, the supernatant was carefully transferred and the absorption (at λ = 532 nm and λ = 600 nm) was measured. The obtained absorbance at λ = 600 nm was subtracted from λ = 532 nm in to adjust non-specific turbidity. Extinction coefficient of 1.55 $m^{-1}Mv^{-1}cm^{-1}$MDA was used in the study. Lipid peroxidation was reported as MDA formed and expressed in nmol g^{-1} FW (Pandey et al., 2010; Dixit et al., 2001).

2.7. Quantification of superoxide radical ($O_2^{\bullet-}$)

Superoxide radical ($O2^{\bullet-}$) assay was conducted in HS, DS and RS tissues of *E. brachyphylla*by using the method of Able et al. (1998). Decrease in XTT was measured in the presence of $O^{2\bullet-}$ radicle. 50mg leaf tissues were homogenised in 1.5mL Tris–HCl (pH 7.5) and then the homogenate was centrifuged 10 min at 5000 g. Assay mixture contained 50 μg leaf extract and 0.5mM XTT in 50mM Tris–HCl (pH 7.5). The supernatant was collected and the absorbance was measured at λ = 470 nm and the rate of $O2^{\bullet-}$ radical production obtained was reported in nmol g^{-1} FW (Extinction coefficient of XTT = $2.16 \times 10^4 M^{-1}cm^{-1}$) (Pandey et al., 2010).

2.8. Measurement of membrane permeability

Relative electrolyte leakage (REL) technique was used to testmembrane integrity of leaf tissues of *E. brachyphylla in* hydrated, desiccated and rehydrated states. Approximately 20 leaf discs (0.3 cm in diameter) from each state were wholly immersed in 60 mL of Milli-Q water and shaken overnight. Later, the sample was degassed for 20 min. The electrical conductivity of the solution was measured as E1 by using an electrical conductivity device SKU-5501 (Countron, India). The suspension containing leaf discs was boiled at 100 °C for 15 min in a water bath and allowed to cool at room temperature. The solution was thoroughly homogenisedfor 5 min and maximum conductivity of the solution measured as E2. The electrical conductivity of Milli-Q water (E3) was also measured. The REL was calculated using the equation: REL (%)= (E1 - E3)/(E2 - E3) × 100% (Wang et al., 2010; Chen et al., 2017).

2.9. Determination of sucrose and starch

E. brachyphylla tissues from hydrated, desiccated and rehydrated plants were finely ground in a pre-cooled mortar with ice-cold 1M perchloric acid. These extracts were centrifuged at 12,000 g for 4 min at 4 °C. The supernatants were neutralised with 5M Potassium carbonate (K_2CO_3). The supernatants and the pellets were kept separately on ice. Sucrose was spectrophotometrically assayed in supernatants whereas starch was assayed in pellets. The supernatants were resuspended in 0.2 ml buffer. The suspension was treated with 14 units amyloglucosidase and 0.4 units amylase at 55 °C for 2 h (or overnight at room temperature). Reaction buffer consisted of 100 mM HEPES pH 7.0, 5 mM $MgCl_2$, 0.5 mMdithiothreitol, 0.02% (w/v) bovine serum albumin. In a photometric cuvette, 1.0 mM ATP and 0.4 mM NADP and 50 μï extract were added. The absorbance was recorded at 340 nm. Further, 0.5 units glucose - 6-phosphate dehydrogenase, 0.5 units phosphoglucose isomerase, 3 units hexokinase and 100 units invertase were added and the absorbance measured at 340 nm. Difference in the readings gives the concentration of specific glucose which appeared after its liberation from sucrose hydrolysis catalysed by invertase. The sucrose concentration was then calculated using 6.22 $10E^3M^{-1}cm^{-1}$as extinction coefficient of NAD (P) H. In this assay, glucose hydrolysed from starch was measured as an indicator of the concentration of starch. The pellet obtained from the extraction was washed and homogenized three times in 0.2 M MES buffer (pH 4.5) and was then added 0.2 ml buffer. The suspension was treated with 14 units amyloglucosidase and 0.4 units amylase at 55 °C for 2 h (or overnight at room temperature). The reaction mixture was centrifuged and glucose in the supernatant as below: Reaction buffer consisted of 100 mM HEPES pH 7.0, 5 mM $MgCl_2$, 0.5 mMdithiothreitol 0.02% (w/v) bovine serum albumin. To the cuvette, 1.0 mM ATP and 0.4 mM NADP and 50 μï extract was added. The absorbance was recorded at λ = 340 nm. Further, 0.5 units glucose - 6-phosphate dehydrogenase was added and the absorbance measured at λ = 340 nm. The difference in the readings gave the concentration of glucose hydrolysedfrom starch which was calculated using 6.22 as extinction coefficient of NAD (P) H (at 1mM and 1cm) (Coombs et al., 1985).

