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RESEARCH ARTICLE

Drought memory in *Acanthostachys strobilacea*, a CAM epiphytic bromeliad

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Keywords

Bromeliaceae; chlorophyll; denitrosylase; drought recovery; nitric oxide; priming; stress imprint.

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ABSTRACT

- Stress memory is the development of altered responses to stress due to previous exposure, which might result in increased tolerance. Biochemical and physiological parameters shown to be positively affected by stress memory include those of the antioxidant and nitrosative metabolism, photosynthetic pigments and osmolyte content. Epiphytic bromeliads likely present stress memory since they experience frequent droughts in the canopies. Thus, we aimed to evaluate if the epiphytic bromeliad *Acanthostachys strobilacea* (Schult. & Schult.f.) Klotzsch shows improved metabolic stress defence responses to a second drought and rewatering cycle compared to a single exposure.
- In a controlled environment chamber, 90-day-old plants were exposed to one or two drought–rewatering cycles of 14 days without irrigation and 5 days of rewatering each. Sampling occurred after the final drought and rewatering periods for one or two cycles treatments.
- The free amino acid, chlorophyll, and carotenoid levels and S-nitrosoglutathione reductase (GSNOR) activity were higher at the second drought than at the first exposure. The rise in nocturnal acidification (indicative of increased CAM activity) caused by the initial drought persisted through the second drought–rewatering cycle, implying a lasting memory effect on CAM activity. Furthermore, the second recovery did not induce glutathione accumulation, as in the first rewatering event, suggesting the pre-exposure to drought reduced this thiol's demand during a later recovery.
- Our results evidence metabolic changes related to drought stress memory in *A. strobilacea*, supporting this mechanism might be involved in the tolerance of epiphytic bromeliads to intermittent droughts.

INTRODUCTION

It is extensively supported that plants can alter responses to stress after recovering from a past exposure – a process known as "stress memory". Overall, those responses can lead to improved plant performance under repeated stress (Thellier & Lüttge 2013; Galviz *et al.* 2020). In that scenario, memory formation results from the priming effect of a first stress event (Bruce *et al.* 2007; Hilker & Schmülling 2019).

Drought stress memory has been shown to improve several defence mechanisms in response to recurrent drought, including osmolytes accumulation, such as proline (Pro) (Alves *et al.* 2020; Khan *et al.* 2021; Wang *et al.* 2021), photosynthetic traits, such as chlorophyll content, and antioxidant activity (Alves *et al.* 2020; Lukić *et al.* 2020, 2023; Khan *et al.* 2021). The regulation of drought memory was shown to involve nitric oxide (NO), as Wang *et al.* (2021) reported that NO is partly responsible for the higher Pro accumulation at a second drought in wheat seedlings (*Triticum aestivum* L.). Hence, the nitrosative metabolism, which also includes S-nitrosothiols (SNO) and denitrosylase enzymes, is possibly involved in drought memory formation, as shown for several abiotic stress responses (Begara-Morales *et al.* 2019). However, to our knowledge, the effects of reiterated drought on those nitrosative parameters are still unknown.

Stress memory enables the plant to rapidly acclimate to changing environmental conditions (Crisp *et al.* 2016). Therefore, it is likely present in epiphytic species, since they are exposed to frequent droughts due to the intermittent water supply (Benzing 2000). Bader *et al.* (2009) reported evidence of drought priming in seedlings of the epiphytic bromeliad *Tillandsia flexuosa* Sw. Plants pre-treated with mild and moderate drought had higher growth and carbohydrate levels after severe drought than plants not previously exposed to stress (Bader *et al.* 2009). However, no further studies were found on the effects of reiterated drought over other traits in epiphytic bromeliads. Such research is crucial for understanding how these ecologically important plants will be affected by climate change (Cach-Pérez *et al.* 2014), which could increase the frequency and severity of droughts in the future (Trenberth *et al.* 2014).

Many epiphytic bromeliads present crassulacean acid metabolism (CAM), generally associated with drought tolerance (Winter & Smith 1996; Benzing 2000; Crayn *et al.* 2015). Classical CAM plants have reduced water loss through transpiration because the stomata open exclusively at night for CO_2 uptake when evaporative demand is low, remaining closed during the daytime (Winter & Smith 1996). At night, CO_2 is assimilated by phosphoenolpyruvate carboxylase into malic acid, which is stored in the vacuoles. During the day, malic acid is transferred to the cytosol and decarboxylated to release CO_2.

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This CO_2 is then re-fixed through the C3 cycle when stomata are closed. As a result, CAM plants exhibit a distinctive pattern of nocturnal acidification in the cells (Winter & Smith 1996). The pattern of stomatal opening/closure in the CAM diurnal cycle is highly flexible and adjusts according to water availability, thus regulating water loss through transpiration (Dodd *et al.* 2002; Lüttge 2010). This flexibility of CAM plants facing variable humidity suggests that memory plays a role in the drought tolerance of these species, as proposed for the CAM plant *Aptenia cordifolia* (L.f.) Schwantes (Fleta-Soriano *et al.* 2015). When *A. cordifolia* plants were exposed to two cycles of drought, alterations in gibberellin and abscisic acid levels were consistent with memory formation, although CAM activity was not affected (Fleta-Soriano *et al.* 2015). It remains unclear whether the CAM cycle itself is part of drought memory responses.

