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Ferns and Lycophytes as new challenges

Physiological and biochemical changes in desiccation and rehydration cycles of *Selaginella convoluta* (Selaginellaceae)

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Abstract

Vegetative desiccation tolerance (VDT) is a biological process that allows resurrection plants to complete desiccation-rehydration cycles and survive without permanent damage. Physiological and biochemical changes related to the VDT were evaluated in the lycophyte *Selaginella convoluta* from the Caatinga biome, a seasonally dry tropical forest of northeastern Brazil. As representative of the oldest division of extant vascular plants, the investigation of VDT mechanisms in this species serves as an opportunity to provide information for research communities interested in functional traits related to water-limited conditions. The experiments were performed with plants in their natural habitat, in dry and rainy seasons, and in pots under shade house conditions. Relative water content, chlorophyll *a* fluorescence, gas exchange, activity of antioxidant enzymes, concentration of chlorophylls, proteins, total amino acids, proline, and soluble carbohydrates in leaf tissues were evaluated throughout the process of complete dehydration and subsequent re-watering of intact plants. The overall results reveal the capacity of *S. convoluta* to tolerate extreme dehydration and rapidly recover upon rehydration. This species minimizes potential damages during the desiccation-rehydration cycle due to the presence of inductive mechanisms (photochemical preventing, sucrose accumulation) and constitutive mechanisms (antioxidant activity of catalase and ascorbate peroxidase), which share similarities with the VDT of both angiosperms and bryophytes.

Key words: caatinga, dehydration resistance, functional traits, resurrection plant, survival strategy.

Resumo

Tolerancia à dessecação vegetativa (TDV) é um processo biológico que permite que plantas revivescentes sobrevivam a ciclos completos de dessecação-reidratação sem danos permanentes. Alterações fisiológicas e bioquímicas relacionadas à TDV foram avaliadas na licófita *Selaginella convoluta* do bioma Caatinga, em uma floresta tropical sazonalmente seca do nordeste do Brasil. Como representante da mais antiga divisão de plantas vasculares não extintas, a investigação de mecanismos de TDV nesta espécie serve como uma oportunidade para fornecer informações para comunidades de pesquisa interessadas em características funcionais relacionadas às condições limitantes de água. Os experimentos foram conduzidos com plantas em seu habitat natural, nas estações seca e chuvosa, e em potes sob casa de vegetação sombreada. Conteúdo relativo de água, fluorescência da clorofila *a*, trocas gasosas, atividade de enzimas antioxidantes, concentração de clorofilas, proteínas, aminoácidos totais, prolina e carboidratos solúveis de tecidos foliares foram avaliados ao longo dos processos de completa dessecação e subsequente reidratação de plantas intactas. Os resultados revelam a capacidade de *S. convoluta* para tolerar desidratação extrema e rapidamente recuperar após a reidratação. Esta espécie minimiza danos potenciais durante o ciclo de dessecação-reidratação devido à presença de mecanismos protetivos dos tipos indutivos (inibição fotoquímica, acúmulo de sacarose) e constitutivos (atividade antioxidante de catalase e ascorbato peroxidase), mostrando similaridades com a TDV de angiospermas e briófitas.

Palavras-chave: caatinga, resistência à desidratação, características funcionais, planta da ressureição, estratégia de sobrevivência.

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Introduction

Vegetative desiccation tolerance (VDT) is a biological process found in living plant tissues as a whole, to survive in a hygroscopic equilibrium state with dry air. This process is predominantly observed in algae, lichens, and bryophytes, while it is uncommon in the vascular clades of ferns, fern allies, and angiosperms and completely absent in gymnosperms (Oliver *et al.* 2000).

The initial evolution of VDT was likely a crucial step in the transition from aquatic to terrestrial life for plants, explaining its widespread occurrence within nonvascular species (Bewley 1979; Bewley & Krochko 1982; Oliver *et al.* 2000; Hilhorst & Farrant 2018). As vascular plants evolved, marked by an increasing structural and morphological complexity, VDT ability was lost. However, there have been cases of independent re-evolution within groups of vascular land plants, resulting in the reappearance of some degree of VDT (Oliver *et al.* 2000). Most VDT studies focus on bryophytes and angiosperms. In bryophytes, VDT is primarily characterized by the presence of constitutive cellular mechanisms as a protective/repair strategy to recover from desiccation, which is not dependent on inductive mechanisms by drying (Gao *et al.* 2017; Oliver *et al.* 2000). The consistent presence of compounds such as sucrose and rehydrins in stable concentrations during desiccation and rehydration phases serves as examples of constitutive mechanisms through which VDT bryophyte tissues protect themselves from damage (Oliver *et al.* 2005).

In angiosperms, the VDT process is primarily controlled rather by a set of inductive protections that are mainly activated during the dehydration phase (Farrant 2007; Moore *et al.* 2009; Bartels & Hussain 2011). These plants employ various inductive mechanisms to protect tissues from the potentially harmful effects of desiccation, such as the accumulation of solutes in response to osmotic stress, the synthesis of compounds that maintain membrane and macromolecule integrity, and the activation of antioxidant systems (Hoekstra *et al.* 2001; Rascio & La Rocca 2005; Farrant *et al*. 2015; Costa *et al.* 2017; VanBuren 2017).

Selaginella convoluta (Arn.) Spring is a VDT species characterized by its ability to recover the greenish appearance in its branches when rehydrated after losing more than 90% of their relative water content (Rawitscher *et al.* 1952; Gaff 1987; Xavier 2007). This herbaceous, terrestrial, or rupicolous species with a neotropical distribution is the most abundant pteridophyte in the tropical dry forest of the Brazilian Caatinga biome (Xavier 2007). As a lycophyte, it represents the oldest division of extant vascular plants (Iturriaga *et al*. 2006; Banks 2009; Gechev *et al*. 2021).

Despite being a rare phenomenon among vascular species, VDT species are typically found in high abundance in the vegetation of rupestrian fields and rocky outcrops. These ecosystems, particularly in the biomes of tropical regions, are considered centers of diversity for VDT species (Porembski & Barthlott 2000). Common characteristics of these environments include substrates lacking deep soil and easy drainage, which are favorable for the establishment of VDT species. In addition to soil islands on rocky outcrops, *Selaginella convoluta* is also found covering soils in plain areas beneath the shrubby-arboreal stratum of the hyperxerophilic vegetation of Caatinga, in the Depressão Sertaneja geoenvironmental unit (Silva *et al.* 1993). The occurrence of rain in the semi-arid climate of this region is characterized as irregular and poorly distributed in space and time (Moura *et al.* 2007; Teixeira 2010), causing short and recurring periods of water availability. Ferns are recognized by their adaptations to extreme environments (Rathinasabapathi 2006), making them potentially important for biotechnological purposes in order to improve crop resistance to stress potential factors such as water-limited conditions (Rathinasabapathi 2006). *Selaginella*, in particular, has become a target of extreme interest for research in comparative plant genomics, biochemistry, and development, as it was chosen as one of the non-crop plants for genome sequencing (Zhu *et al*. 2017; VanBuren *et al*. 2018; Xu *et al*. 2018). Analyses of *Selaginella* have uncovered novel transcripts not found in angiosperms (Weng *et al*. 2005; Zhu *et al*. 2017; Xu *et al*. 2018).

This study was conducted to identify protective mechanisms related to the extreme capacity of *Selaginella convoluta* to tolerate desiccation. Understanding these mechanisms in the context of the climate can help explain why this species thrives abundantly in one of the driest and hottest ecosystems in South America. We evaluated the physiological and biochemical responses of intact plants to complete desiccation and rehydration cycles in their natural habitat within a preserved Caatinga vegetation and in pots under a shaded house condition. This study is valuable to plant ecophysiology and biotechnology research communities interested in functional traits to extreme environments and gene-transfer technologies to improve stress crop resistance.

Material and Methods

In situ experiment

Physiological and biochemical evaluations were performed with spontaneously occurring adult individuals of *Selaginella convoluta*. This experiment was carried out with physiological and biochemical measurements under the diurnal and seasonal variations in the environmental factors of its natural habitat in a hyperxerophilic Caatinga vegetation at Caatinga Experimental Field, Embrapa Semi-Arid, Petrolina, PE, northeastern Brazil (09º04'21.97"S, 40º20'12.15"W). The climate in the Caatinga biome is characterized as warm and dry tropical semi-arid, with an average annual temperature ranging from 25 to 28 °C, and the volume of rainfall typically below 500 mm (Correia *et al.* 2011). Meteorological data for the studied area were monitored at a climate station located within 500 m of the experimental site (Embrapa Semiárido 2015). The soil in the studied area, according to the Brazilian Soil Classification System (Santos *et al.* 2018), was classified as Yellow Argisol, with medium to medium-clayey texture and flat relief, with a slope of 1–2. The soil is stony, rocky and well drained. Experimental evaluations were carried out during the transition from the rainy to the dry season on 21-Feb-2014 and 15-May-2014. Additional assessments were carried out after the rainy season resumed, with the recovery capacity assessment on 26-Jan-2015. The measurements were performed in different individuals of the same population.