2.10. Statistical analyses

Values presented in this manuscript have been obtained from mean of three independent scientific experiments. The legends shows mean ± SE. Student's t-test was used to analyse the significant differences between hydrated, desiccated and rehydrated states of *E. brachyphylla.*

3. Results

3.1. Relative water content analysis

Fully hydrated (Figure 2a) plants were subjected to desiccation and subsequent rehydration. Our studies showed that the time taken to rehydrate is vital for the potential of plants to resurrect. During desiccation (48 h), RWC of plants reached an air-dry state (8 % RWC) accompanied by leaf folding and in-curling. Further, the adaxial surface of the leaf was not exposed to light and only the abaxial side exposed to light, the colour of the leaves turned in to light green/yellow (Figure 2b). Upon rehydration, 92% of the RWC was regained by the plants when compared to control after 12 h. Also, the rehydrated plants regained the original morphology (Figure 2c).

3.2. Ultrastructure

Scanning electron microscopy (SEM) images of hydrated (Figure 3A and B stem. Figure 3C leaf), desiccated (Figure 3D and E stem. Figure 3F leaf), and rehydrated (Figure 3G & H stem. Figure 3I leaf) at different magnifications (Figure 3A, D and G at 200 x; Figure 3B, E and, H 500x; Figure 3 C, F and I at 1000x) show distinguishable morphological changes (Figure 3). Considerable changes occurred in the cross-section of leaves and stem of *E. brachyphylla* in HS, DS and RS states. Alterations in the tissues and cellular structures were observed due to desiccation stress. Cross-sections showed perfect anatomical features, such as spongy mesophyll layer, epidermis and palisade mesophyll layer with vascular cells. In desiccated tissues, anatomical dis-organisationwas observed due to cell wall folding during desiccation resulting in a wrinkled, compacted appearance of cells. Rehydrated tissues showed typical anatomical features as that of the hydrated tissue. Hence, it can be inferred that desiccated stem and leaves tissues recovered to itsoriginal morphology upon rehydration.

3.3. Pigment content and photosynthesis analysis

The total chlorophyll content (Chl a + b) in hydrated, desiccated and rehydrated leaves have been depicted in Figure 4a. Our results indicate that there was a significant difference in the total chlorophyll content among HS, DS and RS tissues. The Chl a + b during hydrated state was found to be 18.11 mg/g FW and in the desiccated state, it dropped to 10 mg g^{-1} FW. Hence, 42% of total chlorophyll content of the fully hydrated plants was lost during the desiccation. However, after rehydration the total chlorophyll content increased to 16.5 mg/g FW. CO_2exchange rate (CER) in hydrated plants was 1.367 umol m^{-2} s^{-1}. Desiccation had a dramatic inhibitory effect on photosynthesis leading to a negative CER of-0.3 umol m^{-2} $s^{-1.}$ However, rehydrated plants could recover to a positive CER of 0.833 umol m^{-2} s^{-1} (Figure 4b). We observed that anthocyanins in the hydrated state was found to be 0.21 mg/ml/g DW and