The epiphytic bromeliad *Acanthostachys strobilacea* (Schult. & Schult.f.) Klotzsch presents several drought resistance traits from early to adult life stage including CAM activity (Crayn *et al.* 2015; Menezes *et al.* 2020; Carvalho *et al.* 2021). In this study, we tested the hypothesis that juvenile *A. strobilacea* plants exhibit improved biochemical and physiological responses to a secondary event of drought and rewatering compared to a single cycle, as indicative of stress memory. For this purpose, we exposed 90-day-old plants to mild drought stress in which recovery would be achieved quickly after rewatering. We evaluated parameters associated with CAM activity, photosynthetic integrity, nitrosative metabolism, antioxidant system and osmotic adjustment, since previous research evidenced that these mechanisms are part of short-term drought defence in juvenile *A. strobilacea* (Carvalho *et al.* 2021) and/or in the drought memory of other species (Alves *et al.* 2020; Lukić *et al.* 2020, 2023; Khan *et al.* 2021; Wang *et al.* 2021).

MATERIAL AND METHODS

Plant material and treatments

Seeds of *A. strobilacea* (record number A0A27A1 at the Brazilian Genetic Diversity bank - SisGen, *Ministério do Meio Ambiente*) were cultivated *in vitro* following Carvalho *et al.* (2014) and maintained for 15 days in a culture room adjusted to 25 °C, a 12-h photoperiod, and a photon flux density (PFD) of 100 µmol $m^{-2}{\cdot}s^{-1}$ provided by white tubular 6500K LED lamps. Subsequently, plants were transferred to plastic trays (35 plants each), containing approximately 1 l of fine sterilized commercial *Pinus* bark. Subsequently, the trays were placed in a walk-in environmental chamber under 25 °C/16 °C (day/night), 60%/70% relative air humidity, a 12-h photoperiod, and a PFD of 300 µmol $m^{-2}{\cdot}s^{-1}$ provided by LED panels with a light spectrum as in Figure S1. Plants remained under these conditions for 90 days, when they reached 18.8 ± 2.3 cm in height, on average (N = 6, mean ± SD). During the growth period, plants were irrigated twice a week with distilled water by spraying leaves and substrate until drainage was detected. Fertilization was performed in the same manner biweekly with 1 $g{\cdot}l^{-1}$ commercial fertilizer of 20:20:20 composition (total N:P:K; Plant-Prod, Canada) until 1 week before the onset of experiments.

Trays were randomly separated according to exposure to drought-rewatering (D-R) cycles (Fig. 1): D1 – single D-R: 14 days without irrigation and 5 days of rewatering; D2 – double D-R: 14 days without irrigation and 5 days of rewatering, followed by another 14 days without irrigation and 5 days of rewatering. The control plants (CT) were kept under irrigation through the 37 days of the experiment. The irrigation of CT, D1 and D2 plants followed the schedule described in Figure S2. Sampling of CT, D1 and D2 leaves for biochemical and physiological assays took place at 32 and 37 days (Fig. 1). Substrate samples were collected on days 1, 14, 19, 32 and 37 to assess te progression of substrate water content (SWC) during the experiment (Figures S2 and S3). For assessment of nocturnal acid accumulation (ΔH^+) indicative of CAM activity, sampling of biological triplicates (three plants each) took place 2 h after onset of the light period (dawn) and 2 h before the end of the light period (dusk). A sampling of biological triplicates with six plants each was performed at the middle of the light period for the remaining analyses. After removing approximately 3 cm of leaf bases and tips, part of the leaf pool was immediately frozen in liquid nitrogen and maintained at −80 °C, whereas another part was utilized fresh for other analyses, as described below.

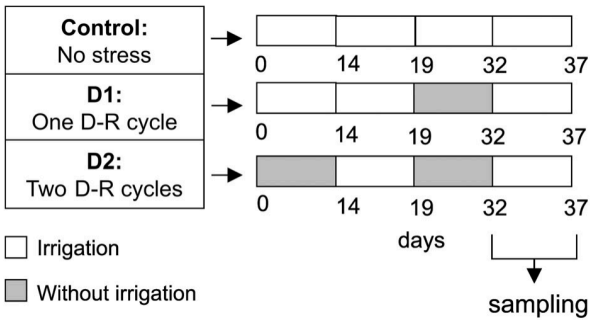


Fig. 1. Experimental design and sampling days. D-R, drought-rewatering.

Biometric, water status, CAM, and chlorophyll fluorescence measurements

We measured the number of leaves, dead leaves, and plant height (measured from the base of plants to the tip of the longest leaf) of a batch of six plants without removing them from the substrate, to assess growth. Relative water content (RWC) and osmotic potential were analysed from fresh leaf disks 0.5 cm in diameter, following Fleta-Soriano *et al.* (2015) and Rigui *et al.* (2019), respectively. SWC was assessed from aliquots obtained from three separate trays of each treatment, following Carvalho *et al.* (2021). CAM activity was assessed as the titratable acidity of samples obtained at dawn and dusk, according to Menezes *et al.* (2020). ΔH^+ was obtained by subtracting dusk from dawn values per replicate. Maximum potential quantum efficiency of PSII (Fv/Fm) was measured in the longest leaf of four intact plants using a modulated fluorometer (PAM 2500; Walz, Germany) after being kept for at least 15 min in leaf clips for dark adaptation.

Free amino acid (AA) content

Amino acids and Pro were quantified in the same extracts of 100 mg frozen powdered leaves in 1 ml ethanol 40% (v/v). After remaining at 8 °C for 24 h, extracts were centrifuged at

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16,100×*g* for 5 min (adapted from Carillo *et al.* 2005). AA content was estimated following Yokoyama & Hiramatsu (2003). Pro levels were quantified with the cuvette spectrophotometer protocol detailed in Carillo & Gibon (2011).