Leaf gas exchange (LGE) measurements were performed with an infrared gas analyzer (IRGA Li-6400, LI-COR, Lincoln, NE, USA) to evaluate photosynthetic assimilation of carbon dioxide (A, mmol CO_2 $m^{-2}s^{-1}$), stomatal conductance to water vapor (g_s, mol H_2O $m^{-2}s^{-1}$), transpiration (E, mmol H_2O $m^{-2}s^{-1}$), and instantaneous water-use efficiency, represented by the A/E ratio (mmol CO_2 / mmol H_2O). These evaluations were performed on fully expanded young branched stems along which the microphylls are arranged (referred to only as leaves onwards) without signs of disease or pest attack. The sampled leaf completely covered the circular sampling area (2 cm^2) of the leaf chamber fluorometer light source (LCF Li-6400-40, LI-COR, Lincoln, NE, USA).

On each evaluation date, measurements were taken every two to three hours during the daytime, between 7h00 and 16h00 on sunny days. By checking the instantaneous measurements of the external photosynthetically active radiation (*PAR*) sensor of the IRGA, the photosynthetic photon flux density was prefixed before each set of hourly gas exchange measurements using the leaf chamber fluorometer light source (LCF Li-6400-40, LI-COR, Lincoln, NE, USA), according to the approximate incident radiation at the leaf level. Additionally, the average values of air temperature (T_{air}) and leaf-to-air vapor pressure deficit (VPD_l) were monitored for each set of leaf gas exchange measurements. However, leaf gas exchange assessment could not be performed on 15 May 2014 due to leaf curling caused by desiccation, preventing proper sampling by the IRGA leaf chamber.

Soil samples for moisture determination were collected at two depths (0–10 cm and 10–20 cm), with a horizontal distance of 10 cm from each plant previously evaluated by LGE measurements. The fresh mass (FM), saturated mass (SM), and dry mass (DM) after 48 h in an oven at 80 °C were determined. Subsequently, the relative water content of the soil sample (RWC_s, %) was calculated according to Turner (1981) as [RWC = ((FM - DM) / (SM - DM)) * 100]. Leaf water status was monitored by the relative water content (RWC_l, %) in the same leaves used for LGE measurements, using the same equation as for RWC_s determination. The fresh mass (FM), saturated mass (SM) after 24 h of hydration in distilled water, and dry mass (DM) after 36 h in an oven at 70 °C were determined.

After collecting leaf samples for the RWC_l measurements, new leaf samples were collected for the biochemical evaluations, immediately frozen in liquid nitrogen, and stored in an ultra-freezer at -80 °C, until processing. For the total amino acids and proline analysis, 0.3 g of fresh leaf mass was macerated in 80% (v/v) ethanol. The resulting extract was incubated at 70 ºC for 90 minutes and subjected to centrifugation of 15,000 g for 10 minutes at room temperature. The ethanol-soluble fraction (supernatant) was used to quantify the concentrations of total amino acids (Moore & Stain 1948) and proline (Bates *et al.* 1973).

Soluble carbohydrates were extracted from the leaves of each treatment in 80% (v/v) ethanol. The plant material was incubated at 70 °C for

90 minutes and subjected to two centrifugations (15,000 g, 10 min). The ethanol-soluble fraction was used to quantify hexose (glucose + fructose) and sucrose concentrations (Praxedes *et al.* 2006).

The extraction of antioxidant enzymes Superoxide Dismutase (SOD = EC 1.15.11), Ascorbate Peroxidase (APX = EC 1.11.1.1), and Catalase (CAT = EC 1.11.1.6) was based on Rolão (2010) with modifications: 0.3 g of leaf tissue was macerated in an extraction buffer containing 100 mmol L^{-1} Tris-HCl (pH 7.5), 2 mmol L^{-1} EDTA, 14 mmol L^{-1} mercaptoethanol, 150 mmol L^{-1} ascorbic acid, 10 mmol L^{-1} DTT, 20 mmol L^{-1} sodium metabisulfite, 200 mmol L^{-1} sodium tetraborate, 10 mmol L^{-1} KCl, 0.1% Triton X-100 (v/v), 7% sucrose and 50% (w/w) PVPP. The activity of antioxidant enzymes was determined as described by Fornazier *et al.* (2002) and Lima *et al.* (2002). The same extract was used to quantify total proteins (Bradford 1976).

The experiment was carried out with three biological replicates independently (using different individuals with the same apparent characteristics and environmental conditions) on each evaluation day. For physiological and biochemical variables, replicates comprised the average of two measurements per plant performed on two different leaves.

Statistical differences between the sampling moments throughout the experimental period were evaluated by *ANOVA*, followed by the Tukey test ($p < 0.05$), using the Sisvar statistical package (Ferreira 2011).

Ex-situ experiment

Intact adult plants of *Selaginella convoluta* were collected from the Caatinga Experimental Field, Embrapa Semi-Arid, Petrolina, PE, which is the same area used for field studies *in situ*. The individuals in the desiccated state were transplanted without natural soil substrate into 200 mL pots containing a commercial peat and vermiculite mixture (Plantmax, Eucatex, Brazil). The plants were kept under shaded house conditions (75%) in the Caatinga Experimental Field, with daily irrigation via micro-sprinklers (70 L/h) for 1 minute every 40 minutes. The irrigation was maintained for 60 days until the beginning of the experiment when the plants displayed unfolded turgid leaves with their normal greenish adaxial surface completely expanded and exposed to ambient light. To assess VDT, the plants were separated into two groups: one with suspended watering until complete plant desiccation followed by rehydration (Desiccation/Rehydration treatment- DR) and the other group kept irrigated (Control Irrigated treatment - CI).

The suspension of watering for the DR treatment was maintained for 9 days after the last irrigation event. Afterward, irrigation was resumed and maintained for 7 days until the conclusion of the experiment. Irrigation was kept daily for the CI group throughout the experimental period. During this period, the following variables were evaluated every two to three days in intact plants: RWC_l, A, g_s, and E, as described above. Additionally, measurements of the ratio between the intercellular and atmospheric concentration of CO_2 (C_i/C_a), electron transport rate (*ETR*), photosynthetic quantum efficiency of light-adapted leaves (F_v'/F_m') and effective of photosystem II (Φ_{PSII}), were taken using the LCF coupled to the infrared gas analyzer, along with measurements of *a*, *b* and total chlorophyll (CFL 1030, Falker). During the experiment, air temperature ranged from 32 to 37 ºC, air humidity from 55 to 65%, and VPD_l from 2.5 to 3.5. *PAR* was fixed at 1200 mmol photons $m^{-2}s^{-1}$ for all LGE and photochemical measurements.

Measurements were consistently performed in three biological replicates (composed by the average of two measurements per plant performed on two different leaves) for each treatment independently on each evaluation day. The Shapiro-Wilk test was used to check the normality of the data, and the t-test was employed to determine differences between the means of the two groups along the experimental period.

Results

Selaginella convoluta branches curled towards the central bud of the rosette due to desiccation within a few days after water depletion. The greenish adaxial surface of the microphylls becomes protected on the inner face of the curled branch, while the opposite paleaceous surface was exposed to the surrounding environment during the desiccated state (Fig. 1). In this condition, the plant typically tolerates desiccation for at least several months in the field until water becomes available again. Within a few hours of rehydration, the plant branches uncurled, "covering the soil like a green carpet" (Braga 1960 apud Andrade-Lima 1989), standing out visually in the still leafless Caatinga vegetation composed of predominantly deciduous species.