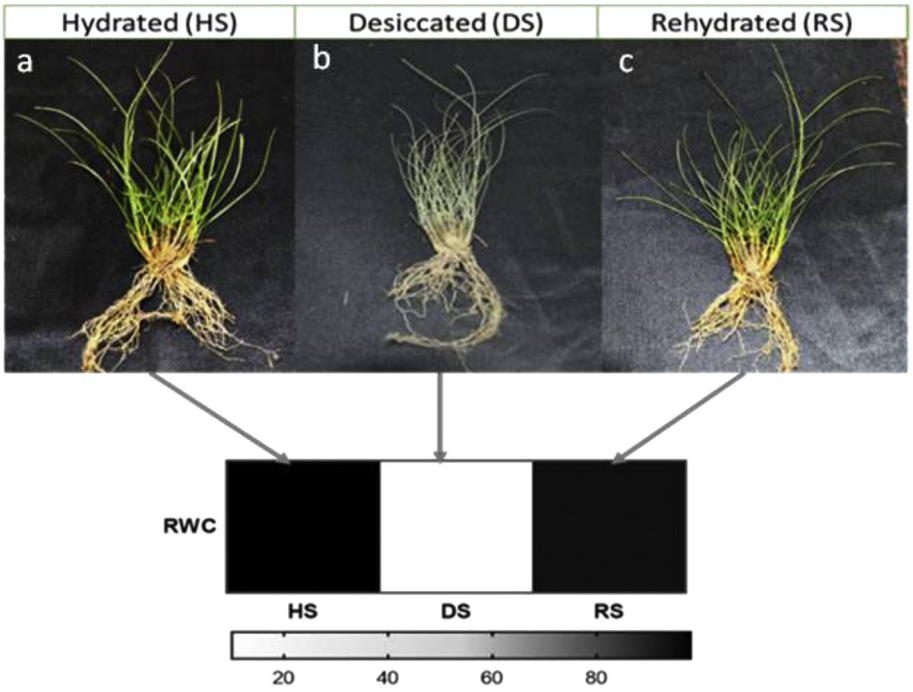

Figure 2. *E. brachyphylla* resurrection grass, a. Well-watered (relative water content:100 %). b. Desiccated (relative water content:8 %) after two days of withholding water. c. Rehydrated for 12 h post-hydration (relative water content:92%). The heat map below represents the rehydration potential (ability to rehydrate) of the resurrection grass *E. brachyphylla*. **HS,** Well-watered (relative water content: 100%). **DS**, Desiccated (relative water content 8 %) after two days of withholding water. **RS,** Condition 24 h post-hydration (relative water content:92%). with three replicates ($n = 3$).

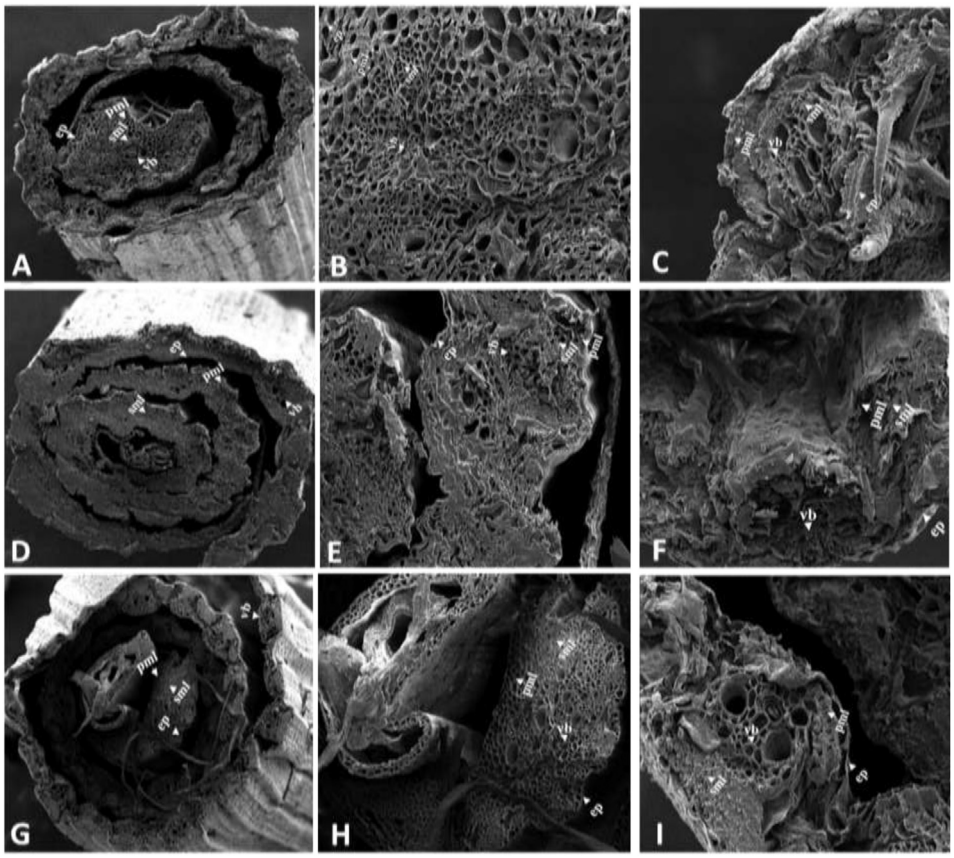

Figure 3. Scanning electron microscopic images of hydrated (A& B stem, C leaf), desiccated (D & E stem, F leaf), and rehydrated (G & H stem, I leaf) at different magnifications (A, D and G 200x; B, E and H 500x; C, F and I 1000x).

significantly increased to 0.85 mg/ml/g DW during desiccation. It reducedto the values similar that of the control (0.19 mg/ml/g DW in RS tissues) (Figure 4c). Carotenoid levels in hydrated fronds were found to be 5.21 mg/ml/g DW which decreased to 1.3 mg/ml/g DW in dehydrated fronds, and in the rehydrated fronds the concentration of the carotenoids recovered to 3.78 mg/ml/g DW (Figure 4c).