Photosynthetic pigments, antioxidants, and lipid peroxidation

Chlorophylls (Chl) *a* and *b* and carotenoids (Car) were quantified using extracts made of 80 mg frozen powdered leaves homogenized in 2 ml alkaline acetone 80% (v/v), followed by 10 min mixing in an ice bath. After centrifugation at 16,100×*g* for 10 min at 4 °C, extraction was repeated with the pellets using 1.5 ml alkaline acetone 80% as described earlier. Supernatants were then mixed and used for spectrophotometry, with final pigment concentrations calculated according to Lichtenthaler & Wellburn (1983). The Chl *a*:*b* ratio was also determined.

The activities of superoxide dismutase (SOD), ascorbate peroxidase (APX) and glutathione reductase (GR) were quantified following Carvalho *et al.* (2017). Total glutathione, reduced (GSH) and oxidized (GSSG) fractions were quantified with the enzymatic recycling assay as described in Carvalho *et al.* (2021). The glutathione redox state was inferred by calculating percentage of the GSH pool.

The estimation of lipid peroxidation (*i.e.*, membrane damage) was evaluated by quantifying hydroperoxide-conjugated dienes (HPCD) following Pignata *et al.* (2002), using extracts obtained from 40 mg frozen powdered leaves homogenized in 2 ml 96% ethanol (v/v).

Content of SNO and S-nitrosoglutathione reductase (GSNOR) activity

Total SNO was estimated with Saville's colorimetric method, according to Frungillo *et al.* (2013), with modifications described in Carvalho *et al.* (2021). GSNOR extraction was performed on 200 mg fresh leaf disks ground in 1 ml 0.1 mol·l^{-1} phosphate buffer pH 7.2 with a mortar and pestle in an ice bath (adapted from Frungillo *et al.* 2013). After centrifugation at 12,000×*g* and 4 °C for 15 min, supernatants were used for kinetic assays following Kubienová *et al.* (2016).

Statistical analyses

The experimental design was completely randomized. Means of each treatment (CT, D1 and D2) per period were subjected to a one-way ANOVA and post-hoc Tukey's tests ($P < 0.05$), using the GraphPad Prism 5.01 software. A principal components analysis (PCA) using a correlation matrix was performed with the Past3 software to summarize effects of one and two D-R cycles. The significance of each PC was determined with the broken-stick method (Vítolo *et al.* 2012).

RESULTS

Growth, water status and CAM activity

Plants of all conditions had similar sizes (height and leaf number) by the end of the experiment (Table S1). However, the number of dead leaves in D1 and D2 plants significantly surpassed those of CT by 64%, on average (Table S1). Despite the intense decline in SWC, the first and second droughts caused mild and statistically similar reductions in the RWC compared to the CT, reaching an average of 74% (Fig. 2a,b). Rewatering allowed complete SWC and RWC recovery in D1 and D2 to CT levels. The osmotic potentials did not differ between D1 and D2 (Fig. 2c), supporting the occurrence of mild drought. The first and second droughts significantly increased ΔH^+ (indicative of CAM activity) at similar rates, being 28% higher than the CT (Fig. 2d). The same pattern was maintained after rewatering.

Free AA, Pro and pigment pools

The total free AA content was progressively and significantly increased by the number of droughts and was maintained after the final rewatering events (Fig. 3a). A similar response occurred for Pro, although no difference was detected between D1 and D2 (Fig. 3b). The content of Chl *a*, Chl *b* and Car significantly intensified in relation to the number of droughts (Fig. 3). The first drought caused increases of 53% and 79% in Chl *a* and *b* compared to the CT, respectively (Fig. 3c,d). The second drought increased Chl *a* and *b* more in D2 than D1 (99% and 135% increase for Chl *a* and *b*, respectively, compared to CT). Means of Car were significantly higher by 46% and 82% in D1 and D2 at 32 days than in CT, respectively (Fig. 3f). After rewatering, Chls and Cars remained higher in D1 and D2 than in CT, although D1 and D2 were statistically similar. These changes did not significantly affect the Chl *a*:*b* ratio (Fig. 3e).

Photosynthesic status, antioxidant activity and nitrosative metabolism

Although D1 and D2 altered pigment levels, Fv/Fm did not change significantly (Fig. 4a). Accordingly, no significant HPCD accumulation from lipid peroxidation was detected (Fig. 4b). SOD, APX and GR activities were significantly higher by 30%, 77% and 76%, on average, respectively, than in CT, but were statistically similar between D1 and D2 (Figures 4c,d and 5a). Nevertheless, average APX and GR activities in D2 where higher than in D1. The activities of these enzymes were statistically similar between CT, D1 and D2 plants after rewatering.

Drought exposure did not affect GSH levels, although rewatering lead to an increase of 30% in D1 compared to CT and D2 (Fig. 5b). Meanwhile, GSSG was unaltered by D-R cycles (Fig. 5c). Accordingly, glutathione redox state was not affected by D-R cycles and was sustained in a highly reduced state of at least 85% (Fig. 5d). SNO, indicative of nitrosative status, accumulated at statistically similar levels between one and two droughts (Fig. 5e, 48% and 81% increase in D1 and D2, respectively, compared to CT). SNO was restored completely to CT levels after rewatering periods in D1 and D2. GSNOR activity in D2 was significantly higher than after D1, with increases of 32% and 16% more than in CT, respectively (Fig. 5f). After rewatering, GSNOR values of D2 recovered to CT values.

