VICTÓRIA DE CARVALHO

Tolerância e memória à seca na bromélia epífita *Acanthostachys strobilacea* (Schult. & Schult.f.) Klotzsch

Tese apresentada ao Instituto de Pesquisas Ambientais da Secretaria de Infraestrutura e Meio Ambiente, como parte dos requisitos exigidos para a obtenção do título de DOUTOR em BIODIVERSIDADE VEGETAL E MEIO AMBIENTE, na Área de Concentração de Plantas Vasculares em Análises Ambientais.

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RESUMO

Bromélias epífitas dependem da água da atmosfera como fonte hídrica por se sustentarem sobre árvores e não haver contato direto entre suas raízes e o solo; portanto, são naturalmente expostas à seca intermitente. Plantas jovens de bromélias epífitas podem ser vulneráveis à seca pois têm alta predisposição à perda de água e pelo fato de diversas adaptações morfológicas à seca não estarem desenvolvidas por completo. A avaliação das respostas à seca e reidratação em plantas jovens de bromélias pode promover maior entendimento sobre sua tolerância aos episódios de seca, que podem se intensificar devido às alterações climáticas. Importantes componentes da defesa à seca em plantas incluem o sistema antioxidante, o ajuste osmótico e a regulação de permeabilidade de membranas através das aquaporinas. Muitas destas respostas são reguladas por vias de sinalização compostas por espécies reativas de oxigênio e nitrogênio (RNS, ROS) e o ácido abscísico (ABA), ativadas em curto prazo de exposição à seca. A bromélia epífita *Acanthostachys strobilacea* (Schult. & Schult.f.) Klotzsch, nativa da Mata Atlântica e Cerrado, apresenta diversas adaptações à seca (*e.g.* metabolismo ácido das crassuláceas, CAM), sendo um adequado modelo de estudo para a avaliação de mecanismos de defesa à seca em plantas jovens de bromélias epífitas. O objetivo deste trabalho foi avaliar três hipóteses quanto à resposta à seca e recuperação em plantas juvenis de *A. strobilacea*, sendo elas: (i) as primeiras horas de exposição à seca levam ao aumento no teor dos sinalizadores RNS, ROS e ABA e, subsequentemente, à ativação de mecanismos de defesa; (ii) as plantas mostram respostas bioquímicas de defesa mais intensas após exposição a um segundo ciclo de seca-reidratação, indicando formação de memória; (iii) existe modulação no ritmo diurno da fotossíntese CAM dentre a seca e reidratação em curto prazo, associada a alterações na expressão gênica de aquaporinas, padrão anatômico e parâmetros hídricos. Para a avaliação das duas primeiras hipóteses, foram quantificadas moléculas sinalizadoras (ROS, RNS e ABA) e mecanismos de defesa bioquímicos (*e.g.* atividade antioxidante e conteúdo de osmólitos). Para a validação da terceira hipótese, avaliaram-se trocas gasosas, parâmetros hídricos, anatômicos e expressão gênica de aquaporinas. A primeira linha de estudo mostrou que o aumento em atividade de enzimas antioxidantes, prolina e ajuste osmótico ocorreu ao longo de 24 horas de seca concomitantemente a picos no teor de RNS, sugerindo o envolvimento destas moléculas nas vias de sinalização à seca. Ao contrário do esperado, o acúmulo de ABA ocorreu posteriormente às 72 horas de exposição, estando potencialmente relacionado à regulação de respostas mais tardias à seca. O segundo estudo mostrou que o conteúdo de pigmentos, aminoácidos totais e atividade da S-nitrosoglutationa redutase aumentaram após uma segunda exposição à seca de 14 dias, realizada após um ciclo de seca de 14 dias e reidratação por 5 dias, confirmando a

segunda hipótese. Por fim, a terceira hipótese foi confirmada pois a exposição à seca e reidratação por 14 dias e um dia, respectivamente, induziu de modo reversível a remobilização de água no hidrênquima (tecido armazenador de água), ajustes na expressão gênica de aquaporinas e na fotossíntese CAM. O presente estudo fornece evidências de mecanismos fisiológicos, bioquímicos, moleculares e morfológicos de resistência à seca em plantas jovens de *A. strobilacea*, demonstrando pronunciada capacidade de defesa desde estágios ontogenéticos iniciais nesta espécie. Este trabalho cria subsídios para futuros estudos sobre a resposta à seca em epífitas visando aprofundamento do papel das RNS e ABA em vias sinalizadoras, da formação de memória e da contribuição das aquaporinas na dinâmica hídrica entre o hidrênquima e clorênquima, típico de suculentas. Portanto, os resultados deste trabalho aliados a futuras pesquisas na área podem auxiliar na compreensão de como as alterações climáticas podem afetar bromélias epífitas, essenciais ao equilíbrio ecológico das florestas tropicais.

Palavras-chave: Bromeliaceae, déficit hídrico, estresse oxidativo, memória a estresse, óxido nítrico, suculência

ABSTRACT

Epiphytic bromeliads depend on the atmosphere as a water source because they sustain themselves on trees, without direct contact between their roots and soil; therefore, they are naturally exposed to intermittent drought. Young plants of epiphytic bromeliads might be vulnerable to drought because they have a high predisposition to water loss and because several morphological adaptations to drought are not fully developed. Research on responses to drought and rewatering of juvenile epiphytic bromeliads can promote greater understanding of their tolerance to drought periods, which might be intensified due to climate change. Important components of plant drought defence include the antioxidant system, osmotic adjustment, and membrane permeability regulation through aquaporins. Many of these responses are regulated by signalling pathways composed of reactive oxygen and nitrogen species (RNS, ROS) and abscisic acid (ABA), activated during early drought. The epiphytic bromeliad *Acanthostachys strobilacea* (Schult. & Schult.f.) Klotzsch, native to the Atlantic Forest and Cerrado, presents several drought adaptations (*e.g.*, crassulacean acid metabolism, CAM), being an appropriate model for the evaluation of drought defence mechanisms in juvenile epiphytic bromeliads. The objective of this work was to evaluate three hypotheses regarding the response to drought and subsequent recovery in juvenile plants of *A. strobilacea*, which are: (i) the first hours of drought exposure lead to an increase in the content of RNS, ROS and ABA and, subsequently, to the activation of defense mechanisms; (ii) plants show more intense biochemical defense responses after exposure to a second drought-rewatering cycle, indicating memory formation; (iii) short-term drought and rewatering result in modulation of the daily rhythm of CAM photosynthesis, associated with changes in aquaporin genes expression, anatomical pattern and water status. For the evaluation of the first two hypotheses, signalling molecules (*e.g.*, RNS and ABA) and biochemical defence mechanisms (*e.g.*, antioxidant activity and osmolyte content) were quantified. Gas exchange, water status parameters, and gene expression of aquaporins were evaluated for the validation of the third hypothesis. The first line of study showed that the increase in antioxidant enzymes activities, proline and osmotic adjustment occurred during 24 hours of drought concomitantly with peaks in the RNS content, suggesting the involvement of these molecules in drought signalling pathways. Contrary to expectations, ABA accumulation occurred later at 72 hours of exposure, being potentially related to the regulation of late responses to drought. The second study showed that the content of pigments, total amino acids and S-nitrosoglutathione reductase activity were intensified after a second exposure to a 14-day drought, performed after a cycle of 14 days of drought and 5 days of rewatering, confirming the second hypothesis. Finally, the third hypothesis was confirmed because a 14-day drought

followed by one day of rewatering reversibly induced water remobilization in the hydrenchyma (water storage tissue), adjustments in aquaporins gene expression and in CAM photosynthesis. The present study provides evidence of physiological, biochemical, molecular, and morphological mechanisms of drought resistance in young *A. strobilacea* plants, demonstrating that a pronounced defence capacity is present from early ontogenetic stages in this species. This work provides information for future studies on the drought response of epiphytes aiming to better understand the roles of RNS and ABA in signalling pathways, memory formation and aquaporins contribution to the water dynamics between hydrenchyma and chlorenchyma, typical of succulents. Therefore, the results of this study combined with future research in the area can help in understanding how climate change might affect epiphytic bromeliads, essential to the ecological balance of tropical forests.

Keywords: Bromeliaceae, nitric oxide, oxidative stress, stress memory, succulence, water deficit

LISTA DE ABREVIATURAS

AA: aminoácidos

ABA: ácido abscísico

ANOVA: análise de variância

APX: ascorbato peroxidase

AQPs: aquaporinas

ATP: adenosina trifosfato

CAM: metabolismo ácido das crassuláceas

Car: carotenoides

CAT: catalase

Chl: clorofila

DAB: 3,3'-diaminobenzidina

FC: capacidade de campo

FW: peso fresco

GR: glutationa redutase

GSH: glutationa reduzida

GSNOR: S-nitrosoglutationa redutase

GSSG: dissulfeto de glutationa

HPCD: dienos conjugados de hidroperóxidos

NADPH: fosfato de dinucleótido de nicotinamida e adenina

NBT: nitrotetratzólio azul

NIPs: proteínas intrínsecas semelhantes à nodulina26

NO: óxido nítrico

Ns: não significativo

PCA: análise de componentes principais

PEPC: fosfoenolpiruvato carboxilase

PFD: densidade de fluxo de fótons

PIPs: proteínas intrínsecas de membrana plasmática

Pro: prolina

PSII: fotossistema II

RBCV-SP: Reserva da Biosfera do Cinturão Verde de São Paulo

RH: relative humidity

RNA: ácido ribonucleico

RNS: espécies reativas de nitrogênio

ROS: espécies reativas de oxigênio

RT-qPCR: reação em cadeia da polimerase *via* transcriptase reversa quantitativa

RWC: conteúdo relativo de água

SIPs: pequenas proteínas intrínsecas básicas

SNO: S-nitrosotióis

SOD: superóxido dismutase

SWC: conteúdo de água do substrato

TIPs: proteínas intrínsecas do tonoplasto

XIPs: proteínas intrínsecas não categorizadas

WUE: eficiência no uso da água

SUMÁRIO

INTRODUÇÃO GERAL

1 Resistência à seca em plantas

A seca é considerada o fator de estresse abiótico mais importante que afeta negativamente o crescimento e desenvolvimento vegetal. Este estresse ocorre quando o nível de água disponível para ser absorvido por uma planta é reduzido, podendo por fim levar à deficiência hídrica, que ocorre quando a perda de água pela transpiração da planta excede o volume sendo absorvido pela mesma (Salehi-Lisar & Bakhshayeshan-Agdam 2016).

Devido ao aumento da temperatura global induzido pela ação humana, a expectativa é de que ocorra um aumento geral na evapotranspiração, o que fará com que períodos de seca se estabeleçam de forma mais rápida, intensa e frequente, resultando em estiagens de maior duração (Trenberth *et al.* 2014) – o que foi confirmado em relatório recente do IPCC (Painel Intergovernamental sobre Mudanças Climáticas 2021). Neste cenário, diversos biomas podem ser afetados quanto à composição de espécies (Walther *et al.* 2005), o que depende diretamente do nível de adaptação e tolerância de cada uma.

1.1 Mecanismos de resistência à seca

1.1.1 Metabolismo ácido das crassuláceas

O metabolismo ácido das crassuláceas (CAM) é um tipo fotossintético diretamente associado à resistência à seca, pois promove alta eficiência no uso da água (WUE; razão de CO_2 assimilado: água transpirada) em relação a outras vias fotossintéticas, sendo seis vezes maior do que no mecanismo C_3 (Nobel 1996, Winter & Smith 1996). A alta WUE em plantas CAM deriva, no geral, de dois fatores associados a este metabolismo: (i) a abertura estomática para captação de CO_2 atmosférico ocorre no período noturno, quando há menor demanda evaporativa; (ii) a alta capacidade de concentração de carbono inorgânico que ocorre durante a manhã quando os estômatos estão fechados, otimizando a atividade fotossintética (Cushman 2001, Lüttge 2002, 2010). Assim, o CAM está presente principalmente em espécies de ambientes semiáridos (*e.g.* climas mediterrâneos), áridos (*e.g.* desertos) ou com disponibilidade intermitente de água (*e.g.* habitat epifítico tropical), representando cerca de 5% das plantas vasculares (Winter & Smith 1996, Cushman 2001). Famílias com predominância de metabolismo CAM incluem Crassulaceae, Cactaceae, Agavaceae, Orchidaceae e Bromeliaceae (Winter & Smith 1996, Lüttge 2010).

O ciclo diário de CAM, de acordo com Osmond (1978), é tipicamente dividido em quatro fases (figura 1). A fase I compreende o período noturno, quando ocorre a captação de CO_2 pelos estômatos, que é fixado principalmente como malato através da enzima

fosfoenolpiruvato carboxilase (PEPC). O malato é então estocado como ácido málico nos vacúolos das células, o que ocasiona a diminuição do pH celular no período noturno. A fase III ocorre ao longo do dia, quando os estômatos se fecham e o ácido málico é liberado ao citosol e descarboxilado para fornecer CO_2 à Rubisco para fixação em carboidratos de reserva, aumentando o pH celular neste período. As fases II e IV representam breves períodos de abertura de estômatos no início e final do período diurno, quando há uma transição entre a fixação de carbono pela PEPC e Rubisco (fase II), e vice-versa (fase IV) (ver CAM em figura 1). Assim, o consumo de ácidos durante o dia seguido por seu acúmulo à noite é uma das principais características que diferenciam as plantas CAM daquelas que aplicam a via C_3 para fixação de carbono, condição em que tal flutuação não é observada (Osmond 1978, Cushman & Bohnert 1999, Borland *et al.* 2011).

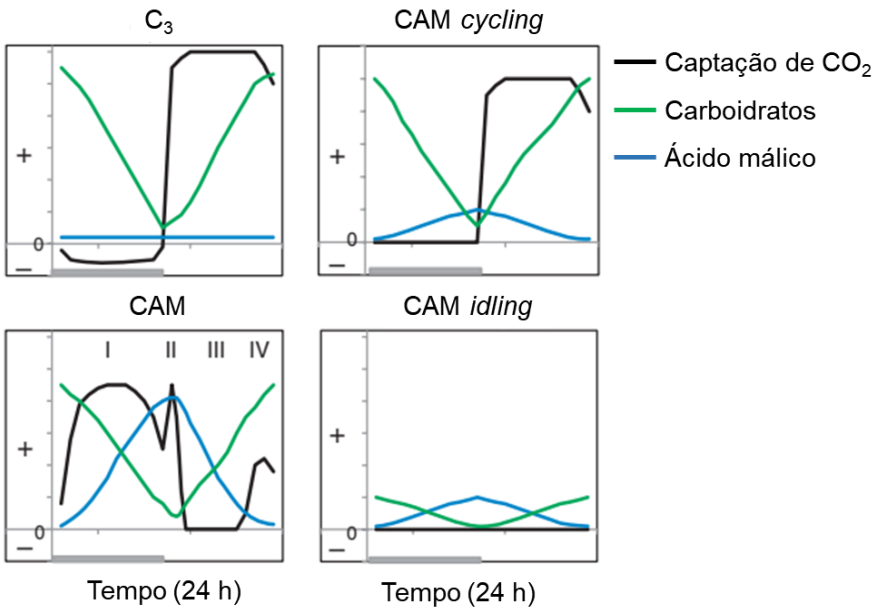

Figura 1. Tipos de fotossíntese CAM e seus ciclos diários em relação à captação de CO_2, conteúdo de ácido málico e carboidratos. A barra cinza na base dos gráficos indica o período noturno. Os numerais de I a IV indicam as fases do CAM. Adaptado de Borland *et al.* (2011).

O ciclo diário de CAM é altamente plástico, de forma que o tempo de ocorrência de cada fase é ajustável de acordo com as condições ambientais e o desenvolvimento das plantas, para otimizar o ganho de carbono e conservação de água (Cushman & Bohnert 1999, Dodd *et al.* 2002, Borland *et al.* 2011). Esta plasticidade levou à definição de tipos adicionais de CAM, baseados no padrão de abertura estomática e na fonte de CO_2 para síntese de malato (Ting 1985, Cushman & Bohnert 1999, Lüttge 2004, Borland *et al.* 2011, Winter 2019). O modo CAM *cycling* descreve plantas que apresentam padrões de trocas gasosas semelhantes a plantas com

o sistema C_3, em que os estômatos abrem durante o dia, mas ainda assim acumulam malato à noite, sintetizado a partir da reciclagem de CO_2 respiratório (figura 1). O CAM *idling* é caracterizado pelo fechamento contínuo dos estômatos durante as 24 horas do dia associado à acidificação noturna em baixa magnitude (figura 1), sendo que o CO_2 respiratório é utilizado como substrato para síntese de malato (Ting 1985, Cushman & Bohnert 1999, Borland *et al.* 2011, Winter 2019). O modo CAM *cycling* é detectado em plantas hidratadas (Martin 1994), sendo considerado uma forma mais fraca deste metabolismo (Cushman & Bohnert 1999, Borland *et al.* 2011, Winter 2019). Assim, existe a hipótese de que o CAM *cycling* permite que uma planta ative rapidamente o CAM tradicional quando a seca se estabelece para promover maior conservação da água, sendo vantajoso em habitats nos quais a seca pode se estabelecer em poucas horas (*e.g.* epifítico; Ting 1985, Cushman & Bohnert 1999, Silvera *et al.* 2010). Já o CAM *idling* é considerada a forma mais intensa de CAM (Lüttge 2004), ativada em condições de seca severa de modo a preservar o balanço de carbono e aparato fotossintético, o que acaba por estender a sobrevivência das plantas em condições altamente adversas (Ting 1985, Cushman & Bohnert 1999). Além disso, existem as plantas CAM facultativas, que apresentam o mecanismo C_3 ou C_4 em condições bem hidratadas, mas ativam o CAM em seca de modo reversível (Winter 2019). Independentemente do modo de expressão de CAM, plantas que têm capacidade de utilizar este metabolismo usualmente apresentam células com grandes vacúolos que comportam o armazenamento de ácidos orgânicos – parâmetro relacionado à suculência dos tecidos (Lüttge 2004, 2010, Males 2018).

1.1.2 Adaptações anatômicas: suculência

De acordo com Eggli & Nyffeler (2009), a suculência é definida como "o armazenamento de água utilizável em tecidos vivos de uma ou várias partes da planta, de forma a permitir que a planta seja temporariamente independente de fontes externas de água, podendo reter pelo menos alguma atividade fisiológica". Desta forma, plantas suculentas podem utilizar a água estocada em seus tecidos de reserva durante períodos de seca de modo a evitar quedas em sua atividade metabólica (Eggli & Nyffeler 2009, Males 2017). No geral, o mesofilo de folhas e caules suculentos é composto por grandes células dominadas por grandes vacúolos, dispostas de forma justa com baixa presença de espaço de ar intercelular (Smith *et al.* 1996, Nelson & Sage 2008). Esta adaptação anatômica está comumente presente em plantas de ambientes semiáridos e áridos, e majoritariamente com fotossíntese CAM (Griffiths & Males 2017). De fato, os grandes vacúolos de células suculentas possibilitam o armazenamento substancial de ácidos orgânicos resultantes de CAM (Lüttge 2010). No entanto, estudos mostram que nem sempre a suculência está diretamente associada à intensidade do CAM

(Nelson & Sage 2008, Herrera 2020), o que acrescenta ao questionamento sobre se a suculência seria um pré-requisito à evolução de CAM (Males 2017). De qualquer forma, a suculência pode ser detectada em diversos grupos de gimnospermas e angiospermas, sendo considerada um exemplo clássico de evolução convergente (Griffiths & Males 2017, Males 2017).

Folhas e caules suculentos podem ser classificados de acordo com dois tipos de arranjo anatômico (figura 2):

i) Suculência *all-cell*, em que o armazenamento de água ocorre em células expandidas do clorênquima, que ocupam maior parte do órgão fotossintético;

ii) Suculência de armazenamento (*storage succulence*), em que o estoque de água é mantido em tecido especializado do mesofilo denominado de hidrênquima, que difere do clorênquima por suas células não apresentarem clorofila e atividade fotossintética. Plantas com este tipo de suculência podem mostrar diferentes distribuições e transições dentre o hidrênquima/clorênquima nas folhas e caules, conforme a figura 2 (Griffiths & Males 2017, Males 2017).

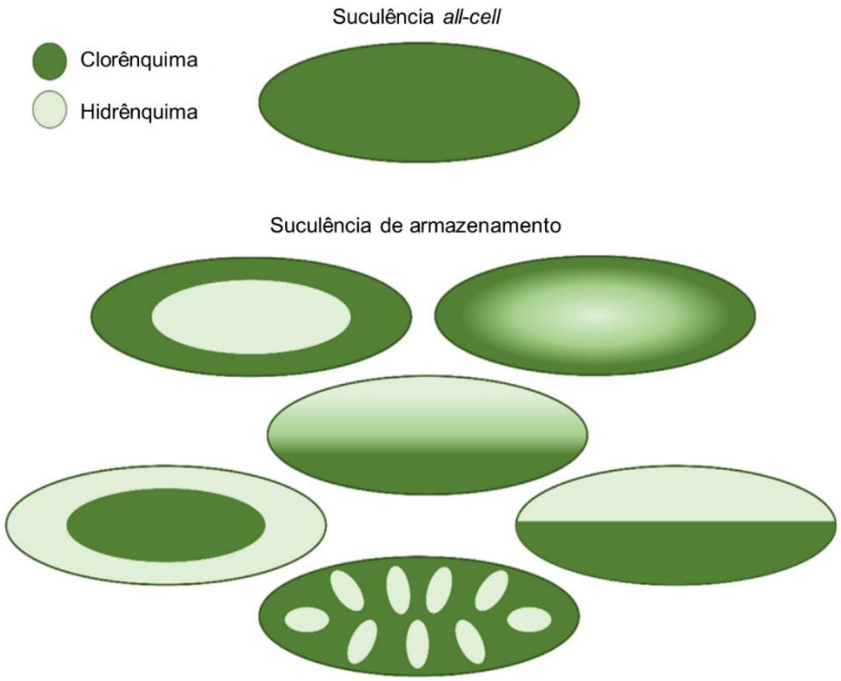

Figura 2. Tipos de distribuição de clorênquima e hidrênquima em suculentas do tipo *all-cell* e de armazenamento, incluindo a transição gradual ou abrupta dentre os tecidos. Reproduzido de Griffiths & Males (2017).

Plantas com suculência de armazenamento apresentam uma estratégia singular no uso de água em ocasiões de seca, quando ocorre a translocação da água armazenada no hidrênquima ao clorênquima, mantendo o potencial hídrico neste último tecido. Isto permite que a atividade fotossintética do clorênquima seja preservada por períodos mais longos de seca (Goldstein *et*

al. 1991, Nowak & Martin 1997, Nobel 2006, Males 2017). A água do hidrênquima é então recuperada nos eventos de maior disponibilidade hídrica, o que foi reportado ocorrer de modo rápido no cacto figo-da-índia, *Opuntia ficus-indica* L. Miller (Scalisi *et al.* 2016). É dito que esta dinâmica de entrada e saída de água no hidrênquima é facilitada pela alta elasticidade das paredes celulares, evitando danos nas células durante a translocação de água (Goldstein *et al.* 1991, Nowak & Martin 1997, Nobel 2006, Males 2016). Esta característica fornece maior capacitância às células do hidrênquima em relação às do clorênquima, termo definido como "a mudança no volume do tecido ou célula para uma certa alteração no potencial hídrico" (Ogburn & Edwards 2012); deste modo, as células do hidrênquima apresentam alta variação de volume frente a pequenas alterações de potencial hídrico (Goldstein *et al.* 1991, Ogburn & Edwards 2012) – o que permite rápida resposta às variações de disponibilidade de água.

Além da estocagem de água, os grandes vacúolos dos tecidos suculentos podem acumular solutos osmoticamente ativos (osmólitos) que auxiliam na manutenção do turgor (Nikalje *et al.* 2018). Desta forma, o ajuste osmótico tem grande relevância na defesa à seca de plantas suculentas.

1.1.3 Ajuste osmótico

Um importante mecanismo de defesa ao déficit hídrico em plantas é o ajuste osmótico, que ocorre através do acúmulo de osmólitos para reduzir o potencial de água das células, promovendo absorção de água do ambiente pelas raízes e a manutenção do turgor celular (ou seja, ação osmorreguladora; Salehi-Lisar & Bakhshayeshan-Agdam 2016, Zivcak *et al.* 2016). O ajuste osmótico, portanto, permite a manutenção da atividade metabólica apesar de quedas no potencial hídrico celular (Chaves *et al.* 2003). Os osmólitos são considerados solutos compatíveis por não afetarem as vias metabólicas e estrutura celular quando acumulados, e incluem moléculas pequenas, neutras, com alta solubilidade e baixa toxicidade como aminoácidos (*e.g.* prolina) e carboidratos (*e.g.* açúcares solúveis) (Kido *et al.* 2019). No caso de plantas CAM, foi verificado que os ácidos orgânicos acumulados no período noturno também têm atividade osmótica, auxiliando na manutenção de turgor frente à maior transpiração neste período (Smith & Lüttge 1985, Smith *et al.* 1987). De fato, o tipo de osmólito e a contribuição de cada um ao ajuste osmótico é altamente variável e depende das condições ambientais e da espécie (Zivcak *et al.* 2016). Por exemplo, o aminoácido prolina, um dos mais estudados, pode mostrar aumentos acima de cem vezes em plantas de Solanaceae sob déficit hídrico, enquanto muitas outras espécies não mostram acúmulo suficiente para uma ação osmótica ativa (Hare & Cress 1997, Forlani *et al.* 2019). Além da função osmorreguladora, alguns solutos compatíveis também apresentam ação osmoprotetora, que consiste na

estabilização de enzimas, proteínas e membranas, promovendo maior integridade da célula e funcionamento metabólico durante o estresse (Zivcak *et al.* 2016, Forlani *et al.* 2019). No caso da prolina, trabalhos sugerem que esta promove maior atividade antioxidante enzimática, por proteger as enzimas contra a desnaturação (revisado em Forlani *et al.* 2019).

1.1.4 Regulação da permeabilidade de membranas por aquaporinas

As proteínas intrínsecas denominadas aquaporinas (AQPs) também estão envolvidas no ajuste hídrico e manutenção de turgor por facilitarem o movimento de água através das membranas celulares (Chaumont & Tyerman 2014). Particularmente em folhas, as AQPs auxiliam no transporte extravascular de água aos tecidos internos através da via simplástica e, portanto, têm efeito direto sobre as relações hídricas das plantas (Tyerman *et al.* 1999, Heinen *et al.* 2009, Chaumont & Tyerman 2014, Maurel *et al.* 2015, Afzal *et al.* 2016). As AQPs de plantas superiores mostram grande variedade de genes (35 genes em *Arabidopsis thaliana* (L.) Heynh., 55 em *Populus trichocarpa* Torr. & A. Gray, 71 em *Gossypium hirsutum* L., entre outros; revisado em Maurel *et al.* 2015). As AQPs são classificadas em cinco subfamílias, baseadas principalmente em sua localização subcelular: as proteínas intrínsecas de membrana plasmática (PIPs), as proteínas intrínsecas do tonoplasto (TIPs), as proteínas intrínsecas semelhantes à nodulina26 (NIPs), as pequenas proteínas intrínsecas básicas (SIPs) e as proteínas intrínsecas não categorizadas (XIPs) (Maurel *et al.* 2015). As PIPs são a maior subfamília de AQPs nas plantas e estão localizadas principalmente na membrana plasmática de órgãos sob altos fluxos de água. Elas são ainda divididas em dois subgrupos, sendo PIP1 mais permeável que PIP2 (Kapilan *et al.* 2018). As TIPs também são altamente abundantes em plantas e estão presentes no tonoplasto (ou seja, na membrana do vacúolo). Devido à alta abundância de TIPs no tonoplasto, a permeabilidade do vacúolo supera a da membrana plasmática e facilita assim o equilíbrio osmótico entre o citosol e o vacúolo, implicando seu papel na regulação do turgor celular (Maurel *et al.* 2008, Kapilan *et al.* 2018, Kurowska 2021a). A maioria das PIPs e TIPs são altamente permeáveis à água, mas também transportam solutos adicionais como peróxido de hidrogênio (H_2O_2) e CO_2 para PIPs, ou amônia e ureia para TIPs (Maurel *et al.* 2015, Groszmann *et al.* 2017). Em contraste, a subfamília NIP, localizada nas membranas plasmáticas e intracelulares, apresenta baixa permeabilidade da água e realiza principalmente o transporte de moléculas pequenas e não carregadas como metaloides, amônia, H_2O_2 e glicerol (Pommerrenig *et al.* 2015).

A expressão gênica e abundância de AQPs é altamente variável de acordo com a isoforma, intensidade do estresse, órgão e espécie (Afzal *et al.* 2016, Shivaraj *et al.* 2021). Por exemplo, estudos com indivíduos transgênicos com aumento na expressão de diferentes AQPs

mostram tanto aumento quanto queda na tolerância à seca (Aharon *et al.* 2003, Xu *et al.* 2014). Diferentes trabalhos também ilustram padrões de queda ou aumento na expressão de genes de AQPs frente à seca (Alexandersson *et al.* 2005, Kurowska *et al.* 2019, Đurić *et al.* 2021). Supõe-se que a redução na expressão gênica de AQPs em seca reduz a permeabilidade da membrana e condutância hidráulica das folhas, levando à maior retenção de água nas células (Galmés *et al.* 2007, Maurel *et al.* 2015, Afzal *et al.* 2016, Chaumont & Tyerman 2017, Kurowska 2021a, b). Já o aumento na expressão gênica das AQPs poderia auxiliar no movimento hídrico foliar e na manutenção fisiológica das plantas durante a seca (Shivaraj *et al.* 2021). A ação de AQPs no transporte de água foliar também se mostrou importante para a recuperação do estado hídrico de plantas após a seca ter cessado (Ohrui *et al.* 2007, Galmés *et al.* 2007, Laur & Hacke 2014, Grondin *et al.* 2016, Secchi *et al.* 2017). Por exemplo, foi visto que plantas de arroz (*Oryza sativa* L.) reidratadas pós-seca com água contendo azida – um inibidor da respiração usado para bloquear AQPs – não recuperaram o potencial osmótico foliar aos níveis do pré-estresse, além de mostrarem inibição nas taxas de transpiração em relação às plantas reidratadas sem azida (Grondin *et al.* 2016). No entanto, o uso da azida como inibidor de AQPs deve ser avaliado com cautela por apresentar efeitos tóxicos e não ter especificidade a estas proteínas (Maurel *et al.* 2015, Chaumont & Tyerman 2017). De qualquer modo, plantas que tiveram redução de expressão gênica de AQPs foliares durante a seca mostraram recuperação ou mesmo aumento em diferentes genes após reidratadas, conforme observado em videira (*Vitis* sp., Galmés *et al.* 2007), *P. trichocarpa* (Laur & Hacke 2014), e na bromélia epífita *Tillandsia ionantha* Planchon (Ohrui *et al.* 2007). Desta forma, as evidências sugerem que a ação das AQPs na regulação da permeabilidade de membranas é essencial para o ajuste hídrico em resposta à seca e posterior recuperação, visando otimização no uso da água.

1.1.5 O sistema antioxidante como estratégia de defesa à seca

O sistema antioxidante tem a função de evitar e reduzir o estresse oxidativo decorrente da falta de água. De fato, um dos efeitos da seca é a formação excessiva de espécies reativas de oxigênio (ROS). As ROS são formas mais reativas do O_2 como o radical hidroxila ($^{\bullet}OH$), superóxido ($^{\bullet}O_2^-$) e H_2O_2. Quando produzidas de forma exacerbada, as ROS podem oxidar e inativar diversas estruturas celulares e, consequentemente, alterar o estado redox (ou seja, o balanço dentre moléculas oxidantes e redutoras da célula) e levar ao estresse oxidativo (Apel & Hirt 2004, Cruz de Carvalho 2008, Gill & Tuteja 2010). O sistema antioxidante age diretamente na eliminação das ROS através da ação de compostos não-enzimáticos, como o ácido ascórbico e glutationa, e enzimáticos, como superóxido dismutase (SOD), catalase (CAT), ascorbato peroxidase (APX) e glutationa redutase (GR; Apel & Hirt 2004, Gill & Tuteja 2010). Estes

componentes agem de forma integrada. Inicialmente, a enzima SOD realiza a remoção de $^{\bullet}O_2^-$, liberando H_2O_2. Este, por sua vez, pode ser removido pela CAT e APX. As enzimas APX e GR compõem o ciclo ascorbato-glutationa, sendo responsáveis por regenerarem o ácido ascórbico e a glutationa reduzida (Gill & Tuteja 2010, Groß *et al.* 2013). Estes metabólitos são cruciais ao balanço redox pois realizam a eliminação dos radicais $^{\bullet}OH$, uma das ROS mais reativas e danosas (Foyer & Noctor 2011). Outra categoria de antioxidantes não enzimáticos são os carotenoides presentes nos complexos antena, que previnem a peroxidação lipídica das membranas dos cloroplastos, protegendo assim os fotossistemas (Smirnoff 2007). É estabelecido que a tolerância ao déficit hídrico tem relação direta com o potencial antioxidante da planta, apesar do padrão de resposta variar por espécie e intensidade de estresse (Cruz de Carvalho 2008).