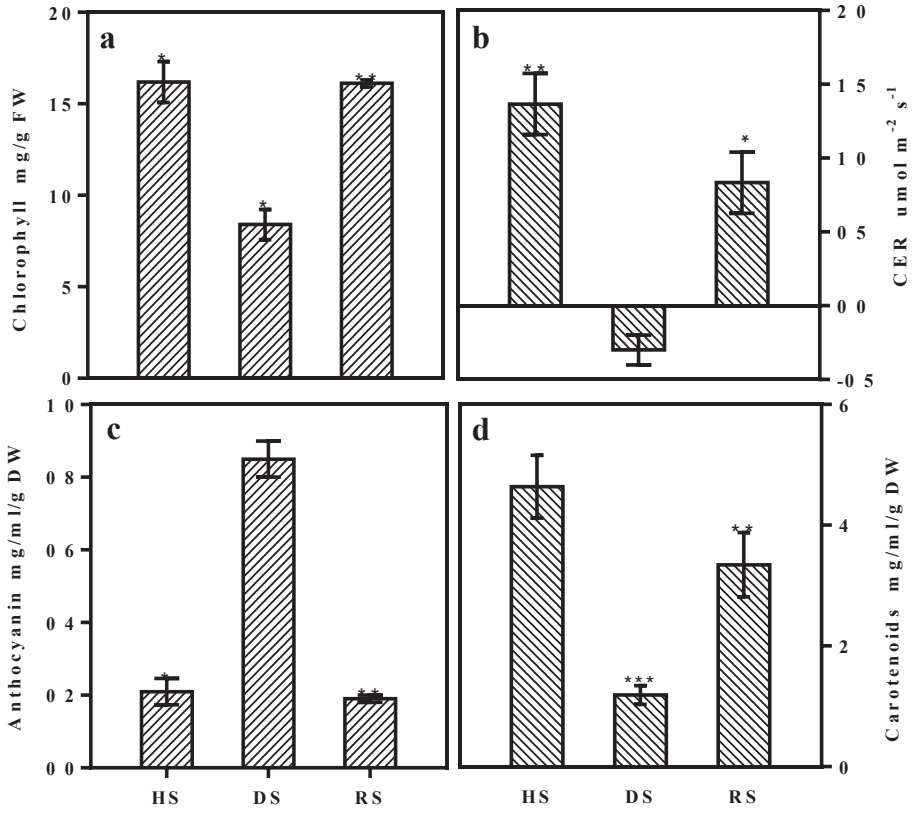

Figure 4. Pigment composition in *E. brachyphylla,* (a) Total chlorophyll, (b) CO_2 exchange rate [CER], (c) Anthocyanin and (d) Carotenoids estimated during Hydrated (HS), Desiccated (DS) and rehydrated (RS) states. Data represent the means ± SE of three separate experiments. *P ≤ 0.01, **P ≤ 0.001.

3.4. Antioxidant enzyme activity

Catalase, superoxide dismutase, peroxidase and glutathione reductase were assayed to determine the anti-oxidative enzyme activities during desiccation stress. SOD activity which is responsible for the scavenging of the $O2^{\bullet}$ increased in the desiccated state by 74% (13 in HS to 22.7 units/mg protein in DS) (Figure 5a). Upon rehydration the activity of SOD significantly decreased when compared to desiccated state to 16 units/mg protein. CAT activity also increased by 92 % during desiccation (15.25 in HS to 30.5 units/mg protein in DS) and when rehydrated it recovered to the similar levels as that of the control (17.25 units/mg protein) (Figure 5b). The POD activity ($H_2O_2+AH_2 \rightarrow 2H_2O$ + A) enhanced by 58.20 % (222.7 μ mol/min/mg protein in HS to 353 in DS) and reached the control levels upon rehydration (Figure 5c). Similar results were observed for GR, where the activity of the GR in the hydrated state was found to be 0.265 units/mg protein (Figure 5d), in desiccated state GR activity increased to 0.636 units/mg protein, which decreased to 0.217 units/mg protein in rehydrated plants.

3.5. Estimation of proline, lipid peroxidation, superoxide radical ($O_2^{\bullet -}$) and membrane permeability

From our experiment, it was evident that the concentration of proline drastically increased in the desiccated state. In the hydrated state proline concentration was found to be 3.51 μ mol g^{-1}FW, which increased in the desiccated state to 9.45 μ mol g^{-1} FW and during recovery phase concentration of proline further decreased to 4.61 μ mol/g^{-1}FW (Figure 6a). Relative electrolyte leakage (REL) was measured in all states to determine the membrane integrity by measuring the levels of permeability of the *E. brachyphylla* cell membrane. In the desiccated state the relative electrolyte leakage increased drastically to 86 % from 15% in the HS and in the RS it regained to the control levels and was found to be 16% (Figure 6b). Lipid peroxidation levels expressed in the form of MDA was found to be 29.2% more in the DS of *E. brachyphylla* when compared to HS and RS showed similar levels to that of the control (Figure 6c). Superoxide concentrations (Figure 6d) were quantified in HS, DS and RS tissue of *E. brachyphylla* species. Increase in superoxide concentration was evident during the desiccation stateas superoxide concentrationwas found to be 0.661 nmol g/FW when compared to 246 nmol g/FW in the hydrated state and 0.255 nmol g/FW rehydrated state. Superoxide radical concentration increased more than two folds in DS when compared to control levels.