Principal components analysis (PCA)

The PCA assessed global effects of one and two D-R cycles (Fig. 6). The first and second axes (PC1 and PC2) were

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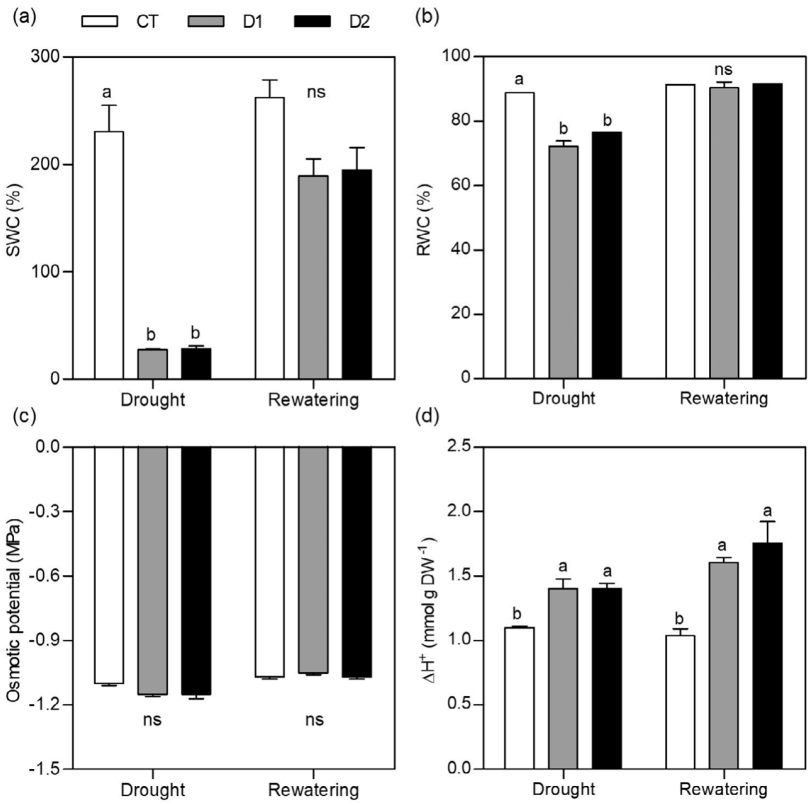


Fig. 2. Substrate water content (SWC) (a), leaf water status (b, c) and CAM activity (d) in *A. strobilacea* plants exposed to well-watered conditions (control, CT), one (D1) and two (D2) drought-rewatering events. 'Drought' and 'rewatering' bars represent means obtained at 32 and 37 days of the experiment, respectively (Fig. 1). Values are the mean ± SE (N = 3). Different lowercase letters indicate significant differences between groups per period (one-way ANOVA and post-hoc Tukey tests, $P < 0.05$). DW, dry weight; ns, not significant; RWC, relative water content; ΔH^+, nocturnal acid accumulation.

significant according to the broken-stick method (Figure S4) and represented 49% and 30% of data variance, respectively. The samples fell into six groups representing each treatment. Samples acquired at 32 days were separated mainly along PC1, with the control (CT-D) and drought samples (D1 and D2) placed at opposite sides of the axis; in addition, D2 was located at a more positive site than D1. The correlations of each variable to PC1 support that a second drought exposure is associated with accumulation of AA, Pro, Chl, SNO, increases in antioxidant enzyme activities (mainly APX, GR) and, to some extent, GSNOR and CAM activity than one drought stress (Table S2). In contrast, data obtained at 37 days, *i.e.*, after D1 and D2 plant rewatering, were mostly separated along PC2. D1-R and D2-R samples were closer to each other than to CT-R, suggesting plant metabolism after rewatering did not entirely recover to pre-stress conditions. The correlation values of the variables to PC2 support that rewatering is related to increases in GSH, Pro, SOD activity and CAM levels (Table S2), especially in D1-R plants.

DISCUSSION

The water status (RWC and osmotic potentials) and growth of *A. strobilacea* plants responded similarly to one or two D-R cycles, indicating that reiterated drought did not affect these parameters. Nevertheless, we observed some metabolic adjustments supporting a drought priming effect on *A. strobilacea*.

Plants exposed to one or two droughts exhibited increased nocturnal acid accumulation (ΔH^+) at similar rates, suggesting that drought intensified CAM activity. However, in both treatments, ΔH^+ did not return to pre-stress levels after rewatering. This indicates that the higher CAM activity induced by the first drought persisted through the second D-R cycle, implying a lasting memory effect on CAM activity (Crisp *et al.* 2016; Schneider *et al.* 2019; Jacques *et al.* 2021). Plants of the facultative CAM species *A. cordifolia* subjected to one or two droughts displayed similar rates of gas exchange parameters related to CAM activity (daytime stomatal conductance and CO_2 assimilation; Fleta-Soriano *et al.* 2015). However, these authors did not assess the extent of nocturnal CO_2 uptake or acidification in plants, which could determine whether repeated droughts altered the intensity of CAM (Winter 2019).

In the present study, a second drought exposure led to higher AA levels than only one exposure, which was sustained after the final rewatering event. Therefore, we consider that drought exposure promoted a downregulation in AA catabolism (Begum *et al.* 2020) and/or increased proteolysis, two well-established responses to dehydration (Hildebrandt 2018; Heinemann *et al.* 2020). Such strategies enable the redirection of free AA to synthesize diverse stress-related molecules, such as secondary metabolites, signalling molecules, hormones, and antioxidants such as GSH (Hildebrandt *et al.* 2015; Hildebrandt 2018). The higher AA/Pro levels for both droughts promote turgor maintenance, improve membrane and protein