1.2 Sinalizadores envolvidos na ativação de mecanismos de resistência à seca

A ativação de mecanismos de resistência à seca em uma planta ocorre através de vias de sinalização desencadeadas após curtos períodos de exposição ao fator de estresse, em uma ordem de segundos a horas. Estas respostas são essenciais para prevenir danos irreversíveis e permitir que a planta atinja um estado aclimatado após períodos de estresse mais longos (Kollist *et al.* 2019, Dubois & Inzé 2020). Os sinais podem ser de caráter elétrico, hidráulico e químico. Nesta última categoria, se enquadram compostos como fitormônios, espécies reativas de oxigênio e nitrogênio (RNS; Huber & Bauerle 2016). O acúmulo do fitormônio ácido abscísico (ABA) é uma das respostas iniciais à seca (Finkelstein 2013, Cai *et al.* 2015, Urano *et al.* 2017, Dubois & Inzé 2020), estando envolvido na indução do fechamento estomático e na regulação da expressão de genes de resposta ao estresse (Fujita *et al.* 2011), como os do sistema antioxidante e síntese de osmólitos (Jiang & Zhang 2004, Sharma *et al.* 2019). Adicionalmente, sabe-se que um mesmo tipo de mecanismo de tolerância à seca pode ser ativado por vias sinalizadoras independentes de ABA em diferentes espécies (Stewart & Voetberg 1987, Bellaire *et al.* 2000, Verslues & Bray 2006, Yamaguchi-Shinozaki & Shinozaki 2006).

Evidências indicam o envolvimento de ABA, ROS e RNS em vias sinalizadoras de mecanismos de defesa à seca (Prakash *et al.* 2019), como o fechamento estomático (Bright *et al.* 2006) e estimulação da atividade antioxidante (Jiang & Zhang 2002, Lu *et al.* 2009, Tanotra *et al.* 2019). As RNS compõem o metabolismo nitrosativo, e incluem o radical gasoso óxido nítrico (NO) e diversos outros compostos que derivam de sua reação com moléculas distintas, como o peroxinitrito ($ONOO^-$), dióxido de dinitrogênio (N_2O_3), dióxido de nitrogênio (NO_2) e S-nitrosotióis (SNO) (Corpas *et al.* 2011). Em particular, os SNO são formados através da S-nitrosação, que consiste na adição covalente de NO ao grupo tiol (-SH) de uma cisteína reativa,

de forma reversível. Este processo é uma modificação pós-traducional que propaga o sinal do NO nas células, através da regulação da estrutura e função proteica. Além de alterar proteínas, a S-nitrosação pode ocorrer em moléculas de baixo peso molecular, mais notadamente a glutationa reduzida (GSH; Kolbert *et al.* 2019, Begara-Morales *et al.* 2019). Esta reação do NO com o grupo tiol da GSH resulta na S-nitrosoglutationa (GSNO), considerada o principal reservatório de NO em plantas (Begara-Morales *et al.* 2019). Assim, o GSNO é uma ligação direta entre o sistema nitrosativo e antioxidante (figura 3). Os níveis de GSNO estão sob controle da denitrosilase S-nitrosoglutationa redutase (GSNOR), que converte GSNO em dissulfeto de glutationa (GSSG) e amônia. Consequentemente, esta enzima controla indiretamente a ocorrência de S-nitrosação e níveis de SNO, reduzindo a disponibilidade de NO (Malik *et al.* 2011, Lindermayr 2018, Begara-Morales *et al.* 2019). Este controle é o que permite a regulação dos efeitos sinalizadores de NO nas plantas (Malik *et al.* 2011, Kolbert *et al.* 2019). Foi visto que a regulação do movimento estomático em células guarda de *A. thaliana* em resposta à seca envolve a S-nitrosação da proteína quinase SnRK2.6 (*open stomata 1 (OST1)/sucrose nonfermenting 1 (SNF1)-related protein kinase 2.6*) (Wang *et al.* 2015), porém a caracterização detalhada do envolvimento desta modificação nas respostas à seca ainda exige maiores estudos.

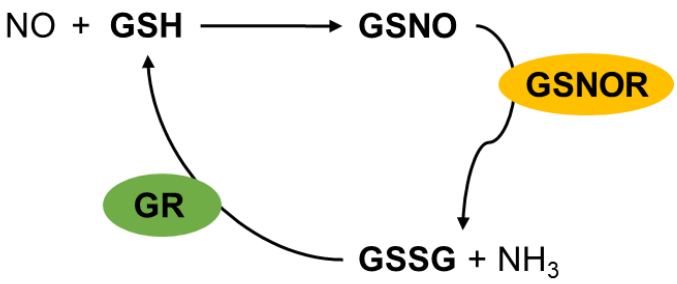

Figura 3. Ciclo de produção de GSNO e recuperação de GSH (Corpas & Barroso 2013).

2 Recuperação do metabolismo após a reidratação

A retomada metabólica após a recuperação da disponibilidade hídrica do ambiente afeta diretamente a capacidade de sobrevivência pós-seca. No entanto, até o momento, os efeitos da reidratação sobre o metabolismo em geral das plantas foram menos explorados do que as respostas à seca em si, conforme discutido em revisões sobre o assunto (Xu *et al.* 2010, Crisp *et al.* 2016, Ruehr *et al.* 2019). Sabe-se que a reidratação pode levar tanto à recuperação completa do metabolismo às condições anteriores, ou à uma nova homeostase – o que dependerá do tempo e intensidade da seca prévia, assim como do nível de resistência da espécie (Xu *et al.* 2010, Crisp *et al.* 2016, Ruehr *et al.* 2019, Maia Júnior *et al.* 2020). Este último fator pode, inclusive, determinar a rapidez de recuperação metabólica de uma espécie. Por exemplo,

plantas suculentas de armazenamento têm potencial para retomar o conteúdo hídrico rapidamente após reidratadas devido à alta plasticidade do hidrênquima, conforme mencionado anteriormente. Por exemplo, plantas de *O. ficus-indica* mostraram recomposição do hidrênquima em pouco mais de um dia após a irrigação, feita após um período de três meses de seca (Scalisi *et al.* 2016). No entanto, a recuperação do estado hídrico ocorre em taxas mais rápidas que a metabólica ou fotossintética (Ruehr *et al.* 2019), tornando essencial a avaliação de parâmetros fotossintéticos e bioquímicos quanto à recuperação pós-seca de uma espécie. Além disso, avaliar a resposta de plantas à reidratação torna-se ainda mais relevante ao se considerar que a recuperação do estado hídrico após um primeiro período de seca pode afetar as respostas de uma planta a secas subsequentes – processo denominado de memória.

3 Memória à seca

A exposição a um primeiro episódio de estresse não-letal, ou seja, que permita que a planta se recupere quando o estresse cessa, pode alterar suas respostas a estresses subsequentes, potencialmente tornando o indivíduo mais tolerante – processo denominado de "memória" ao estresse (Thellier & Lüttge 2013, Fleta-Soriano & Munné-Bosch 2016, Galviz *et al.* 2020). A aquisição dessas memórias na primeira exposição ao estresse ocorre pelo processo de *priming* (Galviz *et al.* 2020). Esta formação de memória está diretamente relacionada à duração do período de recuperação após o primeiro estresse, que pode levar tanto à consolidação quanto ao "esquecimento" de uma resposta em particular (Crisp *et al.* 2016, Jacques *et al.* 2021). O efeito da memória é observado em nível epigenético (*e.g.* metilação de DNA; Sun *et al.* 2021), transcricional (Avramova 2019), pós-traducional (*e.g.* fosforilação de proteínas; Zhang *et al.* 2020), metabólico (Fleta-Soriano *et al.* 2015, Schwachtje *et al.* 2019) e fisiológico (*e.g.* taxas fotossintéticas; Virlouvet *et al.* 2018).

O efeito da memória à seca ganha importância frente à previsão de aumento na frequência das estiagens, podendo ser explorado visando o desenvolvimento de estratégias de melhoramento em produtividade de espécies cultivadas em condições de seca (Choudhary *et al.* 2021). Além disso, estudos de memória à seca podem auxiliar na compreensão dos efeitos das alterações climáticas sobre espécies nativas de habitats ameaçados, principalmente aqueles expostos a frequentes variações de disponibilidade hídrica. Dentre as respostas bioquímicas mencionadas anteriormente e mais afetadas pela seca reiterada, destaca-se o estímulo do sistema antioxidante (Alves *et al.* 2020, Lukić *et al.* 2020, Khan *et al.* 2021, revisado em Jacques *et al.* 2021). Por exemplo, aumentos nos níveis de GSH e atividade de GR têm sido frequentemente relatados como respostas de memória à seca (Selote & Khanna-Chopra 2006, Wang *et al.* 2018, Khan *et al.* 2020, 2021). Em relação ao sistema nitrosativo, foi recentemente

relatado que o NO é parcialmente responsável por estimular o maior acúmulo de prolina em uma segunda exposição à seca em mudas de trigo (*Triticum aestivum* L.) (Wang *et al.* 2021). No entanto, os efeitos da seca reiterada sobre os níveis de SNO e sua manutenção pela atividade da GSNOR ainda são desconhecidos.

4 Adaptações e respostas à seca reportadas em bromélias epífitas

O hábito epifítico é caracterizado por plantas que se sustentam sobre outras (mais comumente em árvores), sem contato com a vasculatura da hospedeira (Benzing 2000, Zotz 2016). O grupo Bromeliaceae apresenta o segundo maior número de epífitas após Orchidaceae, de forma que as bromélias de hábito epifítico são importantes componentes da biomassa de florestas tropicais e assim, cruciais para o balanço ecológico de seu ecossistema (Zotz 2013). Essas plantas dependem da água atmosférica; assim, estão expostas à disponibilidade intermitente de água e, consequentemente, a secas frequentes (Benzing 2000, Zotz 2016). Desta forma, as bromélias epífitas apresentam diversas adaptações de resistência à seca, visando a preservação de seu estado hídrico e atividade metabólica nestas condições (Matiz *et al.* 2013, tabela 1).

Tabela 1. Exemplos de adaptações morfológicas, fisiológicas, bioquímicas e moleculares de resistência à seca de bromélias epífitas e espécies que as apresentam.

Morfológicas	**Espécies**	**Referências**
Tanque/fitotelma	*Vriesea gigantea* Gaudich	Gobara *et al.* (2020)
	Aechmea bracteata (Sw.) Griseb	Benzing (2000)
	Guzmania monostachia (L.) Rusby ex Mez var. *monostachia*	Freschi *et al.* (2010), Rodrigues *et al.* (2016)
Tricomas	*Tillandsia* sp.	Proença & Sajo (2007), Derwidueé & Gonzalez (2010)
	Billbergia distachia (Vell.) Mez, *Billbergia porteana* Brongn, *Vriesea* sp.	Proença & Sajo (2007)
Suculência *all-cell*	*Tillandsia recurvata* (L.) L.	Proença & Sajo (2007)
Suculência de armazenamento	*Tillandsia pohliana* Mez, *Tillandsia tenuifolia* L.	Proença & Sajo (2007)
	Tillandsia ionantha Planchon	Nowak & Martin (1997)
	G. monostachia var. *monostachia*	Rodrigues *et al.* (2016)
	Acanthostachys strobilacea (Schult. & Schult.f.) Klotzsch	Proença & Sajo (2007), Derwidueé & Gonzalez (2010)
Fisiológicas		
Intensificação na atividade do CAM	*Aechmea tessmannii* x *Aechmea fasciata*	Ceusters *et al.* (2009)
	G. monostachia var. *monostachia*	Freschi *et al.* (2010)
Ajuste osmótico	*Tillandsia utriculata* L.	Stiles & Martin (1996)
	T. ionantha	Nowak & Martin (1997)
Bioquímicas		
Aumento na atividade antioxidante	*Tillandsia brachycaulos* Schltdl	González-Salvatierra *et al.* (2010)
Acúmulo de ABA e/ou NO	*G. monostachia* var. *monostachia*	Mioto & Mercier (2013)
	Vriesea sanguinolenta Cogn. & Marchal	Zotz *et al.* (2004)
Acúmulo de carboidratos	*Tillandsia flexuosa* Sw.	Bader *et al.* (2009)
	Aechmea tessmannii x Aechmea fasciata	Ceusters *et al.* (2009)
Moleculares		
Redução na expressão gênica de AQPs	*T. ionantha*	Ohrui *et al.* (2007)
	G. monostachia	North *et al.* (2019)

Dentre as adaptações morfológicas, destaca-se a estrutura do tanque ou fitotelma formada pela sobreposição das bases de folhas largas que permite o armazenamento de água da chuva a ser utilizada em momentos de seca, e os tricomas que são células epidérmicas absorventes de água nas folhas. A presença destes tricomas é mais abundante em espécies que não apresentam o tanque e, portanto, têm maior dependência na captação de água atmosférica – estas espécies são denominadas de "atmosféricas" (Males 2016). Este tipo de bromélia compreende principalmente espécies de Tillandsioideae (Benzing 2000). Outras adaptações morfológicas das bromélias atmosféricas incluem a alta suculência e uso de água conservador (Males 2016). Inclusive, diferentes espécies de *Tillandsia* apresentam suculência por armazenamento (Nowak & Martin 1997, Proença & Sajo 2007), o que foi associado à prolongada sobrevivência frente à seca (Nowak & Martin 1997).

A maior eficiência no uso da água das bromélias epífitas em geral se dá principalmente pelo uso da fotossíntese CAM (Matiz *et al.* 2013), estando inclusive presente em todas as espécies de *Tillandsia* (Crayn *et al.* 2004) – o gênero mais representativo das atmosféricas (Benzing 2000). A expressão deste metabolismo mostra-se bastante diverso dentre as bromélias, havendo relatos de atividade de CAM *cycling* e *idling* (Martin 1994, Ceusters *et al.* 2009). O ajuste osmótico, identificado pela redução no potencial osmótico foliar, também foi associado à resistência de bromélias epífitas à seca, em que auxilia na manutenção fotossintética (Stiles & Martin 1996, Nowak & Martin 1997, Ceusters *et al.* 2009). Similarmente, a importância da regulação osmótica em períodos de maior demanda evaporativa foi observada em um estudo sobre traços de resistência à seca em bromélias de diferentes hábitos (Males & Griffiths 2017). Conforme citado anteriormente, as AQPs são fundamentais para a regulação hídrica e osmótica nas células. Pouco foi explorado sobre a contribuição de AQPs para a tolerância de bromélias epífitas à seca. No entanto, os estudos disponíveis mostram uma tendência de baixa na expressão de *PIP*s sob seca nas folhas da bromélia epífita tanque *G. monostachia* (North *et al.* 2019) e da atmosférica *T. ionantha* (Ohrui *et al.* 2007), e uma subsequente recuperação nos níveis de expressão quando reidratadas pós-seca. Também foi relatado que plantas CAM (como as bromélias citadas anteriormente) têm baixo conteúdo de AQPs em folhas, o que auxiliaria na redução da perda de água no ambiente árido que essas plantas habitam (Ohshima *et al.* 2001, Lüttge 2004). Estes dados, apesar de preliminares, sugerem a importância do ajuste no fluxo de água *via* AQPs nas folhas de bromélias epífitas durante a seca e subsequente recuperação hídrica.

Devido à exposição constante à seca e ao ambiente luminoso que bromélias epífitas atmosféricas habitam (Graham & Andrade 2004), supõe-se que estas apresentam mecanismos antioxidantes de rápida resposta para evitar danos oxidativos frente às condições ambientais

estressantes. Pouco se conhece sobre o perfil de atividade antioxidante em bromélias atmosféricas; no entanto, existe o relato de que plantas de *T. brachycaulos* presentes em ambiente de floresta decídua seca tiveram maior atividade antioxidante nos períodos de maior seca e luminosidade (González-Salvatierra *et al.* 2010). Além disso, bromélias de diferentes hábitos expostas à seca e outros estresses como frio, poluição e metais pesados mostraram ativação de mecanismos antioxidantes como atividade enzimática, acúmulo de glutationa e ácido ascórbico (Kováčik *et al.* 2012, 2014, Abreu *et al.* 2018, Carvalho *et al.* 2019, Pereira *et al.* 2018), demonstrando a importância desta estratégia em Bromeliaceae.

O conhecimento da sinalização à seca visando a ativação de mecanismos de defesa em bromélias epífitas ainda é escasso. De qualquer modo, foi reportado que ABA, NO e H_2O_2 estão envolvidos na ativação de CAM em resposta a sete dias de déficit hídrico imposto com solução de polietilenoglicol em folhas destacadas de plantas tanque de *G. monostachia* var. *monostachia* (Mioto & Mercier 2013). Outro trabalho mostrou que o acúmulo de ABA ocorre de modo rápido em plantas jovens da bromélia epífita *V. sanguinolenta*, sendo detectados valores dobrados após um dia sem irrigação (Zotz *et al.* 2004). Apesar de terrícola, foi visto que a exposição de plantas da bromélia *Nidularium minutum* Mez a baixas temperaturas (10°C) causou dois picos de produção de NO após 24 e 72 horas (Carvalho *et al.* 2019). Adicionalmente, existem evidências preliminares de que o ABA controla o movimento estomático de plantas do tipo CAM (Zhang *et al.* 2019, Chomthong & Griffiths 2020). Portanto, a ação dos sinalizadores ABA, NO e H_2O_2 aparenta estar diretamente relacionada à modulação de mecanismos de resistência ao estresse em bromélias no geral, inclusive epífitas.

Além da função dos mecanismos previamente citados na resistência à seca, supõe-se que estes também fornecem a capacidade de recuperação em bromélias epífitas. Isto pode ser observado em bromélias epífitas atmosféricas que expressam o CAM, apresentam hidrênquima e abundantes tricomas. Neste sentido, os tricomas permitem que as plantas rapidamente absorvam a água em períodos sazonais de maior umidade, que por sua vez é estocada no hidrênquima. Inclusive, a ação das AQPs auxilia no transporte da água absorvida pelos tricomas (Ohrui *et al.* 2007). A recuperação do estado hídrico também pode levar a mudanças no tipo de CAM sendo expresso, visando otimizar a fixação de carbono em função da água disponível (Cushman & Bohnert 1999, Dodd *et al.* 2002, Borland *et al.* 2011). Considerando a frequente variação entre períodos de maior e menor umidade nas condições de dossel em que as bromélias epífitas se encontram, supõe-se também que elas apresentam mecanismos de memória para melhorar sua performance neste habitat. Bader *et al.* (2009) mostraram indícios deste mecanismo em plantas jovens da bromélia epífita atmosférica *T. flexuosa*, cujo crescimento após período de seca severa foi maior em plantas pré-tratadas com seca de menor intensidade

em relação àquelas que não passaram por um tratamento prévio. Adicionalmente, foram reportadas evidências que sugerem o envolvimento de ABA na sinalização da modulação do estado redox em resposta à seca reiterada em outra espécie CAM, *Aptenia cordifolia* (L.f.) Schwantes (Aizoaceae; Fleta-Soriano *et al.* 2015).

Parte destes mecanismos de defesa de bromélias epífitas são desenvolvidos por completo em estágios mais avançados de desenvolvimento, principalmente as adaptações morfológicas à seca (*e.g.* tanques e tricomas), tornando as plantas jovens mais suscetíveis ao déficit hídrico do que as adultas. Além disso, plantas jovens têm alta superfície/volume nas folhas, o que resulta em maior predisposição à perda de água nestas plantas (Schmidt *et al.* 2001, Bader *et al.* 2009). Sendo assim, é possível supor que existam principalmente adaptações fisiológicas que habilitem as plantas jovens a tolerarem os episódios de falta de água. Nos estudos disponíveis, foi relatado um considerável grau de tolerância em jovens de *T. flexuosa* (Bader *et al.* 2009), *G. monostachia* (Beltrán *et al.* 2013, Rodrigues *et al.* 2016, Carvalho *et al.* 2017), *Guzmania lingulata* (L.) Mez e *Werauhia sanguinolenta* Cogn & Marchal J.R. (Beltrán *et al.* 2013), estando associado a mecanismos como CAM, acúmulo de carboidratos e suculência. Apesar disso, existe uma insuficiência em estudos que avaliem como estas plantas menores de bromélias epífitas suportam condições de seca e se recuperam das mesmas, o que é necessário tendo em vista que as alterações climáticas podem agravar as condições de seca (Trenberth *et al.* 2014).

4.1 Bromélia epífita *Acanthostachys strobilacea* (Schult. & Schult.f.) Klotzsch: modelo para estudos de tolerância à seca

O gênero *Acanthostachys* pertence à subfamília Bromelioideae, e é composto por três espécies – *A. strobilacea* (Schult. & Schult.f.) Klotzsch, *A. pitcarnioides* (Mez) Rauh & Barthlott e *A. calcicola* Marcusso & Lombardi –, sendo que *A. calcicola* foi primeiramente descrita em 2020, tendo sido localizada em região de Cerrado do Tocantins, Brasil (Eggli 2020, Marcusso *et al.* 2020). As duas primeiras espécies podem apresentar o hábito epifítico ou saxícola (Eggli 2020), enquanto *A. calcicola* foi encontrada somente como saxícola até o momento (Marcusso *et al.* 2020). Espécies de *Acanthostachys* apresentam características pouco usuais em relação a maioria das outras bromelioides (*e.g. Aechmea* sp.; Evans *et al.* 2015), como poucas folhas que não formam tanque e brácteas alongadas nas bases das inflorescências (Marcusso *et al.* 2020). Outras características morfológicas incluem a presença de espinhos nas folhas, que são grossas e podem atingir 1 m de comprimento (Eggli 2020, Marcusso *et al.* 2020), agregando valor ornamental a estas espécies. O aspecto morfológico geral do gênero é exemplificado por imagens de *A. strobilacea* na figura 4.

A bromélia *A. strobilacea* foi a primeira a ser descrita no gênero (Eggli 2020) e tem sido modelo em análises filogenéticas de Bromeliaceae, utilizada como principal representante de *Acanthostachys* (Schulte *et al.* 2009, Givnish *et al.* 2011, Evans *et al.* 2015). Árvores consenso baseadas em caracteres morfológicos, marcadores nucleares e plastidiais realizadas nestes estudos definem *Acanthostachys* como grupo irmão das *core* bromelioides (Schulte *et al.* 2009, Givnish *et al.* 2011, Evans *et al.* 2015), apesar da posição filogenética e monofilia do gênero permanecer incerta (Schulte *et al.* 2009, Evans *et al.* 2015). A bromélia *A. strobilacea* tem ampla distribuição, localizando-se no Brasil, Argentina e Paraguai (Govaerts *et al.* 2020). No Brasil, está presente nos domínios de Cerrado e Mata Atlântica (Monteiro 2020). Neste último local, foi descrita no Corredor Central (sul da Bahia e Espírito Santo) e no Corredor da Serra do Mar (Rio de Janeiro e região sul e litorânea de São Paulo) (Martinelli *et al.* 2008), que inclui a Reserva da Biosfera do Cinturão Verde de São Paulo (RBCV-SP; Victor *et al.* 1994). Portanto, *A. strobilacea* tem importante valor ecológico, por estar presente em dois *hotspots* de biodiversidade expostos à grande ameaça de perda de habitat e extinção de espécies vegetais (Myers *et al.* 2000). Apesar de ter dimensões maiores que as *Tillandsia* sp. (figura 4), pode-se considerar que *A. strobilacea* possui características similares a bromélias atmosféricas pois não contém tanque, apresenta numerosos tricomas, alta suculência fornecida por um volumoso hidrênquima e expressão de CAM (Proença & Sajo 2007, Derwidueé & Gonzalez 2010, Crayn *et al.* 2015, Males 2016). Portanto, *A. strobilacea* apresenta diversas adaptações xeromórficas (Benzing 2000), o que a constitui como uma espécie altamente adaptada à seca e potencial modelo de estudo sobre as estratégias de tolerância a este estresse em bromélias epífitas.

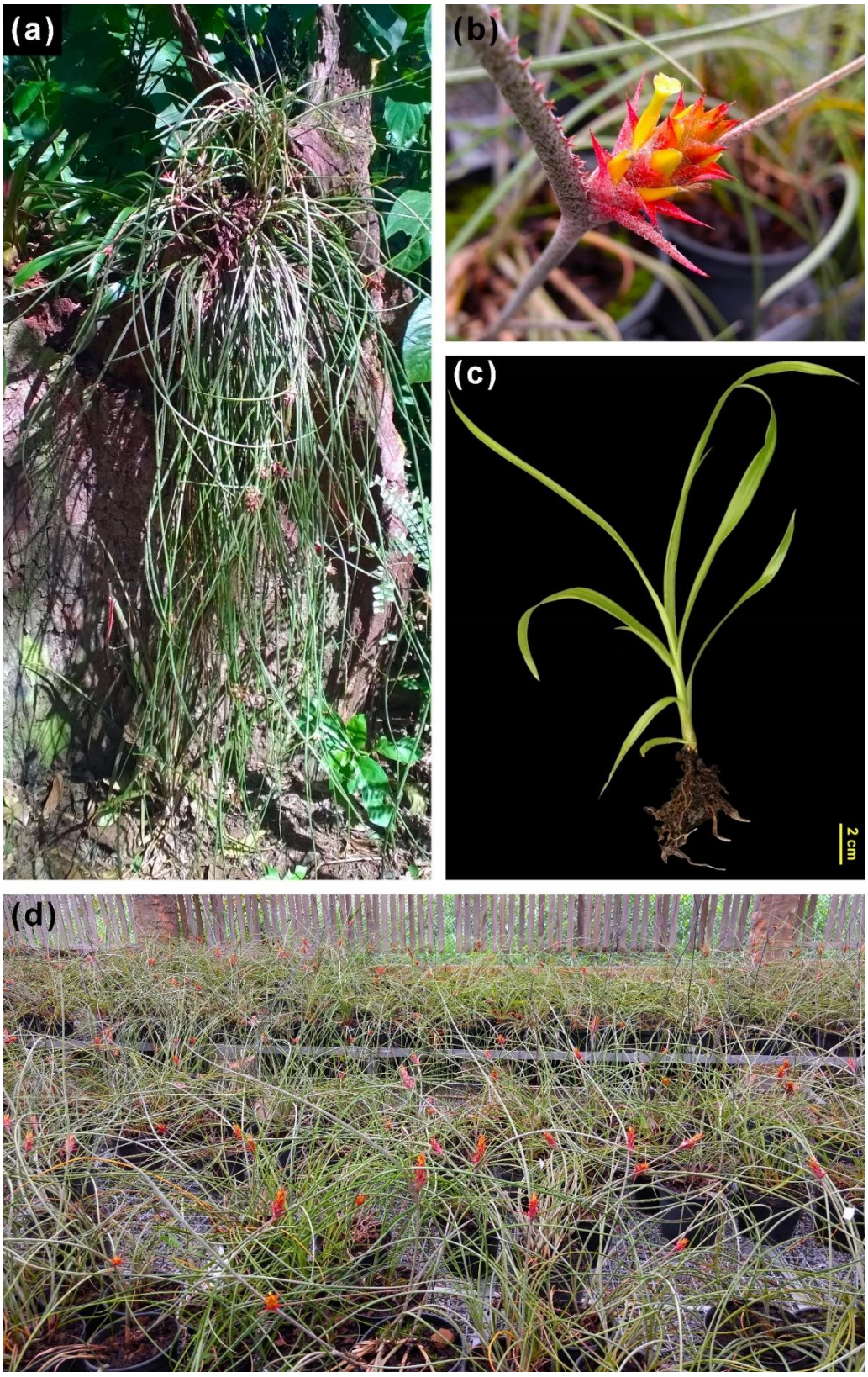

Figura 4. Aspecto morfológico de plantas de *A. strobilacea*. (a) Indivíduo adulto; (b) detalhe de inflorescência; (c) planta jovem de três meses cultivada em condições de ambiente controladas; (d) coleção de indivíduos adultos em casa de vegetação do Instituto de Pesquisas Ambientais, obtidos de sementes provenientes de população natural em reserva biológica de Mogi Guaçu, São Paulo, Brasil. Fotos: Victória de Carvalho.

Durante os últimos 10 anos, nosso grupo de pesquisa tem se dedicado a estudar os mecanismos de resistência ao estresse abiótico em plantas jovens de *A. strobilacea* (Carvalho *et al.* 2014, Santos *et al.* 2017, Menezes *et al.* 2020). Atualmente, o grupo também desenvolve estudo genômico comparativo com *A. strobilacea* e *A. pitcarnioides* (FAPESP 20/11908-7), o que possibilitará a determinação de genes relacionados aos mecanismos de tolerância ao estresse de *A. strobilacea* elucidados até o momento. Estes trabalhos foram possibilitados devido ao desenvolvimento de um protocolo de germinação de sementes *in vitro* e micropropagação pelo grupo (Santos *et al.* 2010), que permitiu a obtenção de plantas para os experimentos e a formação de uma coleção *ex situ* de indivíduos produtores de sementes viáveis (figura 4d). Dentre os primeiros estudos, foi relatada tolerância a temperaturas baixas em plantas de *A. strobilacea* cultivadas *in vitro*, que tiveram uma taxa de 100% de sobrevivência após três meses de cultivo a 10 e 15°C (Carvalho *et al.* 2014). Um experimento piloto desta tese indicou que plantas de três meses de idade têm resistência e capacidade de recuperação à seca. Neste experimento, plantas mostraram queda de 39% no conteúdo relativo de água (RWC) foliar nos primeiros 10 dias sem irrigação, atingindo uma média de 60% (figura 5a). A partir deste ponto, a redução em RWC foi menos intensa − cerca de 26% após outros 60 dias de tratamento. Por fim, foi registrada taxa de mortalidade de 100% (ausência de folhas verdes) apenas aos 110 dias de suspensão de rega. Adicionalmente, plantas mantidas por até 21 dias sem água e reidratadas por 14 dias recuperaram seu RWC a níveis similares aos de plantas bem-irrigadas (figura 5b). Estes resultados indicaram o potencial de plantas jovens de *A. strobilacea* para uso em estudos sobre tolerância à seca em bromélias e epífitas no geral. Desta forma, o grupo objetivou investigar mais profundamente quais os mecanismos que poderiam ser ativados durante a exposição inicial à seca, quando ocorreu a perda de água mais intensa nessas plantas, e que possivelmente auxiliam na resistência a períodos mais longos sem água – linha de pesquisa em que esta tese se inseriu.

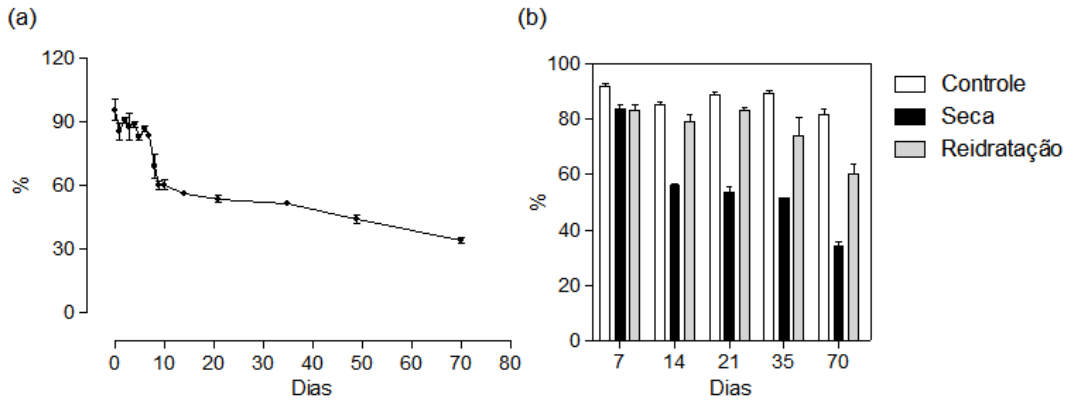

Figura 5. Valores de RWC das folhas de plantas de três meses de idade de *A. strobilacea* cultivadas em bandejas contendo casca de *Pinus*, em sala climatizada, submetidas à suspensão de irrigação (a). Após certos períodos de seca (indicados no eixo x), plantas foram reidratadas por 14 dias e avaliadas quanto ao RWC (b). Plantas do controle foram mantidas bem irrigadas. Os valores são as médias ± desvio padrão de três réplicas biológicas. O método de realização da análise seguiu Fleta-Soriano *et al.* (2015).