3.6. Determination of sucrose and starch

Sucrose levels in the HS was found to be 0.28 μ $molg^{-1}$ FW which increased to 0.79 μ $mol.g^{-1}$ FW in DS. Further, the sucrose level decreased to 0.36 μ mol g^{-1} FW in the RS (Figure 7a). The starch concentration was found to be 0.71 μ $molg^{-1}$ FW in HS which reduced to 0.18 μ $molg^{-1}$ FW in DS which reached 0.58 μ mol g^{-1} FW in the RS (Figure 7b). In this study, Starch content decreases 3.9 folds in the DS when compared to hydrated state and sucrose content increased 2.8 folds in the DS when compared to HS.

4. Discussion

4.1. Plant relative water content and ultrastructure

Fully hydrated plants were desiccated and subsequently rehydrated in our studies. Desiccated plants reached an air-dry state (8 % RWC) in this condition folding and in-curling of leaf take place. Further, the adaxial surface of the blade was not exposed to light and only the abaxial side exposed to light, the colour of the leaves turned in to light green-yellow. Upon rehydration (92% RWC) plants regained the original morphology.

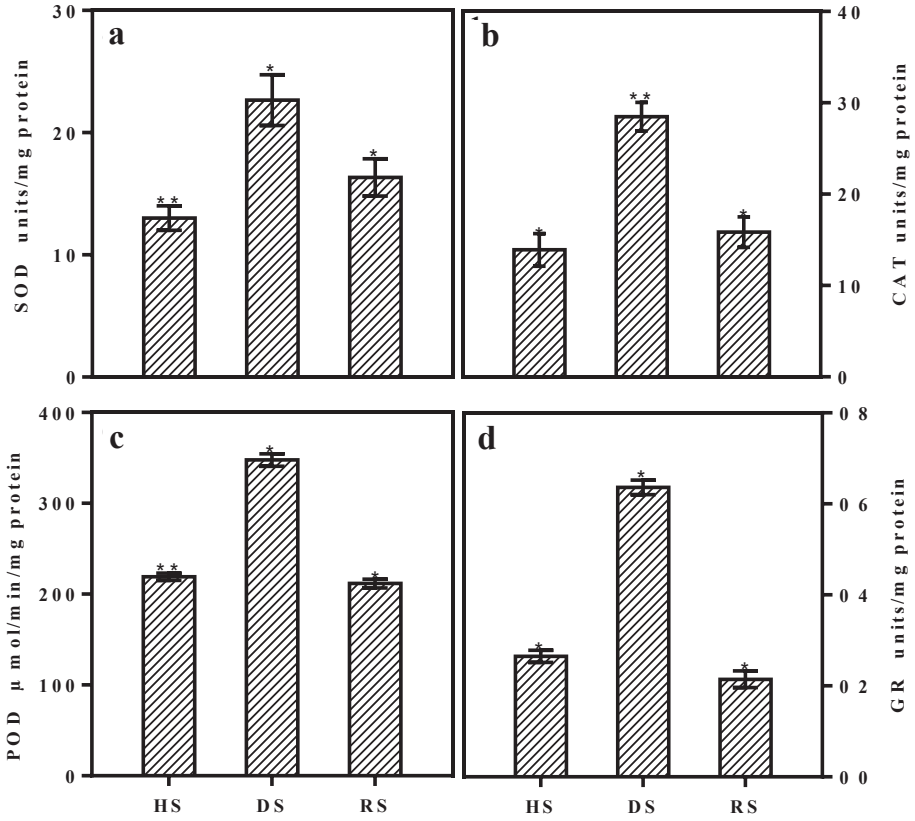

Figure 5. Activities of antioxidant enzymes in *E. brachyphylla*. (a) Superoxide dismutase (SOD); (b) Catalase (CAT), (c) Peroxidase (POD) and (d) Glutathione reductase (GR) in Hydrated (HS), Desiccated (DS) and rehydrated (RS) states. Data represent the means ± SE of three biological replicates. *P ≤ 0.01, **P ≤ 0.001.