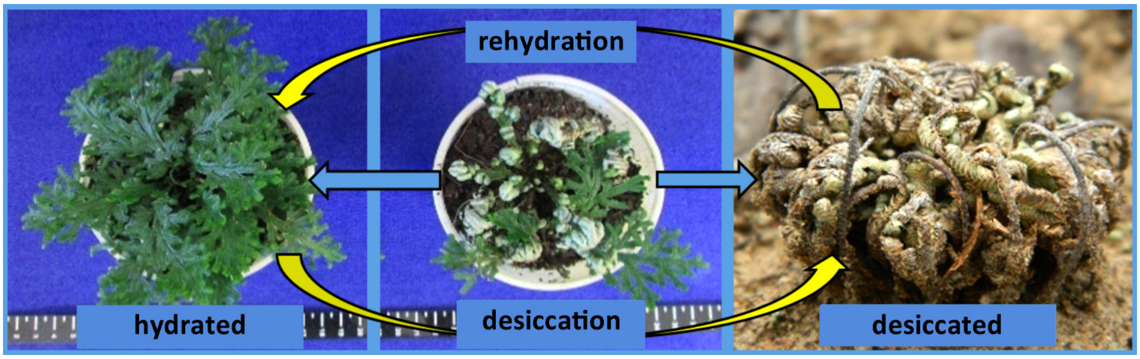

Figure 1 – Visual aspects of *Selaginella convoluta* branches along the cyclic process of desiccation and rehydration. Intermediary phases of dehydration or rehydration can be reversed to rehydration or desiccation depending on the occurrence of changes in the conditions of water availability in the environment.

Field evaluation *in situ*

Climate conditions

Historical climate diagrams of the *in situ* study for the periods 2014 to 2022, July 2013 to June 2014, and July 2014 to June 2015, according to Walter & Lieth (1960), are presented in Figure 2a-c. These diagrams indicate the periods corresponding to the wet ones (P > 2T). The period between January and March is indicated as the most humid in the longer time series (Fig. 2a). However, for the annual periods in which the experiments were conducted, two wetter periods are indicated, between May and June and between October and November (Fig. 2b-c). This implies that there were approximately seven to eight dry months in each of these annual periods.

It is important to note that the indicated dry months correspond to those in which the return of water to the atmosphere is potentially greater than the rainfall and not necessarily indicating less rainfall (Coutinho 2016). Even in rainy months, a few days to a week of drought can be sufficient to initiate vegetative desiccation of *Selaginella convoluta.* This happens due to the relatively high evapotranspiratory demand driven by high air temperatures. These conditions lead to the relatively rapid dehydration of the most superficial soil layer where the roots of this species predominantly extend (up to 10 cm). After a certain critical level of soil dehydration in dry air, the plants start losing water to the environment until complete desiccation. Figure 3 highlights the climatic context in which the assessments were carried out in the field experiment (*in situ*), specifically in relation to the rainfall regime. On the dates of 21-Feb-2014 and 26-Jan-2015 (Fig. 3a-b), the plants were visually well-hydrated with fully expanded leaves. These assessment dates were carried out 1 day after a rainfall of 6 mm and 4 days after a rainfall of 12 mm, respectively (Fig. 3a-b). On 15 May 2014 (Fig. 3a), the plants were completely desiccated, 13 days after a rainy week. Thus, a sparse distribution of rainfall over time in hot weather causes short periods of drought potentially capable of causing complete desiccation of *S. convoluta* in its natural habitat.

Soil and leaf water status

Between 21-Feb-14 and 15-May-14, the RWC_s in the 0–10 cm soil layer decreased on average from 45% to 4.6%, and from 15.8% to 7% in the 10-20 cm deep layer of soil (Fig. 4a). The RWC_l was 97.5% on 21-Feb-2014 and decreased to 9.3% on 15-May-14 (Fig. 4b). On 26-Jan-2015, when the plants' recovery capacity was evaluated, RWC_s was 36% and 13.3% in the 0–10 cm and 10–20 cm deep layers, respectively (Fig. 4a), and the RWC_l had resumed to 95% (Fig. 4b).

The lower soil humidity in the deepest layer compared to the most superficial layer (Fig. 4a) indicates that the volumes of precipitation that occurred in the area in the days prior to collections were enough to hydrate only the superficial soil layer and still promote plant hydration close to water saturation (Fig. 4b).

Leaf gas exchange

The different *PAR* values used for leaf gas exchange measurements *in situ* on 21-Feb-2014 and 26-Jan-2015 reflected, respectively, the highest and lowest leaf coverage of deciduous trees above the *Selaginella convoluta* environment

(Fig. 5a-b). Due to higher radiation exposure, T_{air} and VPD_l showed broader variation on 26 Jan 2015, reaching maximum values around 13h20 on both dates (Fig. 5c-f). Under these natural conditions, on both dates, a decrease in stomatal conductance was observed around 10h00, with greater restriction on A than on E (Fig. 6a-f). Consequently, the water use efficiency estimated by the A/E ratio followed the trends observed for A (Fig. 6g-h).

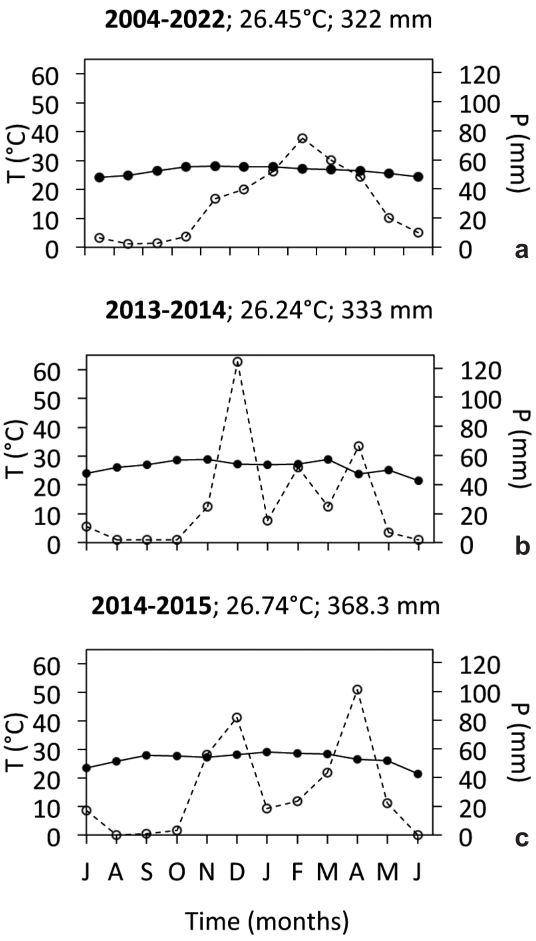

Figure 2 – a-c. Climate diagrams, according Walter & Lieth (1960), of the Caatinga Experimental Field, Embrapa Semiárido, Petrolina, PE, Brazil, the location of the experimental site (09º04'21.97"S, 40º20'12.15"W). Elevation: 382 m above sea level. Monthly average temperatures (T, °C) (closed circles and solid lines) and monthly precipitation (P, mm) (open circles and dashed lines) from July (J) to June (J) – a. 2004 to 2022 (historical period), annual average temperature 26.45 °C, total precipitation 322 mm; b. 2013 to 2014, annual average temperature 26.24 °C, total precipitation 333 mm; c. 2014 to 2015, annual average temperature 26.74 °C, total precipitation 368.3 mm. Climate data were recorded by a meteorological station established at 500 m from the experimental site, approximately.

Biochemical analysis

During the transition from the rainy to dry season, from February to May 2014, the leaf hexose content decreased from 35.1 to 20.0 mmol g^{-1} DM. The sucrose content increased from an undetectable value to 6.8 mmol g^{-1} DM in the same period. Physiological recovery measurements of the plants during the subsequent rainy season (26-Jan-2015) showed that the hexose content was 16.2 mmol g^{-1} DM, and the sucrose content was 5.9 mmol g^{-1} DM (Tab. 1).