5 Justificativa e hipóteses

O estabelecimento mais rápido e intenso de seca ocasionado pelo aumento da temperatura global pode afetar principalmente espécies vegetais que não tem acesso ao solo e dependem basicamente da obtenção de água da atmosfera, como é o caso de bromélias epífitas. A intensificação da seca será inevitavelmente uma ameaça para plantas jovens destas bromélias, o que pode afetar sua população a longo prazo. Desta forma, o estudo da influência da seca sobre o metabolismo e morfologia de jovens bromélias epífitas é essencial para averiguar sua capacidade de adaptação às futuras condições ambientais e chances de permanência no ambiente. A espécie *A. strobilacea* é um adequado modelo para este tipo de estudo, pois apresenta diversas adaptações clássicas de bromélias epífitas. Além disso, estudos preliminares mostraram que plantas jovens desta espécie sobrevivem a longos períodos sem água e têm capacidade de recuperação pós-seca, sugerindo uma alta tolerância. No entanto, mais estudos se faziam necessários para ampliar o conhecimento sobre os mecanismos de defesa à seca dessas plantas jovens. A avaliação dos ajustes rápidos à seca, ou seja, horas após a suspensão hídrica, permitem elucidar potenciais componentes das vias de sinalização e os primeiros mecanismos de tolerância ativados. Adicionalmente, é importante avaliar os eventos fisiológicos, bioquímicos, moleculares e morfológicos associados à reidratação, inclusive a ocorrência de memória, pois estes determinam a capacidade de sobrevivência pós-seca em

plantas. Com estas estratégias de estudo, é possível definir a importância de ajustes rápidos e mecanismos como a memória à seca para bromélias epífitas juvenis, evidenciando a resiliência destas plantas frente às alterações climáticas.

O principal objetivo deste estudo foi avaliar a ativação de mecanismos fisiológicos, bioquímicos, moleculares e morfológicos de defesa em plantas juvenis de *A. strobilacea* durante exposição em curto prazo à seca e subsequente recuperação, a partir de três linhas de pesquisa relevantes quanto à dinâmica da seca em ambientes epifíticos, sendo elas:

a) O estudo das respostas de sinalização e defesa durante as horas iniciais de exposição à seca;
b) A avaliação dos efeitos da exposição reiterada a ciclos de seca e reidratação, visando aferir a presença de memória;
c) A regulação da fotossíntese CAM e estado hídrico após a seca e reidratação em curto prazo.

A estratégia de estudo das duas primeiras linhas foi a de delinear o perfil bioquímico, referente a moléculas sinalizadoras (ROS, RNS e ABA) e mecanismos de defesa como ação antioxidante, ação de denitrosilases, conteúdo de osmólitos e pigmentos fotossintéticos. Tal estratégia se baseou no fato de que plantas jovens devem depender principalmente de ajustes fisiológicos e bioquímicos como forma de tolerância à seca. Para a terceira linha de estudo, a estratégia foi a de avaliar parâmetros hídricos, trocas gasosas, ajustes anatômicos e expressão gênica de AQPs de modo a entender a relação entre os ajustes da fotossíntese CAM e relações hídricas em uma maior escala. Deste modo, este trabalho visou testar as seguintes hipóteses:

I. As primeiras horas de exposição à seca levam ao aumento no teor dos sinalizadores RNS, ROS e ABA e, subsequentemente, à ativação de mecanismos de defesa, revelando resposta rápida em plantas jovens de *A. strobilacea*.
II. Plantas jovens de *A. strobilacea* mostram respostas bioquímicas de defesa mais intensas após exposição a um segundo ciclo de seca-reidratação. Ou seja, apresentam indícios de formação de memória em nível bioquímico.
III. Existe modulação no ritmo diurno da fotossíntese CAM dentre a seca e reidratação em curto prazo, acompanhada de alterações na expressão gênica de AQPs, padrão anatômico e parâmetros hídricos.

A avaliação de cada hipótese está apresentada na tese no formato de artigo científico. O artigo referente à primeira pergunta encontra-se publicado e é apresentado na íntegra no capítulo 1. Os capítulos 2 e 3 se referem aos artigos das hipóteses II e III, respectivamente. Todos os coautores que colaboraram na elaboração destes dois últimos artigos são indicados nos capítulos 2 e 3.

Este projeto está inserido no Plano de Desenvolvimento Institucional do Instituto de Pesquisas Ambientais, financiado pela FAPESP (processo 17/50341-0), cujo objetivo é avaliar o impacto das ações antrópicas e das mudanças climáticas sobre florestas urbanas e peri-urbanas de modo a propor e/ou validar modelos preditivos para a conservação da biodiversidade e restauração de ecossistemas terrestres e aquáticos do Estado de São Paulo. O projeto tem como modelo a RBCV-SP, área de Mata Atlântica sujeita a efeitos da ação antrópica que potencializarão o aumento de temperatura e períodos de estiagem (Follador *et al.* 2018) – local em que a bromélia *A. strobilacea* está presente, sendo, portanto, modelo da flora epifítica desta região.

MATERIAL E MÉTODOS GERAIS

Nos experimentais visando o estudo das três hipóteses, foram utilizadas plantas de três meses de idade de *A. strobilacea* cultivadas em substrato. Sob esta condição de cultivo, a absorção de água em bromélias epífitas jovens ocorre através das raízes (Vanhoutte *et al.* 2017) – portanto, a seca foi induzida pela interrupção do fornecimento de água no substrato e, consequentemente, nas raízes. Desta forma, o experimental para o teste de cada hipótese consistiu, de forma geral, em:

1. Hipótese I

A avaliação das respostas de plantas jovens de *A. strobilacea* aos primeiros momentos de seca foi realizada a partir da indução do estresse após a transferência das plantas para substrato previamente seco. Os parâmetros de sinalização e mecanismos de defesa foram avaliados após 2, 5, 10, 24, 48, e 72 horas de estresse (figura 6).

2. Hipótese II

No segundo experimental, as plantas foram submetidas a um ou dois ciclos de seca-reidratação. A seca foi induzida pela suspensão da irrigação. A partir de experimento piloto, foi verificado que 14 dias de seca ocasionava os primeiros indícios de murcha nas folhas, enquanto o período de reidratação de cinco dias permitia a restauração completa do conteúdo hídrico foliar a níveis pré-estresse. Assim, foram elaborados os tratamentos D1 – 14 dias sem irrigação e reidratação em cinco dias; e D2 – 14 dias sem irrigação e cinco dias de reidratação, seguido por mais um ciclo similar (figura 6). Plantas do controle foram mantidas em condições bem hidratadas ao longo do experimento.

3. Hipótese III

Para avaliar a hipótese III, as plantas também foram submetidas à suspensão de rega por 14 dias. Ao 15º dia, as plantas em seca foram reidratadas e avaliadas 24 horas depois (figura 6). Plantas do controle foram mantidas em condições bem hidratadas ao longo do experimento.

Maiores detalhes sobre cada experimental encontram-se nas seções de material e métodos dos respectivos capítulos.

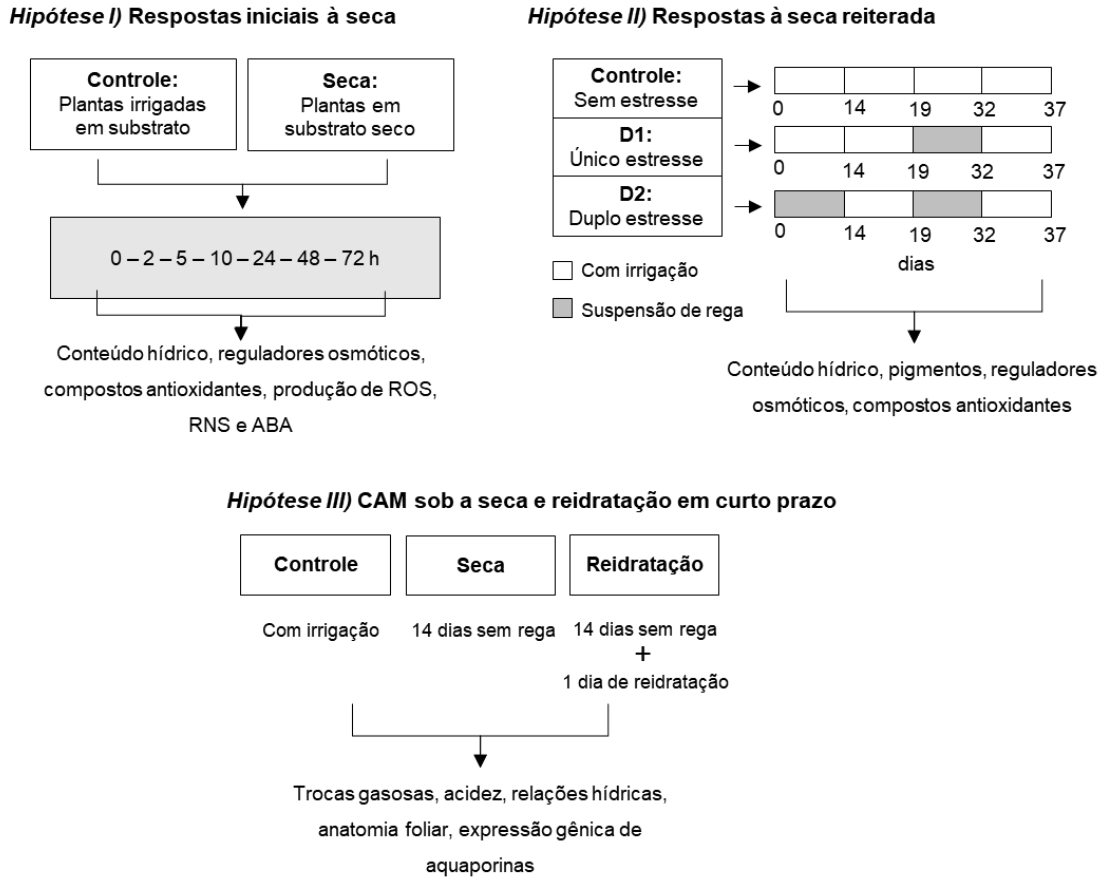

Figura 6. Visão geral do experimental de avaliação das três hipóteses do trabalho.

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CAPÍTULO 1

Short-term drought triggers defence mechanisms faster than ABA accumulation in the epiphytic bromeliad *Acanthostachys strobilacea*

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ABSTRACT

Epiphytic bromeliads might experience drought after a few hours without water, which is especially critical during early life stages. Consequently, juvenile epiphytic bromeliads probably rely on short-term activation of drought tolerance strategies, although the biochemical processes involved are still poorly understood. In this study, we aimed to evaluate the short-term drought response of juvenile plants of the epiphytic bromeliad *Acanthostachys strobilacea* (Schult. & Schult. f.) Klotzsch. We hypothesized that short-term drought would induce the accumulation of abscisic acid (ABA) and secondary messengers such as reactive oxygen and nitrogen species (ROS and RNS, respectively) before the activation of defence mechanisms. Three-month-old plants were transferred from well-watered to dry substrates and stress markers were assessed at 0, 2, 5, 10, 24, 48, and 72 h. Drought caused a 27.3% decrease in relative water content compared to the well-watered control at 72 h. A nearly 5-fold increment in the ABA content occurred at 72 h of stress, which was about two days after the first detection of peaks in RNS levels and defence mechanisms activation. Indeed, ascorbate peroxidase (EC 1.11.1.11) activities and proline content increased after 10 h, whereas after 24 h a higher catalase (EC 1.11.1.6) activity and osmotic adjustment occurred. Oxidative stress markers and photochemical efficiency of photosystem II indicated no significant damage induced by drought. We concluded that defence mechanisms activation during early drought in juvenile *A. strobilacea* might be regulated initially by ABA-independent pathways and RNS, while ABA-induced responses are triggered at subsequent stages of stress.

1. Introduction

Epiphytic plants are de-coupled from the soil and dependent on water absorption from the atmosphere (Zotz, 2016). Hence, they are constantly exposed to drought, which might occur after a few hours without water (Zotz and Hietz, 2001). This condition is especially critical during early ontogenetic stages because most adaptations that prevent water loss are only developed at later stages (Benzing, 2000; Bader et al., 2009). Consequently, juvenile epiphytes probably rely on tolerance strategies to resist short-term drought (Bader et al., 2009). However, few studies have analysed the tolerance response to short drought periods, i.e. from hours to days (Chaves et al., 2003), in juvenile epiphytes (Zotz et al., 2001, 2004). This research is important because the survival and establishment of epiphytic species might be threatened by the effects of drought episodes due to climate change (Benzing, 1998; Cach-Pérez et al., 2014), which will probably become more frequent and severe in the future (Winkler et al., 2005; Trenberth et al., 2014).

In plants, the development of early responses immediately after the stress onset is crucial to prevent the irreversible damage and to activate defence mechanisms that lead to an acclimated state for longer stress periods (Kollist et al., 2019; Dubois and Inzé, 2020). One of the early responses to drought periods is the accumulation of the hormone abscisic acid (ABA) (Finkelstein, 2013; Cai et al., 2015; Urano et al., 2017; Dubois and Inzé, 2020). Under osmotic stress conditions, ABA can mainly induce stomatal closure to promote water conservation and regulate the expression of several stress-related genes (Fujita et al., 2011), such as those involved in the antioxidant system and adjustment of osmolyte accumulation (Jiang and Zhang, 2004; Sharma et al., 2019).

Reactive oxygen and nitrogen species (ROS and RNS, respectively) also participate in the activation of defence mechanisms during the initial stages of stress (Noctor et al., 2018; Sami et al., 2018). There is evidence supporting that ABA, ROS, and RNS are crucial in drought tolerance, as evidenced by their role in stomatal closure (Bright et al., 2006) and stimulation of antioxidant activity (Jiang and Zhang, 2002;

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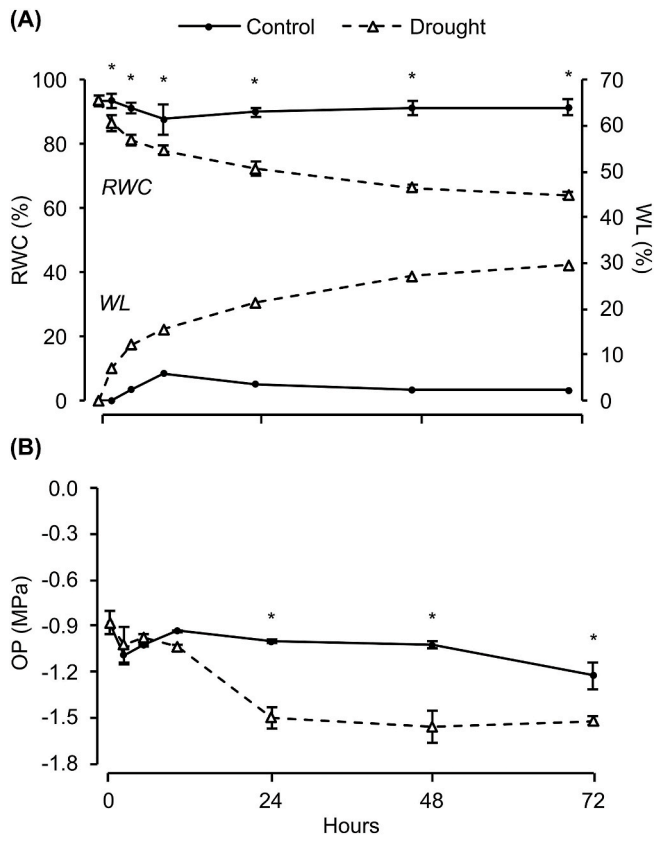

Fig. 1. Leaf water status of *A. strobilacea* plants after 0, 2, 5, 10, 24, 48, and 72 h of well-watered (control) and drought conditions. RWC and OP values are means ± s.e. calculated from four and three replicates, respectively. Significant differences for RWC and OP between groups were tested by *two-way* ANOVA, with asterisks comparing control and treated plants per period ($P < 0.05$). The WL is the RWC mean of each period subtracted from the 0 h.

Lu et al., 2009; Tanotra et al., 2019). The role of these molecules in the drought response has been described in adult individuals of the epiphytic bromeliad *Guzmania monostachia* (Mioto and Mercier, 2013). In fact, the biochemical processes during the initial periods of drought exposure in juvenile epiphytes are still poorly understood. Up to date, there are no studies about the response of juvenile epiphytes to hour periods of drought, although the ABA accumulation after one or two days of irrigation withholding has been reported in young plants of the epiphytic orchid *Dimerandra emarginata* (Zotz et al., 2001) and the epiphytic bromeliad *Vriesea sanguinolenta* (Zotz et al., 2004).

The epiphytic bromeliad *Acanthostachys strobilacea* is an unusual species because it has few thickened, slender, and pendulous leaves that can reach 1 m in length (Eggli, 2020; Fig. S1). This species is distributed in Argentina, Paraguay, and Brazil (Govaerts et al., 2020). In Brazil, it can be found in regions of savanna vegetation (Cerrado biome; Monteiro, 2020), where plants can be exposed to three to five months of little or no rainfall and relative humidity (RH) levels of approximately 15% (Coutinho, 2002). Adult plants of *A. strobilacea* endure these conditions due to the presence of diverse drought adaptations e.g., succulence (Eggli, 2020), absorptive foliar trichomes (Derwidueé and Gonzalez, 2010), and crassulacean acid metabolism (CAM), which optimize water conservation in the tissues (Crayn et al., 2015). Although juveniles of *A. strobilacea* (i.e. plants with 10–20% of the maximum leaf length of adults; Meisner et al., 2013) do not have the same succulence and notorious formation of trichomes as adult plants (V. Carvalho, *personal communication*), we have previously evidenced a considerable degree of drought resistance in these young plants, which makes this species adequate for the evaluation of stress-tolerance strategies (Menezes et al., 2020).

Our previous results indicated that three-month-old plants of *A. strobilacea* performed CAM under conditions of daily irrigation and progressive soil drying, and showed increased starch mobilization and activity of antioxidant enzymes after four and eight days of stress, respectively (Menezes et al., 2020). However, the early sensing and signalling mechanisms leading to drought tolerance are not yet known in this species. Therefore, in this study, we tested the hypothesis that short-term drought would induce the accumulation of ABA and secondary messengers involved in drought responses (ROS and RNS), followed by the stimulation of defence mechanisms such as osmotic adjustment and antioxidant activity.

2. Materials and methods

2.1. Plant material and treatment

Seeds of *Acanthostachys strobilacea* (Schult. & Schult. f.) Klotzsch (Bromelioideae, Bromeliaceae; record number A0A27A1 at the Brazilian Genetic Diversity bank - SisGen, *Ministério do Meio Ambiente*) were harvested from the plant collection of *Núcleo de Pesquisa em Plantas Ornamentais*. These plants came from seeds obtained from the natural population located at *Núcleo de Pesquisa Reserva Biológica de Mogi Guaçu* in Mogi Guaçu, São Paulo, Brazil (22°15′04.2″S and 47°09′56.8″W).

Seeds were cultivated *in vitro* as described by De Carvalho et al. (2014) and maintained in a culture room with temperature adjusted to 23 °C, a 16 h-photoperiod and a photon flux density of 100 μmol m^{-2} s^{-1}. After 15 days, plants were transferred to plastic (PET) trays of 30:150:30 mm with drainage holes in the bottom, containing approximately 800 mL of fine sterilized commercial *Pinus* bark (De Carvalho et al., 2014). Each tray contained 30 plants and was maintained under the same conditions described above for 90 days until plants reached at least 20 cm in height (Fig. S1). Plants were irrigated twice a week by spraying leaves and substrate with distilled water until drainage was detected. Plants were fertilized biweekly with 1 g L^{-1} of a commercial fertilizer of 20:20:20% composition (total nitrogen:phosphorous:potassium; Plant-Prod) until one week before the onset of experiments.

Experiments were performed by transferring plants to dry soil (Dubois and Inzé, 2020). With this method, the onset of stress is precise and allows a more accurate temporal evaluation of early responses than progressive soil drying. Since water uptake is performed by roots in juvenile epiphytic bromeliads grown in horticultural substrates (Vanhoutte et al., 2017), stress was applied by transferring intact individuals to a dried bark substrate. On the first day of the experiment, all plants were irrigated as described above at approximately 1 h after the onset of the light period (07:00 a.m.). One hour later (08:00 a.m.), drought was applied to plants by transferring them to new trays containing approximately 800 mL of a *Pinus* bark substrate that was previously dried for seven days in an oven at 60 °C, reaching 0.77 ± 0.15% (n = 3, mean ± s.d.) of water content, which ensured that roots had almost no contact with water. Substrate water content (SWC) was assessed using the equation:

SWC (%) = $(FW - DW)/DW \times 100$; where FW: fresh weight; DW: dry weight.

Control plants were sprayed with distilled water as described previously every 24 h, at 07:00 a.m. (well-watered conditions). Samples from control and drought treatment were collected by removing plants from the original substrate at 0, 2, 5, 10, 24, 48, and 72 h after the end of the transfer procedure. During the experiment, the environmental conditions in the growth room were measured at 08:00 a.m. using a thermo-hygrometer (model 00325; AcuRite). The vapor pressure deficit (VPD) was calculated with means of minimum, maximum temperatures, and RH data according to Allen et al. (1998). The daily mean conditions were as follows (n = 16, mean ± s.d.): 22.9 ± 1.5 °C; 63.4 ± 9.6% of RH and 0.96 kPa of VPD.

At each time of harvesting, all leaves were removed at approximately

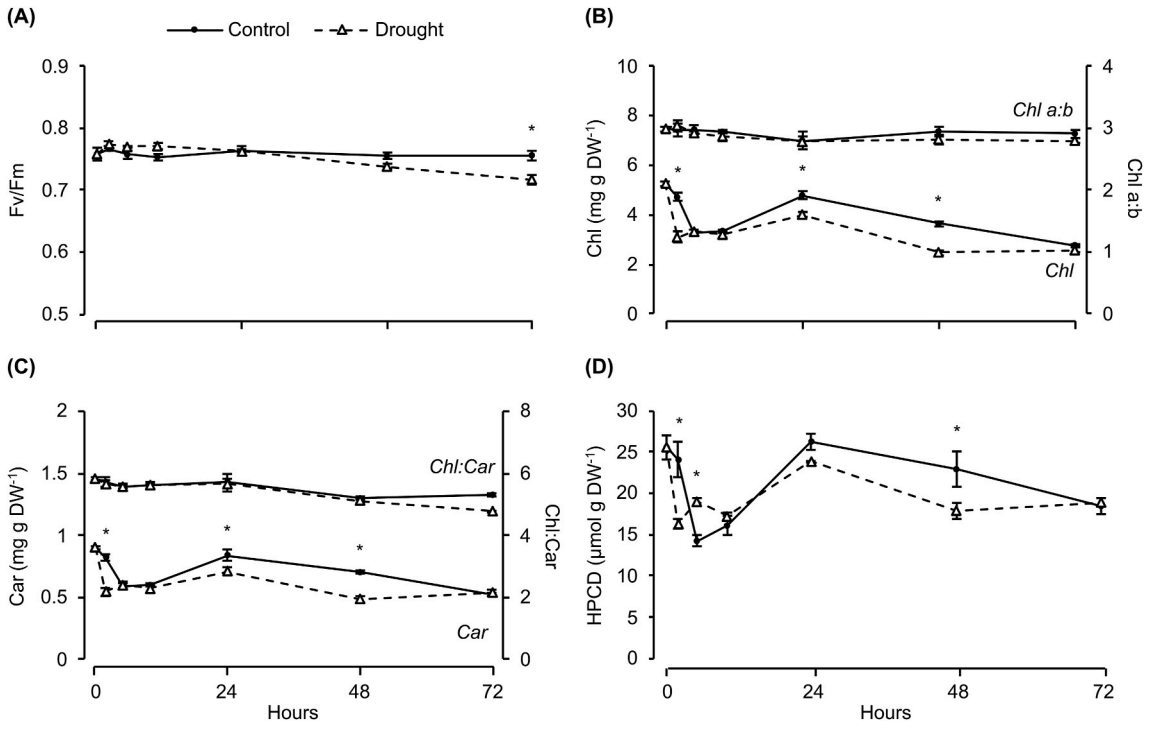

Fig. 2. Photosynthetic (A–C) and membrane damage (D) parameters of *A. strobilacea* plants exposed to 0, 2, 5, 10, 24, 48, and 72 h of well-watered (control) and drought conditions. Values are means ± s.e. calculated from three replicates. Significant differences between groups were tested by *two-way* ANOVA, with asterisks comparing control and treated plants per period ($P < 0.05$).

3 cm of their bases. Part of the pool of leaves was immediately frozen in liquid nitrogen and maintained at −80 °C while another part was utilized fresh for certain analyses, as described below. Four and three replicates containing leaves from seven plants from each treatment were used for relative water content (RWC) and remaining analyses, respectively.

2.2. RWC, water loss rate (WL) and osmotic potential (OP)

The RWC was estimated using 200 mg of fresh leaves cut into pieces of similar sizes (approx. 0.3 cm) and processed according to Fleta-Soriano et al. (2015). The WL was calculated by subtracting RWC mean values of each period and treatment from the values at the 0 h, using the equation:

WL (%) = $RWC_{zh} - RWC_{fh}$; where RWC_{zh}: mean RWC at 0 h, RWC_{fh}: mean RWC at 2–72 h period.

OP analyses were made with 200 mg of fresh leaves subsequently frozen in liquid nitrogen and processed according to Rigui et al. (2019).

2.3. Photosynthetic parameters and membrane damage assessment

The estimation of the maximum potential quantum efficiency of the photosystem II (Fv/Fm ratio) was performed in the longest leaf of three intact plants with a modulated fluorometer (PAM 2500; Walz). Leaves were dark-adapted by leaf clips for at least 15 min prior to readings.

Pigment determination was performed according to Pignata et al. (2002), using extracts from 40 mg of frozen pulverized plant material homogenized in 2 mL of 96% (v/v) ethanol. Absorbance readings and calculations of chlorophyll (Chl) and carotenoid (Car) contents were made according to Lichtenthaler and Wellburn (1983). The ratio of Chl a and b (Chl a:b) and of total Chl to total Car (Chl:Car) were also calculated.

The estimation of membrane damage by ROS was evaluated by quantifying hydroperoxide conjugated dienes (HPCD) with the same extracts used for pigment determination, following Pignata et al. (2002).

2.4. Osmolyte quantification

The osmolyte proline (Pro) was quantified by homogenizing 100 mg of frozen leaves in 1 mL of 40% (v/v) ethanol. Extracts were maintained at 8 °C for 24 h. After centrifugation at 16100 g for 5 min, supernatants were used in analyses adapted for microplate readers as described by Carillo and Gibon (2011), with calculations based on a Pro standard curve.

Total soluble sugars (Sug) were quantified following Menezes et al. (2020), modified as follows. An aliquot of 50 mg of frozen pulverized leaves was homogenized in 500 µL of 80% (v/v) ethanol and incubated at 80 °C for 15 min. Extracts were centrifuged at 16100 g for 10 min. The remaining pellets were homogenized in 500 µL of ultrapure water and incubated at 60 °C for 15 min, followed by centrifugation at 16100 g for 10 min. This procedure was repeated once more with the pellets. The pooled supernatants were vacuum dried and resuspended in 125 µL of ultrapure water. Sug levels were calculated based on a glucose standard curve.

2.5. Antioxidant activities

The activity of superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), ascorbate peroxidase (APX, EC 1.11.1.11), and glutathione reductase (GR, EC 1.6.4.2) was quantified following Carvalho et al. (2017). Protein content was determined from the antioxidant enzyme extracts using Bradford reagent (Sigma-Aldrich) with bovine serum albumin as a standard.

Reduced (RedA) and total ascorbic acid (AA) were analysed using the chromatographic method described by Brandão et al. (2017). Dehydroascorbate (oxidized form, DHA) levels were estimated based on the difference between AA and RedA contents. The redox state of AA was inferred by calculating the percentage of the RedA pool.

Total glutathione (TGSH), and the reduced (GSH) and oxidized (GSSG) fractions were quantified using the enzymatic recycling assay. TGSH extraction was made with 100 mg of powdered frozen leaves in

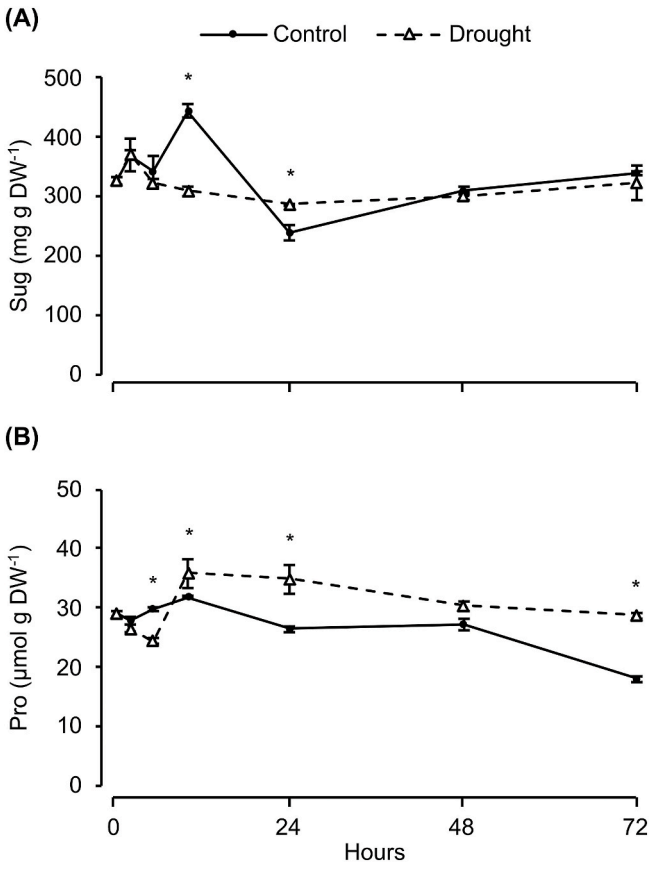

Fig. 3. Osmolyte content of *A. strobilacea* plants exposed to 0, 2, 5, 10, 24, 48, and 72 h of well-watered (control) and drought conditions. Values are means ± s.e. calculated from three replicates. Significant differences between groups were tested by *two-way* ANOVA, with asterisks comparing control and treated plants per period ($P < 0.05$).

0.1% (w/v) sulfosalicylic acid. Samples were centrifuged at 13400 g and 4 °C for 20 min (adapted from Israr et al., 2006). To prevent artefactual oxidation of GSH to GSSG in the samples, a separate extraction was made for the analysis of GSSG, following Giustarini et al. (2013) with modifications. Briefly, 100 mg of powdered frozen leaves were mixed with 1 mL of a solution containing 50 mM Tris buffer (pH 8.0) and 31 mM N-ethylmaleimide (NEM). Samples were acidified with 7 µL of 60% (w/v) trichloroacetic acid (TCA) and centrifuged at 16100 g and 4 °C for 2 min. To remove NEM excess, 1.5 mL of dichloromethane was added to 500 µL of extract and the mixture was shaken at 800 rpm and room temperature for 5 min. Mixtures were centrifuged at 14000 g for 30 s and the supernatants maintained on ice for later use. For TGSH and GSSG assays, 100 µL aliquots of the extracts were added to 900 µL of 0.5 mM sodium EDTA, 50 µL of 0.3 mM 5,5′dithio-bis-(2-nitrobenzoic acid) and 50 µL of 0.4 mM NADPH, all diluted in 100 mM potassium phosphate buffer (pH 7.0). Reactions were started with the addition of 1 µL of GR. Mixtures were kept under light for 20 min. Absorbances were read in a spectrophotometer at 412 nm and estimations were made based on a standard curve of GSH (adapted from Israr et al., 2006). The GSH content was calculated by subtracting the GSSG from the TGSH content. The TGSH redox state was inferred by calculating the percentage of the GSH pool.