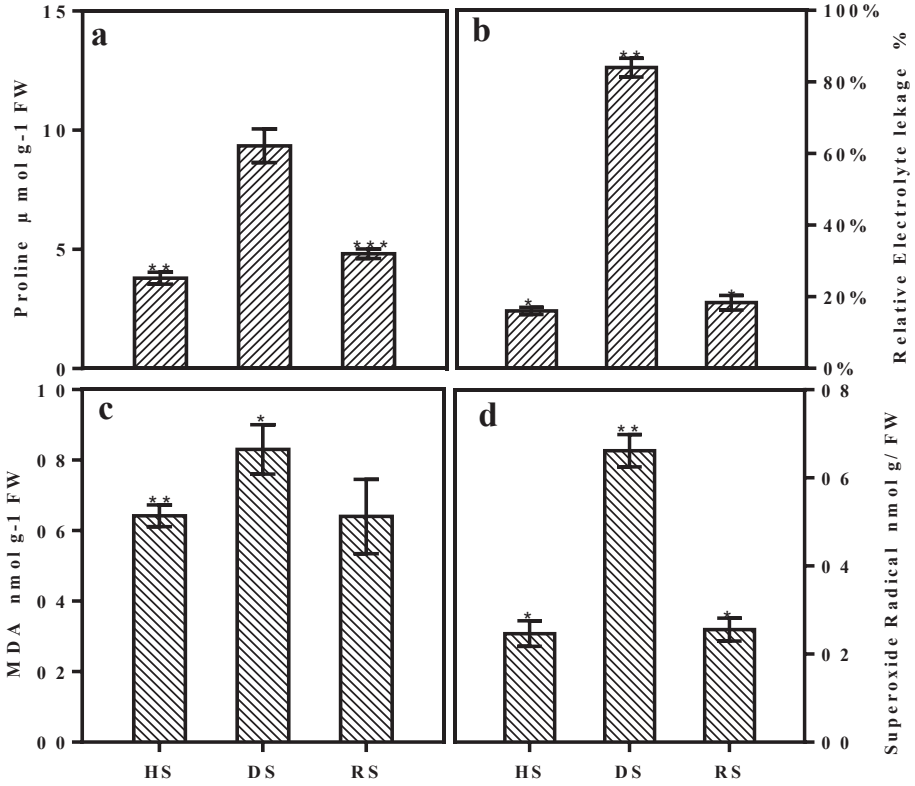

Figure 6. Proline accumulation (a), REL (b), Changes in the levels of MDA (c) and Quantity of superoxide radical (d) in Hydrated (HS), Desiccated (DS) and rehydrated (RS) states of *E. brachyphylla*. Data represent the means ± SE of three separate experiments. *P ≤ 0.01, **P ≤ 0.001. ***P ≤ 0.0001.

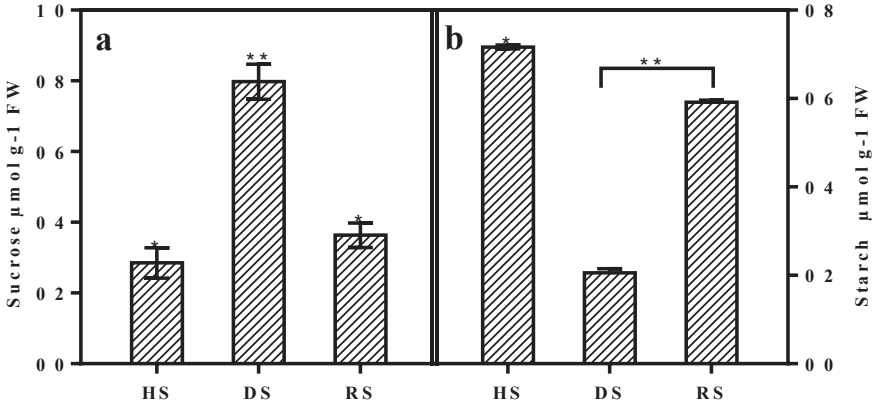

Figure 7. Changes in the level of sucrose (a) and of starch (b) in HS, DS and RS of *E. brachyphylla*. Data represent the means ± SE of three separate experiments. *P ≤ 0.01, **P ≤ 0.001. ***P ≤ 0.0001.

Morphological changes in the form of curling and folding in plants is an essential response to desiccation stress. DT plants expose only the abaxial side to sunlight and minimise the light absorption on the adaxial surface upon desiccation (Vicré et al., 2004). Similar mechanisms were observedin *X. humilis*, *C. wilmsii,M. flabellifolius* (Farrant et al., 2003). In our study, SEM images of hydrated, desiccated and rehydrated leaf and stem tissues confirmed that cellular damage was minimised due to cell wall folding induced due to desiccation stress DT plants reduce the active transpiring surface of the leaf during the early stages of dehydration by the folding to minimise the impact of radiation (Vicré et al., 2004). Its is clearly evident from our study that decreased RWC during desiccation does not have a major impact on the plant as morphological changes prevent the plant from complete dehydration.