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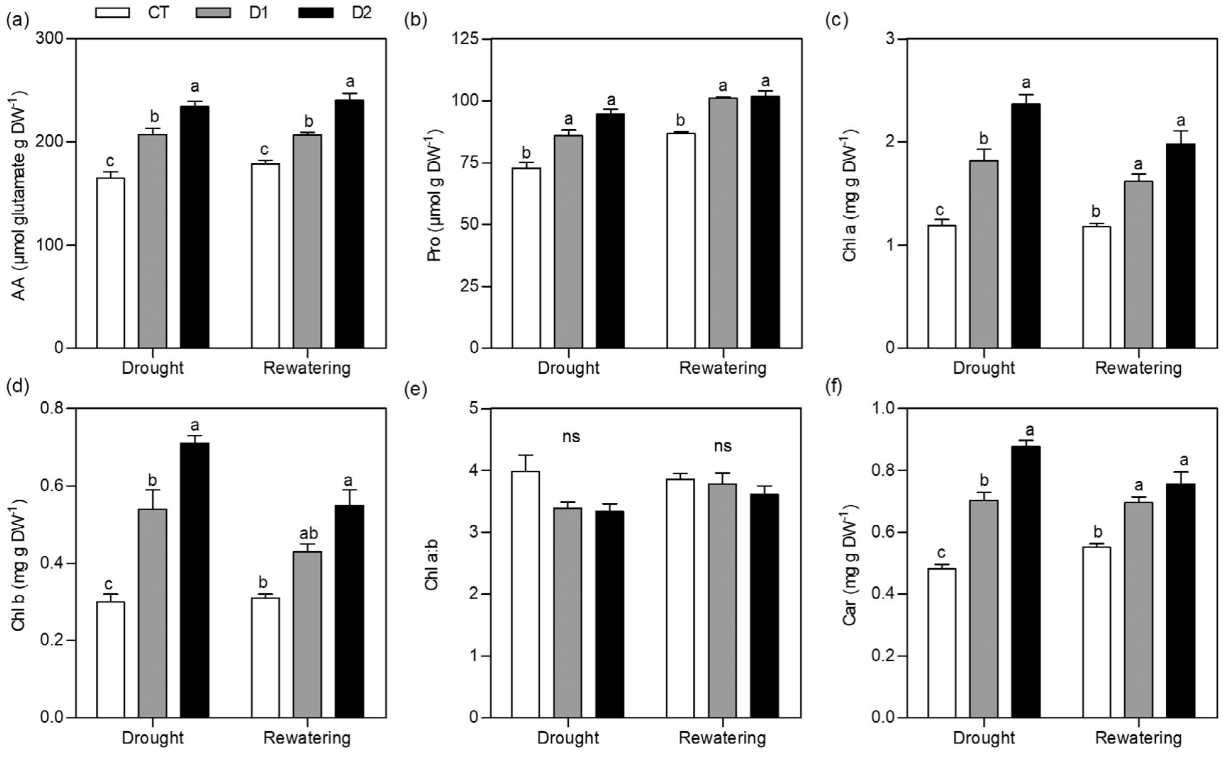


Fig. 3. Content of total free amino acids (AA) (a), proline (Pro) (b), chlorophylls (Chl) (c-e) and carotenoids (Car) (f) in leaves of *A. strobilacea* exposed to well-watered conditions (control, CT), one (D1) and two (D2) drought-rewatering events. 'Drought' and 'rewatering' bars represent means obtained at 32 and 37 days of the experiment, respectively (Fig. 1). Values are the means ± SE (N = 3). Different lowercase letters indicate significant differences between groups per period (one-way ANOVA and post-hoc Tukey tests, $P < 0.05$). DW, dry weight; Ns, not significant.

stabilization, serve as organic nitrogen storage and as alternative respiratory substrates for ATP production (Hildebrandt *et al.* 2015; Slama *et al.* 2015). Similarly, Pro and other osmoprotectants in *Dipteryx alata* Vogel, a species from a tropical dry forest, accumulated at higher rates under exposure to three D-R cycles rather than one. The authors concluded that increased osmoprotection enabled triple-stressed plants to maintain their physiological performance similar to that of unstressed plants (Alves *et al.* 2020).

Likewise, Chl content progressively increased during the two D-R cycles, which might result from intensified Chl synthesis or reduced Chl degradation. Although Chl accumulation is an unusual drought resistance trait among plants, it has been reported scarcely but commonly in species from arid areas, including *Calotropis procera* (Aiton) W.T. Aiton (Rivas *et al.* 2020) and *Acacia saligna* (Labill.) H.Wendl (Nativ *et al.* 1999). In the perennial grass *Alopecurus pratensis* L., Chl accumulation was promoted after repeated drought (Lukić *et al.* 2020), as observed herein. Lukić *et al.* (2020) observed that exposing *A. pratensis* plants to a 2-year drought, followed by a 3-week recovery, and a subsequent 2-week drought led to a 57% increase in Chl *b* compared to plants exposed only to the 2-week stress. Similarly, Lukić *et al.* (2023) observed that *Plantago lanceolata* L. plants exposed to drought in offspring and maternal generations had higher Chl concentrations than those that underwent stress only in the offspring generation. The consensus is that these accumulated pigments might promote higher photosynthetic rate by optimizing the light-harvesting process (Nativ *et al.* 1999; Lukić *et al.* 2020, 2023; Rivas *et al.* 2020) and, consequently, the ATP and NADPH supply for intensive stress metabolite synthesis. This optimization might also have occurred in *A. strobilacea* since Fv/Fm indicates that PSII functionality was preserved at CT levels during both D-R cycles. The assumed higher electron transport rate resulting from the Chl increase could be a potential source of reactive oxygen species (ROS) formation, especially in D2 plants, which was possibly compensated by increased Car that prevent photooxidative damage in the photosynthetic apparatus (Uarrota *et al.* 2018). Thus, Chl and Car accumulation is presumably highly advantageous for maintaining photosynthesis under drought in the epiphytic environment of dry forests, such as those where *A. strobilacea* is present, which is associated with a high light incidence due to the frequent tree defoliation (Cervantes *et al.* 2005).