2.6. ROS quantification

For hydrogen peroxide (H_2O_2) extraction, 20 mg of fresh leaves were cut into pieces of approx. 0.3 cm and homogenized in 1 mL of 0.1% (w/v) TCA using a chilled mortar and pestle. Extracts were centrifuged at 16100 g and 4 °C for 10 min. In a 96-well microplate, 60 µL of the extracts were mixed with 240 µL of a solution containing 1% (v/v) ethanol, 0.1 mM xylenol orange, 25 mM sulphuric acid, and 0.25 mM ferrous ammonium sulphate. After the mixture was shaken at 200 rpm for 15 min in the dark, absorbances were read in a microplate reader at 550 and 800 nm. Final absorbance values were calculated as *A550 - A800*, and the H_2O_2 content calculated based on a standard curve (adapted from Cheeseman, 2006).

Superoxide anion ($^{\bullet}O_2^-$) production rate was measured by monitoring the nitrite (NO_2^-) formation from hydroxylamine in the presence of $^{\bullet}O_2^-$ following Menezes et al. (2020).

The hydroxyl radical ($^{\bullet}OH$) content was estimated based on the degradation of 2-deoxy-D-ribose by $^{\bullet}OH$, which produces a mixture in which malondialdehyde (MDA) is the most abundant. An aliquot of 200 mg of powdered frozen leaves were mixed with 1.5 mL of a 6 mM potassium phosphate buffer (pH 7.4) with 15 mM 2-deoxy-D-ribose and centrifuged at 5000 g and 4 °C for 20 min. Supernatants were incubated at 37 °C for 2 h (adapted from Beligni and Lamattina, 2002). For MDA detection, equal parts of the incubated extracts were mixed with 1% (w/v) thiobarbituric acid in 50 mM NaOH and 2.8% (w/v) TCA and heated in a boiling water bath for 20 min. After cooling, absorbances were read at 532 and 600 nm in a spectrophotometer (adapted from Halliwell et al., 1988). MDA concentration was calculated as follows (Heath and Packer, 1968):

$$(\text{nmol MDA/mL}) = [(A532 - A600)/155000]*10^6$$

2.7. RNS metabolism assessment

The anion NO_2^-, one of the main NO precursors (Astier et al., 2018), and *S*-nitrosothiols (SNO), mediators of NO signalling effects (Begara-Morales et al., 2019), were estimated according to Frungillo et al. (2013), with modifications. An aliquot of 200 mg of powdered frozen leaves was homogenized in 1 mL of 100 mM phosphate buffer at pH 7.2, followed by centrifugation at 12000 g and 4 °C for 15 min. In a 96-well microplate, 50 µL of the extracts were added to 50 µL of solution A (1% (w/v) sulphanilamide in 0.5 M HCl) or solution B (solution A plus 0.2% (w/v) $HgCl_2$) followed by incubation for 7 min. An aliquot of 100 µL of 0.02% (w/v) N-(1-naphthyl) ethylenediamine dihydrochloride was added to all wells and incubated for 7 min. The absorbance was measured at 540 nm in a microplate reader. The NO_2^- content resulting from the reaction of the extracts with solution A was quantified based on a $NaNO_2$ standard curve. The SNO content was calculated by subtracting the absorbance values of the reaction with solution A to those obtained with solution B and estimated based on a standard curve using *S*-nitrosoglutathione.

2.8. ABA quantification

ABA quantification followed Rodrigues et al. (2016) with modifications described by Zörb et al. (2013). Aliquots of 100–200 mg of powdered frozen leaves were lyophilized and homogenized in 1 mL of isopropanol:acetic acid (95:5), followed by the addition of 0.5 µg of [$^2H_6$]-ABA as the internal standard. Samples were vortexed for 10 min at room temperature then maintained under agitation on ice for 70 min and protected from light. Samples were then mixed on a vortex for 15 min and centrifuged at 25000 g and 4 °C for 20 min. Supernatants were vacuum-dried until about 50 µL remained in the tube and then acidified to an approximate pH of 2.5 with 1–2 µL of 1 N HCl. An aliquot of 500 µL of ethyl acetate was added to the remaining supernatants, vortexed for 10 min, and centrifuged for 5 min at 25000 g and 4 °C. This process was further repeated twice with the remaining precipitates and the resultant supernatants were pooled together in the same tube. Extracts were vacuum-dried and mixed with 100 µL of methanol in a vortex for 10 min and then transferred to vials. Samples were vacuum-dried and mixed with 30 µL of methanol in a vortex for 20 min. For the derivatization, 30 µL of trimethylsilyldiazomethane was added to samples and mixed for

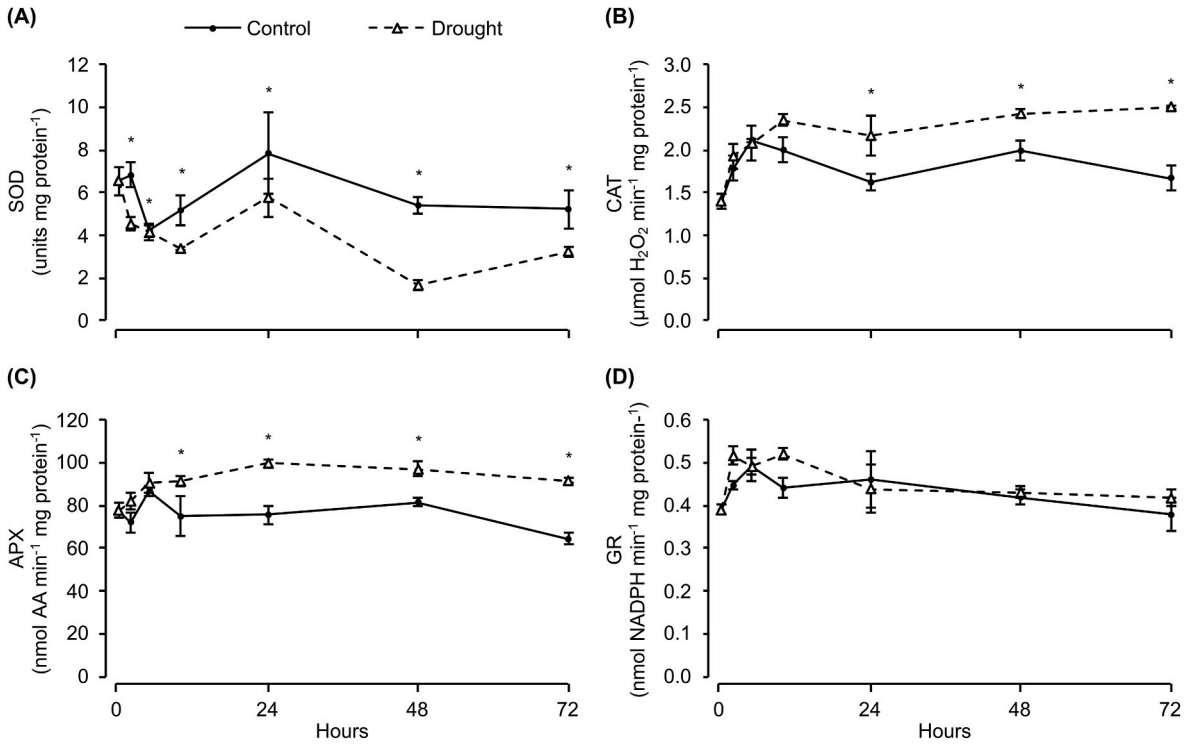

Fig. 4. Antioxidant enzyme activities of *A. strobilacea* plants exposed to 0, 2, 5, 10, 24, 48, and 72 h of well-watered (control) and drought conditions. Values are means ± s.e. calculated from three replicates. Significant differences between groups were tested by *two-way* ANOVA, with asterisks comparing control and treated plants per period ($P < 0.05$). In A, one unit is the amount of enzyme required to inhibit the reduction in nitrobluetetrazolium by 50%.

30 min in a vortex. Derivatized extracts were dried under nitrogen flow followed by the addition of 30 µL of methanol. Derivatized extracts were analysed in a gas chromatograph coupled to a mass spectrometer (Shimadzu QP2010SE) with a Restek RTx-1701 column (30 m × 0.25 mm, 5 µm particle diameter) and helium as the carrier gas at a flow rate of 1 mL min^{-1}. The initial running condition was 150 °C for 5 min, followed by a gradient up to 270 °C at 10 °C min^{-1} and 3 min of holding. The injector, ion source, and quadrupole temperatures were 150 °C, 230 °C, and 150 °C, respectively. The monitored ions for endogenous ABA were those with mass/charge ratio (m/z) of 190, 134, and 162; and those with m/z of 194, 138, and 166 for [$^2H_6$]-ABA. Endogenous ABA levels were calculated based on chromatograms at m/z 190 and 194.

2.9. Statistical analyses

The experimental design was completely randomized. Means were subjected to a *two-way* analysis of variance (ANOVA) with time and watering conditions (control and drought) as factors ($P < 0.05$), using the SISVAR 5.6 software. When the interaction was significant, an ANOVA with unfolding degrees of freedom of the interaction was performed to identify the effects of each factor on the parameters analysed.

3. Results

3.1. Leaf water status during drought exposure

Leaf RWC and OP were negatively affected in a time-dependent manner by exposure to drought (significant interaction, Table S1). After 2 h of stress, treated plants showed a significant reduction in RWC compared to the control (Fig. 1A). During the first 24 h, the WL was reduced by 21% compared to the initial period. From that point on, the WL was less intense and more stable, showing a 6 to 2% decrease between 24-48 h and 48–72 h, respectively (Fig. 1A). OP significantly decreased by 0.44 MPa on average between 24 and 72 h of stress compared to control plants (Fig. 1B), which was suggestive of osmotic adjustment. Despite these changes in RWC and OP, the morphological aspect of plants was largely similar under control and drought conditions at 72 h (Fig. S2).

3.2. Photosynthesis and membrane damage during drought exposure

The Fv/Fm ratio decreased slightly but significantly during drought exposure (Table S1), being 1.05 times lower in stress-treated plants than in the control at 72 h (Fig. 2A). Chl and Car were negatively affected by time and stress conditions (Table S1), although drought caused mild reductions in pigment contents at 2, 24, and 48 h compared to the control (Fig. 2B and C). Nevertheless, the Chl a:b and Chl:Car ratios were unaltered by drought (Table S1, Fig. 2B and C). Watering conditions significantly affected the degree of membrane damage (HPCD content) mostly under interaction with time (Table S1). There was a 1.3 times increase in HPCD values of drought-treated plants at 5 h compared to the respective control; however, in the remaining periods, means were similar between watering conditions (10, 24 and 72 h) or 1.5 and 1.3 times lower under drought at 2 and 48 h, respectively (Fig. 2D). These results indicate that photoinhibition, membrane damage and pigment degradation were mostly avoided during drought exposure in *A. strobilacea* plants.

3.3. Modulated defence mechanisms during drought exposure

Of the osmolyte parameters, Sug were significantly affected by the treatment and time interaction (Table S1), although differences were slight between drought-treated and control plants (Fig. 3A). Meanwhile, Pro content was overall positively affected by drought, although there was a decrease at 5 h of stress compared to the control (Fig. 3B). The Pro content of drought-treated plants at 10, 24, and 72 h significantly increased by 3.9, 8.4, and 10.7 µmol g DW^{-1} compared to controls, respectively.

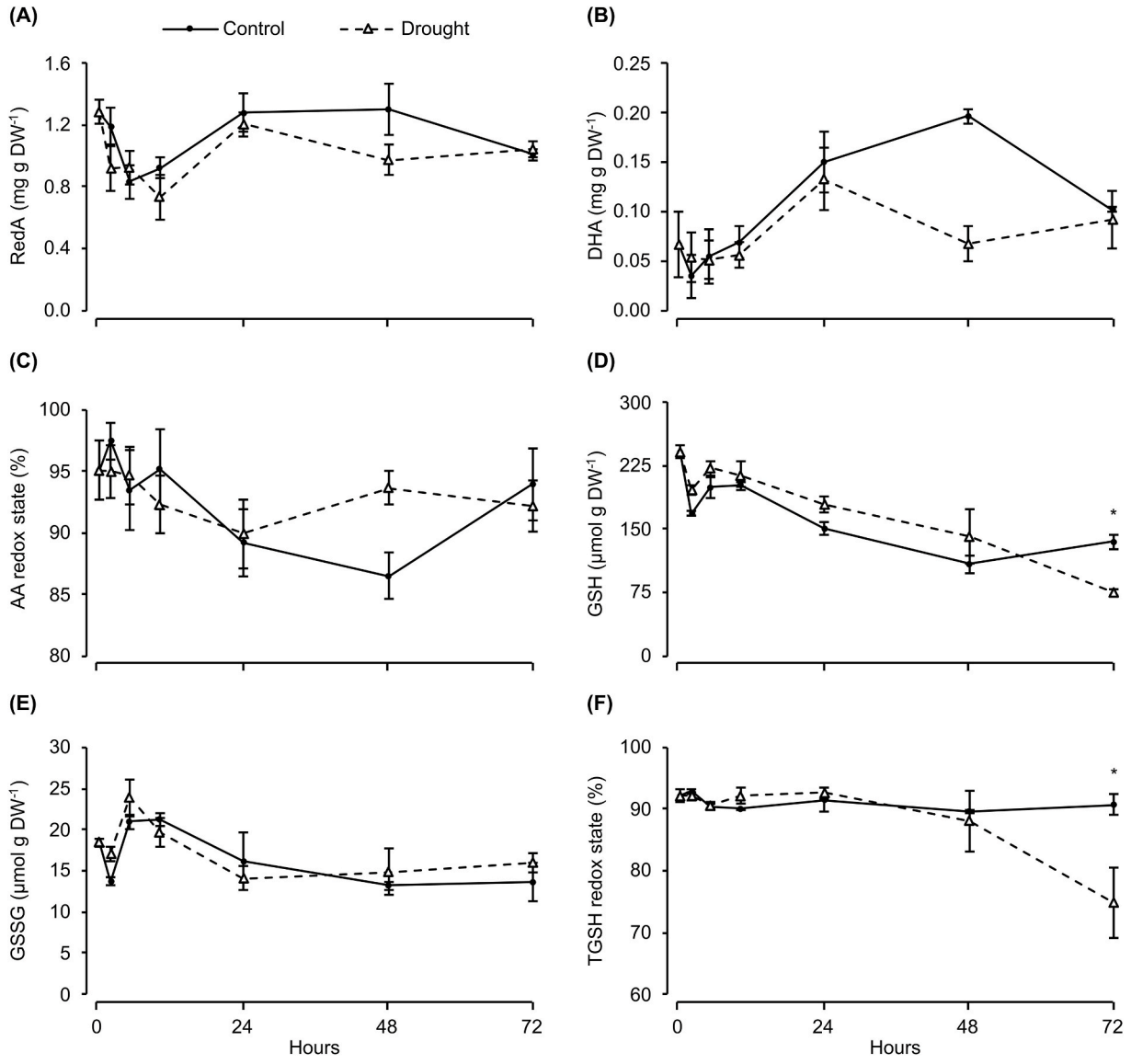

Fig. 5. Non-enzymatic antioxidants content of *A. strobilacea* plants exposed to 0, 2, 5, 10, 24, 48, and 72 h of well-watered (control) and drought conditions. Values are means ± s.e. calculated from three replicates. Significant differences between groups were tested by *two-way* ANOVA, with asterisks comparing control and treated plants per period ($P < 0.05$).

The SOD activity was negatively affected by drought regardless of time (Table S1, Fig. 4A). The lowest levels, which were 3.2 times lower than the control, occurred at 48 h of drought. On the other hand, CAT and APX activities (Fig. 4B and C) were increased by drought in a time-dependent manner (significant interaction, Table S1). Specifically, APX and CAT activities were significantly higher in treated plants from 10 and 24 h, respectively. By 72 h, CAT and APX activities were, on average, 1.5 times higher than the control. GR activities were significantly affected by the time factor alone (Table S1), indicating that the functions of this enzyme were preserved despite drought conditions (Fig. 4D).

The *two-way* ANOVA showed no significant effects of drought on AA content and redox state (Table S1). Nevertheless, there was a decrease in DHA and RedA at 48 h in stressed plants (Fig. 5A and B) and therefore, the percentage of RedA surpassed control levels at that point by 7.2% (Fig. 5C). In fact, this antioxidant remained in a highly reduced state during drought (at least 90%, Fig. 5C).

Regarding glutathione forms, the GSSG content was not significantly affected by drought (Table S1, Fig. 5E). On the contrary, GSH levels of treated plants were 1.8-fold lower than the control at 72 h (Fig. 5D), which was reflected in a 15.9% reduction of the redox state at the same period (Fig. 5F).

3.4. Drought effects on secondary messengers (ROS and RNS) and ABA

Excepting H_2O_2, the secondary messengers ($^{\bullet}O_2^-$, $^{\bullet}OH$, NO_2^-, SNO) were significantly affected by the interaction of time and treatment (Table S1, Fig. 6A). The $^{\bullet}O_2^-$ production rate differed between control and drought conditions only at 48 h, when it was 2 times lower under drought conditions (Fig. 6B). Similarly, $^{\bullet}OH$ levels were progressively reduced in drought-treated plants mainly between 10 and 48 h, reaching values that were 1.5 times lower than the control (Fig. 6C). Nevertheless, $^{\bullet}OH$ content was restored to control levels after 72 h. These results suggest high antioxidative maintenance in plants during drought.

The levels of NO_2^- content peaked twice during treatment, being 2.7 and 1.8 times higher than the control at 5 and 48 h, respectively (Fig. 6D). The SNO also showed two transient peaks throughout drought exposure, although a similar pattern was observed for the control (Fig. 6E). The SNO peaks in drought-treated plants were 1.4 and 2.4 times higher at 5 and 24 h than controls, respectively. For control plants,

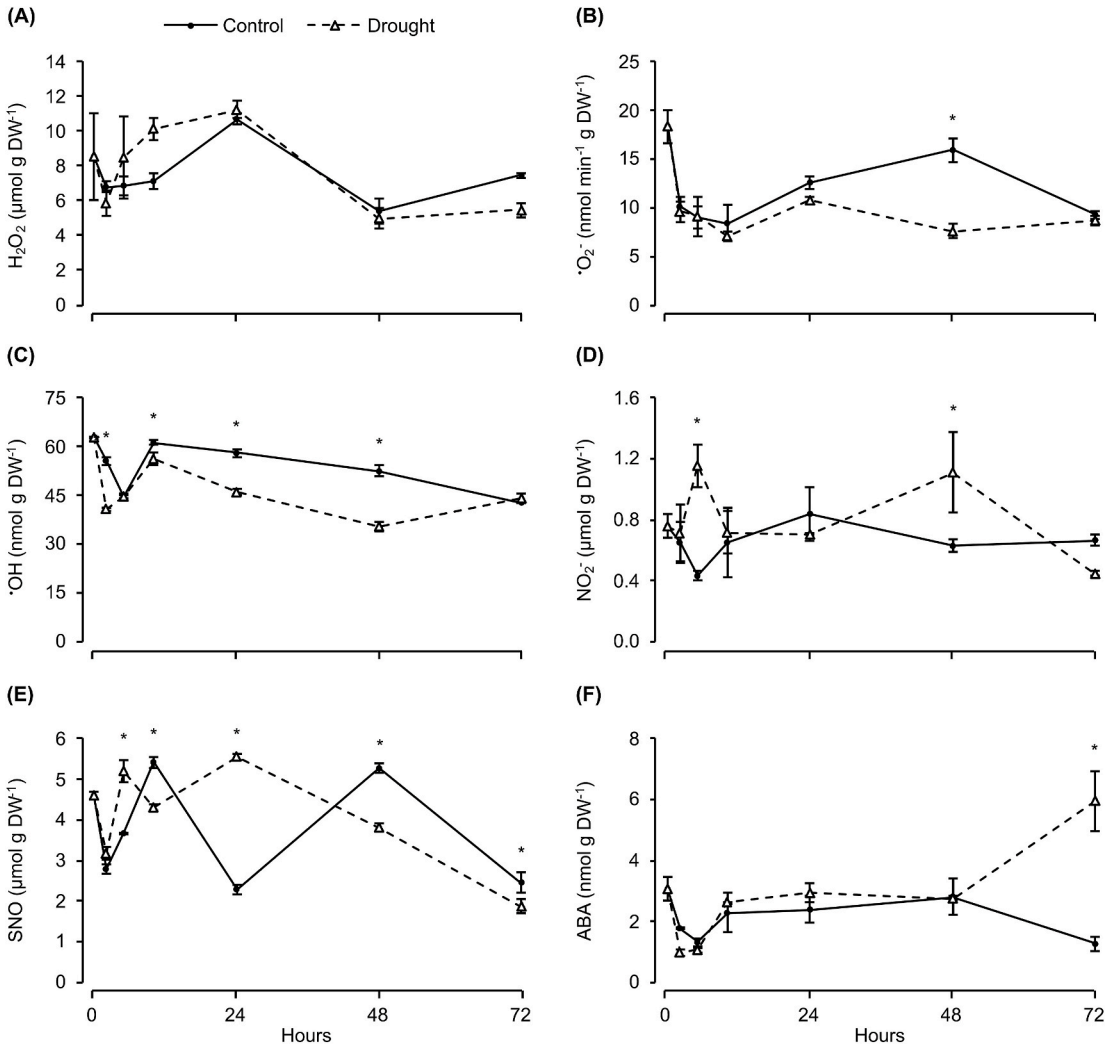

Fig. 6. Secondary messengers (A–E) and ABA content (F) of *A. strobilacea* plants exposed to 0, 2, 5, 10, 24, 48, and 72 h of well-watered (control) and drought conditions. Values are means ± s.e. calculated from three replicates. Significant differences between groups were tested by *two-way* ANOVA, with asterisks comparing control and treated plants per period ($P < 0.05$).

these peaks occurred later at 10 and 48 h (1.2 and 1.4 times higher than treated plants, respectively), and values remained 1.3 times higher than in treated plants at 72 h.

The ABA content was significantly affected by time and treatment (Table S1, Fig. 6F). No significant differences were found between 2 and 48 h; however, there was a 4.7-fold increase in ABA levels at 72 h of drought compared to the control (Fig. 6F).

4. Discussion

Despite the WL caused by short-term drought in *A. strobilacea* plants, the photosynthetic apparatus was generally preserved, and the excessive lipid peroxidation was prevented. This was possibly achieved by the turgor maintenance and the robust and plastic antioxidant capacity that promoted ROS and membrane damage control, as it will be discussed in this section.

Osmotic adjustment, as evidenced by the decrease in OP (Stiles and Martin, 1996), occurred concurrently with lower WL in *A. strobilacea* plants, i.e. between 24 and 72 h of stress. This suggests that turgor maintenance, which might have aided to prevent photosynthetic damage during drought (Blum, 2017), was promoted in that period (Verslues et al., 2006). Other studies also indicated that osmotic adjustment is related to photosynthetic maintenance in epiphytic bromeliads (Stiles and Martin, 1996; Nowak and Martin, 1997; Ceusters et al., 2009), while a comprehensive trait analysis corroborated the importance of an osmotically-mediated tolerance in bromeliads during the periods of high evaporative demand (Males and Griffiths, 2017). However, the reduced OP described here contrasted with the unaltered values reported for *A. strobilacea* plants exposed to eight days of soil drying (Menezes et al., 2020), leading to the assumption that the more intense drought imposed herein activated distinct metabolic responses in plants (Dubois and Inzé, 2020).

Pro showed a moderate accumulation, while Sug, which are commonly associated with osmotic adjustment (Forlani et al., 2019), did not accumulate in *A. strobilacea* during drought, as reported previously in other CAM species (*Sedum* sp.; Koźmińska et al., 2019). Nevertheless, from 10 h of drought, Pro might have aided in the oxidative maintenance in *A. strobilacea* plants, since this amino acid stabilizes antioxidant enzymes, promotes alternative ROS detoxification pathways, and prevents ROS formation by providing the electron acceptor $NADP^+$ throughout its synthesis (Szabados and Savouré, 2010).

Drought exposure caused a general decrease in SOD activity but it did not lead to $^{\bullet}O_2^-$ or $^{\bullet}OH$ accumulation in *A. strobilacea*, since both ROS were at control or lower levels in drought-treated plants. It is possible that $^{\bullet}O_2^-$ scavenging was supplemented by the redox buffers AA and TGSH during drought exposure (Foyer and Noctor, 2011), further

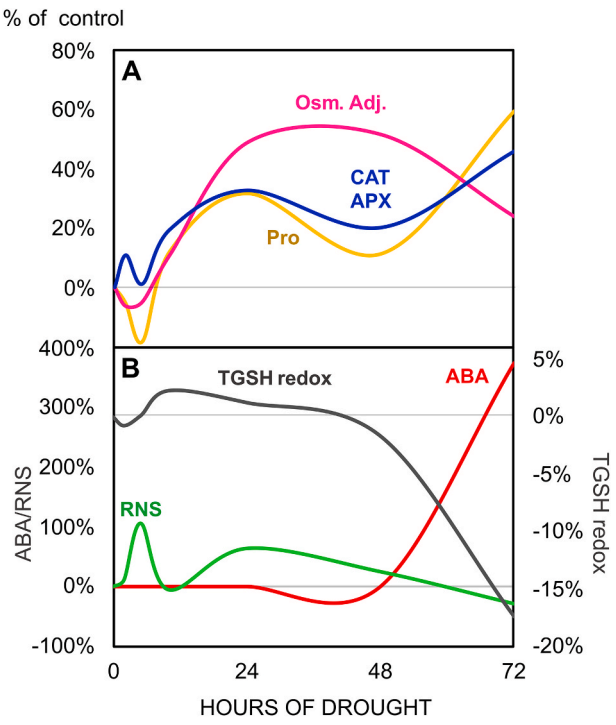

Fig. 7. Summary of the main defence (A) and regulatory (B) responses observed during exposure of *A. strobilacea* plants exposed to 0, 2, 5, 10, 24, 48, and 72 h of well-watered (control) and drought conditions. Values are the mean percentages of increase/reduction observed in stress-treated plants compared to the respective controls at each sampling point. Osmotic adjustment indicates OP. RNS values are the mean percentages of NO_2^- and SNO. CAT and APX values are the mean percentages of both enzymes.

preventing $^{\bullet}OH$ formation from $^{\bullet}O_2^-$, which is the most reactive and damaging ROS (Apel and Hirt, 2004), especially to membranes (Møller et al., 2007). Indeed, the greatest decrease in the levels of $^{\bullet}OH$, $^{\bullet}O_2^-$ and SOD activity occurred at 48 h of drought, concurrent to reduced membrane damage and increased AA redox state in comparison to the controls. Moreover, AA was kept at a highly reduced state throughout stress, which suggests an effective performance of the AA-TGSH cycle, as indicated by the sustained APX and GR activities. The TGSH showed a similar response to AA during most of the stress treatment, although at 72 h its redox state was more oxidized. This was concomitant to the slight decrease in Fv/Fm, suggesting drought began to negatively affect the photosystem II at 72 h. Under such conditions, the demand for antioxidant protection to preserve the photosynthetic apparatus would be increased. Hence, the GSH depletion could be due to a more intense consumption in oxidant metabolism pathways at later stress, including $^{\bullet}O_2^-$ scavenging and AA recovery (Noctor et al., 2012). Additionally, it seems CAT and APX were activated as a compensatory mechanism to SOD (Apel and Hirt, 2004), which most likely improved H_2O_2 scavenging and maintained this ROS under control levels from 10 to 72 h of stress in *A. strobilacea*. The two former enzymes seem particularly important for the H_2O_2 maintenance in *A. strobilacea*, since a similar response was observed in this species after eight days of progressive soil drought (Menezes et al., 2020), also suggesting that this is a conserved response to drought. Pro may have stabilized or activated CAT and APX between 10 and 72 h of drought and contributed to maintain antioxidant status (Guan et al., 2020; Forlani et al., 2019; De Carvalho et al., 2013; Szabados and Savouré, 2010). It is likely that such control over the H_2O_2 content aided in minimizing $^{\bullet}OH$ production, since the latter can be generated by the interaction of $^{\bullet}O_2^-$ and H_2O_2 (Hajiboland, 2014). Ultimately, these antioxidant adjustments were probably necessary to the regulation of $^{\bullet}O_2^-$ and H_2O_2 levels and, consequently, to reduce $^{\bullet}OH$ content and prevent membrane damage and photoinhibition in *A. strobilacea* during drought.

A similar ROS and photosynthetic maintenance were observed in juvenile plants of other epiphytes. For example, juvenile plants of the epiphytic bromeliad *G. monostachia* showed stable H_2O_2 levels after eight days of soil drying despite reduced SOD, APX, and CAT activities (Carvalho et al., 2017), while young plants (1–5 cm of leaf length) of the epiphytic bromeliad *V. sanguinolenta* showed mild reductions in Fv/Fm (6–13%) despite a 34–47% decrease in RWC after three days without irrigation (Zotz et al., 2004). Hence, ROS balance and photosynthetic maintenance might be important tolerance strategies of epiphytic bromeliads to the intense drought in early ontogenetic stages (Bader et al., 2009).

The WL in juvenile *A. strobilacea* plants was not accompanied by a progressive accumulation of ROS and RNS, which suggested that plants were not under nitro-oxidative stress during drought (Begara-Morales et al., 2019), corroborating the results discussed above. In fact, higher $^{\bullet}OH$, $^{\bullet}O_2^-$, and SNO levels detected in plants under well-watered conditions (controls) might indicate an overall tolerance to nitro-oxidative stress in *A. strobilacea*. As a result, we could not infer the involvement of early ROS accumulation in the short-term drought response of *A. strobilacea*. Nevertheless, we did detect two transient peaks in NO_2^- at 5 and 48 h of drought. A similar two-peak pattern in NO content was observed in the terricolous bromeliad *Nidularium minutum* after 24 and 72 h under 10 °C (Carvalho et al., 2019). Transient peaks in NO_2^-/NO have also been observed in tobacco cells after 2 h (Ke et al., 2013) and after 30 min in leaf cell suspensions of *Arabidopsis thaliana* (Ederli et al., 2019), both under PEG-induced osmotic stress. The transient elevations in NO_2^- levels might have led to higher NO formation in *A. strobilacea* (Astier et al., 2018), which in turn might have promoted post-translational modifications (PTMs) to several defence-related proteins, contributing to drought tolerance (Begara-Morales et al., 2019; Nabi et al., 2019).

SNO, a product of the PTM *S*-nitrosation, presented two peaks at 5 and 24 h of drought, although a similar response was observed in control plants at different periods as well. It is known that SNO levels are under tight regulation by denitrosylases (Begara-Morales et al., 2019). Hence, we might assume that, rather than a general increase in SNO content, drought possibly shifted the temporal pattern of *S*-nitrosation/denitrosylation balance of proteins and other thiol-containing molecules to adjust their activities in *A. strobilacea* plants. Additionally, we cannot assume that the SNO peaks in *A. strobilacea* plants were a product of NO_2^--derived NO since there was no increase in NO_2^- under control conditions. In fact, SNO accumulation without a concurrent NO increase has been previously described for *A. thaliana* and the sunflower (Feechan et al., 2005; Chaki et al., 2011). This independence between SNO and NO might partly derive from the distinct regulatory functions these molecules might have, as evidenced in *A. thaliana* plants with mutated NO- and SNO-related genes exposed to pathogen stress (Feechan et al., 2005; Yun et al., 2016).

After the increase in RNS, we detected an almost 5-fold rise in ABA levels by 72 h of drought. Similarly, juvenile plants of the epiphytic orchid *D. emarginata* (sized 7.0–13.0 cm) showed a tenfold increase in ABA levels after two days of water withholding (Zotz et al., 2001), while plants with a leaf length of 1.0 cm of the epiphytic bromeliad *V. sanguinolenta* showed a twofold increase in ABA after one day of soil drying (Zotz et al., 2004). Hence, our results support that the ABA has an early role in the induction of drought tolerance in juvenile epiphytes.