The concentrations of proteins and total amino acids in leaves were lower in the desiccated state, at 18.0 mg g^{-1} DM and 16.2 mmol g^{-1} DM, respectively (15-May-2014), while in the hydrated state (21-Feb-2014 and 26-Jan-2015) they were around 30.0 mg g^{-1} DM and 30.0 mmol g^{-1} DM,

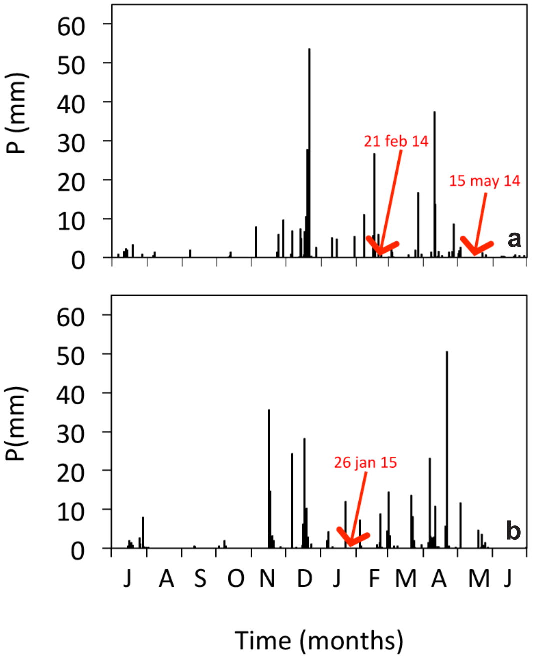

Figure 3 – a-b. Daily precipitation (P, mm) between the months of July (J) to June (J) during the annual cycles – a. 2013 to 2014; b. 2014 to 2015. Red arrows indicate the dates of evaluations (21-Feb-14, 15-May-14, and 26-Jan-2015). Embrapa Semiárido climate station, Caatinga Experimental Field, Petrolina, PE, Brazil.

respectively (Tab. 1). Leaf proline concentration decreased from 3.0 to 1.8 mg g^{-1} DM during the transition from the hydrated to the desiccated state (21-Feb-2014 and 15-May-2014). Following rehydration in the subsequent rainy season (26-Jan-2015), proline concentration was 1.7 mg g^{-1} DM (Tab. 1).

The SOD's activity decreased with desiccation, measuring 29.4 unit min^{-1} mg^{-1} protein on 21-Feb-2014 (rainy season), and decreasing to 20.3 unit min^{-1} mg^{-1} protein on 15-May-2014 (desiccated condition under dry season). However, after rehydration on 26-Jan-2015 (following the rainy season), SOD activity was significantly higher compared to the dry season, reaching 38.5 unit min^{-1} mg^{-1} protein. No variation in the activity of CAT and APX enzymes was observed between hydrated and desiccated plants during the experimental period (Tab. 1).

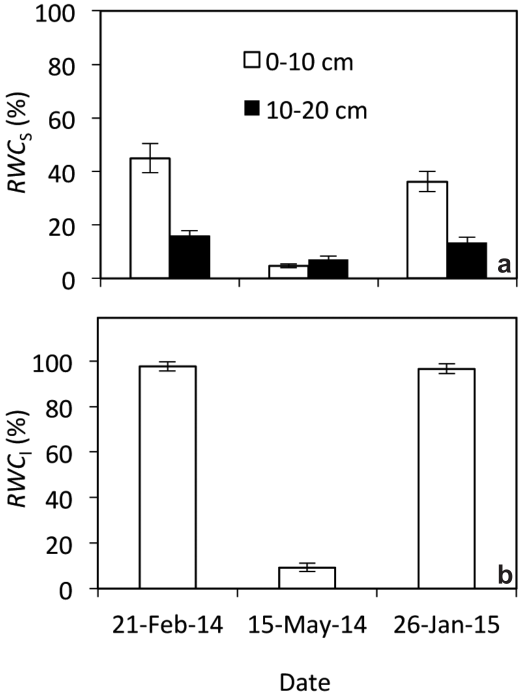

Figure 4 – a. Soil relative water content (%) in 0–10 cm (white columns) and 10–20 cm (black columns) deep layers. b. leaf relative water content (RWC_l, %) of *Selaginella convoluta* in the transition from rainy to dry season (21-Feb-14 to 15-May-14) and in the following rainy season (26-Jan-2015). Field experiment, Embrapa Semiárido, Caatinga Experimental Field, Petrolina, PE, Brazil. Each column represents the mean ± standard deviation (N = 3).

Pot experiment *ex-situ*

Leaf water status

In the DR group of pot experiment *ex-situ*, the RWC_l decreased from 93.2% to 12.4% from an initial condition of substrate water saturation over nine days of watering suspension (Fig. 7). During this process, the rolling of the leaves becomes the first visual indicator that characterizes the initial process of progressive leaf tissue moisture loss. Subsequently, the leaves have become completely curled in the desiccated state, hiding their adaxial surface. The recovery of leaf water status was demonstrated with an increase in RWC_l to 92.5% within five days of daily saturation of substrate hydration (Fig. 7). Although this measurement was not performed on a shorter time scale, this vegetative resaturation process typically occurs within 24 hours. In comparison, the CI group maintained RWC_l between 91% to 95% throughout the entire experiment.

Leaf gas exchange

Differences in gas exchange measurements between the DR and CI groups became evident when RWC_l decreased to 72% on average (Fig. 8a), corresponding to 5 days after stopping irrigation (DASI). The g_s of the DR group decreased by approximately 50% compared to that of the CI group (Fig. 8a) when the RWC_l was at 72%. As dehydration progressed, the values of g_s, in comparison to the CI group, decreased in the DR group by approximately 75% and 90%, under RWC_l of 43.2% and 12.4%, respectively. After rehydration, g_s showed incomplete recovery despite RWC_l increasing above to 90% (Fig. 8b), even after 7 days of rehydration (DAR) (Fig. 7). *E* and *A* presented a similar trend to g_s during dehydration, with a significant decrease under 72% of RWC_l (Fig. 8c,e). As observed to g_s, *E* values were close to zero at 12.4% of RWC_l (Fig. 8c), while *A* decreased to values close to zero at 43.2% of RWC_l (Fig. 8e). These values close to zero observed for *A* corresponded to the CO_2 compensation point, and then, slightly negative values were observed under 12.4% of RWC_l (Fig. 8e). *E* also exhibited incomplete recovery after rehydration under RWC_l above 90% of RWC_l, corresponding to 70% of the value obtained for the control group (Fig. 8d). Although the DR group presented an absolute mean value lower than the CI group, *A* showed a complete recovery after seven DAR when RWC_l was above 90% (Fig. 8f).

The C_i/C_a ratio remained stable and close to 0.7 until dehydration reached 72% of RWC_l (5 DASI). Then, the DR group showed an increase of 21% and 97% in C_i/C_a ratio under 43.2% and 12.4% of RWC_l (7 and 9 DASI), respectively, in relation to the CI group (Fig. 8g). However, these values were completely recovered after rehydration (Fig. 8h).

Photochemical analysis

Until 72% of RWC_l during the dehydration phase, *ETR* and Φ_{PSII} remained stable, while F_v'/F_m' did not differ from that in the CI group until 43.2% of RWC_l (Fig. 9a-f). Under an RWC_l of 12.4%, photochemical variables assumed values close to zero (Fig. 9a,c,e). The DR group recovered total *ETR* and Φ_{PSII} after rehydration, with RWC_l above 90% (Fig. 9b,f). Normally, this recovery can occur before the fifth DAR. However, for F_v'/F_m', complete recovery was observed only two days later under similar RWC_l (Fig. 9d) compared to the CI group.

Chlorophyll index

The concentration of chlorophylls remained stable during the complete dehydration-rehydration cycle, characterizing the homoiochlorophyllous behavior of *Selaginella convoluta*. On average, the total chlorophyll index of the DR treatment group was 35 ± 4.08 (standard deviation, SD), and it did not differ from the hydrated control group (37 ± 6.4 SD). Chlorophylls *a* and *b* averaged 27.5 (± 4.5 SD) and 7.3 (± 1.1 SD) for the DR group and 28.5 (± 4.96 SD) and 8.1 (± 1.9 SD) for the CI group, respectively.