Accordingly, there was an overall increase in enzymatic antioxidant responses under drought, although at similar levels between D1 and D2, which likely helped prevent photosynthetic damage or lipid peroxidation in both conditions. The higher levels of AA in the second drought might have also improved ROS maintenance, *e.g.*, by stabilizing antioxidant enzymes and promoting alternative detoxification pathways (Slama *et al.* 2015). Additionally, the glutathione redox state was preserved in D1 and D2 plants, with some changes after rewatering. While the first recovery period induced some GSH accumulation, this did not occur at the second rewatering, suggesting that pre-exposure to drought reduced demand for this thiol during later recovery. GSH has also been shown to increase after

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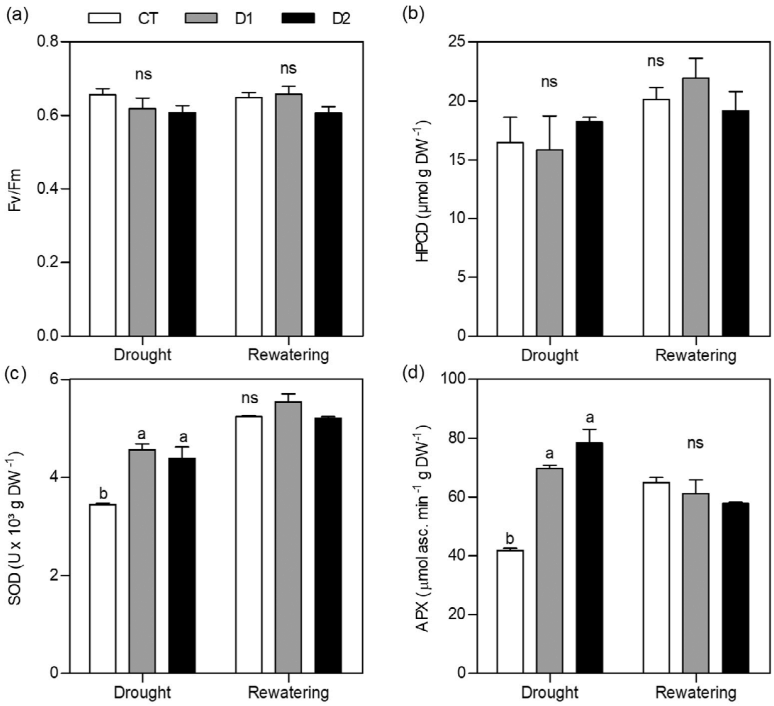


Fig. 4. Maximum potential quantum efficiency of PSII (Fv/Fm) (a), lipid peroxidation (b) and antioxidant enzyme activity (c, d) in leaves of *A. strobilacea* plants exposed to well-watered conditions (control, CT), one (D1) and two (D2) drought-rewatering events. 'Drought' and 'rewatering' bars represent means obtained at 32 and 37 days of the experiment, respectively (Fig. 1). Values are the mean ± SE (N = 3). Different lowercase letters indicate significant differences between groups per period (one-way ANOVA and post-hoc Tukey tests, $P < 0.05$. In (c), one unit (U) is the amount of enzyme required to inhibit the reduction in nitrobluetetrazolium by 50%. APX, ascorbate peroxidase; Asc., ascorbate; HPCD, hydroperoxide conjugated dienes; ns, not significant; SOD, superoxide dismutase.

rewatering in wheat seedlings (*T. aestivum* cv. C306) exposed to severe water stress. However, contrary to our findings, such increase was higher in plants pre-acclimated with mild stress than non-acclimated plants (Selote & Khanna-Chopra 2006).

The total SNO content suggests a higher nitrosative status in D1 and D2 than in CT. It is possible that part of the GSH pool reacted with excess NO and increased GSNO levels, which represent the largest fraction of low-molecular SNO in plant cells (Begara-Morales *et al.* 2018). The higher denitrosylase GSNOR activity in D2 suggest that repeated exposure induced higher defence action against such SNO accumulation (Corpas & Barroso 2013). In that case, an increase in GSSG, a product of GSNOR activity, should be expected in D2, although there was no alteration. We might assume that GSH recovery from the GSSG pool was compensated by the higher GR activity in D2, preventing GSSG accumulation and maintaining the redox ratio. SNO and GSNOR regulate several abiotic stress responses, such as the enzymatic antioxidant system (Begara-Morales *et al.* 2019; Li *et al.* 2021). Enzymes involved in Chl synthesis and AA metabolism are also potentially regulated by S-nitrosation (Hu *et al.* 2015; Silva *et al.* 2019). Furthermore, SNO formation *per se* might be a stress defence mechanism since it prevents oxidation of critical cysteine residues in proteins into irreversibly oxidized forms (Begara-Morales *et al.* 2019). Research on the involvement of S-nitrosation in reiterated drought responses, as suggested by our results, is still lacking. Nevertheless, a recent report showed that higher Pro accumulation as a result of drought hardening in wheat seedlings (*T. aestivum* Yangmai 16) induced by two subsequent periods of polyethylene glycol treatment was dependent on NO (Wang *et al.* 2021), whose bioactivity is exerted mainly through PTMs, such as S-nitrosation (Begara-Morales *et al.* 2018). Thus, it might be worth investigating if S-nitrosation and its regulation by GSNOR are involved with the reiterated drought effects in pigments and AA content in *A. strobilacea*.