It is widely known that ABA can activate several stress resistance mechanisms (Fujita et al., 2011), such as the antioxidant system and osmolyte accumulation (Jiang and Zhang, 2004; Sharma et al., 2019). Accordingly, increased ABA levels are overall detected prior or paralleled to the activation of such mechanisms during drought (Jiang and Zhang, 2002; Urano et al., 2009; Kowitcharoen et al., 2015; He et al., 2015; Xing et al., 2016; Xu et al., 2017). Unexpectedly, higher CAT and APX activities, Pro content, and osmotic adjustment occurred within the first two days prior to ABA accumulation during the short-term drought

exposure of *A. strobilacea* plants, in contrast to our original hypothesis. This might suggest that an ABA-independent signalling pathway is involved in the regulation of early responses to drought. In fact, previous studies show that osmotic adjustment, higher antioxidant activity, and Pro accumulation might also be induced independently of ABA in response to osmotic stress in other species (Stewart and Voetberg, 1987; Bellaire et al., 2000; Verslues and Bray, 2006). Considering that the RNS peaks detected in the present study occurred concurrently with those mechanisms, it might be worth investigating if RNS was related to that modulation, as demonstrated in several other species exposed to osmotic stresses (Ke et al., 2013; Hasanuzzaman et al., 2017; Sahay et al., 2019; see review by Nabi et al., 2019). Indeed, the RNS accumulation pattern we observed is characteristic of early regulatory responses such as the Ca^{+2} and ROS waves that, despite being highly transient, are crucial for defining the long-term acclimation response (see reviews by Kollist et al., 2019; Dubois and Inzé, 2020). The main defence and regulatory responses of *A. strobilacea* plants submitted to drought for 72 h are summarized in Fig. 7.

We might also assume that the ABA increase at 72 h was involved in the up and downregulation of several other mechanisms in *A. strobilacea*, since this phytohormone affects the expression of several stress-related genes (Fujita et al., 2011). The slight decrease in Fv/Fm detected at 72 h might suggest that some of these ABA-induced mechanisms might have been associated to the protection of photosystem II in face of longer stress exposure (Yang et al., 2006; Qian et al., 2020). Furthermore, the simultaneous decrease in the redox state of TGSH at 72 h might be involved in the ABA-induced responses, since this imbalance has been related to the regulation of ABA and other phytohormones' signalling pathways (Mhamdi et al., 2013; Noctor et al., 2018). Although there is some evidence that ABA regulates stomatal movement in constitutive CAM plants (Zhang et al., 2019; Chomthong and Griffiths, 2020), the involvement of this phytohormone in the regulation of other drought responses in these plants remain to be explored. Similarly, the specific functions of ABA in juvenile epiphytic plants have not yet been described.

5. Conclusions

Our results demonstrated that juvenile plants of *A. strobilacea* showed the activation of defence mechanisms and regulatory responses that might contribute significantly to the maintenance of oxidative levels and photosynthetic capacity during short-term drought. This response might represent an important strategy against the intense drought faced during this ontogenetic stage in the epiphytic environment. Additionally, our results suggest that activation of antioxidant mechanisms and osmotic adjustment during an early drought might be regulated by ABA-independent pathways, while ABA might have a role at later stages of stress. We concluded that RNS might be involved in the regulatory mechanisms to early drought responses in *A. strobilacea.* We believe that further research about (i) the role of RNS in the regulation of antioxidant activity and osmotic adjustment, and (ii) the function of ABA and TGSH redox imbalance in the modulation of stress tolerance in *A. strobilacea* plants might help understand the early drought responses in juvenile epiphytic bromeliads.

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Author contributions

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

M.G. and C.C.N. conceived and designed the experiments, interpreted the data, and revised the paper.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2020.12.030.

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SUPPLEMENTARY MATERIAL

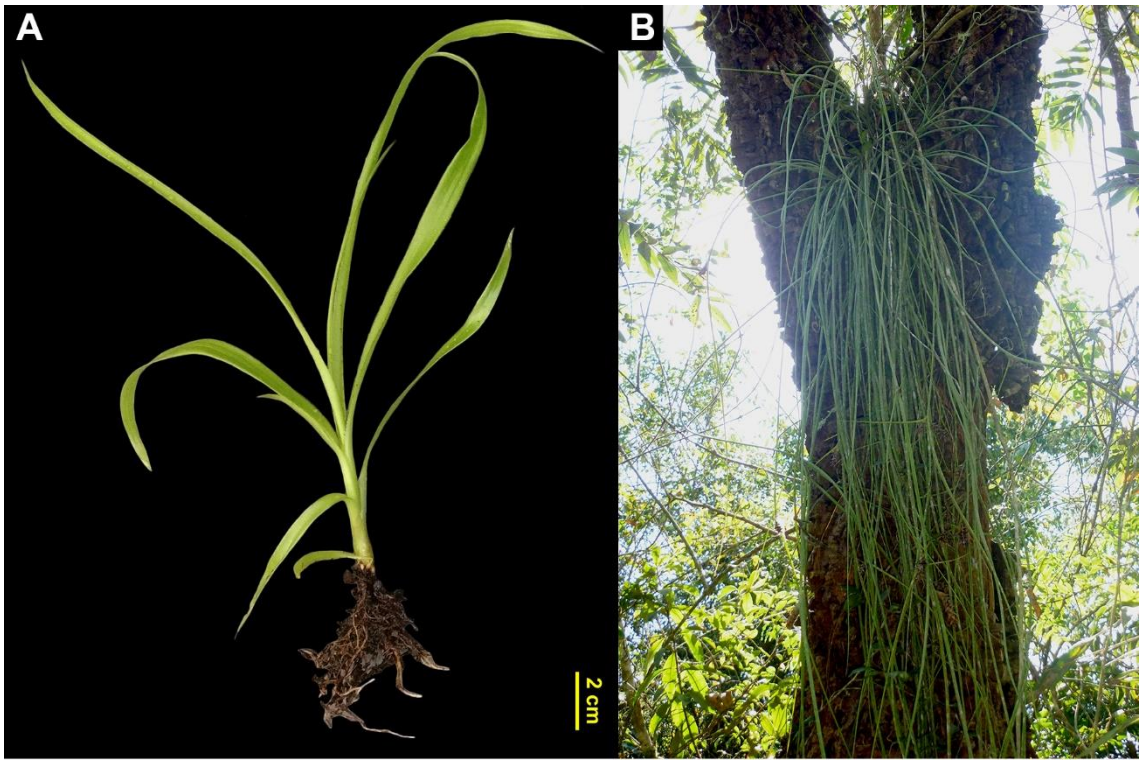

Figure S1. Morphological aspect of *A. strobilacea* plants: (A) three-month-old plant representative of those utilized in the present study; (B) adult specimen from the natural population located at the *Núcleo de Pesquisa Reserva Biológica de Mogi Guaçu* of the *Instituto de Botânica* in Mogi Guaçu, São Paulo, Brazil (22°15’04.2’’S and 47°09’56.8’’W).

Figure S2. Morphological aspect of *A. strobilacea* plants under control (A) and drought conditions (B) after 72 hours.

Table S1. Values of significance (*P* values) resulted from two-way ANOVA with time and watering condition (control and drought) as factors ($P < 0.05$). Ns, not significant.

	Significance		
	Time	Treatment	Interaction
RWC	<0.0001	<0.0001	<0.0001
OP	<0.0001	<0.0001	<0.0001
Fv/Fm	0.0002	ns	0.0026
Chl	<0.0001	<0.0001	<0.0001
Chl a:b	ns	ns	ns
Car	<0.0001	<0.0001	<0.0001
Chl:Car	<0.0001	ns	ns
HPCD	<0.0001	0.0495	0.0003
Sug	<0.0001	0.0226	0.0001
Pro	<0.0001	0.0001	<0.0001
SOD	0.0003	0.0002	ns
CAT	<0.0001	0.0001	0.0225
APX	0.0083	<0.0001	0.0190
GR	0.0066	ns	ns
RedA	0.0012	ns	ns
DHA	0.0009	ns	ns
AA redox state	ns	ns	ns
GSH	<0.0001	ns	0.0085
GSSG	0.0001	ns	ns
TGSH redox state	0.0023	ns	0.0068
H_2O_2	0.0016	ns	ns
$^{\bullet}O_2^-$	<0.0001	0.0086	0.0156
$^{\bullet}OH$	<0.0001	<0.0001	<0.0001
NO_2^-	ns	ns	0.0195
SNO	<0.0001	0.0010	<0.0001
ABA	<0.0001	0.0074	<0.0001

CAPÍTULO 2

Drought memory in a CAM epiphytic bromeliad

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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 osmolytes 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* shows improved metabolic stress defence responses to a second drought and rewatering 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 five days of rewatering each. The free amino acid, chlorophylls, carotenoid levels and S-nitrosoglutathione reductase (GSNOR) activities were higher at the second drought than at the first exposure. The accumulated pigments might optimize the light-harvesting process, with carotenoids improving antioxidant defence. The GSNOR responses suggest the improvement in nitrosative stress prevention due to reiterated drought exposure. Furthermore, the second recovery did not induce reduced 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 evidenced 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.

Keywords: Bromeliaceae, chlorophyll, denitrosylase, drought recovery, reiterated drought, nitric oxide, stress imprint

INTRODUCTION

It is extensively supported that plants can alter responses to stress after recovering from a past exposure – a process known as "stress memory". Those responses can, but not always, lead to improved plant performance under repeated stress (Thellier & Lüttge 2013, Fleta-

Soriano & Munné-Bosch 2016, Galviz *et al.* 2020). In that scenario, memory formation results from the priming effect of a first stress event (Bruce *et al.* 2007, Hilker *et al.* 2015, 2019). However, not all responses elicited by the priming stimulus are consolidated as memories that can be recalled when stress recurs, which is directly related to the duration of the recovery period after the first stress (Crisp *et al.* 2016).

Stress memory might affect parameters ranging from epigenetic, transcriptional, post-translational, metabolic to physiological levels (Fleta-Soriano *et al.* 2015, Virlouvet *et al.* 2018, Avramova 2019, Schwachtje *et al.* 2019, Zhang *et al.* 2020, Sun *et al.* 2021). Among the biochemical parameters, there is a greater accumulation of osmolytes such as the amino acid proline (Pro) due to drought priming in diverse species (Wang *et al.* 2018, 2021, Alves *et al.* 2020, Khan *et al.* 2020, 2021). This accumulation might be related to the importance of osmotic adjustment and the many other stress-protective functions of osmolytes in plant defence against drought (Slama *et al.* 2015).

Photosynthetic-related traits such as chlorophyll content have also improved after repeated drought in the perennial forage grass *Alopecurus pratensis* L. (Lukić *et al.* 2020), suggesting that promoting the photochemical process is prioritised as a tolerance trait due to priming. However, no other reports were encountered detailing the effects of reiterated drought over chlorophyll content and synthesis. It is well established that drought memory improves redox maintenance (Alves *et al.* 2020, Lukić *et al.* 2020, Khan *et al.* 2021). For instance, increased glutathione levels and glutathione reductase (GR) activities have been frequently reported as drought priming responses (Selote & Khanna-Chopra 2006, Wang *et al.* 2018, Khan *et al.* 2020, 2021). Glutathione is the major non-protein thiol in plant cells and GR converts its oxidised (GSSG) form to reduced form (GSH) to maintain a high redox ratio of this antioxidant. Thus, glutathione and its associated enzyme are crucial for maintaining the overall redox state in cells and promoting reactive oxygen species (ROS) detoxification during stress (Gill *et al.* 2013).

The nitrosative metabolism – referent to reactive nitrogen species as nitric oxide (NO) – is also involved in drought memory. Wang *et al.* (2021) recently reported that NO is partly responsible for stimulating higher Pro accumulation during a second drought exposure in wheat seedlings (*Triticum aestivum* L.). NO acts as a regulator of plant metabolism by inducing several post-translational modifications (PTM) in proteins and low-molecular weight thiols (*e.g.*, GSH), such as the reversible S-nitrosation, forming S-nitrosothiols (SNO) (Malik *et al.* 2011, Lindermayr 2018, Begara-Morales *et al.* 2019). The S-nitrosation of GSH leads to S-nitrosoglutathione (GSNO), the main reservoir of NO in plants. Thus, GSNO is a direct link between the nitrosative and antioxidant system. The levels of GSNO are under strict control by

the denitrosylase S-nitrosoglutathione reductase (GSNOR), which converts GSNO to glutathione disulfide (GSSG) and ammonia (NH_3). Consequently, this enzyme indirectly controls S-nitrosation and total SNO content by reducing NO availability (Malik *et al.* 2011, Lindermayr 2018, Begara-Morales *et al.* 2019). The regulation of abiotic stress responses by S-nitrosation and SNO is being increasingly investigated (see review by Begara-Morales *et al.* 2019). Accordingly, the increased activity of GSNOR has been associated to abiotic stress tolerance (Cheng *et al.* 2018, Pissolato *et al.* 2020, Basu *et al.* 2021). However, to our knowledge, the effects of reiterated drought on SNO maintenance in general are still unknown.

Stress memory enables the plant to rapidly acclimate to changing environmental conditions (Crisp *et al.* 2016). Therefore, this tolerance strategy is likely present in species from habitats under frequent environmental alterations, such as the epiphytic niche. Epiphytes, as many of the Bromeliaceae, are detached from the soil and sustain themselves over other trees, and thus depend on atmospheric water (*e.g.*, rain and dew). Consequently, these plants are exposed to frequent droughts as a result of a highly intermittent water supply (Benzing 2000, Zotz 2016), making epiphytic bromeliads interesting models for studying stress memory. Bader *et al.* (2009) reported evidence of drought priming in seedlings of the epiphytic bromeliad *Tillandsia flexuosa* Sw, Plants pre-treated with mild 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 and in other epiphytic bromeliads, thus in-depth knowledge of drought memory in these plants is currently deficient. Such research is crucial for the understanding of how these ecologically important plants will be affected by climate change (Cach-Pérez *et al.* 2014, Benzing 1998), which could increase the frequency and severity of droughts in the future (Trenberth *et al.* 2014).

The epiphytic bromeliad *Acanthostachys strobilacea* (Schult. & Schult.f.) Klotzsch (Bromelioideae, Bromeliaceae) presents several drought resistance traits from early to adult life stage including crassulacean acid metabolism (CAM, 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 an 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 focused on evaluating parameters associated to the photosynthetic integrity, nitrosative metabolism, antioxidant system and osmotic adjustment since previous research evidenced that these mechanisms are part of the short-term drought defence in juvenile

A. strobilacea (Carvalho *et al.* 2021) and in the drought memory of other species (Wang *et al.* 2018, 2021, Alves *et al.* 2020, Lukić *et al.* 2020, Khan *et al.* 2020, 2021).

MATERIALS 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 harvested from the plant collection of *Instituto de Pesquisas Ambientais*, originated from a natural population located at Mogi Guaçu biological reserve, São Paulo state, Brazil (22°15′04.2″S and 47°09′56.8″W). Seeds were cultivated *in vitro* following Carvalho *et al.* (2014) and maintained 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} s^{-1} provided by white tubular 6500K LED lamps. Subsequently, plants were transferred to 1 L plastic (PET) trays with drainage holes in the bottom (35 plants each), containing approximately 1 L of fine sterilised commercial *Pinus* bark (Carvalho *et al.* 2014). The trays were placed in a walk-in environmental chamber under 25/16°C (day/night), 60/70% relative air humidity, a 12 h-photoperiod, and a PFD of 300 μmol m^{-2} s^{-1} provided by 1200 C3 Full Espectro LED panels with a light spectrum as in figure S1 (InstalaFrio, Pinhais - Brazil). Plants remained under these conditions for 90 days, when plants reached 18.8 cm in height, on average. During the growth period, plants were irrigated twice a week with distilled water using a compression sprayer by spraying leaves and substrate until drainage was detected. Fertilization was performed in the same manner biweekly with 1 g L^{-1} of a commercial fertilizer of 20:20:20% composition (total nitrogen:phosphorous:potassium, Plant-Prod, Canada) until one week before the onset of experiments.

From a pilot experiment performed under the same conditions described above, it was verified that 14 days of drought induced by irrigation withholding caused the first indications of wilting in the leaves, while a subsequent rewatering period of five days allowed the complete restoration of relative leaf water content at pre-stress levels. Thus, trays were randomly separated according to exposure to drought-rewatering (D-R) cycles (figure 1): D1 – single D-R: 14 days without irrigation and five days of rewatering, D2 – double D-R: 14 days without irrigation and five days of rewatering, followed by another 14 days without irrigation and five days of rewatering. The first D-R cycle in D1 plants was performed simultaneously with the second cycle in D2 plants to account for any effects of plant growth over the evaluated parameters. The control plants (CT) were kept under constant irrigation through the 37 days of the experiment. The irrigation of CT, D1 and D2 plants was performed as previously described, approximately four hours after the onset of the light period, following the schedule in figure

S2. Sampling of CT, D1 and D2 leaves occurred at 32 and 37 days (figure 1). For the assessment of daily acidity variation (ΔH^+) indicative of CAM activity, sampling of biological triplicates composed of three plants each took place two hours after the onset of the light period (dawn) and two hours 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 utilised fresh for certain analyses, as described below.

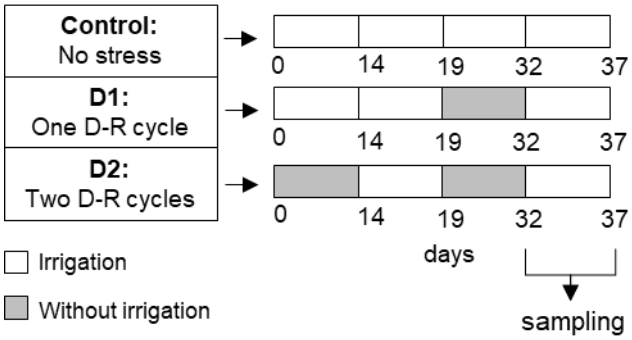

Figure 1. Experimental design and sampling days. D-R, drought-rewatering.

Biometric, water status, CAM, and chlorophyll fluorescence measurements

We measured the number of leaves, dead leaves, and plant height (measured from the base of the plants to the tip of the longest leaf) of a batch of six plants without removing them from the substrate to assess their growth. The relative water content (RWC) and osmotic potential were analysed from fresh leaf disks of 0.5 cm in diameter obtained at the middle of the photoperiod, following Fleta-Soriano *et al.* (2015) and Rigui *et al.* (2019), respectively. Substrate water content (SWC) was assessed from aliquots obtained from three separate trays of each treatment, following Carvalho *et al.* (2021).

CAM activity was assessed by the titratable acidity of samples obtained at dawn and dusk. Aliquots of 100 mg of frozen powdered leaves were homogenised in 1.5 mL of boiled ultrapure water. Extracts were maintained in a boiling water bath for 15 min and centrifuged at 11000 g for 15 min. The supernatant volume was completed to 10 mL with boiled ultrapure water and 10 µL of the pH indicator phenolphthalein 2% (w/v) was added per sample. Titration was performed with 0.02 N NaOH until solutions developed a pink color (*i.e.*, pH 9.0). The sample's acidity was calculated from the utilised NaOH volume and expressed as mmol H^+ g^{-1} dry weight. The ΔH^+ was obtained by subtracting the dusk from the dawn values per replicate (adapted from Freschi *et al.* 2010).

The maximum potential quantum efficiency of the PSII (Fv/Fm ratio) was measured in the longest leaf of four intact plants with a modulated fluorometer (PAM 2500, Walz, Germany), at midday, after being kept for at least 15 min onto leaf clips for dark adaptation.

Free amino acids (AA) content

AA and Pro were quantified in the same extracts, made by homogenizing 100 mg of frozen powdered leaves in 1 mL of ethanol 40% (v/v). After remaining under 8°C for 24 h, extracts were centrifuged at 16100 g for 5 min (adapted from Carillo *et al.* 2005). AA content was estimated with the ninhydrin reagent formulated with the addition of 0.25 g L^{-1} of ascorbic acid and calculated from a glutamic acid standard curve (Yokoyama & Hiramatsu 2003). Pro levels were quantified with the cuvette spectrophotometer protocol detailed in Carillo & Gibon (2011), using a Pro standard curve.

Photosynthetic pigments, antioxidants, and lipid peroxidation

Chlorophylls (Chl) a and b and carotenoids (Car) were quantified using extracts made from 80 mg of frozen powdered leaves homogenised in 2 mL of alkaline acetone 80% (v/v), followed by 10 min of mixing in an ice bath. After centrifugation at 16100 g for 10 min under 4°C, supernatants were reserved on ice, and extraction was repeated with the pellets using 1.5 mL of alkaline acetone 80% as described earlier. Supernatants were then mixed and used for analysis in a spectrophotometer, with final pigment concentrations calculated according to Lichtenthaler & Wellburn (1983). The Chl a:b and total Chl:Car ratios were also determined.

The activities of superoxide dismutase (SOD, EC 1.15.1.1), ascorbate peroxidase (APX, EC 1.11.1.11) and glutathione reductase (GR, EC 1.6.4.2) were quantified following Carvalho *et al.* (2017), utilizing fresh leaf discs for the extraction. Total glutathione, the reduced (GSH) and oxidised (GSSG) fractions were quantified with the enzymatic recycling assay as described in Carvalho *et al.* (2021). The glutathione redox state was inferred by calculating the percentage of the GSH pool.

The estimation of lipid peroxidation (*i.e.*, membrane damage) was evaluated by quantifying hydroperoxide conjugated dienes (HPCD), representative of the initial reaction product in the peroxidation of polyunsaturated fatty acids (Gasparovic *et al.* 2017). HPCD was measured following Pignata *et al.* (2002), using extracts obtained from 40 mg of frozen powdered leaves homogenised in 2 mL of 96% ethanol (v/v).

SNO content and GSNOR activity

Total SNO was estimated by Saville's colorimetric method based on the SNO hydrolysis in the presence of mercuric salts, according to Frungillo *et al.* (2013), with modifications described in Carvalho *et al.* (2021).

For assessing GSNOR activity, extraction was performed with 200 mg of fresh leaf disks ground in 1 mL of 0.1 mol L^{-1} phosphate buffer pH 7.2 in a mortar and pestle on an ice bath (adapted from Frungillo *et al.* 2013). After centrifugation under 12000 g and 4°C for 15 min, supernatants were used for kinetic assays in a cuvette spectrophotometer following Kubienová *et al.* (2016).

Statistical analyses

The experimental design was completely randomised. The means of each treatment (CT, D1 and D2) per period were subjected to a one-way analysis of variance (ANOVA) and post-hoc Tukey's tests ($P < 0.05$), using the GraphPad Prism 5.01 software. A principal component analysis (PCA) using a correlation matrix was performed with the Past3 software to summarise the 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 showed similar size (plant 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 the 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 value of 74% (figure 2a, b). Statistics indicated that rewatering allowed complete SWC and RWC recovery in D1 and D2 to CT levels. The osmotic potentials did not differ between D1 and D2 (figure 2c), supporting the occurrence of mild droughts in plants.

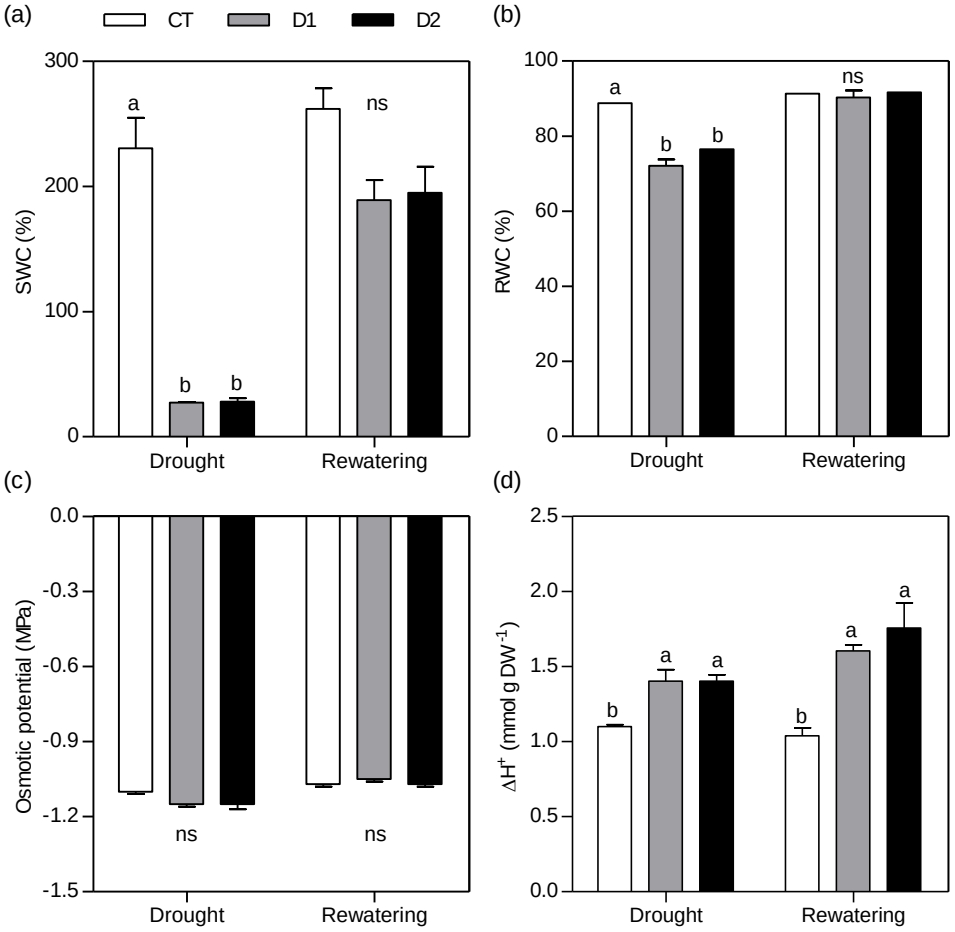

Figure 2. Substrate water content (a), leaf water status (b, c) and CAM activity (d) in *A. strobilacea* plants exposed to well-watered conditions (control, CT), one (D1) and two (D2) drought-rewatering events. 'Drought' and 'rewatering' bars represent means obtained at 32 and 37 days of the experiment, respectively (figure 1). Values are the means ± s.e. (n = 3). Different lowercase letters indicate significant differences between groups per period (one-way ANOVA and post-hoc Tukey's tests, $P < 0.05$). Ns, not significant.

The first and second droughts significantly increased ΔH^+ (indicative of CAM activity) at similar rates, being 28% higher than the CT (figure 2d). The same pattern was maintained after rewatering, although the increase was 62% on average.

Free AA and Pro pools

The AA means were progressively and significantly increased by the number of droughts, which was maintained after the final rewatering events (figure 3a). A similar response occurred for Pro, although no difference was detected between D1 and D2 (figure 3b).

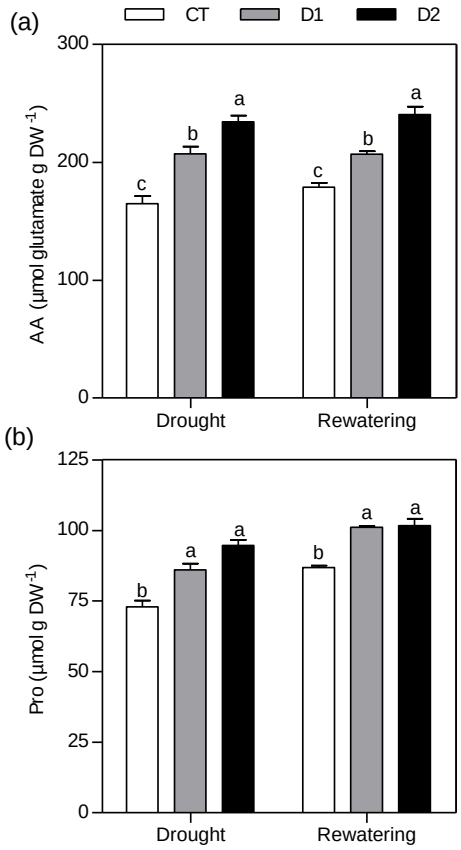

Figure 3. Total free amino acids (a) and proline content (b) in leaves of *A. strobilacea* exposed to well-watered conditions (control, CT), one (D1) and two (D2) drought-rewatering events. 'Drought' and 'rewatering' bars represent means obtained at 32 and 37 days of the experiment, respectively (figure 1). Values are the means ± s.e. (n = 3). Different lowercase letters indicate significant differences between groups per period (one-way ANOVA and post-hoc Tukey's tests, $P < 0.05$). Ns, not significant.

Pigment content

Like free AA, Chl a, Chl b and Car means were significantly intensified according to the number of droughts (figure 4). The first drought caused increases of 53% and 79% in Chl a and b compared to the CT, respectively (figure 4a, b). The second drought intensified Chl a and b at higher rates in D2 than D1 (a 99% and 135% increase for Chl a and b, respectively, compared to the CT). Means of Car were significantly higher by 46% and 82% in D1 and D2 at 32 days than the CT, respectively (figure 4d). After rewatering, Chls and Car remained higher in D1 and D2 than CTs, although D1 and D2 were statistically similar between them. These changes did not significantly affect the Chl a:b ratio (figure 4c).

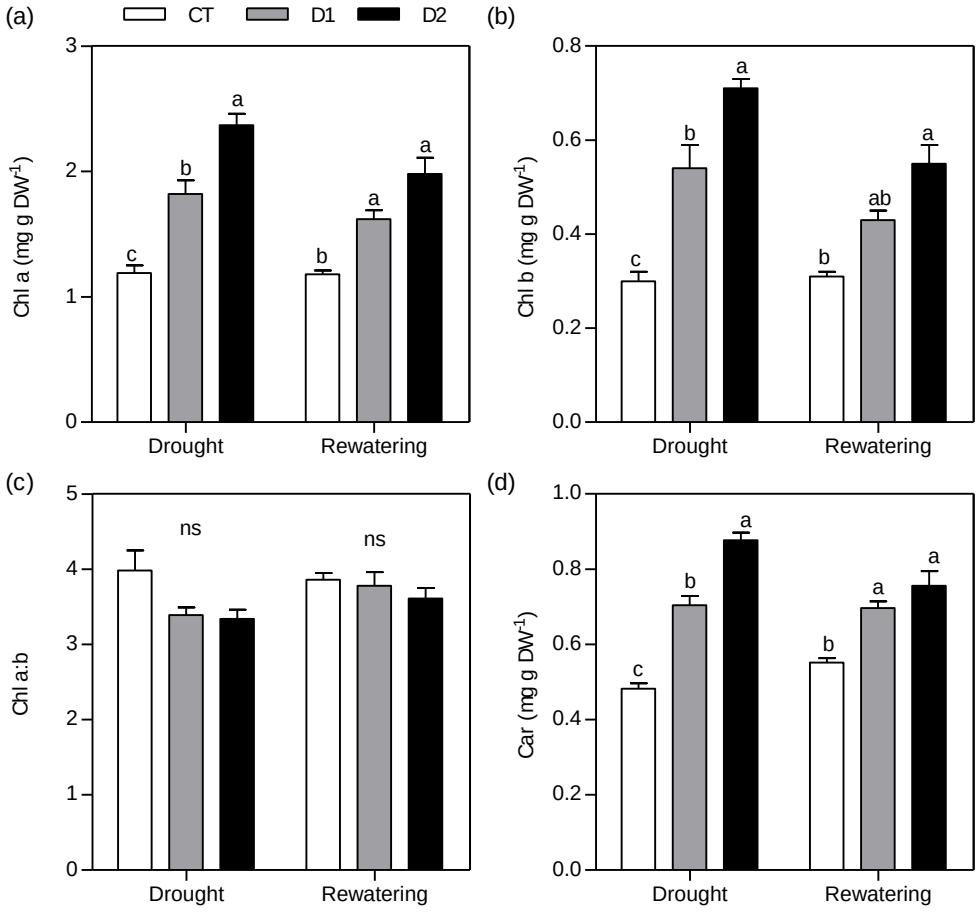

Figure 4. Content of chlorophylls (a-c) and carotenoids (d) in leaves of *A. strobilacea* plants exposed to well-watered conditions (control, CT), one (D1) and two (D2) drought-rewatering events. 'Drought' and 'rewatering' bars represent means obtained at 32 and 37 days of the experiment, respectively (figure 1). Values are the means ± s.e. (n = 3). Different lowercase letters indicate significant differences between groups per period (one-way ANOVA and post-hoc Tukey's tests, $P < 0.05$). Ns, not significant.

Photosynthetic status, antioxidant activity and nitrosative metabolism

Although D1 and D2 altered pigment levels, the Fv/Fm did not change significantly (figure 5a). Accordingly, no significant HPCD accumulation due to lipid peroxidation and oxidative stress was detected during the D-R cycles (figure 5b).