4.2. Pigment content and photosynthesis

Our results indicate that significant damage to the total chlorophyll content as 42 % is lost during desiccation as in most of the poikilochlorophyllous desiccation-tolerant plants (Sherwin and Farrant, 1998; Bhatt et al., 2009). The same mechanism has been observed in *X. viscosa* (Farrant et al., 2015; Ingle et al., 2007) *X. humilis* (Beckett et al., 2012) *X. retinervis* (Bhatt et al., 2009). In poikilochlorophyllous desiccation-tolerant plants (PDTs), photosynthetic apparatus with its pigments and thylakoids is broken down, which is regained upon rehydration. Incontrast, the homiochlorophyllous (HDTs) desiccation-tolerant plant species preserve their photosynthetic apparatus in the desiccated state such that its recovery is rapid upon rehydration (Bewley, 1979; Csintalan et al., 1996; Bhatt et al., 2009). *E. brachyphylla* disassembles (42 %) its chlorophyll content on drying and resynthesises it again on rehydration. Here we report for the first time the poikilochlorophyllous nature of *E. brachyphylla*. After rewatering, plants recovered its morphological features comparable to that of the HS. To avoid damage caused by reactive oxygen species, anthocyanins act as a 'sunscreen' and reflects photosynthetically active radiation during desiccation. Increase in anthocyanin content is responsible for the lower rate of cell death in leaves (Sherwin and Farrant, 1998). Increase in anthocyanins during desiccation is responsible for masking of chlorophyll which acts as a filter to prevent additional light absorption by the leaf (Farrant et al., 2015). They have also been suggested to play an antioxidant role (Sadak et al., 2019; Larson, 1988). The accumulation of anthocyanins has been confirmed during dehydration of both *C. wilmsii* and *X. viscosa* resulting in the reduction of light absorption and free-radical quenching. The retention of anthocyanins during rehydration of *X. viscosa* may lead to additional protection against light damage during the reassembly of thylakoid membranes and *de novo* synthesis of chlorophyll. In rehydrating *C. wilmsii*, where such reconstitution is not required, anthocyanin contents declined rapidly which has also been observed in our results (HS 0.21 mg/ml/g DW, DS 0.85 mg/ml/g DW and RS 0.19 mg/ml/g DW). From our results it can be inferred that *E. brachyphylla* has a very efficient photoprotection system to overcome desiccation stress.

4.3. Proline accumulation

In our study, proline concentration increased during the desiccated state. Proline is a proteinogenic multifunctional amino acid and is essential for primary metabolism and acts as a compatible solute (Szabados & Savouré, 2010). Numerous studies have shown that the proline content in resurrection plants increases under desiccation stress and the examples include *Eragrostis nindensis* (Vander Willigen, 2004), *Selaginella bryopteris* (Pandey et al., 2010), *Selaginella tamariscina* (Wang et al., 2010), *Paraisometrum mileense* (Li et al., 2014), *Sprobolus stapfianus* (Martinelli et al., 2007), *Tripogonlo liiformis* (Asami et al., 2019), *Craterostigma wilmsii, Myrothamnus flabellifolius, Xerophyta humilis and viscosa* (Vicré et al., 2004). It is crucial in mechanically stabilising the cells and membranes in the desiccated state (Pandey et al., 2010; Vander Willigen, 2004). Proline plays many protective roles to help plants adapt to water stress they are (1) scavenging free radicals, (2) plays a significant role in stabilising sub-cellular structures, (3) as a non-water electron donor to PSII and buffering cellular redox potential under stress conditions (Ashraf and Foolad, 2007; Wang et al., 2015; De Ronde et al., 2004; Parvaiz & Satyawati, 2008; Gururani et al., 2015). (4) Proline confers a protective effect by inducing activity of other stress-protective proteins (Khedr, 2003). Proline also acts as an alleviating cytoplasmic acidosis and protein compatible hydrotrope. Proline accumulates in the small vacuoles bundle cover cells in resurrection grass *E. nindensis* (Pandey et al., 2010). Our results show that proline could have played a major role as an osmolyte during desiccation due to significant increase in its concentration during desiccation.

4.4. Role of antioxidant enzymes

Regulation of reactive oxygen species such as hydrogen peroxide (H_2O_2), superoxide anions ($O_2^{\bullet-}$), hydroxyl radical ($OH\bullet$) and singlet oxygen ($^1O_2$), occurs by enzymatic and non-enzymatic methods (Abdalla et al., 2010). Antioxidants play a crucial role in plants defense against desiccation stress (You and Chan, 2015). Antioxidant systems of resurrection plants effectively deal with ROS generated by stress related metabolism. Our results show that (Figure 5 a-d), activities of antioxidant enzymes CAT, SOD, POD and GR increased during desiccation and reached normal levels upon rehydration. Hence, it can be inferred that antioxidation mechanisms might havebeen induced by oxidative burst during desiccation. light-mediated overexcitation of chlorophyll leading to ROS generation when encountered by dehydrationthe main cause for cell death in desiccation sensitive plants (Wang et al., 2010; Apel & Hirt,