The PCA corroborated that double-stressed *A. strobilacea* plants had more intense responses than single-stressed plants. It also clarified that rewatered D1 and D2 plants show a distinct metabolic response compared to unstressed plants (controls), but somewhat similar between them, supporting that they were not completely restored to the pre-stress condition. This results from AA, pigments and ΔH^+, which remained higher than in the control after rewatering. The pattern of metabolite accumulation that lingers after rewatering and results in higher accumulation at a second stress might be considered a memory response (Bruce *et al.* 2007; Thellier & Lüttge 2013; Crisp *et al.* 2016; Jacques *et al.* 2021). We consider that the non-recovery of pigments and AA might indicate an energy-saving strategy in these plants. Despite rewatering, it would be more efficient to sustain Chl/Car/AA levels rather than engaging in energy-consuming degradation pathways. A similar pattern to Chl/Car/AA was detected for ΔH^+, which did not return to pre-stress levels after rewatering but was maintained at similar rates between D1 and D2. The sustained higher CAM activity during rewatering might keep plants prepared for any rapid drought recurrence, which could occur within a few hours in the

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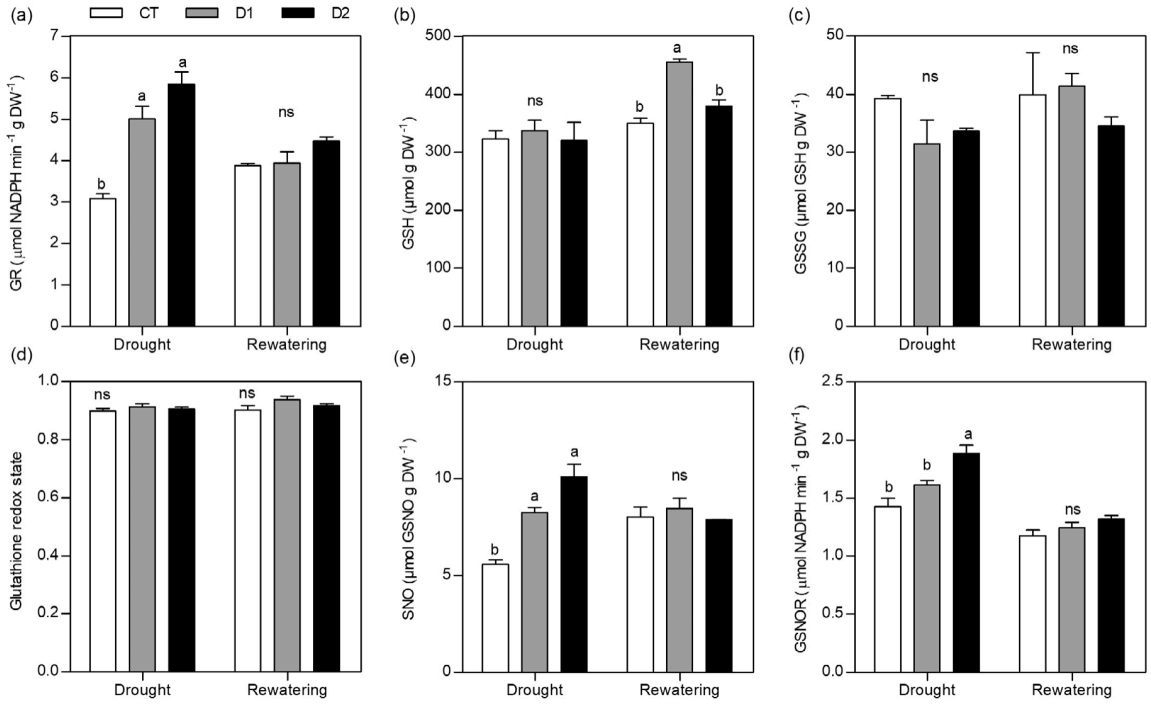


Fig. 5. Activity of glutathione reductase (GR) (a), glutathione levels (b–d) and nitrosative parameters (e, f) in leaves of *A. strobilacea* plants exposed to well-watered conditions (control, CT), one (D1) and two (D2) drought-rewatering events. 'Drought' and 'rewatering' bars represent means obtained at 32 and 37 days of the experiment, respectively (Fig. 1). Values are the mean ± SE (N = 3). Different lowercase letters indicate significant differences between groups per period (one-way ANOVA and post-hoc Tukey tests, $P < 0.05$). GSH, reduced glutathione; GSNOR, S-nitrosoglutathione reductase; GSSG, oxidized glutathione; ns, not significant; SNO, S-nitrosothiols.