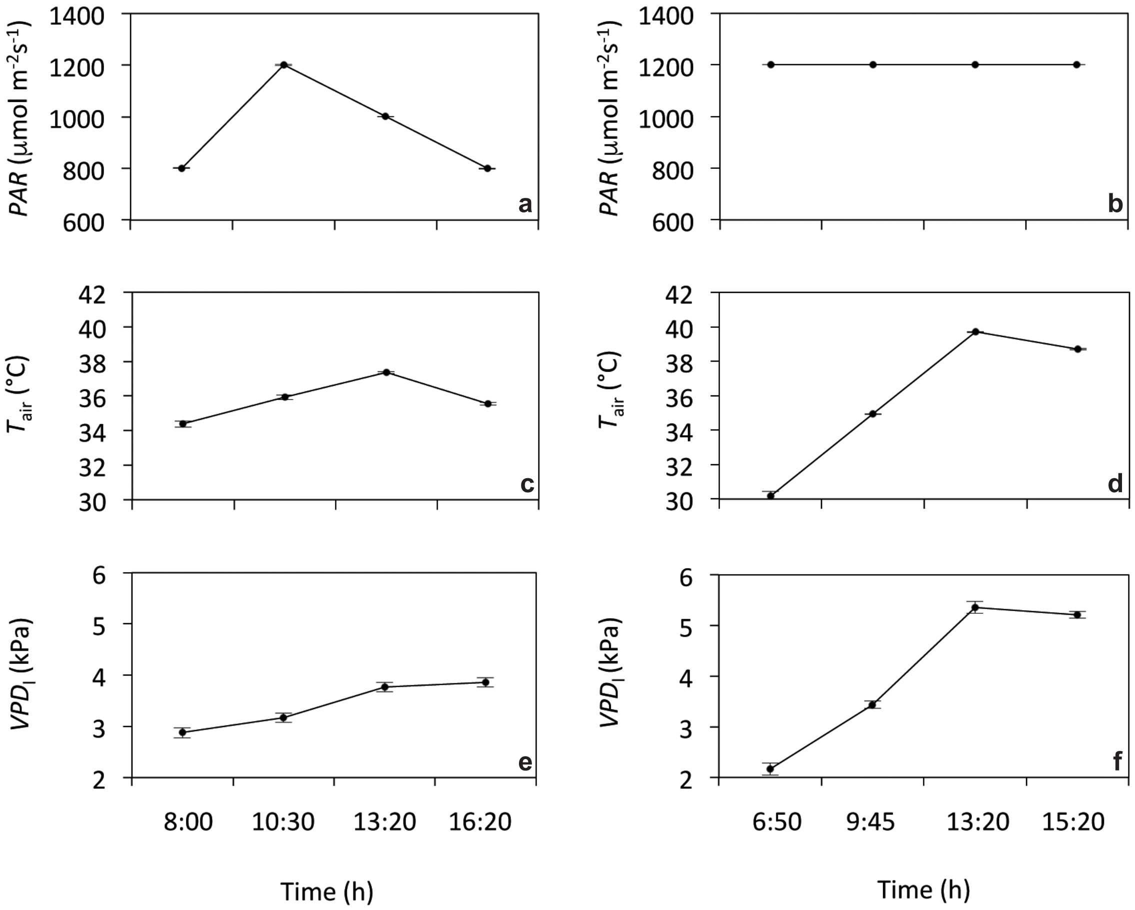

Figure 5 – a-b. Photosynthetically active radiation (*PAR*, mmol photons $m^{-2}s^{-1}$). c-d. air temperature (T_{air}, °C). e-f. leaf-to-air vapor pressure deficit (VPD_l, kPa) of *Selaginella convoluta*. [a,c,e. before desiccation (21-Feb-14). b,d,f. after rehydration (26-Jan-2015)]. Field experiment, Embrapa Semiárido, Caatinga Experimental Field, Petrolina, PE, Brazil. Each point represents the mean ± standard deviation (N = 3).

Discussion

VDT is understood as a complex of responses across all orders of the plant hierarchical organization, including changes in ultrastructural anatomy, morphology, physiology, and metabolism (Shivaraj *et al.* 2018). These responses constitute a central feature of the *Selaginella convoluta* successful fitness in its natural habitat, characterized by extreme variability in water availability.

Under initial water deficit conditions, this species conserves water by closing stomata to keep tissues physiologically active. However, the rapid transition to severe water depletion in the soil due to high evaporative demand prevents prolonged water retention in its herbaceous tissues. During this process, photosynthesis appears to be inhibited by diffusive limitations, potentially making the plant vulnerable to respiratory depletion of reserves and photooxidative damages

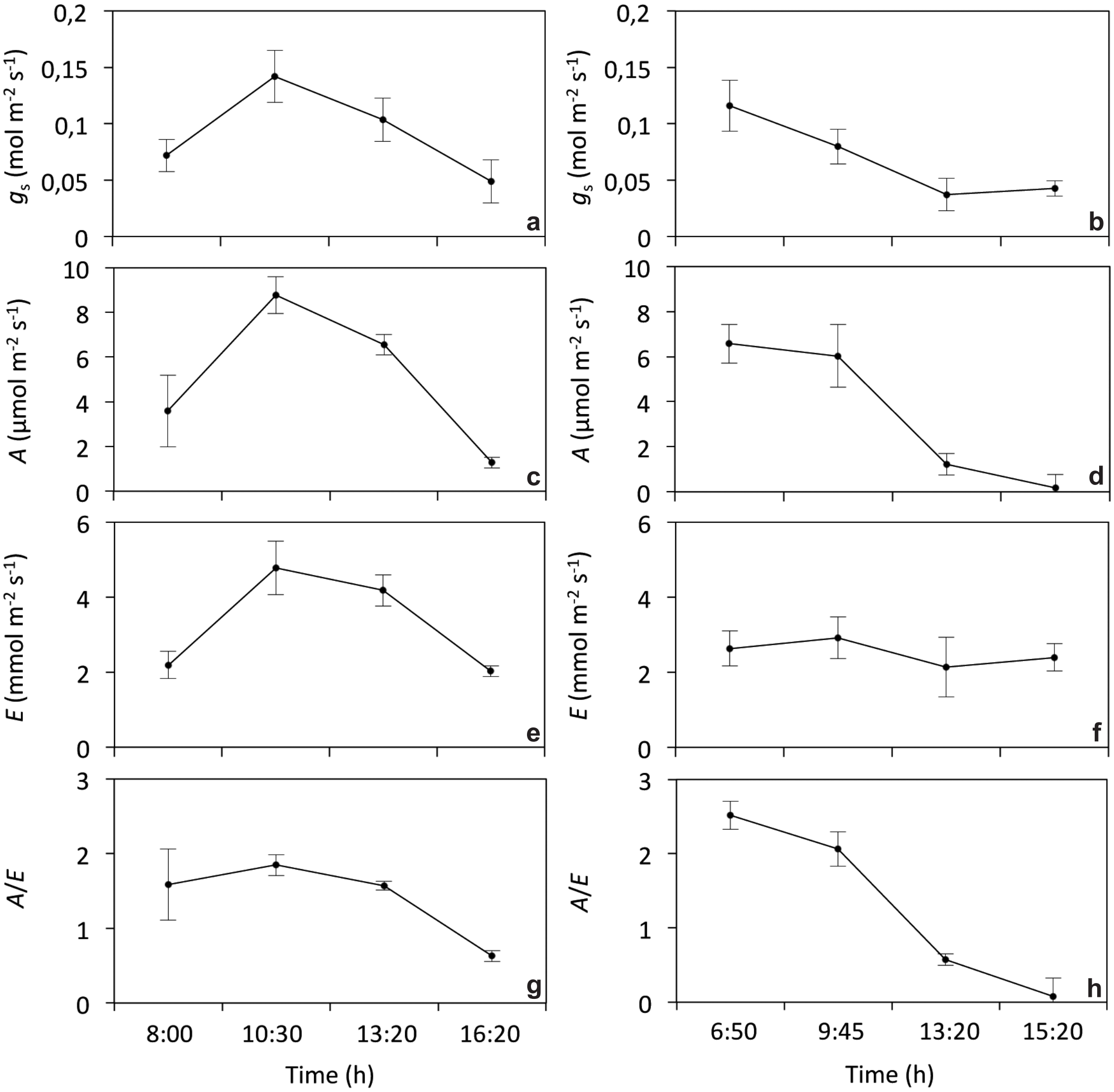

Figure 6 – a-b. Stomatal conductance (g_s, mol m^{-2}s^{-1}). c-d. photosynthesis (*A,* mmol m^{-2}s^{-1}). e-f. transpiration (*E*, mmol m^{-2}s^{-1}). g-h. intrinsic water use efficiency (*A*/*E*). [a,c,e,g. before desiccation (21-Feb-14). b,d,f,h. after rehydration (26-Jan-2015)]. Leaves of *Selaginella convoluta* in field experiment. Embrapa Semiárido, Caatinga Experimental Field, Petrolina, PE, Brazil. Each point represents the mean ± standard deviation (N = 3).

Table 1 – Concentration of hexoses (glucose + fructose) (mmol g^{-1} DM), sucrose (mmol g^{-1} DM), protein (mg g^{-1} DM), total amino acids (mmol g^{-1} DM), proline (mg g^{-1} DM), activity of the enzymes Catalase (CAT), Ascorbate Peroxidase (APX), and Superoxide Dismutase (SOD) (unit min^{-1} mg^{-1} protein) in leaves of *Selaginella convoluta* under field experiment in the hydrated (21-Feb-14), desiccated (15-May-14) and rehydrated condition (26-Jan-2015). Embrapa Semiárido, Caatinga Experimental Field, Petrolina, PE, Brazil. Values represent mean ± standard deviation (N = 3). Means followed by the same capital letters on the lines do not differ significantly by the Tukey test ($p < 0.05$). DM, dry mass.