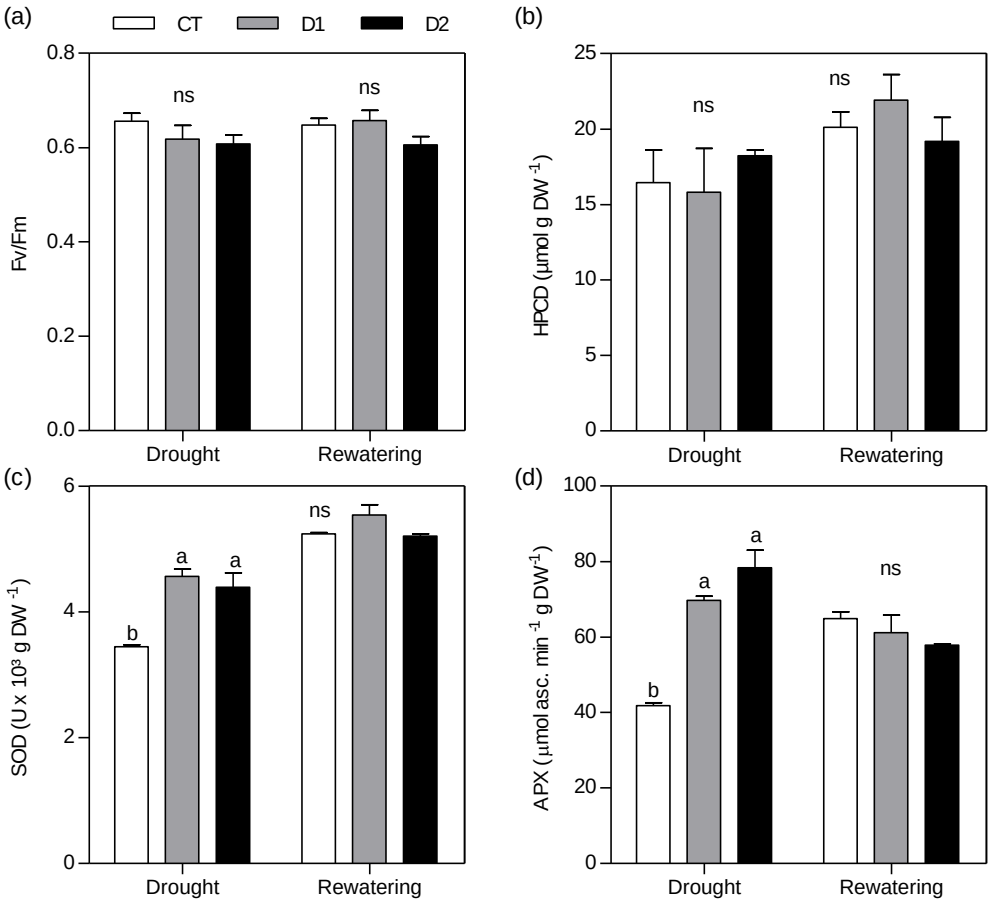

Figure 5. The maximum potential quantum efficiency of the PSII (a), lipid peroxidation (b) and antioxidant enzyme activities (c, d) in leaves of *A. strobilacea* plants exposed to well-watered conditions (control, CT), one (D1) and two (D2) drought-rewatering events. 'Drought' and 'rewatering' bars represent means obtained at 32 and 37 days of the experiment, respectively (figure 1). Values are the means ± s.e. (n = 3). Different lowercase letters indicate significant differences between groups per period (one-way ANOVA and post-hoc Tukey's tests, $P < 0.05$. In (c), one unit (U) is the amount of enzyme required to inhibit the reduction in nitrobluetetrazolium by 50%. Asc., ascorbate; ns, not significant.

The exposure to D-R cycles significantly affected antioxidant enzyme activities. The SOD, APX and GR activities were statistically similar between the first and second drought periods and significantly higher by 30%, 77% and 76% on average, respectively, than the CT (figure 5c, d, 6a). The activities of these enzymes were statistically similar between CT, D1 and D2 plants after rewatering.

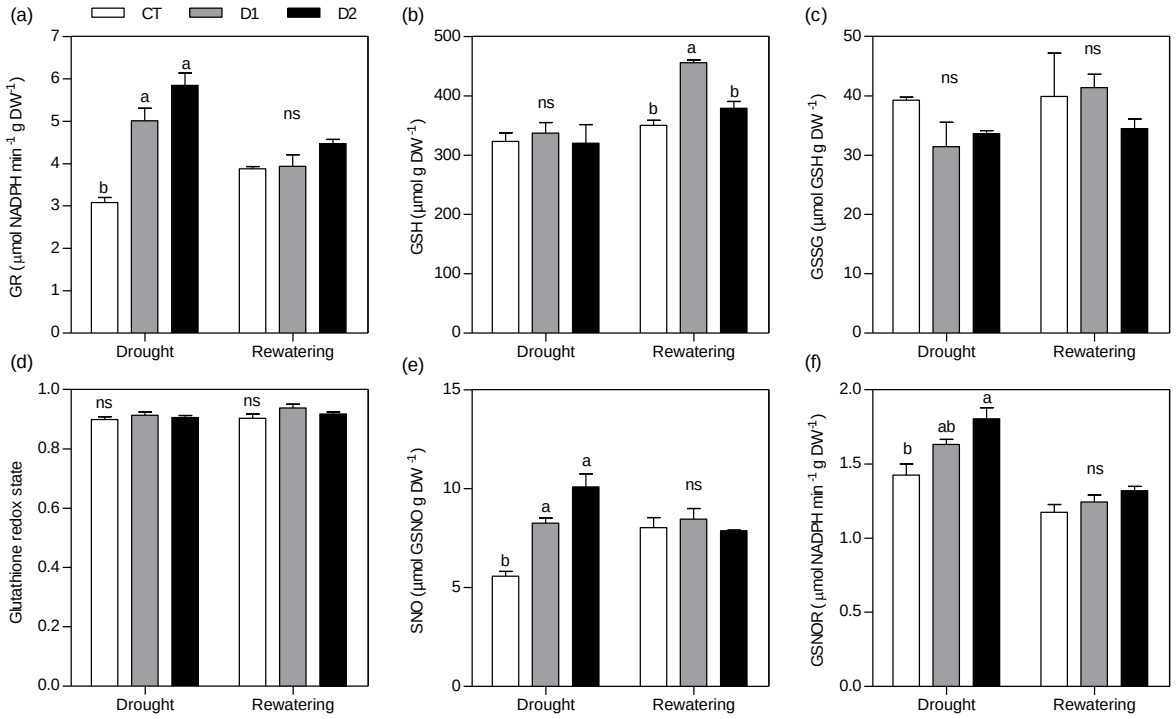

Figure 6. Activities of glutathione reductase (a), glutathione levels (b-d) and nitrosative parameters (e-f) in leaves of *A. strobilacea* plants exposed to well-watered conditions (control, CT), one (D1) and two (D2) drought-rewatering events. 'Drought' and 'rewatering' bars represent means obtained at 32 and 37 days of the experiment, respectively (figure 1). Values are the means ± s.e. (n = 3). Different lowercase letters indicate significant differences between groups per period (one-way ANOVA and post-hoc Tukey's tests, $P < 0.05$). Ns, not significant.

The drought exposure did not affect GSH levels, although rewatering lead to an increase of 30% in D1 compared to CT and D2 (figure 6b). Meanwhile, GSSG was unaltered by D-R cycles (figure 6c). Accordingly, the glutathione redox state was not affected by D-R cycles and was sustained at a highly reduced state of at least 85% (figure 6d).

The SNO content, indicative of nitrosative status, accumulated at statistically similar levels between one and two droughts (figure 6e, 48% and 81% increase in D1 and D2, respectively, compared to CT). The SNO was restored completely to CT levels after the rewatering periods in D1 and D2. The GSNOR activities in D2 were slightly higher than D1, with rates 14% and 27% higher than the CT, respectively (figure 6f). After rewatering, GSNOR values of D1 and D2 were recovered to CT values.

PCA

The PCA assessed the global effects of one and two D-R cycles over the main affected parameters (figure 7). The first and second axes (PC1 and PC2) were significant according to

the broken-stick method (figure S3) and represented 49% and 30% of the data variance, respectively. The samples were distributed into six groups representing each treatment. The 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 correlation values of each variable to the PC1 support that a second drought exposure is associated with accumulation of AA, Pro, Chl, SNO, increase in antioxidant enzymes activities (mainly APX, GR) and, to some extent, GSNOR activities and CAM activity than one stress only (table S2).

In contrast, the data obtained at 37 days, *i.e.*, after D1 and D2 plants' rewatering, were mostly separated along PC 2. The D1-R and D2-R samples were closer to each other than to the CT-R, suggesting the metabolism of plants after rewatering was not entirely recovered 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.

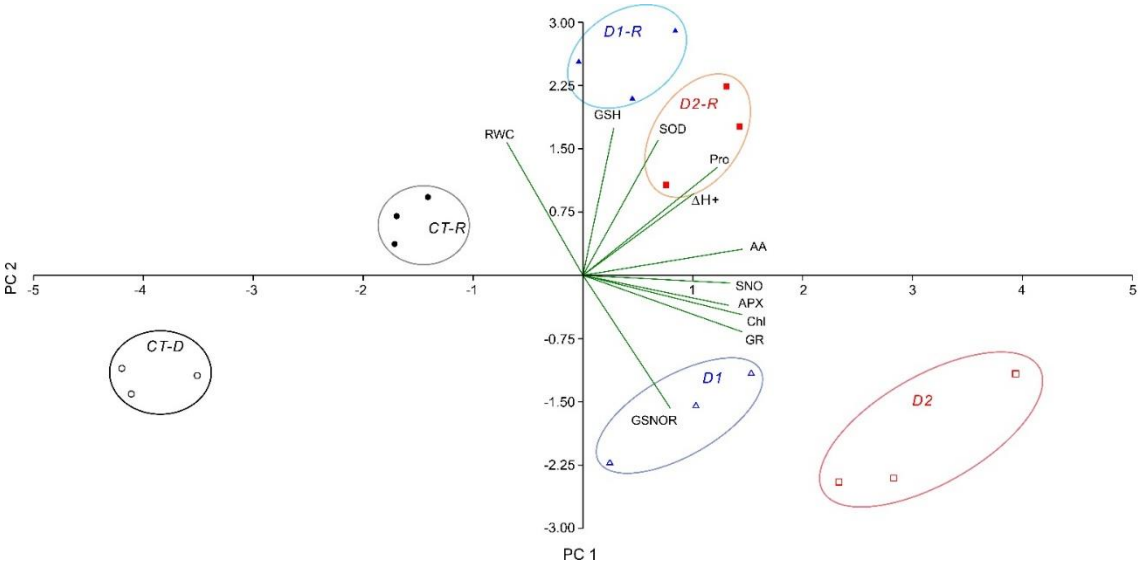

Figure 7. Principal component analysis (PCA) of means obtained at 32 (CT-D, D1 and D2) and 37 days (CT-R, D1-R and D2-R) from leaves of *A. strobilacea* plants exposed to well-watered conditions (control, CT), one (D1) and two (D2) drought-rewatering events. Both PC1 and PC2 are significant according to the broken-stick method (figure S3). Circles indicate the clusters formed according to the treatments. CT-D, D1 and D2: values of the control, one and two droughts obtained at 32 days, respectively, CT-R, D1-R and D2-R: values of the control and rewatering performed after one and two droughts at 37 days, respectively. Total Chl mean values were used in the analyses.

DISCUSSION

The water status (RWC and osmotic potentials), growth and CAM activity of *A. strobilacea* plants responded similarly with one and 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,* which will be detailed in this section.

A second drought exposure led to higher AA levels than only one, which was sustained after the final rewatering event. Therefore, we assume 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 accumulation and redirection of free AA to synthesise diverse stress-related molecules, such as secondary metabolites, signalling molecules, hormones, and antioxidants as GSH (Hildebrandt *et al.* 2015, Hildebrandt 2018). The higher AA/Pro levels at both droughts also imply that its stress-defensive functions were promoted, including turgor maintenance, membrane and protein stabilization improvement, serving as organic nitrogen storage and 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, were accumulated at higher rates under exposition to three D-R cycles rather than one. The authors concluded that osmoprotectant accumulation enabled triple-stressed plants to maintain their physiological performance similar to unstressed plants (Alves *et al.* 2020).

Likewise, Chls were progressively increased during the two D-R cycles, which might result from an intensified Chl synthesis or reduced degradation. The tetrapyrrole (chlorophyll, heme, siroheme, and phytochromobilin) synthesis starts with the conversion of glutamate into aminolevulinic acid (Tanaka *et al.* 2011). Considering the paralleled Chl and AA increase rates during D-R cycles, we might assume that part of the glutamate from the free AA pool released during drought was routed to the tetrapyrrole synthesis in *A. strobilacea* and gains importance at the second D-R exposure. Although the ninhydrin assay utilised in this study quantifies the total free AA pool, proteomic studies with *Arabidopsis thaliana* (L.) Heynh. show that glutamate is among the two highest accumulating AA in the free form under unstressed conditions (Hildebrandt 2018, Heinemann *et al.* 2020), thus corroborating the previous assumption.

The Chl accumulation is an unusual drought resistance trait among plants. It has been reported scarcely but commonly in highly resistant species, including *Calotropis procera* (Aiton) W.T. Aiton, an evergreen species from Asian and African arid areas (Rivas *et al.* 2020), *Acacia saligna* (Labill.) H.Wendl, an Australian species adapted to arid zones (Nativ *et al.*

1999), and in another epiphytic bromeliad, *Guzmania monostachia* (L.) Rusby ex Mez var. *monostachia* (Freschi *et al.* 2010). In another recent work with the perennial grass *A. pratensis*, Chl was promoted after repeated drought (Lukić *et al.* 2020), as observed herein. Lukić *et al.* (2020) observed that exposing *A. pratensis* plants to a two-year drought, three-week recovery, and two-week drought period lead to a 57% increase in Chl b compared to plants exposed only to the two-week stress exposure. The consensus is that the accumulated pigments might promote a higher photosynthetic rate by optimizing the light-harvesting process (Nativ *et al.* 1999, Lukić *et al.* 2020, Rivas *et al.* 2020) and, consequently, the ATP and NADPH supply for the intensive stress metabolite synthesis. This optimization might also have occurred in *A. strobilacea* since the Fv/Fm indicates that the 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 D2 plants, which was possibly compensated by the increased Car that prevents photooxidative damage in the photosynthetic apparatus (Uarrota *et al.* 2018). The positive effects of Chl accumulation on photosynthesis and senescence prevention have been confirmed in a study with a Chl-accumulating SlOFP20-overexpressing tomato line, which presented increased and repressed photosynthesis- and senescence-related genes, respectively (Zhou *et al.* 2019). Thus, the Chl and Car accumulation is presumably highly advantageous for the photosynthetic efficiency maintenance under drought at the epiphytic environment of dry forests as those where *A. strobilacea* is present, which is usually associated with a high light incidence due to the frequent tree defoliation (Cervantes *et al.* 2005).

Due to the threatening aspect of increased Chl formation over the oxidative status, cells are expected to engage an efficient antioxidant response so that Chl accumulation is advantageous during stress events. Our data shows an overall increase in antioxidant responses under droughts, which likely prevented photosynthetic damage or lipid peroxidation in *A. strobilacea* plants. Besides increased Car levels, as mentioned previously, both droughts lead to higher antioxidant enzyme activities than CT plants, although at similar levels between one and two drought exposures. The glutathione maintenance was also preserved due to the sustained redox ratio in D1 and D2 plants. While the first recovery period induced some GSH accumulation, this did not occur at the second rewatering, suggesting the pre-exposure to drought reduced this thiol's demand during a later recovery. The GSH has also been shown to increase after rewatering in wheat seedlings (*T. aestivum* cv. C306) exposed to severe water stress. However, contrarily to our findings, such increase was higher in plants pre-acclimated with mild stress than non-acclimated plants (Selote & Khanna-Chopra 2006). Furthermore, the higher levels of AA in the second drought might have also improved ROS maintenance, *e.g.*,

by stabilising antioxidant enzymes and promoting alternative detoxification pathways (Slama *et al.* 2015).

Our results indicate that the total SNO content increased in *A. strobilacea* after one and two droughts, suggesting a higher nitrosative stress condition than CT. However, the denitrosylase GSNOR activities showed a slightly more intense response at D2, suggesting the repeated exposure stimulated a higher defensive action against SNO accumulation (Corpas & Barroso 2013). The GSNOR can indirectly control S-nitrosation and total SNO content by processing GSNO, since it represents the largest fraction of low-molecular SNO in plant cells (Malik *et al.* 2011, Begara-Morales *et al.* 2018, Lindermayr 2018). The SNO and GSNOR were also shown to regulate several abiotic stress responses, most notably the control of redox homeostasis through the enzymatic antioxidant system (Begara-Morales *et al.* 2019, Li *et al.* 2021). Enzymes involved in Chl synthesis are also potentially regulated by S-nitrosation (Hu *et al.* 2015), as those of the AA metabolism. It was recently shown that the first enzyme of nitrogen assimilation into AA, glutamine synthetase, is negatively regulated by S-nitrosation (Silva *et al.* 2019). Furthermore, the SNO formation *per se* might be a stress defensive mechanism since it prevents the oxidation of critical cysteine residues in proteins to an irreversibly oxidised form (Begara-Morales *et al.* 2019). Research on the involvement of S-nitrosation in reiterated drought responses, as suggested by our results, is still lacking. Still, a recent report showed that the higher Pro accumulation resultant of drought hardening in wheat seedlings (*T. aestivum* Yangmai 16) induced by two subsequent periods of polyethylene glycol treatments was dependent on nitric oxide (Wang *et al.* 2021), whose bioactivity is exerted mainly through PTMs as S-nitrosation (Lindermayr 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 corroborates that double-stressed *A. strobilacea* plants showed more intense responses than single-stressed plants. It also clarifies that rewatered D1 and D2 plants show a distinct metabolic response compared to unstressed plants (control), but somewhat similar between them, supporting they were not completely restored to pre-stress conditions. This results from the AA and pigments, which remained higher than the control after rewatering. Such a 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 might assume 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 the Chl/Car/AA levels rather than engaging in energy-consuming degradation pathways. The GSNOR activity response is also indication of

memory, since higher activities were detected at the second drought despite complete restoration to the CT after the first rewatering (Jacques *et al.* 2021). The same might be said for the GSH, since this thiol was reduced at the second rewatering compared to the first, possibly due to previous drought exposure.

Few studies have discussed reiterated drought stress in epiphytes. Still, 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 four-month-old seedlings pre-treated with drought (irrigation per one or 1.5 weeks) for two months had increased growth and carbohydrate accumulation after exposition to severe drought (irrigation every three weeks for three months) than plants irrigated four times a week during the experiment. Therefore, it suggests that pre-conditioned plants would persist longer in the face of future droughts. Additional studies would also be required to assess how the drought priming effects described herein are regulated. These effects' regulation in other plants involve phytohormones such as gibberellin and abscisic acid (Fleta-Soriano *et al.* 2015), transcriptional regulation (Avramova 2019), epigenetic alterations such as DNA methylation (Sun *et al.* 2021), and post-translational modifications as protein phosphorylation (Zhang *et al.* 2020).

CONCLUSIONS

Our data confirms that juvenile *A. strobilacea* plants present stress memory responses, as indicated by the increase in pigments, free AA pools and GSNOR activities after two droughts, and the reduction in GSH after the second recovery. Based on these findings, we believe that further research about the regulatory mechanisms involved in S-nitrosation, Chl synthesis and antioxidant mechanisms during a reiterated drought might provide substantial information on the stress memory development in this epiphyte. The performance of experimental designs with longer rewatering periods are also necessary to clarify the duration of memory formation. Nevertheless, our results provide a solid starting point for in-depth investigations on drought memory in epiphytic bromeliads, and support that this mechanism is involved these plants' high tolerance.

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SUPPLEMENTARY MATERIAL

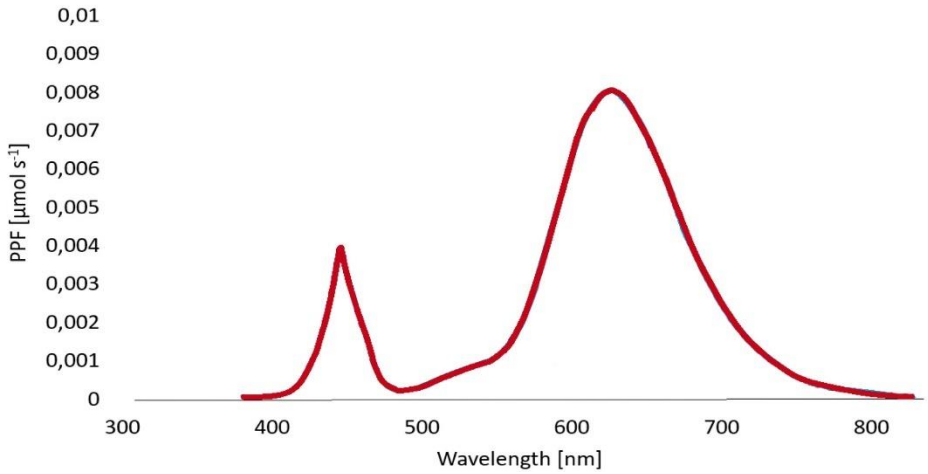

Figure S1. Light spectrum of 1200 C3 Full Espectro LED panels (InstalaFrio, Pinhais - Brazil) installed in the walk-in controlled environment chamber utilised in this study.

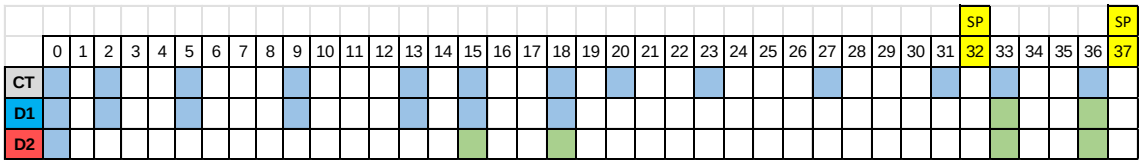

Figure S2. Irrigation and sampling schedule during the 37 days of experimentation. CT – control, D1 – single drought-recovery, D2 – double drought-recovery, SP – sampling point. Light blue cells indicate the irrigation days, green cells indicate the rewatering days in D1 and D2.

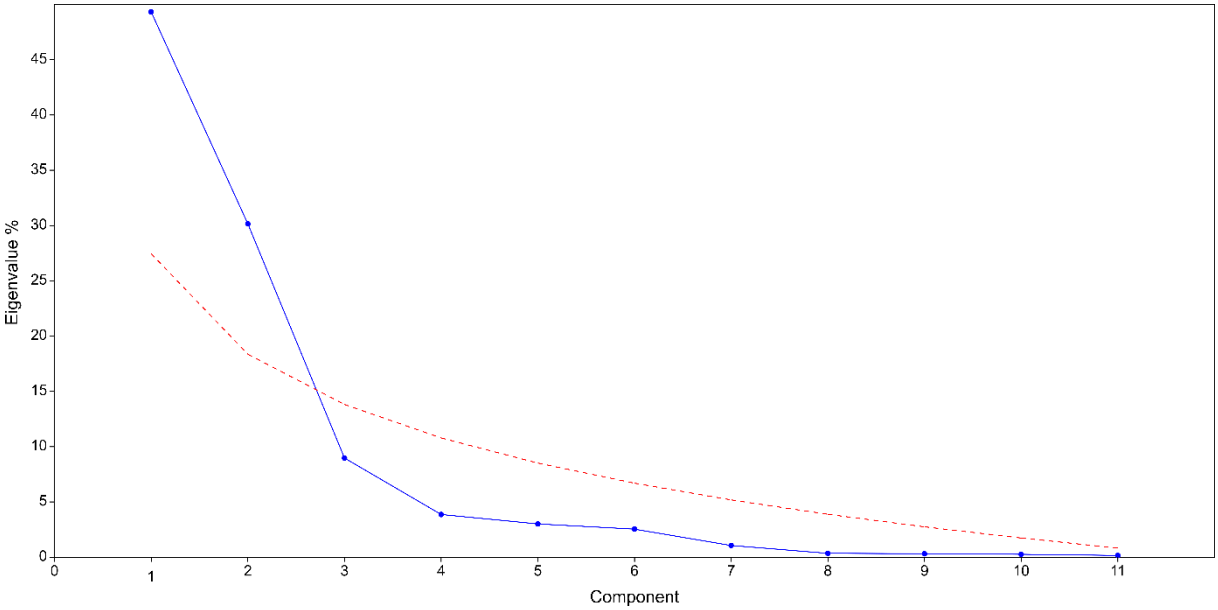

Figure S3. Broken-stick evaluation of the principal component analysis (PCA).

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 means ± s.e. calculated from six biological replicates. Different letters indicate significant differences between groups (one-way ANOVA and post-hoc Tukey's tests, $P < 0.05$). Ns, not significant.

Drought	Leaf number	Dead leaf number	Plant height
CT	8.33 ± 0.33 ns	1.33 ± 0.42 ns	24.13 ± 0.59 ns
D1	7.33 ± 0.42	2.00 ± 0.00	22.73 ± 0.84
D2	7.50 ± 0.32	2.17 ± 0.24	22.63 ± 1.11
Rewatering			
CT	8.50 ± 0.22 ns	1.83 ± 0.17 b	25.07 ± 0.90 ns
D1	8.17 ± 0.31	3.17 ± 0.17 a	25.70 ± 0.72
D2	8.33 ± 0.21	2.83 ± 0.17 a	24.87 ± 1.07

Table S2. Correlation values of variables to the significant components (PC1 and PC2), as calculated in the principal component analysis (PCA), with the Past3 software.

	PC1	PC2
RWC	-0.43	0.76
ΔH^+	0.62	0.47
Pro	0.76	0.62
AA	0.90	0.15
Chl	0.89	-0.22
APX	0.82	-0.17
GR	0.90	-0.32
SOD	0.42	0.77
GSH	0.17	0.84
SNO	0.83	0.04
GSNOR	0.49	-0.76

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CAPÍTULO 3

The water-saving strategy of juvenile epiphytic bromeliad involves leaf succulence, CAM activity and decreased aquaporin expression

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ABSTRACT

Epiphytic bromeliads are under frequent exposition to drought and rewatering cycles in the forest canopies since they are detached from the soil. These plants present adaptations and mechanisms of water preservation such as leaf succulence through a water storage parenchyma (hydrenchyma), crassulacean acid metabolism (CAM) and the regulation of membrane permeability through leaf aquaporins (AQPs) expression. Young epiphytes are susceptible to water loss due to high leaf surface/volume ratio; consequently, they are threatened by drought intensification resultant of climate change, which might affect the epiphytes population in the long-term. Thus, the assessment of drought-rewatering responses of juvenile epiphytes is crucial to determine their adaptability to climate change. The CAM epiphytic bromeliad *Acanthostachys strobilacea* presents drought tolerance from the early stages of development. Thus, we evaluated if a short-term drought and rewatering cycle over young *A. strobilacea* plants would induce changes to the CAM cycle, leaf succulence and AQP gene expression as to promote higher water conservation, in a fully reversible way. Under greenhouse conditions, three-month-old plants were kept for 14 days without irrigation then rewatered for one day. Plants underwent mild water losses, no oxidative stress or photochemical efficiency reductions, which possibly derived from (i) the apparent remobilization of water between the hydrenchyma and chlorenchyma, (ii) adjustments in stomatal behaviour, possibly linked to regulation of CAM intensity, and (iii) modulation in leaf AQP genes expression. All adjustments observed under drought were reversed to control levels after 24 hours of rewatering. Besides confirming our hypothesis, the data suggest that *A. strobilacea* presents a robust water status regulation at early ontogenetic stages. This will likely allow the endurance of this species to drought intensification from climate change.

Keywords: Bromeliaceae, drought recovery, leaf anatomy, membrane permeability, stomatal conductance, water deficit

INTRODUCTION

Epiphytic plants are those which germinate and grow over other plants (most commonly trees), without contacting the vasculature of the host (Benzing 2000, Zotz 2016). The Bromeliaceae is the second most numerous group of epiphytes, being important ecological components of Neotropical forests (Benzing 2000, Zotz 2016). These plants depend exclusively on atmospheric water; thus, they are exposed to intermittent water supply and, consequently, frequent droughts (Zotz 2016). Therefore, epiphytes present several adaptations to drought, aiming at the preservation of its water status. In the case of epiphytic bromeliads, there are those which present a "tank" formed by the overlap of its wide leaf bases to store rainwater, and those which do not but present high succulence and numerous water-absorbing trichomes in the leaves, categorized as "atmospheric" bromeliads (Benzing 2000, Males 2016). Anatomically, many epiphytic bromeliads show "storage succulence", in which the mesophyll presents an adaxial layer of water storage parenchyma (the hydrenchyma), which consists in cells that do not perform photosynthesis but serve as buffers of water under drought situations (Males 2016, Herrera 2020). Indeed, the water from the hydrenchyma might be distributed to the chlorenchyma cells under drought as to preserve photosynthetic activity (Lüttge 2004, Males 2016).

The photosynthetic mode crassulacean acid metabolism (CAM) is widely present among epiphytic bromeliads (Crayn *et al.* 2015) and promotes high water use efficiency due to the diurnal stomatal closure (Matiz *et al.* 2013). The diel cycle of CAM is adjustable to the environmental conditions and plant development as to optimize water conservation (Cushman & Bohnert 1999, Dodd *et al.* 2002). Consequently, two alternate types of CAM have been described (CAM cycling and idling), which differ mainly in stomatal behaviour and source of CO_2 for malate synthesis (Lüttge 2004, Winter 2019). Another important strategy under drought conditions is the regulation of membrane permeability to maintain turgor pressure in leaves. Aquaporins (AQPs), intrinsic proteins of plasma and intracellular membranes, are important players in such process, aiding in the extravascular water transport of inner leaf tissues (Tyerman *et al.* 1999, Chaumont & Tyerman 2014, Maurel *et al.* 2015, Maurel & Prado 2017). The plasma membrane intrinsic proteins (PIPs) are the major AQP subfamily in plants and are mostly located in the plasma membrane of organs under high fluxes of water (Kapilan *et al.* 2018). The tonoplast intrinsic proteins (TIPs) are the second largest subfamily of plant AQPs and are highly abundant in the vacuole, providing it with high permeability that facilitates

osmotic balance with the cytosol, regulating cell turgor (Maurel *et al.* 2008, Kapilan *et al.* 2018, Kurowska 2021a). Many studies with agricultural and model species have shown that leaf AQP expression and abundance is differently regulated to drought, possibly because of the high diversity of AQP in plants (see review by Shivaraj *et al.* 2021). Nevertheless, a common result is the downregulation of leaf AQP expression as to reduce membrane permeability and leaf hydraulic conductance, leading to higher water retention in the cells and vacuoles to prevent further water losses under drought (Alexandersson *et al.* 2005, Maurel *et al.* 2015, Afzal *et al.* 2016, Maurel & Prado 2017, Sade & Moshelion 2017, Kurowska 2021a, b). AQPs are also important for facilitating water transport during rewatering after drought (Ohrui *et al.* 2007, Galmés *et al.* 2007, Laur & Hacke 2014, Grondin *et al.* 2016, Secchi *et al.* 2017). Reports on the effects of drought-rewatering in AQP expression in epiphytic bromeliads show a downregulation trend on the expression of *PIP*s during drought and a subsequent recovery after brief rewatering periods, as detected for leaves of the tank bromeliad *Guzmania monostachia* (L.) Rusby ex. Mez (North *et al.* 2019) and of the atmospheric bromeliad *Tillandsia ionantha* Planch. (Ohrui *et al.* 2007). Besides AQP gene expression regulation, water reabsorption after drought in the epiphytic bromeliads *G. monostachia, T. ionantha* and an *Aechmea* hybrid were shown to involve CAM and antioxidant activity modulation (Ohrui *et al.* 2007, Ceusters *et al.* 2009, Freschi *et al.* 2010, Carvalho *et al.* 2017). Further studies are necessary to detail the mechanisms involved in recovery of epiphytic bromeliads after drought, which is particularly relevant due to the erratic water availability of theses species' habitat.

It is known that young epiphytes are susceptible to water loss because they have a high leaf surface/volume ratio (Schmidt *et al.* 2001). Thus, the predicted drought intensification and frequency resultant of climate change (Trenberth *et al.* 2014) might be a threat to young plants and population of epiphytes in the long-term. This makes the research on drought-rewatering responses of juvenile epiphytes important for the assessment of their resilience to climate change and the species permanence in tropical forests (Cach-Pérez *et al.* 2014).