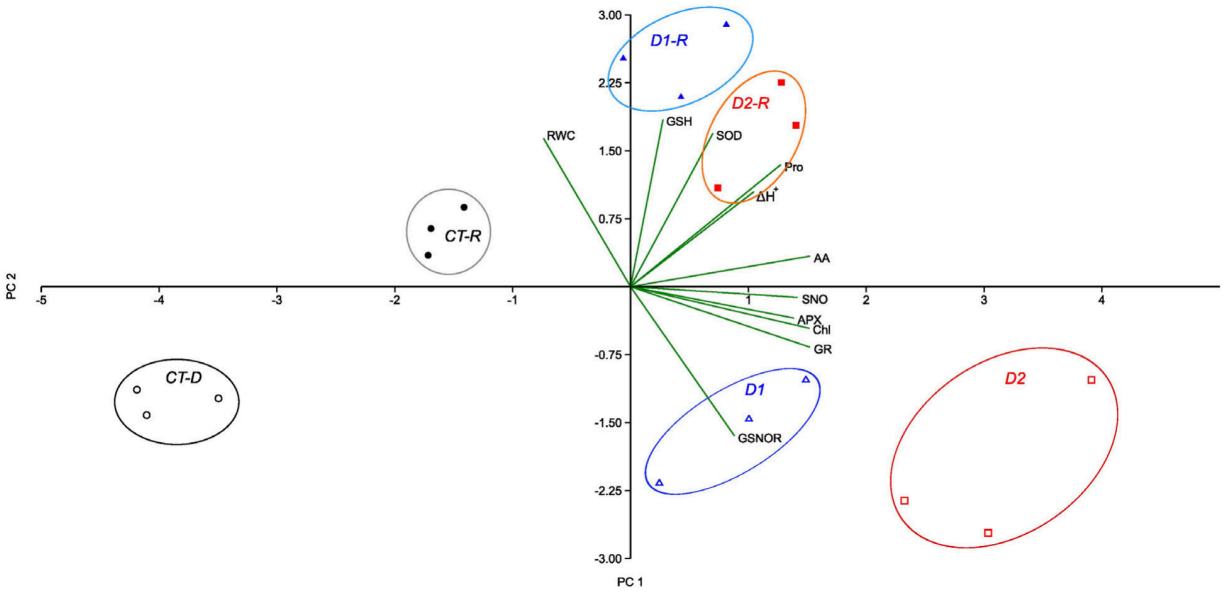


Fig. 6. Principal components analysis (PCA) of means obtained at 32 (CT-D, D1 and D2) and 37 days (CT-R, D1-R and D2-R) from leaves of *A. strobilacea* plants exposed to well-watered conditions (control, CT), one (D1) and two (D2) drought-rewatering events. Both PC1 and PC2 are significant according to the broken-stick method (Figure S4). Circles indicate clusters formed according to treatments. CT-D, D1 and D2: values of control, one and two droughts obtained at 32 days, respectively; CT-R, D1-R and D2-R: values of control and rewatering performed after one and two droughts at 37 days, respectively. Total Chl mean values were used in the analysis. AA, total free amino acids; APX, ascorbate peroxidase; Car, carotenoids; Chl, chlorophylls; GSH, reduced glutathione; GSNOR, S-nitrosoglutathione reductase; Pro, proline; RWC, relative water content; SNO, S-nitrosothiols; SOD, superoxide dismutase; ΔH^+, nocturnal acid accumulation.

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epiphytic environment (Zotz & Hietz 2001). The GSNOR activity response is also an indication of memory, since higher activity was detected at the second drought despite complete restoration to the CT after the first rewatering (Jacques *et al.* 2021). A similar pattern occurred for APX and GR activity, although the difference between D1 and D2 was not confirmed by ANOVA. The same might be concluded about GSH, since this thiol was significantly reduced at the second rewatering compared to the first, possibly due to previous drought exposure. Similar to our study, Bader *et al.* (2009) reported evidence of drought priming in the CAM epiphytic bromeliad *T. flexuosa*, which could point to memory formation. They found that 4-month-old seedlings pre-treated with mild or moderate drought by irrigating once after 1.0 or 1.5 weeks, respectively, for 2 months had increased growth and carbohydrate accumulation after exposure to severe drought (irrigation every 3 weeks for 3 months) than plants pre-treated with abundant irrigation (four times per week). This suggests that pre-conditioned plants would persist for longer in the face of future droughts.

CONCLUSIONS

Our data confirm that juvenile *A. strobilacea* plants have stress memory responses, as indicated by the significant increases in pigments, free AA pools and GSNOR activity after two droughts, and the reduction in GSH after the second recovery. The sustained increase in CAM activity detected from the first to the end of the second drought-rewatering cycle also indicates a memory effect. Based on these findings, we believe that further research on the regulatory mechanisms involved in S-nitrosation, Chl synthesis, antioxidant mechanisms and CAM activity during a reiterated drought might provide substantial information on the stress memory development in this epiphyte.

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AUTHOR CONTRIBUTIONS

All authors conceived and designed the experiments, interpreted the data, and revised the paper. V.C. performed the experiments and analyses, and wrote the paper.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Light spectrum of 1200 C3 Full Espectro LED panels (InstalaFrio, Pinhais - Brazil) installed in the walk-in controlled environment chamber utilized in this study.

Fig. S2. Irrigation and sampling schedule during the 37 days of experimentation. CT – control, D1 – single drought-recovery, D2 – double drought-recovery, SP – sampling point. Light blue cells indicate irrigation days, green cells indicate rewatering days in D1 and D2.

Fig. S3. Substrate water content (SWC) from trays containing *A. strobilacea* plants exposed to well-watered conditions (control, CT), one (D1) and two (D2) drought-rewatering events during the 37 days of experimentation. Values are mean ± SE (N = 3).

Fig. S4. Broken-stick evaluation of the principal components analysis (PCA).

Table S1. Biometric analyses in *A. strobilacea* plants exposed to well-watered conditions (control, CT), one (D1) and two (D2) droughts or droughts and rewatering. Values are mean ± SE, calculated from six biological replicates (N = 6). Different letters indicate significant differences between groups (one-way ANOVA and post-hoc Tukey tests, $P < 0.05$). Ns, not significant.

Table S2. Correlation values of variables to the significant components (PC1 and PC2), as calculated in the principal components analysis (PCA), with the Past3 software. AA, total free amino acids; APX, ascorbate peroxidase; Car, carotenoids; Chl, chlorophylls; GSH, reduced glutathione; GSNOR, S-nitrosoglutathione reductase; Pro, proline; RWC, relative water content; SNO, S-nitrosothiols; SOD, superoxide dismutase; ΔH^+, daily acidity variation.

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