	Hydrated (21-Feb-2014)	**Desiccated (15-May-2014)**	**Rehydrated (26-Jan-2015)**
Hexoses (mmol g^{-1} DM)	35.1 ± 12.0 A	20.0 ± 5.1 B	16.2 ± 5.4 B
Sucrose (mmol g^{-1} DM)	0.0 ± 0.0 B	6.8 ± 2.3 A	5.9 ± 3.2 A
Proteins (mg g^{-1} DM)	29.4 ± 4.6 A	18.0 ± 1.7 B	30.1 ± 4.4 A
Total amino acids (mmol g^{-1} DM)	27.9 ± 5.6 A	16.2 ± 3.3 B	30.0 ± 3.0 A
Proline (mg g^{-1} DM)	3.0 ± 0.9 A	1.8 ± 0.6 B	1.7 ± 0.4 B
CAT (unit min^{-1} mg^{-1} protein)	0.043 ± 0.011 A	0.044 ± 0.012 A	0.053 ± 0.022 A
APX (unit min^{-1} mg^{-1} protein)	0.62 ± 0.12 A	0.59 ± 0.15 A	0.56 ± 0.13 A
SOD (unit min^{-1} mg^{-1} protein)	29.4 ± 8.5 AB	20.3 ± 6.4 B	38.5 ± 13.4 A

DM = dry matter

if relatively prolonged. However, in more complex plant groups, from ferns to angiosperms known as modified desiccation tolerant (Oliver *et al.* 1998), it is understood that the preparation of vegetative tissues for desiccation begins at this stage of water conservation. In this sense, stomatal closure provides the necessary time for the induction of protective mechanisms and acquisition of desiccation tolerance.

The maintenance of solar radiation absorption by leaves under diffusive limitations of photosynthesis caused by water deficit would lead to the production of reduced power compounds in the chloroplast electron transport chains. Consequently, excessive energy absorbed could increase oxidative damage, making protection mechanisms against this process very important during dehydration.

Selaginella convoluta is found in regions prone to water deficits in several Brazilian biomes (Assis & Labiak 2009; Salino & Almeida 2008; Hirai & Prado 2000). In the Caatinga biome, its presence is linked to its adaptability to occupy environments ranging from partially shaded areas beneath larger shrubs and trees to areas that are open or poorly shaded (Xavier 2007), such as the borders of soil island communities on rocky outcrops. Therefore, the species is considered mesophilic to heliophilic (Xavier 2007) and requires efficient mechanisms against photo-oxidation.

The stable activity of the antioxidant enzymes CAT and APX after desiccation can suggest that its protection may be a constitutive mechanism in *Selaginella convoluta*. In SOD, the recovery of its activity to the original levels after rehydration suggests that its protective function is associated

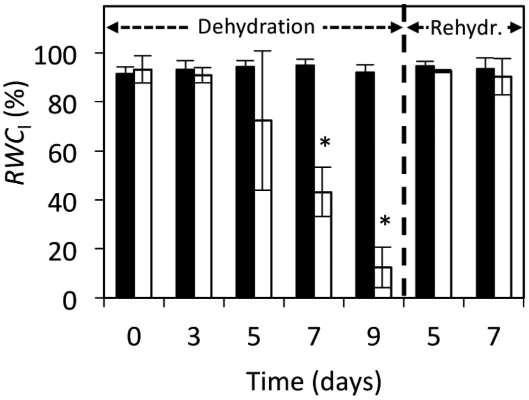

Figure 7 – Leaf relative water content (RWC_l, %) of *Selaginella convoluta* at zero, three, five, seven, and nine days of dehydration and at five and seven days after rehydration (white bars) in comparison to irrigated control (black bars), under pot experiment. Embrapa Semiárido, Caatinga Experimental Field, Petrolina, PE, Brazil. * Statistically different from control group by the Tukey test ($p < 0.05$). Error bars indicate the standard deviation of the means (N = 3).

with tissues exhibiting high RWC_l during the rehydration phase. At least for those enzymes evaluated, dehydration was not an inducible factor for its antioxidant activities in *S. convoluta*. The responses of antioxidant activity in *S. convoluta* differ from other VDT *Selaginella* species, but there

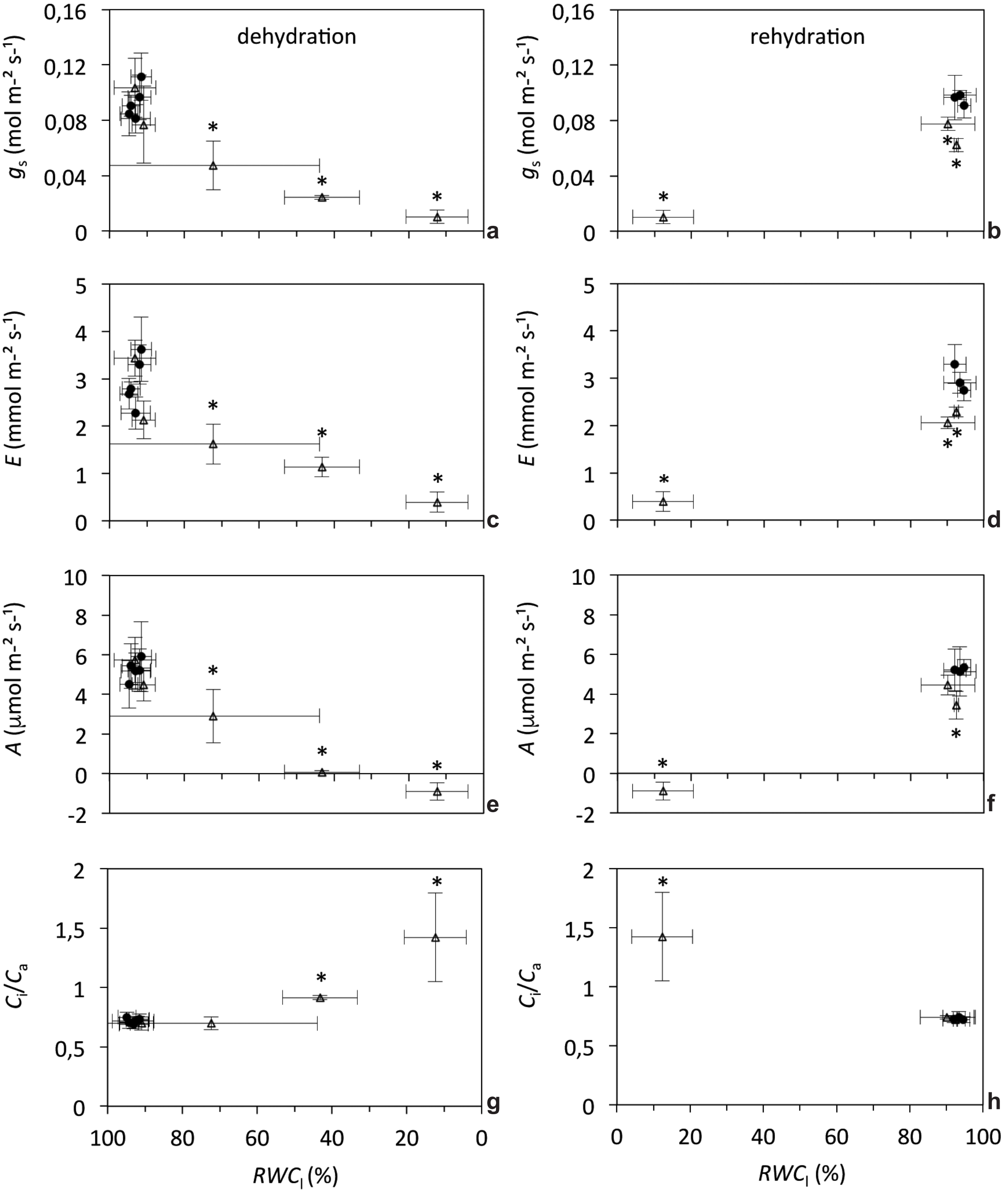

Figure 8 – a,b. Stomatal conductance (g_s, mol m^{-2}s^{-1}). c,d. transpiration (E, mmol m^{-2}s^{-1}). e,f. photosynthesis (A, mmol m^{-2}s^{-1}). g,h. C_i/C_a ratio. Leaves of *Selaginella convoluta* under pot experiment. (a,c,e,g. dehydration phase. b,d,f,h. rehydration phase). Closed circles = irrigated plants. Opened triangles = dehydrated plants. Embrapa Semiárido, Caatinga Experimental Field, Petrolina, PE, Brazil. * Statistically different from control group by the Tukey test ($p < 0.05$). Error bars indicate the standard deviation of the means (N = 3).

is no single pattern of responses reported. Some species induce the activity of antioxidant enzymes along with dehydration (Alejo-Jacuinde *et al.* 2020), while in others this process was described to occur in the desiccated state (Wang *et al.* 2010; Deeba *et al.* 2016; Shivaraj *et al.* 2021). In bryophytes, antioxidant enzymes are primarily transcribed and translated during rehydration to contribute to repair mechanisms in the recovery phase (Oliver *et al.* 2004). Distinct dynamics of antioxidant enzymes have also been observed in angiosperms, with some showing an increase during dehydration and a decline during rehydration, while others exhibit an increase upon rehydration (Sherwin & Farrant 1998). Thereby, a variability of responses of the same group or different antioxidant enzymes along dehydration-rehydration cycles can occur between different species, even of the same genus.