The epiphytic bromeliad *Acanthostachys strobilacea* (Schult. & Schult.f.) Klotzsch (Bromelioideae, Bromeliaceae) is a tankless bromeliad, presenting long (1 m) and succulent leaves with a substantial hydrenchyma (Proença & Sajo 2007, Eggli 2020). *A. strobilacea* is widely distributed in humid and dry forests of South America (Govaerts *et al.* 2020, Monteiro 2020), encountered in epiphytic or saxicolous habits (Versieux & Wendt 2006). This species shows numerous drought adaptations and thus, is suitable for studies evaluating drought tolerance and rewatering in juvenile epiphytic bromeliads. Indeed, 90-day-old plants of *A. strobilacea* were shown to activate the antioxidant system and osmotic adjustment under short-term droughts (Menezes *et al.* 2020, Carvalho *et al.* 2021). These plants also presented

nocturnal acid accumulation regardless of watering conditions, indicative of CAM activity (Menezes *et al.* 2020). However, adjustments in stomatal conductance and net photosynthetic rates to drought and rewatering are not known, as well as the anatomical features and AQP gene expression. Thus, we evaluated if a short-term drought and rewatering cycle over juvenile *A. strobilacea* plants would induce changes to the diurnal CAM cycle, leaf succulence and AQP gene expression as to promote higher water conservation, in a fully reversible way.

MATERIALS AND METHODS

Plant material and treatment

Seeds of *A. strobilacea* (record number A0A27A1 at the Brazilian Genetic Diversity bank - SisGen, *Ministério do Meio Ambiente*) were harvested from the plant collection of *Instituto de Pesquisas Ambientais*, originated from the natural population located at the biological reserve in Mogi Guaçu, São Paulo, Brazil (22°15′04.2″S and 47°09′56.8″W). Seeds were cultivated *in vitro* following Carvalho *et al.* (2014) and maintained 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} s^{-1} provided by white tubular 6500K LED lamps. Subsequently, plants were transferred to 50 mL polystyrene centrifuge tubes with a punctured bottom, containing the same volume of vermiculite and small gravels at the bottom for drainage. Plants were kept at the same culture room, being watered with distilled water at 70% of field capacity (FC) plus the spraying of leaves, twice a week. After 60 days, the tubes containing the plants were transferred to a greenhouse. At that site, plants were watered at 60% FC three times a week and kept at these conditions for 20 more days, prior to the onset of experiment. During all growth, fertilization was performed weekly at the same watering levels with 1 g L^{-1} of a commercial fertilizer of 20:20:20% composition (total nitrogen:phosphorous:potassium; Plant-Prod, Canada).

For the drought treatment (D), water was withheld for 14 days. For the rewatering treatment (R), plants under drought were rewatered at 60% FC at the 15^{th} day of drought. Control plants (C) were watered at 60% FC three times a week during the experiment. The harvesting and analyses took place on the day after the post-drought irrigation of rewatered plants, and the 14^{th} day of drought. The assessment of acidity and osmotic potential was performed with four biological replicates, while AQP gene expression was made with three. Each replicate contained leaves of three plants, which were frozen in liquid nitrogen and kept at -80°C. The gas exchange, chlorophyll fluorescence and water potential analyses were performed with five distinct individuals. The anatomical survey and ROS staining was performed in three biological replicates, containing leaves of one individual each.

Environmental data

The daily data for temperature, relative humidity (RH), evaporation and global irradiation during the experiment was obtained from the Weather Station of the Institute of Astronomy, Geophysics and Atmospheric Science of the University of São Paulo (IAG, USP; 23,6512°S, 46,6224°W), located approximately 1.2 km from the greenhouse in which the experiment was performed (figure S1). The temperature and RH values were also acquired during 24 hours at the day of gas exchange analyses (figure S2). Additional measurements inside the greenhouse were made during that same day using a thermohygrometer (model 00325; AcuRite, USA) placed close the plants (figure S2).

Water relations

Substrate water content (SWC) was assessed from the vermiculite content of six individual tubes from each treatment (Carvalho *et al.* 2021). The relative water content (RWC) was performed with leaf sections of circa 5 cm in length obtained at the midday and processed following Fleta-Soriano *et al.* (2015). Water potential (Ψw) was measured using one fully expanded leaf per plant with a Scholander pressure pump (model 1000; PMS Instrument Co., USA). Osmotic potential (Ψs) analyses were made with leaf sap extracted from leaf samples frozen at -80°C with a vapor pressure osmometer (VAPRO model 5520; Wescor Inc., USA), following Rigui *et al.* (2019). Both Ψw and Ψs were assessed at three time points through the daytime: morning (*ca.* two hours after dawn), midday (*ca.* seven hours after dawn) and afternoon (*ca.* two hours prior to dusk).

Titratable acidity

Nocturnal acid accumulation, indicative of CAM activity, was assessed by evaluating the titratable acidity from samples obtained at morning, midday and afternoon. Aliquots of 100 mg of frozen powdered leaves were homogenized in 1.5 mL of boiled ultrapure water. Extracts were maintained in a boiling water bath for 15 min and centrifuged at 11000 g for 15 min. The supernatants volume was completed to 10 mL with boiled ultrapure water and 10 µL of the pH indicator phenolphthalein 2% (w/v) was added per sample. Titration was performed with 0.02 N NaOH until solutions developed a pink color (*i.e.*, pH 9.0). The acidity of the sample was calculated from the utilized volume of NaOH and expressed as mmol H^+ g^{-1} dry weight. The ΔH^+ (nocturnal acid accumulation) was obtained by subtracting the afternoon from the morning values per replicate (adapted from Freschi *et al.* 2010).

Anatomical survey

For the anatomical analyses, one leaf from each plant was harvested at midday, comprising three replicates per treatment. The C and R samples were fixed in paraformaldehyde 4% (w/v) and dehydrated through an ethanol series (10 - 70%), while D samples were fixed in ethanol 100% to avoid rehydration. After fixation, samples were dehydrated in an n-butyl alcohol series, embedded in (2-hydroxyethyl)-methacrylate (Leica Historesin Embedding Kit, Leica, Germany) (Gerrits & Smid 1983), and sectioned at 5 μm thick on a rotary microtome (RM 2155, Leica) for light microscopy analyses. Sections were stained with periodic acid–Schiff's reagent (PAS) and toluidine blue and mounted on slides using Entellan (Merck, Germany). The images were obtained with a microscope (Zeiss Axioskop 40 HBO 50, Zeiss, Germany) and software AxioVision (Version 4.8.2.0).

Gas exchange and chlorophyll fluorescence

Gas exchange analyses were made with a portable infra-red gas analyser system (LI-6800; Li-Cor Inc., USA) equipped with a 6 cm^2 aperture chamber, at four periods during the daytime (07:00, 10:00, 14:00, 17:00) and one at the early night (20:00), on the longest expanded leaf of each plant. Leaf temperature was set to 25°C and RH to 60% on all analyses. The PFD levels were adjusted according to measurements made at each period with a luxmeter on the same site plants were kept at the greenhouse, after conversion using the factor of 0.0185 μmol m^{-2} s^{-1} per lux valid for sunlight (Apogee Instruments, USA) (figure 5a). The fraction of red and blue light was kept under default settings (90% red with a maximum blue light of 40 μmol m^{-2} s^{-1}). Adjustments of leaf-to-air vapor pressure deficit, CO_2 concentration, flow rate, fan speed and overpressure of the LI-6800 were kept at 1.7 kPa, 400 μmol mol^{-1}, 500 mL min^{-1}, 10,000 rpm, and 0.1 kPa, respectively. Data were recorded after a steady state of photosynthetic assimilation rate (A) was reached. Each round of measurements consisted of one replicate per treatment, finishing all the five replicates after approximately one hour. Besides the rates of A, stomatal conductance (gs) and transpiration (E), the ratio of intercellular to ambient CO_2 concentration (Ci/Ca) and instantaneous carboxylation rate (A/Ci) were also estimated (Zhou & Han 2005, Hsu *et al.* 2015). The raw data was adjusted to the leaf area that occupied the chamber, which comprised on average 2.62 ± 0.08 cm^2 (mean ± s.e., n=15).

The chlorophyll fluorescence analyses were performed on the longest expanded leaf of each plant with the fluorometer attached to LI-6800. Dark fluorescence data was obtained at approximately 13:00, in leaves previously kept for 30 min wrapped in aluminium foil for dark adaptation. Light fluorescence data was taken simultaneously to the gas exchange analyses performed at 14:00, by application of a saturating PFD pulse of 3000 μmol m^{-2} s^{-1}. The

evaluated parameters, as provided by the equipment, included the maximum quantum efficiency of PSII photochemistry (Fv/Fm) and electron transport rate (ETR) (Maxwell & Johnson 2000).

Histochemical ROS staining

Leaf sections of approximately 5 cm in length were obtained from separate plants at midday for each treatment and utilized for histochemical staining of H_2O_2 or superoxide anion ($^{\bullet}O_2^-$), following Kumar *et al.* (2014) with modifications as follows. In 5 mL cryogenic tubes covered in aluminium foil, leaves were completely submerged in solution containing 1 mg mL^{-1} of 3,3′-diaminobenzidine (DAB) or 0.2% (w/v) of nitrobluetetrazolium chloride (NBT) in 50 mM sodium phosphate buffer (pH 7.5) for H_2O_2 and $^{\bullet}O_2^-$ staining, respectively. After approximately 24 h, leaves were bleached as described in Daudi & O'Brien (2012) and digitalized using a white background in a scanner at 400 dpi.

AQP gene expression

The total RNA extraction was performed in aliquots of 100 mg of pulverized frozen leaves with the Invitrap Spin Plant RNA Kit (Stratec, Germany), utilizing the RP solution following the manufacturer's instructions, with the inclusion of a 3 min incubation at 55°C. The extracted RNA was quantified in a nanophotometer (Pearl; IMPLEN, Germany) and its integrity was estimated by electrophoresis in 1% agarose gel in TAE 1X buffer. Subsequently, the samples were treated with DNase I Solution (ThermoFisher Scientific, USA) for the removal of genomic DNA. The reverse transcription to cDNA was performed with the RevertAid H Minus First Strand cDNA Synthesis Kit (ThermoFisher Scientific) using oligo $(dT)_{18}$ as primers, following the manufacturer's instructions. Gene expression was assessed using real-time quantitative PCR (RT-qPCR) with the master mix Maxima SYBR Green qPCR (2X) (ThermoFisher Scientific) in a Mastercycler® ep realplex 2S thermocycler (Eppendorf, Hamburg, Germany), according to the kit's protocol. The reaction conditions were as follows: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C and 1 min at 60°C. The amplification specificity of the primers was confirmed by performing melting curves at the end of the qPCRs, in which the temperature was gradually increased from 55°C to 95°C for 20 min.

The primer pairs utilized in this study were the same as those designed for the epiphytic bromeliad *G. monostachia* (Table S1), based on sequences obtained through transcriptomic analyses with the same species (Pereira 2016). The primer efficiencies for the leaves of *A. strobilacea* were determined by means of a standard curve obtained with an equimolar mixture of all cDNA samples (Table S1). The reference genes stability was assessed with the

BestKeeper worksheet (Pfaffl *et al.* 2004). The qPCRs were performed in technical duplicates and biological triplicates. Gene expression was calculated as described by Vandesompele *et al.* (2002), in which transcript abundance for each gene of interest was normalized against reference genes *FB293* and *IF2A* and expressed as relative values to the control (well-watered conditions). The final means are presented in a log2 basis.

Statistical analyses

The experimental design was completely randomised. The means of each treatment were subjected to a one-way analysis of variance (ANOVA) and post-hoc Tukey's tests ($P < 0.05$) using the GraphPad Prism 5.01 software.

RESULTS

Water status

The 14 days of drought caused an 83% decrease in the SWC (figure 1a), representative of 6% of the vermiculite's FC. Despite the low water availability, the leaf RWC was significantly reduced only by 18% after drought, reaching 78% (figure 1b). The SWC and RWC were fully restored 24 hours after rewatering.

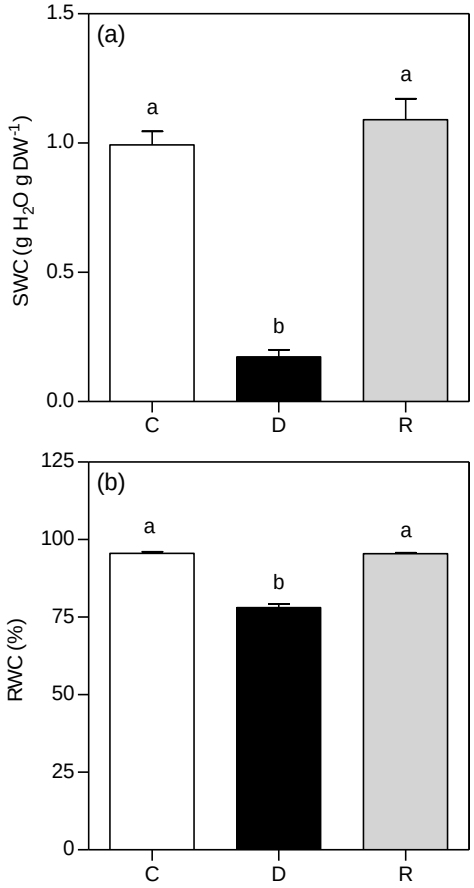

Figure 1. (a) Substrate water content and (b) leaf relative water content of *A. strobilacea* plants exposed to control (well-watered), drought and rewatered conditions. Values are the means ± s.e. (n = 4). Different letters indicate significant differences between groups according to one-way ANOVA and post-hoc Tukey's tests ($P < 0.05$). C, control; D, drought; R, rewatering.

The Ψw was two times lower in D than in C plants, on all periods of the day, reaching an average of -1.0 MPa in the former (figure 2a). The Ψs was distinct between treatments only in the morning, being 14% lower in D (-1.2 MPa) compared to C plants (-1.0 MPa) (figure 2b). As similar to Ψw, the Ψs was increased from the morning to the afternoon in all treatments, but more intensively in D plants. In fact, by midday onwards, the Ψs in D plants reached equivalent levels of C and R plants.

The pattern of acidity during the day and the positive values of ΔH^+ indicates CAM activity in plants of all conditions (figure 2c, d). The acids pool was overall exhausted by midday under all conditions, as it was sustained at similar levels by the afternoon. However, statistics indicated that the level of acidity during the day and the ΔH^+ was not significantly different between conditions.

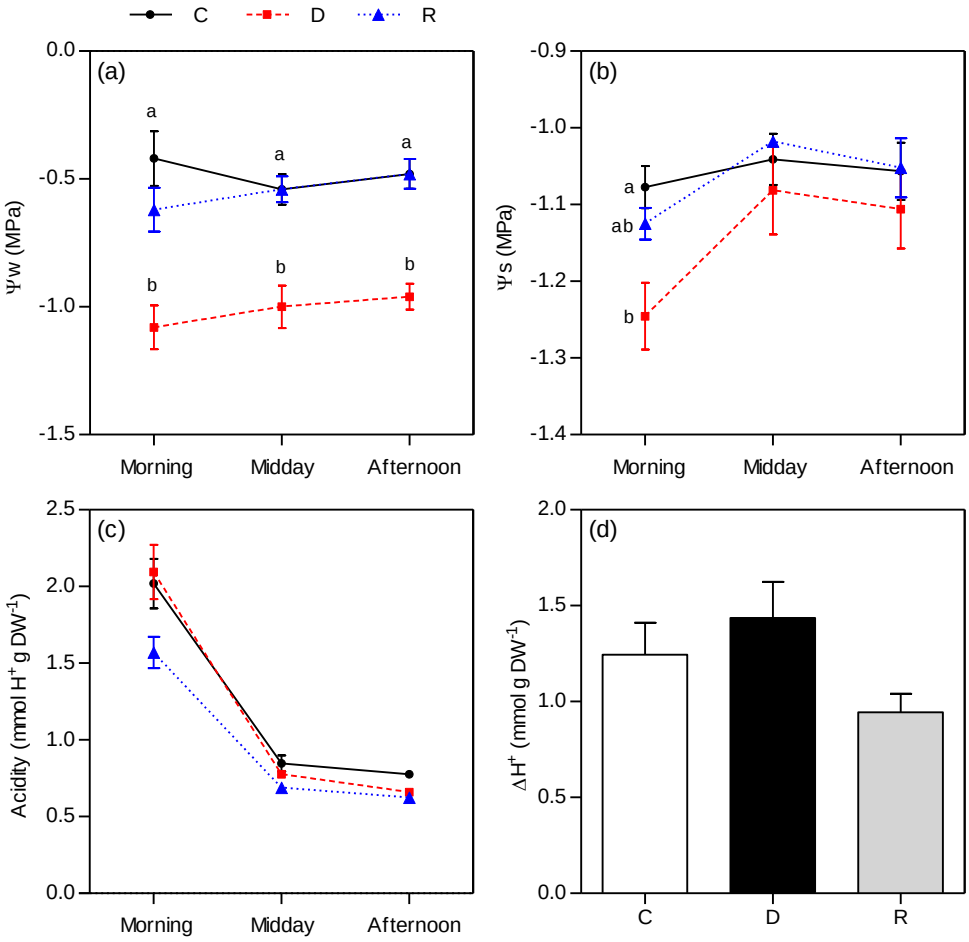

Figure 2. (a) Water potential, (b) osmotic potential and (c) acidity levels during the day and (d) nocturnal acid accumulation in leaves of *A. strobilacea* plants exposed to control (well-watered), drought and rewatered conditions. Values are the means ± s.e. (n = 5 for a; n = 4 for b-d). Different letters indicate significant differences between groups according to one-way ANOVA and post-hoc Tukey's tests ($P < 0.05$). C, control; D, drought; R, rewatering.

Chlorophyll fluorescence and ROS

The chlorophyll fluorescence parameters Fv/Fm and ETR showed no significant differences between C, D and R plants (figure 3a). Indeed, the Fv/Fm was sustained at high levels, suggesting the photosynthetic apparatus was preserved despite water stress. Accordingly, the DAB and NBT staining referent to H_2O_2 and $^{\bullet}O_2^-$ presence, respectively, were not intensified after drought (figure 3b). There was, however, a more intense DAB staining in the median area of the leaf segment of rewatered plants, while no alteration occurred in NBT stained samples.

(a)

	Fv/Fm	ETR
Control	0.78 ± 0.02 ns	24.07 ± 0.90 ns
Drought	0.80 ± 0.03	20.31 ± 1.09
Rewatering	0.77 ± 0.02	20.91 ± 1.15

(b)

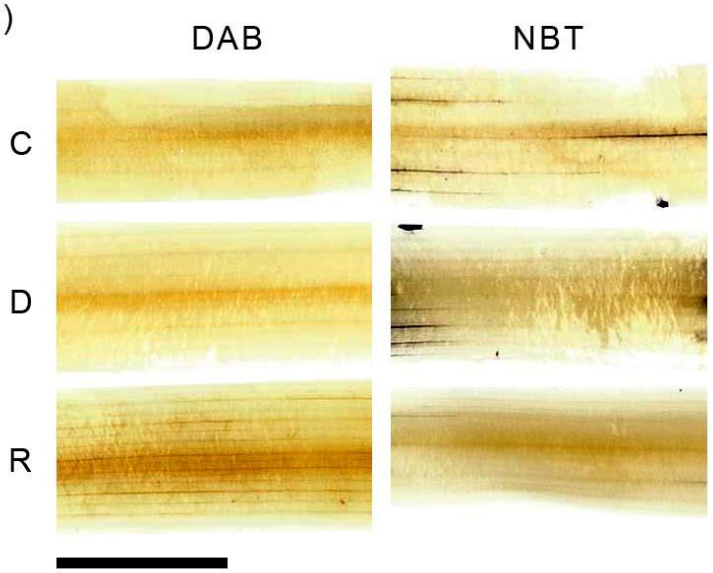

Figure 3. (a) Chlorophyll fluorescence parameters and (b) histochemical staining of H_2O_2 and $^{\bullet}O_2^-$ in leaves of *A. strobilacea* plants exposed to control (well-watered), drought and rewatered conditions. Values in (a) are the means ± s.e. (n = 5). Non significance between groups in (a) was indicated by one-way ANOVA ($P < 0.05$). Scale bar: 1 cm. ETR, electron transport rate; DAB, 3,3′-diaminobenzidine; NBT, nitrobluetetrazolium chloride; C, control; D, drought; R, rewatering.

Anatomical traits of leaves

Overall, the *A. strobilacea* leaves present tissues organized in epidermis, heterogeneous mesophyll, and vascular system (figure 4a, b). The epidermis is unistratified with rectangular cells and thin walls, except for the external periclinal wall, which is slightly thickened (figure 4c-g); the cells on the adaxial face are larger than those of the abaxial face (figure 4c-d, f). The leaf margin cells are larger with papillae (figure 4d-arrow, i). Tetracytic stomata predominate in the abaxial face – eventually on the adaxial face, near the leaf margin – and exhibit large substomatal chambers (figure 4c-f). Uniseriated glandular trichomes (figure 4h) may occur on both sides of the leaf blade and the margin; scales (peltate trichomes) were not observed. The mesophyll is divided in a hypodermis and chlorenchyma on the adaxial and abaxial face, respectively. The hypodermis contains the water storage tissue (hydrenchyma), with large cells and thin walls (figure 4a, c, j). In the central region of the leaf, this tissue contains three layers of cells (figure 4c), reducing to one by the leaf's margin (figure 4d). Contrarily, the

chlorenchyma is formed by smaller, rounded cells (figure 4c-f). Idioblasts containing raphides and polysaccharides (identified with the PAS reagent) were detected in the chlorenchyma and hydrenchyma of all treatments (figure 4c, e-g, i-j). The vascular bundles are collateral and occur in a single row along the lamina (figure 4a-b), surrounded by chlorenchyma cells (figure 4c-f). Starch grains were observed in the chlorenchyma cells of all treatments (figure 4c-f).

Structurally, no changes were observed between C, D and R plants. The chlorenchyma cells were visually unaltered by drought or rewatering. However, drought did induce alterations in epidermal and hydrenchyma tissues. In D leaves, the epidermal cells of the adaxial face showed sinuosities in the internal anticline and periclinal walls (figure 4e, g), not observed in C leaves. The sinuosities observed in the cell walls of the hydrenchyma in C leaves, especially in the central region (figure 4c), are mostly caused by the processing of samples and fragility of the leaves. Still, the signs of dehydration are evident in the hydrenchyma of D plants, as the cells become collapsed and with more prominent sinuosities than the control (figure 4e, g); this promotes a twisted aspect in the leaves, with more ripples in the contour of the blade, which acquires a v-shape in the cross-section (figure 4b). After rehydration, the cells of the hydrenchyma become turgid again, in similarity to the control (figure 4f).

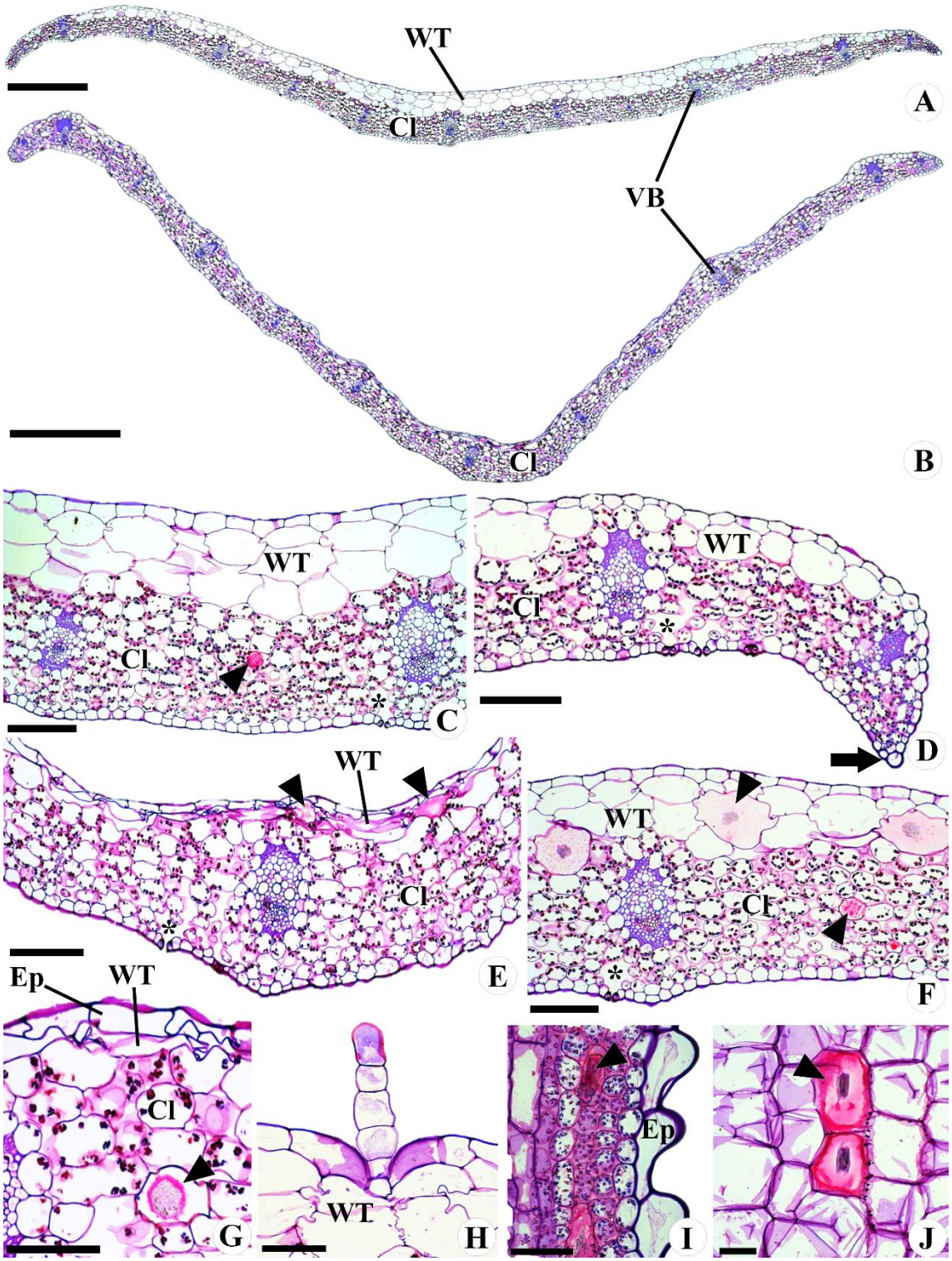

Figure 4. Anatomical aspects of leaves of *A. strobilacea* plants exposed to control (a, c-d, h), drought (b, e, g) and rewatered (f, i-j) conditions, in cross (a-h) and longitudinal (i-j) sections. (a-b) General aspects of the leaf blade. (c,e) Central region of the leaf blade. (d) Leaf margin. (f) Region close to the leaf margin. (g) Detail in a region close to the leaf margin. (h) Glandular trichome on the adaxial surface. (i) Detail of the leaf margin showing the epidermis and chlorenchyma. (j) Detail of the water-storage tissue with idioblasts. (Arrow, epidermal cell on leaf margin; Arrowheads, idioblasts; Ep, epidermis; Cl, chlorenchyma; VB, vascular bundle;

WT, water-storage tissue; *, substomatal chamber). Scale bars: a-b (500 µm), c-f (100 µm), g-j (50 µm).

Gas exchange

The CO_2 assimilation rate (A) observed during the day in C plants mostly followed the PFD evolution (figure 5a, b), in which the former was largely undetected at early morning (07:00) but peaked at 10:00, reaching 0.26 µmol CO_2 m^{-2} s^{-1}. Subsequently, A progressively decreased, reaching negligible values at the night period. The gs and E rates showed similar daily progressions in C plants, being the highest at early morning and decreasing during the day, until reaching a minimum at night (figure 5c, d). The Ci/Ca ratio in C plants showed a depression between 10:00-17:00, increasing again at 20:00 to approximate values to those detected at 07:00, but always at values lower than 1 (*i.e.*, there was always less CO_2 inside the leaves than in the atmosphere; figure 5e). Meanwhile, the A/Ci ratio in C plants followed the same pattern as A (figure 5f).

The gas exchange pattern of D plants was significantly altered compared to the well-watered C plants. Due to drought, the CO_2 assimilation was mostly negligible during the light period, being slightly increased by the early night (figure 5b). Accordingly, the stomatal-related parameters gs and E in D plants were close to zero during the day but reached 1.94 mmol H_2O m^{-2} s^{-1} and 23.93 µmol H_2O m^{-2} s^{-1}, respectively, by 20:00, being statistically higher than the C values (figure 5c, d). The diurnal stomatal closure observed in D plants reflected in a progressive and significant increase in Ci/Ca ratio (figure 5e), peaking at 17:00 when mean values were nearly 4-fold higher than the control. By night, when stomatal aperture and transpiration were increased in D plants, the Ci/Ca decreased again. The A/Ci ratio in D plants followed the same pattern as A (figure 5f). After rewatering of D plants, all gas exchange parameters were mostly restored to C levels.

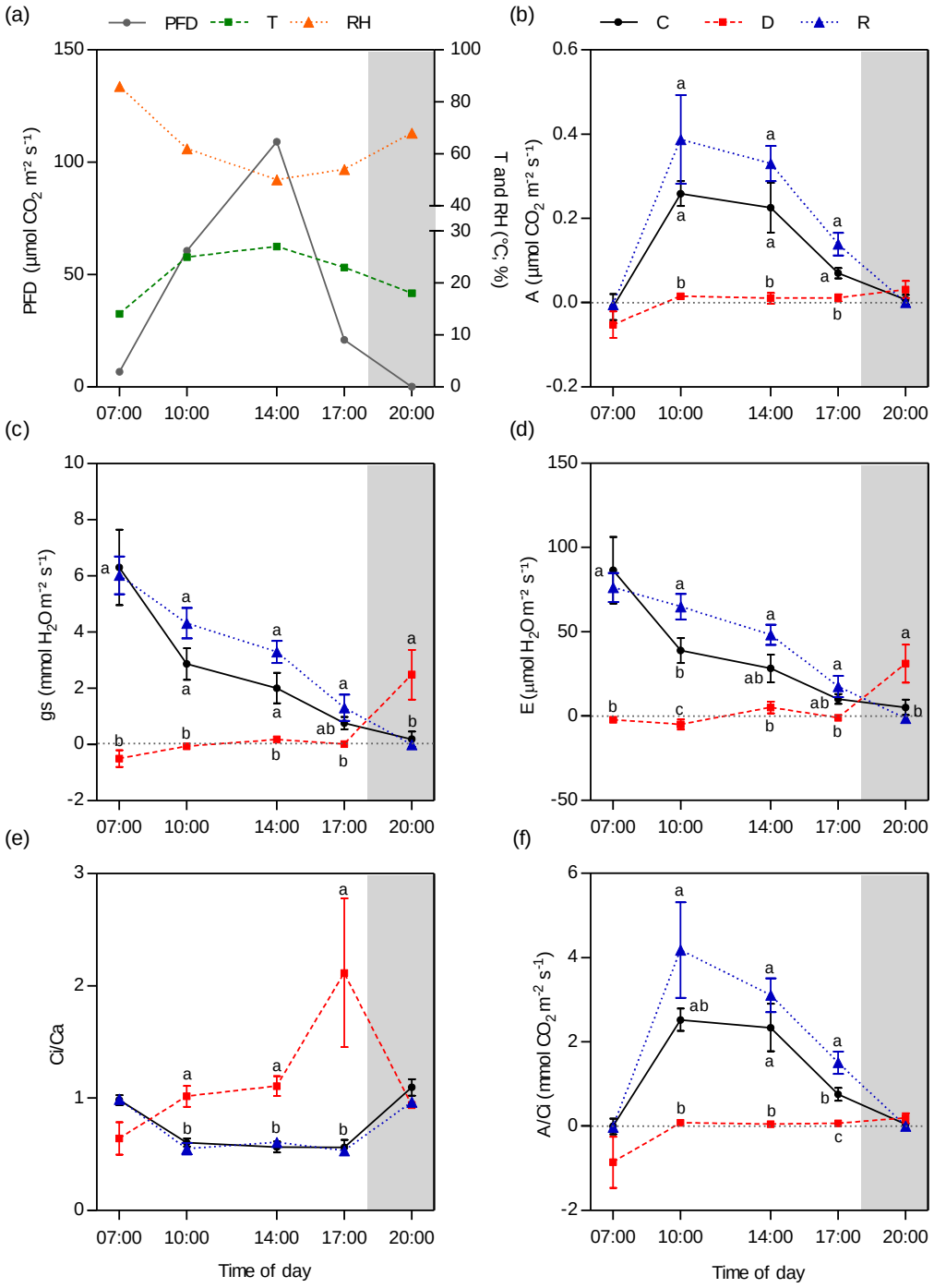

Figure 5. Experiment day environmental conditions (a) and gas exchange parameters (b-f) in plants of *A. strobilacea* exposed to control (well-watered), drought and rewatered conditions. Values in (b-f) are the means ± s.e. (n = 5). Different letters indicate significant differences between groups per time of day according to one-way ANOVA and post-hoc Tukey's tests ($P < 0.05$). C, control; D, drought; R, rewatering; PFD, photon flux density; T, temperature; RH, relative humidity; A, CO_2 assimilation; gs, stomatal conductance; E, transpiration; Ci/Ca, ratio of intercellular to ambient CO_2 concentration; A/Ci, instantaneous carboxylation rate.