2004; Fryer et al., 2003; Blokhina et al., 2003). The cellular damage caused by reactive oxygen species are in the form of degradation of biomolecules like DNA, pigments, lipids, proteins, carbohydrates and ultimately leading to programmed cell death (Das and Roychoudhury, 2014). During desiccation, all the desiccation-tolerant plants increase antioxidant enzymes upon dehydration. Defense against the stress during desiccation in resurrection plants by increased concentrations of anti-oxidant enzymes have been reported in *Craterostigma plantagineum, Craterostigma wilmsii, Xerophyta viscosa, Xerophyta humilis, Sporobolus stapfianus, Eragrostis nindensis, Selaginella bryopteris, Selaginella tamariscina and Selaginella lepidophylla* (Dinakar et al., 2012). Results from our study show that anti-oxidant enzymes are mainly responsible for prevention of cell death and physiological recovery from desiccation in *E. brachyphylla*.

4.5. Lipid peroxidation, super oxide radical and membrane permeability

Desiccation stress can result in lipid destruction and membrane damage due to free radical production. It is known that during lipid peroxidation, its products are polyunsaturated precursors that contain small hydrocarbon fragments such as MDA, ketones andrelated compounds (Garg and Manchanda, 2009; Gill and Tuteja, 2010; Abouzari and Fakheri, 2015). The main cellular components susceptible to damage by free radicals are lipids (peroxidation of unsaturated fatty acids in membranes). Lipid peroxidation indicates that the oxidative stress due to desiccation will result membrane damage and inactivation of enzymes, (Mattos and Moretti, 2016). During desiccation in the resurrection plant, *S. bryopteris,* enhanced production of reactive oxygen species (ROS) was accompanied with increased lipid peroxidation (Pandey et al., 2010). Increased rate of superoxide radical level in DS of *E. brachyphylla* indicates that oxidative stress occurred due to desiccation stress. In our study, significant increase in REL level during desiccation indicates that *E. brachyphylla* losses its membrane integrity and in RS of *E. brachyphylla* membrane permeability is little lower than HS which indicates regain of membrane integrity. Results from our study show that desiccation results in significant increase lipid peroxidation, membrane permeability and free-radical production which requires a significant anti-oxidant response from *E. brachyphylla* in order to maintain cell viability.

4.6. Soluble sugar and starch

Decrease in starch concentration during desiccation and subsequent increase in rehydration has been observed in many of the resurrection plants, e.g., *C. plantagineum, C. wilmsii, X. viscose S. bryopteris, S. tamariscina, S. lepidophylla* (Whittaker et al., 2001; Norwood et al., 2000). Starch is a storage carbohydrate, which is degraded during dehydration (Müller et al., 1997; Whittaker et al., 2007). Besides the degradation of starch desiccation also results in changes in the polysaccharide contents and structure of the cell-wall in resurrection plants (Vicré et al., 2004). Photosynthesis and starch breakdown sustain the simultaneous increase of hexose sugar, sucrose and amino acids. Sugars protect the cells during desiccation by maintaining hydrophilic interactions in proteins and membranes by substituting water from hydroxyl groups of sugars. Hence, through hydrogen–bonding sugars interact with proteins and membrane and prevent denaturation of the protein. Sugars are considered vital contributing factors to vitrification (Vicré et al., 2004; Pandey et al., 2010). In our study, the sucrose content increased and the starch content decreased in desiccated state, indicating that *E. brachyphylla* carefully calibrates its carbohydrate metabolism to counter desiccation stress.

5. Conclusion

Our investigation of morphological, biochemical and physiological responses during hydration, desiccation and rehydration confirmed the occurrence of vegetative desiccation tolerance in *E. brachyphylla*. *E. brachyphylla* recovered its complete RWC and leaf regained its original morphology upon rehydration. *E. brachyphylla* synthesises chlorophyll de novo after desiccation. Further, the total carotenoids and anthocyanins increased during dehydration also protected the plants against radiation induced cell death. Increase in superoxide radicals under water deficit is an indicator of the oxidative stress due to desiccation. Furthermore, antioxidant enzymes SOD, POD, CAT and GR showed increase in concentrations during desiccation. Similarly, signalling and osmoprotective molecule proline was also involved in the acquisition of desiccation tolerance in *E. brachyphylla*. Relative electrolyte leakage and MDA levels reached concentrations equal to that of control on rehydration, giving evidence that membrane functionality recovered. Break down of starch and increasing sugars levels during desiccation indicates that carbohydrate metabolism is controlled during desiccation. The results point out that *E. brachyphylla* is a PDT species which recovers its complete physiological and biochemical activity after rehydration. Hence, we propose that *E. brachyphylla could* be used as a DT model to study mechanisms underlying tolerance to desiccation.

Declaration

Author contribution statement

N.S. Yathisha, P. Barbara, B. Gügi: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

K. Yogendra, S. Jogaiah, D. Azeddine, R.G. Sharatchandra: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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