An alternative explanation for the apparent non-involvement of the tested antioxidant enzymes in the desiccation tolerance of *Selaginella convoluta* may lie in the existence of additional compounds that were not evaluated. In this context, alternative ways to enhance antioxidant

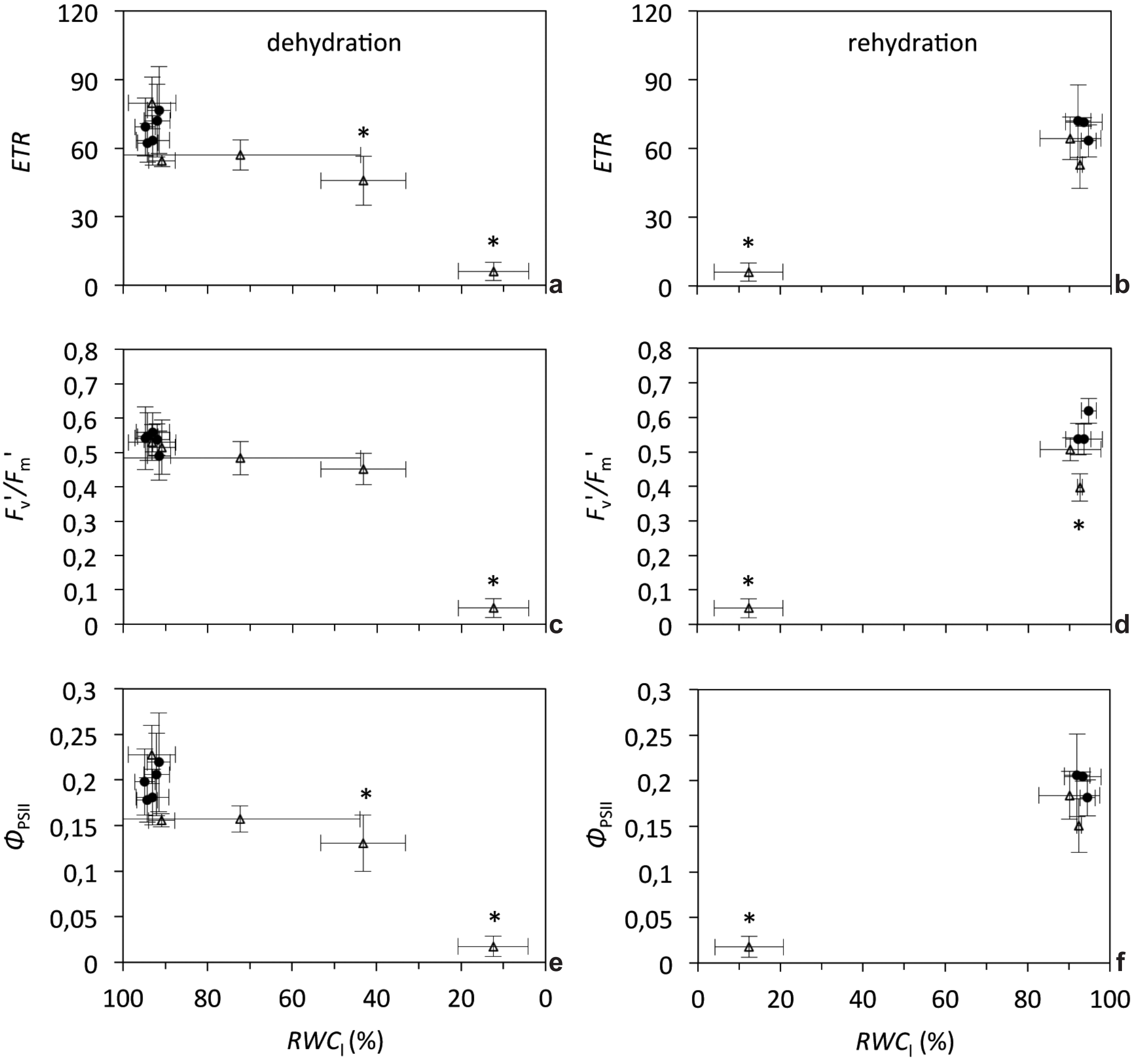

Figure 9 – a,b. Electron transport rate (*ETR*). c,d. potential quantum yield (F_v'/F_m'). e,f. effective quantum yield (Φ_{PSII}). Leaves of *Selaginella convoluta* under pot experiment. (a,c,e. dehydration phase. b,d,f. rehydration phase). Closed circles = irrigated plants. Opened triangles = dehydrated plants. Embrapa Semiárido, Caatinga Experimental Field, Petrolina, PE, Brazil. * Statistically different from control group by the Tukey test ($p < 0.05$). Error bars indicate the standard deviation of the means (N = 3).

ability have been reported in other *Selaginella* species involving phenylalanine metabolism and polyamine catabolism (Gu *et al.* 2019), glutathione metabolism (Yobi *et al.* 2012), vanillate (Yobi *et al.* 2013), and the oleosin and pentatricopeptide gene families (Xu *et al.* 2018).

Associated with the activation of the antioxidant system, photosystem-II activity was inhibited after desiccation. Some possible mechanisms may be related to the light-chlorophyll interaction preventing under-desiccation, as an important characteristic to minimize photooxidation in the persistent chloroplasts. These mechanisms may involve changes in the conformational configuration of antenna pigments or alterations in the chloroplast ultrastructure and/or positioning, which could account for the absence of fluorescence signals in desiccated leaves. Besides this, chlorophyll maskers as pigments, direct inhibition of reaction centers, or quenching by non-photochemical reactions are possible candidates (Choudhury *et al.* 2017). In the DT fronds of *Mohria caffrorum* (L.) Desv. Fv/Fm decreased after RWC_1 dropped below 70% (Farrant *et al.* 2009).

Other desiccation-tolerant vascular species are found in hyperxerophilous Caatinga regions, rocky outcrops, and inselbergs, such as *Selaginella sellowii* Hieron., *Nanuza plicata* (Mart.) L.B.Sm. & Ayensu, *Tripogonella spicata* (Nees) P.M.Peterson & Romasch., and *Anemia* sp. (Gaff 1987, personal communication). Among these species, *N. plicata*, which is restricted to environments directly exposed to solar radiation, undergoes chlorophyll degradation as a protective mechanism against photo-oxidation during desiccation, which characterizes it as a poikilochlorophyllous (Farrant 2007). The other species mentioned conserve the chlorophyll content within chloroplasts in the desiccated state, like *S. convoluta*. In this case, leaf curling seems essential for shielding the chlorophyllated leaf surface from light and protecting it from photooxidation in the desiccated state (Sherwin & Farrant 1998). In addition to the mechanical leaf curling response, the accumulation of photoprotective pigments, such as carotenoids and anthocyanins was observed for other homoiochlorophyllous species (Aidar *et al.* 2017; Charuvi *et al.* 2019). In *Selaginella lepidophylla*, another desiccation-tolerant Selaginellaceae from the southern Texas desert, USA, the increase in xanthophyll content during dehydration was related to the photoprotective non-photochemical quenching of fluorescence (Casper *et al.* 1993).

The decrease in the total content of soluble proteins in *S. convoluta* during dehydration aligns with the results obtained for other VDT species. This change is probably associated with a mobilization process of reserves (Dinakar & Bartels 2013). Despite this, the expression of a set of proteins involved in the decrease of photosynthesis, sugar accumulation, production of antioxidant molecules, or in the increase of the flexibility of cell walls and membranes can be modulated upon dehydration (Dinakar & Bartels 2013).