AQPs relative gene expression

Among the *PIP*s, *PIP1;1* and *PIP1;2b* expression was significantly diminished after drought by a 2.2 and 1.4-fold rate relative to the control, respectively (figure 6). The rewatering restored these genes expression values to pre-stress conditions. A similar pattern occurred at a smaller proportion for *PIP1;2a*, although it was not statistically significant. The *TIP4;4* expression was diminished in both D and R plants by a 0.2-fold rate, but without statistical significance. Such reduction in expression also occurred for *TIP2;1* after drought but at the significant rate of 3.2-fold. The rewatering restored the *TIP2;1* expression only partially to the control, which remained statistically distinct and 1.1-fold smaller. The *NIP2;2* expression was also significantly reduced by 2.5-fold after drought. The subsequent rewatering, however, increased *NIP2;2* expression to levels significantly higher than the control by a 0.6-fold rate. Although *NIP5;1* expression was overall reduced after drought and rewatering by 0.4 and 0.9-fold, respectively, statistics indicated it was not significant.

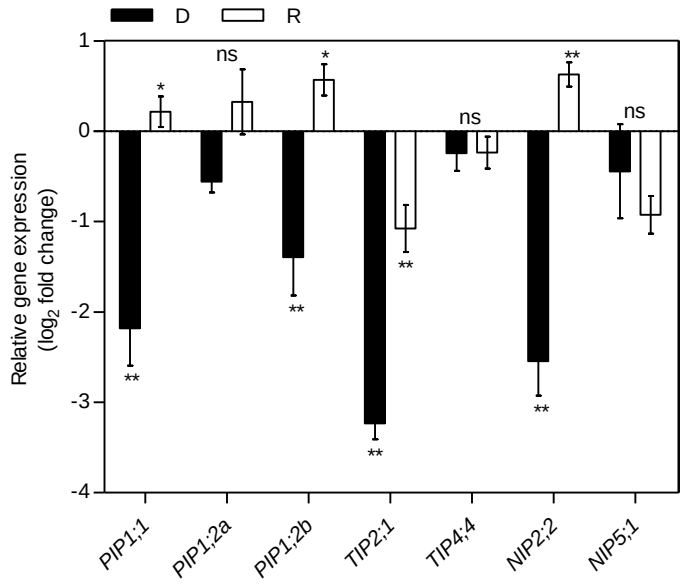

Figure 6. Relative gene expression of *PIP1;1*, *PIP1;2a*, *PIP1;2b*, *TIP 2;1*, *TIP4;4*, *NIP2;2* and *NIP5;1* in leaves of *A. strobilacea* plants exposed to drought and rewatered conditions. Values are means ± s.e. (n = 3). The mean relative expression was normalized against control (well-watered) conditions. Bars with one asterisk show difference from the remaining treatment but not to the control; two asterisks indicate groups which differ from all others according to one-way ANOVA and post-hoc Tukey's tests ($P < 0.05$). D, drought; R, rewatering.

DISCUSSION

The exposure of juvenile plants of *A. strobilacea* to a mild drought was sufficient to cause significant changes to CAM activity, which was fully restored to pre-stress conditions

after only one day of rewatering. The anatomical features and adjustments in AQPs expression also support the high tolerance to drought and rewatering in plants. Through this section, we will elaborate on the role that these characteristics and responses might play under the context of the species' natural habitat.

Although the 14-day drought caused substantial decreases in SWC, water status parameters were mildly reduced, suggesting severe water losses were prevented during that period in juvenile *A. strobilacea*. Previous studies by the group have also shown a similar maintenance in water status to short and middle-term droughts in similar plants (Menezes *et al.* 2020, Carvalho *et al.* 2021). Indeed, the high values of Ψw and Ψs (minimum of -1.0 and -1.2 MPa, respectively) observed herein under drought are commonly found in epiphytic bromeliads even under prolonged water shortage (Martin 1994, Stiles & Martin 1996). It is said that such response is due to the low gas exchange rates and productivity of epiphytes and the presence of significant portion of water storage tissues (Martin *et al.* 2004, Ogburn & Edwards 2012), which is confirmed by the present gas exchange and anatomical analyses. All water status parameters were restored to control levels after the brief rewatering period, supporting the rapid restorative capacity in cells of *A. strobilacea*.

The maintenance of water status observed in juvenile *A. strobilacea* in drought conditions might be partly due to CAM activity. In fact, these plants showed a high level of nocturnal acid accumulation regardless of water conditions, comparable to strong CAM plants (an average ΔH^+ of 122 µmol g FW^{-1} in D plants when converted to a fresh weight (FW) basis, for comparison purposes; Martin 1994, Heyduk *et al.* 2018). The daily consumption of acids in CAM plants are paralleled by an increase in Ψs since they are osmotically active molecules (Smith *et al.* 1985, Lüttge 2004). This pattern occurred more intensively in D plants, although the daily acid oscillation was similar between water conditions. Therefore, it is assumed that the lowest Ψs in D leaves in the morning reflects their lower water content, which made the cell sap more concentrated and caused a steeper daily decrease in Ψs than in well-watered (C and R) plants. This is possibly caused by the absence of Ψs diel oscillation in the hydrenchyma due to the lack of CO_2 fixation in these cells (Smith *et al.* 1987, Goldstein *et al.* 1991, Herrera 2020). In addition, the daily oscillation in Ψw was much milder and stable than that of Ψs, especially in D plants, which has been previously reported for CAM plants and proposed to be caused by a diurnal decrease in pressure potential (Lüttge & Nobel 1984, Ting 1985). These data associated to the gas exchange analyses support the presence of CAM in these plants.

The anatomical analyses show that juvenile *A. strobilacea* leaves present storage succulence (*i.e.*, they present hydrenchyma cells), which is common to bromeliads (Males 2016, Herrera 2020), including in the juvenile stage (Beltrán *et al.* 2013, Rodrigues *et al.* 2016). When

under drought, such cells collapsed in *A. strobilacea*, but the chlorenchyma remained roughly unchanged, which might explain the mild reduction in water status due to drought. The water from the hydrenchyma might have been partly redirected for the chlorenchyma to preserve its turgor and photosynthetic activity, which is a known drought-tolerance strategy in this type of succulent plants (Goldstein *et al.* 1991, Nowak & Martin 1997, Nobel 2006, Males 2016). The water transfer from the hydrenchyma allows the photosynthetic activity of the chlorenchyma to be preserved for longer periods under drought, preventing drought stress effects (Goldstein *et al.* 1991, Nowak & Martin 1997, Nobel 2006, Males 2017). Indeed, no significant damage to the photosynthetic apparatus or excessive ROS after drought was indicated by chlorophyll fluorescence and DAB and NBT staining analyses. The restoration of the hydrenchyma cells volume after 24 hours of rewatering suggest that its cell walls present high elasticity and capacitance (Males 2016). A similar rapid hydrenchyma restoration has been observed previously for the cactus *Opuntia ficus-indica* L. Miller (Scalisi *et al.* 2016). This characteristic is what allows the maintenance of high hydric potentials despite drought (as observed in this study), and the passive transfer of water to the neighbouring chlorenchyma (Ogburn & Edwards 2012). Trichomes, which are cells that perform water and nutrient uptake from atmospheric sources, are abundant in leaves of juvenile plants from several epiphytic bromeliads (Benzing 2000, Schmidt & Zotz 2001, Rodrigues *et al.* 2016). However, no trichomes were detected in young *A. strobilacea*, suggesting an almost exclusive dependence on root water absorption at that stage for this species. Despite this, these plants showed mild water loss under minimal soil water levels and fast recovery capability, which might result from the hydrenchyma water storage and the other mechanisms described here.

The exposure to drought and rewatering induced changes to the gas exchange parameters and, consequently, CAM activity. The A, gs and E rates of the well-watered C plants indicate that exogenous CO_2 capture occurred during the day but not at night – in similarity to C_3 plants' gas exchange patterns. However, C plants showed a significant nocturnal acid accumulation characteristic of CAM. Thus, data suggest these plants might show CAM cycling activity, in which the nocturnal acidification derives from the recycling of respiratory CO_2 (Ting 1985, Cushman & Bohnert 1999). This form of CAM has been described in other Bromeliaceae, epiphytic or terricolous (Martin 1994, Sayed 2001). Although the Ci/Ca ratio suggests no accumulation of CO_2 was detected during the day in C plants, this might be the result of CO_2 assimilation through Rubisco. Accordingly, the A/Ci ratio shows that there was a significant carboxylation rate by Rubisco in C plants. Oppositely, gas exchange data in D plants show their stomata were closed during the daytime and opened at early night, which might support plants shifted to full CAM activity after drought. Accordingly, it is discussed that CAM cycling

performance allows a plant to rapidly engage into full CAM when drought ensues, thus promoting higher water conservation quickly through diurnal stomatal closure – which is a beneficial trait in habitats in which water shortage may set in a few hours (Ting 1985, Cushman & Bohnert 1999, Silvera *et al.* 2010). The increased Ci/Ca ratio during the day suggests that CO_2 was concentrated due to stomatal closure in D plants, while A/Ci was undetectable as an effect of the same process. The A rate was probably null at the night period after drought due to a lagging effect from the recently reopened stomata, as is common in CAM activity (Winter 2019); thus, we assume net photosynthesis rates would increase by late night. Also, since similar levels of ΔH^+ were detected in C and D plants, it is possible that refixation of respiratory CO_2 was partially replaced by atmospheric CO_2 uptake after drought due to the nocturnal stomatal opening in D plants. When rewatered for one day, plants showed almost full recovery of gas exchange parameters to the control, suggesting a shift back to CAM cycling activity. Further analysis of CAM-related parameters (*e.g.*, phosphoenolpyruvate carboxylase activities and malate content) during a 24-hour cycle would be required to best corroborate the types of CAM expressed by *A. strobilacea* plants under drought and well-watered situations. Nevertheless, the present results support that significant alterations in stomatal and photosynthetic behaviour occur despite minor tissue water reductions, which might be involved in the maintenance of water content and photosynthesis in juvenile *A. strobilacea*. Accordingly, the unaffected chlorophyll fluorescence parameters and ROS presence under drought indicate that the photosynthetic apparatus was preserved. The slight increase in DAB staining after rewatering might suggest that water reabsorption leads to higher H_2O_2, although not at the expense of photochemical integrity.

The water status adjustments during drought and rewatering in *A. strobilacea* were also followed by alterations in AQP gene expression. Of the six evaluated genes, four (two *PIPs*, one *TIP* and one *NIP*) showed a reduction in transcript levels after drought in the leaves, like the observed for other epiphytic bromeliads. Leaf blades of adult plants of the tank epiphytic bromeliad *G. monostachia* exposed to 14 days without water had the expression of a *PIP* reduced in a 5-fold rate, consistent to leaf conductance decrease (North *et al.* 2019). Likewise, the expression of *TiPIP2a* in leaves of the atmospheric, CAM and epiphytic bromeliad *T. ionantha* was decreased after a 3-month water shortage (Ohrui *et al.* 2007). The reduction in leaf AQP expression is usually associated with reduced membrane permeability and leaf hydraulic conductance, thus promoting water retention in the cells and vacuoles to prevent further water losses under drought (Maurel & Prado 2017, Sade & Moshelion 2017, Kurowska 2021a, b). Accordingly, it was shown that the CAM succulent *Graptopetalum paraguayense* (NE Br.) E. Walther have low vacuole and plasmatic membranes permeability due to reduced

AQP content when compared with radish, a C_3 representative, which is discussed to be an adaptive characteristic to the arid environment of CAM succulent plants (Ohshima *et al.* 2001). Thus, the reduction in AQP expression observed here might aid in promoting higher water conservation under drought. Such feature would be advantageous for epiphytic bromeliads, which rely on their water reserves to endure the erratic droughts of their environment (Benzing 2000).

In *A. strobilacea*, the most intense reduction in transcript levels was in the *TIP2;1* (3.2-fold), which might be involved in preventing water efflux from the vacuoles as to preserve turgor. This TIP isoform is potentially important in the water content regulation of the vacuole, as a study with *Vitis vinifera* L. showed that *Vv*TnTIP2;1 gating is regulated by membrane tension, being open when cells are flaccid to favour water entrance (Leitão *et al.* 2014) – which would likely allow fast rehydration after rewatering. Contrastingly, *TIP4;4* expression was unaltered by drought and rewatering in *A. strobilacea*. A previous report showed that the *Zea mays* L. ZmTIP4;4 is permeable to urea, with its expression being upregulated by N starvation in expanded leaves, possibly to promote urea unloading from the vacuole for use in N assimilation (Gu *et al.* 2012). Thus, this TIP might be more involved in urea transport than water, which might explain the unresponsive pattern of *TIP4;4* to drought in the present study. The *NIP2;2* was the second gene with the most reduced expression in *A. strobilacea*. The NIP subfamily is known to have low water permeability and be involved mostly in the transport of small, uncharged molecules – *e.g.*, metalloids, NH_3, H_2O_2, glycerol (Pommerrenig *et al.* 2015). Hence, the *NIP2;2* downregulation might be involved in the retention of substrates other than water in the cells that are also metabolically important for drought defence. Although the *NIP5;1* was unaffected by drought and rewatering in *A. strobilacea*, the opposite was observed for the halophyte *Atriplex canescens* (Pursh) Nutt. under polyethylene glycol-induced drought. When overexpressed in *A. thaliana* in the same study, plants showed higher drought tolerance due to faster stomatal closure and reduced transpiration (Yu *et al.* 2015). Considering the involvement of AQPs in stomatal movement (Ding & Chaumont 2020), it is possible that such distinction in the role of NIP5;1 between species in drought defence might derive from the reversed stomatal pattern of CAM plants as *A. strobilacea*. The PIP1 subfamily genes evaluated herein were also downregulated after drought, although at lesser rates than the others. The PIP1s are less permeable to water than the PIP2s but were shown to be crucial for water transport when combined with PIP2s in heterotetramers (Groszmann *et al.* 2017). There are also indications that PIP1s transport CO_2 and thus, are involved in improving mesophyll CO_2 conductance and facilitating its assimilation through Rubisco (Groszmann *et al.* 2017). Therefore, besides retaining water, *PIP1*s downregulation in *A. strobilacea* after drought might

aid in concentrating the CO_2 released from malate decarboxylation at midday in cells (when sampling for molecular analyses was performed), promoting higher efficiency in carbon assimilation through Rubisco.

After rewatering, the *PIP1;1*, *PIP1;2b* and *NIP2;2* expression levels in *A. strobilacea* mostly surpassed those of the control, especially of the latter gene. The recent rewatering event might have stimulated these genes expression to promote water uptake and turgor restoration, potentially in the hydrenchyma. Accordingly, it has been suggested that AQP activity is mostly required during recovery than drought *per se*, as concluded from studies on the AQP involvement in the refilling of xylem vessels after embolism in woody plants (reviewed by Secchi *et al.* 2017). Other reports have also shown the importance of AQP in the water transport during post-drought rewatering (Galmés *et al.* 2007, Laur & Hacke 2014, Grondin *et al.* 2016), including in an epiphytic bromeliad (Ohrui *et al.* 2007). It was shown that soaking *T. ionantha* plants in water for 6 hours after a 3-month drought recovered the expression of *TiPIP2a* to pre-stress levels, in which the authors conclude this AQP is engaged to reinforce and quicken the reabsorption of water (Ohrui *et al.* 2007). In the same study, it was shown that after water is absorbed through leaf trichomes (denominated as phase 1), the intracellular transport is performed by AQPs (phase 2 of leaf water absorption), as confirmed by the inhibition effect of $HgCl_2$ that was reversed by ß-mercaptoethanol. In addition, among the downregulated AQP genes of *A. strobilacea* under drought, only the *TIP2;1* expression was not entirely restored after rewatering, pointing to a distinct regulation between AQPs during water reabsorption in this bromeliad. Similarly, the transcript levels of a *PIP* in *G. monostachia* leaves were not restored to pre-stress conditions when rewatered for 4 days after a 14 day-drought (North *et al.* 2019).

In summary, data show that alterations in anatomical features, photosynthetic activity and AQP genes expression under drought and rewatering enabled the preservation of moderate RWC values and photochemical integrity in *A. strobilacea* despite envi humidity shifts. This agrees with the reported for another atmospheric bromeliad *Tillandsia utriculata* L., in which the stabilization of the RWC and Fv/Fm in adult plants after a 20-day drought under greenhouse conditions was achieved due to adjustments in CAM activity and other traits (Rosado-Calderón *et al.* 2020). The authors concluded *T. utriculata* possesses a fast acclimation capability to water availability due to such adjustments, as might also be the case for juvenile *A. strobilacea*.

CONCLUSIONS

This study indicates that juvenile *A. strobilacea* plants show a preserved hydric and photochemical status in face of drought and rewatering, possibly due to (i) the apparent

remobilization of water between the hydrenchyma and chlorenchyma, (ii) adjustments in stomatal behaviour, possibly linked to regulation of CAM intensity, and (iii) modulation in leaf AQP genes expression. Such robust water status regulation is likely an adaptation to the epiphytic environment, which provides drought tolerance and fast recovery despite the lack of trichomes in young *A. strobilacea* plants. These traits will possibly allow the endurance of this species to drought intensification from climate change.

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SUPPLEMENTARY MATERIAL

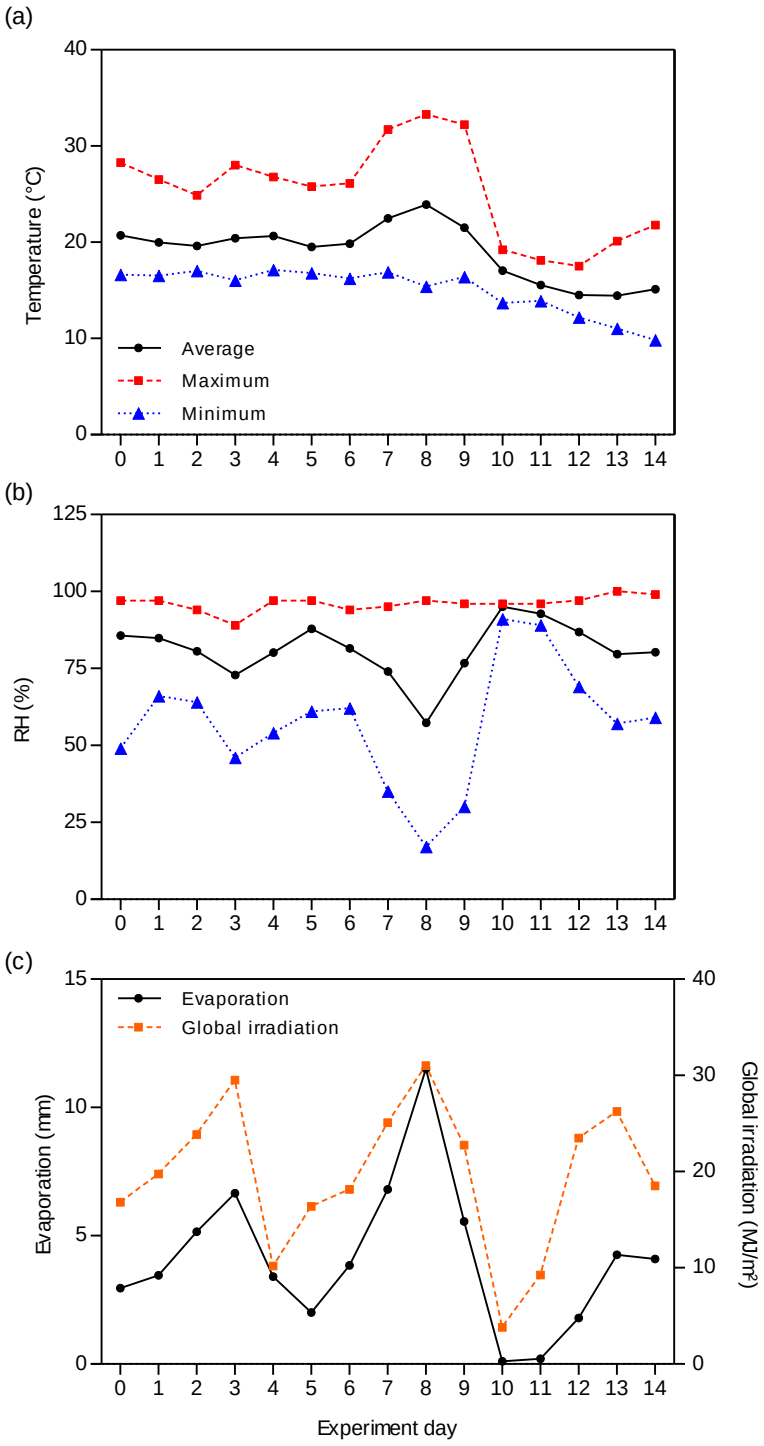

Figure S1. Environmental data of the days of experiment obtained from a weather station. RH, relative humidity.

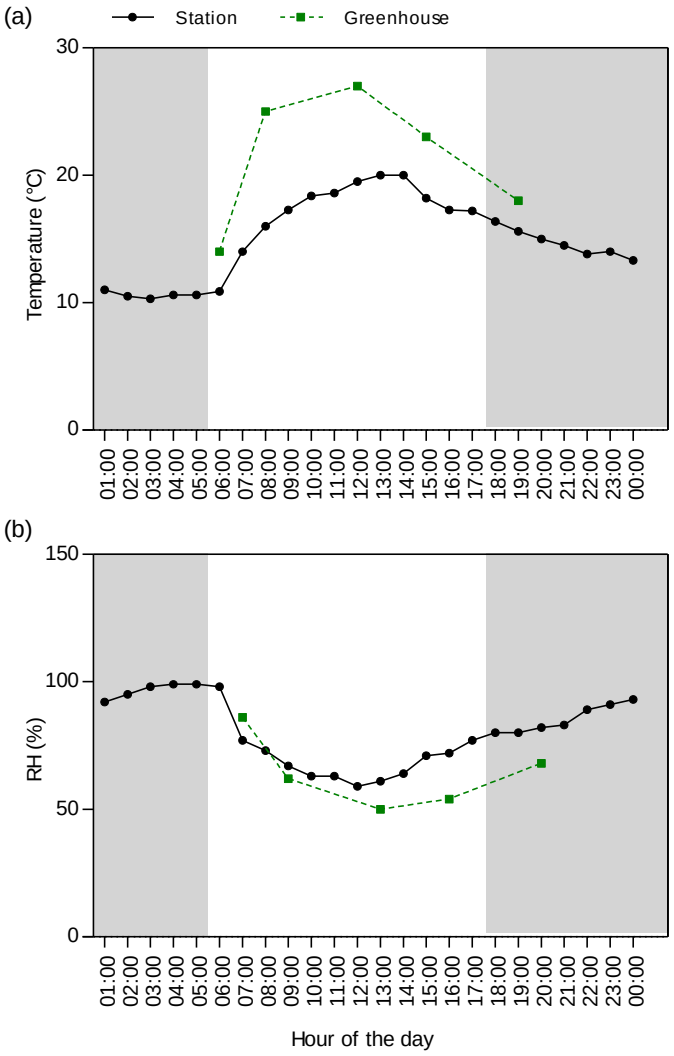

Figure S2. Environmental data for the 24 hours of the day of gas exchange analyses. RH, relative humidity.

Table S1. Aquaporin primers utilized for the qRT-PCR analyses. The efficiencies (E) and linearity (R^2) values were calculated from a standard curve obtained with an equimolar mixture of all obtained cDNAs. F, forward; R, reverse; Tm, melting temperature; *, reference genes.

Annotation	*Primers* sequences	Amplicon length	Tm (°C)	E (%)	R^2
*FB293**	F: 5' CTGAAGATGTGAACAAGCAAATCA 3'	137	52	102	0.9992
	R: 5' CTGCCCAAACAGAAGAAGG 3'				
*IF2A**	F: 5' GATGTCAATGTGGCTTATGAGG 3'	110	52	95	0.9997
	R: 5' CTTTTGCGTTTTCCAGAGGAC 3'				
PIP1;1	F: 5' GTGTGGTGAAGGGGTTCCAA 3'	153	55	93	0.9997
	R: 5' TGGCATCAGTGGCAGAGAAG 3'				
PIP1;2[a]	F: 5' GTGAAGGGGTTCGAGAAGGG 3'	156	54	97	0.9834
	R: 5' CTTGGCATCAGTGGCAGAGA 3'				
PIP1;2b	F: 5' GTCGTCAAGGGCTTCCAGAA 3'	150	55	91	0.9988
	R: 5' CGGAGAAGACGGTGTAGACG 3'				
TIP2;1	F: 5' ATCATCACCGCCACCACCAG 3'	104	57	93	0.9980
	R: 5' TCCTCACCTTCCTCTTCGTCTTCG 3'				
TIP4;4	F: 5' CCGCCATTGCCTACAACAAGTT 3'	133	57	91	0.9868
	R: 5' CGACACCGCCACGAACAG 3'				
NIP2;2	F: 5' GCCTCATCGTAACGGTGATGAT 3'	121	59	101	0.9879
	R: 5' GGCACCTGAATCCAAGGGAA 3'				
NIP5;1	F: 5' GGAGACGCTGATCGGCAATGC 3'	137	60	89	0.9871
	R: 5' CCCAGGGGAAGTGGCGGAG 3'				

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CONSIDERAÇÕES FINAIS E PERSPECTIVAS

O presente estudo fornece evidências de mecanismos fisiológicos, bioquímicos, moleculares e morfológicos de resistência à seca em curto prazo (horas a dias) nas plantas jovens de *A. strobilacea*, demonstrando pronunciada capacidade de defesa desde estágios ontogenéticos iniciais nesta espécie. Os dados também indicam a sensibilidade destas plantas às variações hídricas rápidas do ambiente – característica importante para a sobrevivência no ambiente epifítico, em que o déficit hídrico pode ser imposto horas após redução na umidade (Zotz & Hietz 2001). Tal característica fica clara pela avaliação da hipótese I (capítulo 1), em que a ativação dos mecanismos de defesa, como atividade de enzimas antioxidantes, aumento em prolina e o ajuste osmótico, ocorreu nas primeiras 24 horas de seca. Também foram detectados picos em sinalizadores (RNS e ABA) ao longo das 72 horas de exposição, fornecendo indícios das vias sinalizadoras envolvidas nas respostas à seca destas plantas. A sensibilidade de resposta de plantas jovens de *A. strobilacea* às condições hídricas também foi vista no processo de alternância entre condições de seca e hidratação (capítulos 2 e 3). A exposição das plantas a um ciclo de seca e reidratação otimizou a resposta bioquímica em um segundo ciclo, sugerindo a ocorrência de memória em relação ao conteúdo de pigmentos, metabolismo nitrosativo e da glutationa (capítulo 2). Além disso, foi visto que a seca e reidratação em curto prazo induzem, de modo reversível, a remobilização de água do hidrênquima, intensidade de CAM (indicada pelas trocas gasosas) e expressão gênica de AQPs (capítulo 3).

Em referência ao observado para CAM neste trabalho, notou-se que o aumento significativo no acúmulo noturno de ácidos em resposta à seca ocorreu somente em câmara climática (capítulo 2) e não casa de vegetação (capítulo 3), apesar das análises de trocas gasosas sugerirem intensificação de CAM nestas últimas condições. Supõe-se que a fixação noturna de CO_2 pode ter sido mais intensa nas condições de câmara em função de uma intensidade luminosa três vezes maior do que na casa de vegetação, já que esta intensifica a formação de carboidratos de reserva durante o dia anterior através do ciclo de Calvin-Benson e gliconeogênese, afetando positivamente a síntese de fosfoenolpiruvato e fixação de CO_2 *via* PEPC à noite (Lüttge 2004). Adicionalmente, o ajuste osmótico só foi detectado quando plantas foram expostas à seca de modo abrupto (capítulo 1), sugerindo que a intensidade da imposição do estresse também afeta as respostas fisiológicas. Neste caso, é possível que a provável mobilização de água do hidrênquima para o clorênquima e a redução na expressão gênica de AQPs sob seca mais lenta (ou seja, pela suspensão de rega; capítulo 3) teriam sido suficientes para evitar perda excessiva de água no período em questão, não necessitando do ajuste osmótico

nessas condições. De qualquer modo, estas particularidades são indícios adicionais da sensibilidade das plantas a variações hídricas, e sugerem que o mesmo ocorra para a intensidade luminosa.

É interessante notar que não foi possível detectar, em nenhum dos experimentos, produção exacerbada de ROS à seca, mesmo quantificando diferentes moléculas (H_2O_2, $^{\bullet}OH$, $^{\bullet}O_2^-$, peróxidos de lipídios) com estratégias analíticas distintas (*in situ* e *in vitro*). Vale ressaltar que isto ocorreu mesmo frente aos níveis mínimos de conteúdo hídrico dos substratos aos quais as plantas foram expostas nos três tratamentos de seca, apesar da exposição em curto prazo. Portanto, este trabalho demonstra que plantas jovens de *A. strobilacea* têm alta capacidade de evitar o estresse oxidativo e danos ao aparato fotossintético em seca, o que pode ser resultado da manutenção hídrica celular, indicada pelas baixas perdas de água (capítulos 1 a 3), e do sistema antioxidante, indicado pelo alto estado redutor do ácido ascórbico e glutationa e recrutamento das enzimas antioxidantes (capítulos 1 e 2). Resposta similar foi vista em outro trabalho do grupo, em que plantas de três meses de *A. strobilacea* apresentaram maior atividade de enzimas antioxidantes e inalteração em conteúdo de $^{\bullet}O_2^-$ e peróxidos de lipídios após oito dias sem rega (Menezes *et al.* 2020).

Em suma, este trabalho nos permite concluir que a adaptação de plantas jovens de *A. strobilacea* à seca e reidratação é resultado de estratégia que envolve:

1. Indução de sinalizadores após início da seca (RNS e ABA);
2. Ativação de mecanismos de defesa como ação antioxidante e intensificação de CAM, prevenindo danos ao aparato fotoquímico e otimizando a economia de água;
3. Efeitos de memória em parâmetros como pigmentos, aminoácidos, atividade da S-nitrosoglutationa redutase e glutationa;
4. Regulação hídrica celular dinâmica através da remobilização de água do hidrênquima e ajustes na expressão gênica de AQPs.

Este trabalho cria subsídios para futuros estudos visando: (i) melhor entendimento do papel das RNS, S-nitrosação e ABA na regulação de mecanismos de defesa à seca, inclusive a formação de memória; (ii) elucidar o processo de consolidação da memória de parâmetros bioquímicos sob diferentes períodos de reidratação; e (iii) detalhar a função das AQPs na dinâmica hídrica entre o hidrênquima e clorênquima e na possível absorção hídrica foliar de *A. strobilacea.* Além disso, este trabalho gera subsídios à prospecção de genes de resistência à seca, o que será possibilitado devido à recente conclusão do sequenciamento genômico de *A. strobilacea* pelo grupo (FAPESP 20/11908-7), o que tornará esta espécie um modelo de estudo sobre a resistência à seca ainda mais robusto. Por fim, o presente trabalho aliado a futuras

pesquisas na área pode auxiliar na compreensão de como as maiores temperaturas e seca resultantes das alterações climáticas podem afetar bromélias epífitas, essenciais ao balanço ecológico das florestas tropicais. No contexto do projeto institucional, os dados obtidos fornecem maiores informações sobre o potencial de *A. strobilacea* como biomarcador dos efeitos de estresses ambientais sobre a flora epifítica da Mata Atlântica.

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