The decrease in total amino acids in *S. convoluta*, on the other hand, differs from other resurrection plants, including *Selaginella* species. In these plants, total amino acids typically accumulate during dehydration (Tymms & Gaff 1978; Gaff & McGregor 1979), and function as compatible solutes or as mobile nitrogen reserves for the rehydrating tissues (Martinelli *et al.* 2007; Yobi *et al.* 2012, 2013; Dinakar & Bartels 2013; Singh *et al.* 2021).

The decline in the proline concentration suggests that this compound may not play a direct role in desiccation tolerance acquisition in *S. convoluta*, despite its involvement in the water deficit responses in various plant species (Wang *et al.* 2010; Dar *et al.* 2016; Shivaraj *et al.* 2021). According to different authors, the magnitude of proline increase can vary even for the same species. For instance, Pandey *et al.* (2010) reported an 11-fold increase in proline levels in *Selaginella bryopteris* branches following desiccation, while in Singh *et al.* (2021), this compound increased twofold after 24 h of dehydration.

Decreasing the concentration of hexoses from the hydrated to the desiccated state of *Selaginella convoluta* in field measurements suggests that glycolysis, the tricarboxylic acid cycle, and pentose phosphate phases continue to provide carbon and energy during leaf dehydration, even below the compensation point in the energy production/consumption balance. The higher sucrose content in the desiccated state, compared to an undetectable level in the hydrated state, suggest that hexoses may be alternatively converted into this soluble sugar, which is in agreement with observations reported for other species (Adams *et al.* 1990; Wang *et al.* 2010; Oliveira & Moraes 2015; Shivaraj *et al.* 2021).

The accumulation of sucrose during the VDT process has been reported for several other taxa, where it has been attributed to osmotic and membrane stabilization functions (Farrant *et* *al.* 2012; Dinakar & Bartels 2013). High levels of protoplasmic sucrose accumulation, along with other disaccharides and/or oligosaccharides, can increase the viscosity and form a glassy state that protects the cell integrity of desiccated tissues (Buitink *et al.* 1998). Moreover, as a soluble sugar, the accumulated sucrose constitutes as a readily available energy reserve for the resumption of metabolism after rehydration (Scott 2000).

In a study with *Selaginella tamariscina*, however, trehalose was found to be the major soluble sugar in both hydrated and desiccated states (140 to 134 mg g^{-1} dry weight), while glucose, sucrose, raffinose, and stachyose were undetectable (Liu *et al.* 2008). According to the authors, this behavior of steadily maintaining a high level of disaccharide(s) is similar to the VDT moss *Tortula ruralis* (Hedw.) Gaertn. Mey. & Scherb., but different from higher resurrection plants where the composition of soluble sugars is adjusted during dehydration.

In the subsequent rehydration measurements in *S. convoluta* conducted on 26-Jan-2015 in the field experiment, the even lower level of hexoses and high sucrose content suggest that the plants were still in transition toward complete recovery. In this process, a high respiration resumption likely contributed to maintaining the low hexose content, while sucrose conversion and photosynthesis recovery were still incomplete.

In contrast, in *S. bryopteris* branches, sucrose levels decreased upon desiccation compared to control and starch content, consistently with the increase of soluble acid invertase (Pandey *et al.* 2010). Metabolic profiling of *S. lepidophylla*, indicated the constitutive abundance of trehalose, sucrose, and glucose, along with several glycolysis/gluconeogenesis and tricarboxylic acid (TCA) cycle intermediates close to 100% and to 50% of leaf relative water content (Yobi *et al.* 2012, 2013). A comparative metabolic analysis between the VDT *S. lepidophylla* and its desiccation-susceptible relative *S. moellendorffii* revealed that *S. moellendorffii* contains higher trehalose levels than *S. lepidophylla* (Pampurova & Dijck 2014). Additionally, various sugar alcohols and sugar acids were more abundant in the hydrated state than during dehydration, possibly contributing to water retention during drying and absorption during rehydration, protein stabilization, and ROS scavenging (Yobi *et al.* 2012, 2013). According to Pampurova & Dijck (2014), polyols such as sorbitol and xylitol are 100 times more abundant in *S. lepidophylla* than *S. moellendorffii*.

As discussed, the available data reveal common and species-specific variations in the concentration dynamics of the compounds in response to the desiccation-rehydration cycle. With the exception of antioxidant enzymes, amino acids, and proline, our results generally align with those reported for other DT *Selaginella* species. These results suggest that substantial variation exists when considering the phenotypic plasticity, underlying mechanisms, lineages, populations, and closely related species. Recent works have highlighted the importance of characterizing the variability and natural diversity of desiccation-tolerant phenotypes, as most studies have focused on a conserved response of a more homogeneous group of plants (Marks *et al.* 2021; Alejo-Jacuinde & Herrera-Estrella 2022).

In the pot experiment, the slightly negative values recorded for *A* at the end of the desiccation process may be related to an artifact of the measurement technique or could represent an actual plant response. In the former, an incomplete sealing of the IRGA leaf sampling chamber due to increased leaf roughness in the desiccated state could allow CO_2 to escape into the external environment, resulting in negative values. Alternatively, negative values at the end of the desiccation process may correspond to a phase where respiration continues while photosynthesis is already inactive. Some studies have demonstrated the occurrence of desiccation respiration bursts over dark respiration in VDT plants. In such cases, respiration is transiently maintained after the cessation of photosynthesis to continue supplying the energy needed for the controlled inactivation of metabolism during dehydration, until it becomes undetectable under extreme desiccation (Schwab *et al.* 1989; Tuba *et al.* 1998). However, to maintain a positive carbon balance throughout the complete dehydration-rehydration cycle, the carbon loss phase that follows immediately after the cessation of photosynthesis must be short-lived to enable the ecological success of *S. convoluta* in its natural environment. The relatively rapid cessation of metabolism due to desiccation strategically prevents the depletion of energy reserves or until plant starvation. In this context, the ability of rapid dehydration of *S. convoluta*, ensured by its protection mechanisms, seems to be fundamental for the physiological performance of the species and the success of its desiccation tolerance ability.

A rapid physiological recovery ability from the desiccated state, in its turn, implies the optimized use of water for carbon fixation as soon as it is available. Consequently, maximum physiological potential can be attained within short periods of water availability in the environment. Relatedly, as a homoiochlorophyllous species, chlorophyll and chloroplast membrane conservation during the desiccation-rehydration cycle in *S. convoluta* confers an additional advantage for a rapid recovery of photosynthesis when moisture becomes available (Eickmeier 1980; Péli *et al.* 2012). Conservation of photosynthetic components, such as RuBP carboxylase and protein synthesis apparatus, during the dehydration period, was proposed as the main reason for the rapid recovery of photosynthesis during rehydration in *S. lepidophylla* (Eickmeier 1979).

Therefore, *Selaginella convoluta*'s ability to tolerate desiccation with relatively rapid recovery ensures a positive carbon balance, essential for its growth and survival. The prevalence of VDT species in rock outcrop communities reinforces the ecological advantage of its ability in environments with unpredictable periods of water availability (Gaff 1987; Meirelles *et al.* 1997, 1999; Porembski & Barthlot 2000).

In the Brazilian semi-arid climate of the Caatinga biome, rainfall is characterized as irregular and poorly distributed in space and time (Moura *et al.* 2007; Teixeira 2010). The rapid resumption of a positive carbon balance observed in *S. convoluta* after rehydration suggests a pattern of sporadic utilization of short periods of water availability in an environment where this resource occurs unpredictably.

The presence of inductive responses (such as sucrose and photochemical preventing) and constitutive responses (such as antioxidant activity of CAT and APX) observed during the VDT process of *S. convoluta* aligns with previously published studies with *Selaginella*, where VDT would be achieved by a wide range of constitutive metabolites with some inducible components (Liu *et al.* 2008; Yobi *et al.* 2012, 2013). Due to these constitutive and inductive mechanisms, VDT in *S. convoluta* shares similarities with angiosperms and/or bryophytes (Oliver *et al*. 2000, 2004), which serve to minimize potential damages during dehydration and facilitate repair upon rehydration.

The knowledge of the physiological and biochemical behaviors of *S. convoluta* during the desiccation tolerance process can be used to develop strategies for prospecting target responses using high-throughput approaches. Additionally, the potential of *S. convoluta* for biotechnological applications can be explored further as a source of microbes with growth-promoting functions, as has been proposed for other VDT species (Fernandes-Júnior *et al.* 2015).

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Data availability statement

In accordance with Open Science communication practices, the authors inform that all data are available within the manuscript.

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