VICTÓRIA DE CARVALHO

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

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RESUMO

O metabolismo ácido das crassuláceas (CAM, sigla do inglês) é uma via fotossintética que promove tolerância à seca, permitindo altas taxas de fixação de CO_2 pelas plantas mesmo em condições de escassez de água. Esta via têm alta plasticidade de ajuste quanto alterações na umidade, o que é observado principalmente em espécies C_3-CAM facultativas. Estas podem alternar entre a fixação de CO_2 realizada via C_3 e CAM, o que permite a adaptação de seu metabolismo para períodos de seca e sua recuperação quando o fornecimento de água é restabelecido, retomando seu crescimento e desenvolvimento. Assim, espécies C_3-CAM facultativas podem ser consideradas importantes modelos de estudo para avaliar mecanismos fisiológicos envolvidos na resiliência das plantas à exposição aos ciclos de umidade-seca do ambiente. A ativação de CAM em resposta à seca foi observada na bromélia *Guzmania monostachia* (L.) Rusby ex Mez, o que confere importante adaptação à disponibilidade intermitente de água do ambiente epifítico, natural da espécie. Isto também foi observado em indivíduos jovens atmosféricos desta bromélia, o que pode ser considerado crucial para sua sobrevivência, pois estas não podem acumular água em tanques foliares como as adultas. Adicionalmente, estas plantas também devem tolerar e se restaurar de efeitos consequenciais da seca, como a produção aumentada de espécies reativas de oxigênio (ROS, sigla do inglês) como o peróxido de hidrogênio (H_2O_2), que podem levar a danos nas células. Estudos sobre como plantas CAM realizam o controle da produção de ROS em resposta à seca e reidratação não são conclusivos, no entanto, existe a hipótese de que CAM pode agir como mecanismo de prevenção à produção excessiva de ROS. Desta forma, plantas atmosféricas de *G. monostachia* são adequadas para estudar o metabolismo de ROS quanto a ativação do CAM em resposta à seca e recuperação da atividade fotossintética após a reidratação. O presente trabalho visou avaliar se (i) a expressão de CAM induzida pela deficiência hídrica diminui a produção de ROS e a atividade enzimática antioxidante em plantas atmosféricas de *G. monostachia*; e se (ii) a reidratação destas plantas permitem sua recuperação quanto a atividade fotossintética e metabolismo de ROS. Primeiramente, plantas foram mantidas em irrigação diária (controle) e suspensão de rega por um e oito dias. Em todas as condições, foi observado um acúmulo noturno de malato indicativo de CAM. No entanto, esta via foi intensificada gradualmente ao longo do período de um e oito dias de exposição à seca. Em um dia de tratamento, houve um aumento de 50% no conteúdo total de ROS em comparação ao controle, o que pode estar envolvido na sinalização para ativar mecanismos de aclimatação à perda de água, como a intensificação de CAM. Após oito dias de seca, houve uma queda no conteúdo de ROS a níveis similares ao controle, assim como redução de 38% nas atividades de enzimas antioxidantes, em

média. Estes resultados sugerem que o aumento na expressão de CAM pode ter intensificado seu caráter preventivo à produção de ROS, diminuindo a demanda sobre o sistema antioxidante. Posteriormente, plantas expostas a oito dias de seca foram irrigadas diariamente por mais seis dias. Estas plantas foram capazes de recuperar seu conteúdo hídrico e intensidade de CAM a níveis similares ao controle. No entanto, houve aumento nas atividades de enzimas antioxidantes e alteração no seu ritmo diário em relação ao controle, o que também ocorreu para a produção de H_2O_2. Isto implica que as plantas reidratadas ainda mostravam-se em processo de aclimatação, porém com rápida modulação do sistema antioxidante ao longo do dia em resposta à recente aquisição de água. Este estudo mostrou que a modulação de CAM feita de acordo com o conteúdo de água está estreitamente relacionada com a dinâmica entre a produção de ROS e o sistema antioxidante em plantas atmosféricas de *G. monostachia*. Estes resultados podem auxiliar na formação de subsídios para identificação de genes da modulação da via CAM e metabolismo de ROS envolvidos na tolerância à seca destas plantas.

Palavras-chave: enzimas antioxidantes, epífita, espécies reativas de oxigênio, metabolismo ácido das crassuláceas, reidratação, seca.

ABSTRACT

The Crassulacean acid metabolism (CAM) is a photosynthetic pathway that promotes drought tolerance, allowing plants to have high CO_2 fixation rates even under water shortage conditions. This pathway presents high plasticity in adjustment according to changes in humidity, which is mostly observed in C_3-CAM intermediate species. The CO_2 fixation in these plants can be switched between C_3 and CAM photosynthesis, which allows their metabolism to adapt to drought periods and recover when water supply is restored, resuming its growth and development. Thus, C_3-CAM intermediate species can be considered important models to evaluate physiological mechanisms involved in the resilience of plants to humid-dry cycles. Activation of CAM in response to drought was observed in the bromeliad *Guzmania monostachia* (L.) Rusby ex Mez, which is an important adaptation to the intermittent water availability of the natural epiphytic environment of this species. That was also observed in juvenile atmospheric individuals of this bromeliad, which can be considered crucial for its survival because they cannot store water in leaf tanks as the adults. In addition, these plants must also tolerate and recover from consequential effects to drought, such as increased production of reactive oxygen species (ROS) like hydrogen peroxide (H_2O_2) that can lead to cell damage. Reports of ROS metabolism in C_3-CAM intermediate plants in response to water availability are still scarce; however, it is hypothesized that the CAM pathway may prevent excessive ROS production. Thus, atmospheric plants of *G. monostachia* are suitable to study ROS metabolism in response to CAM activation after drought exposure and photosynthetic recovery after rehydration. This study aimed to verify whether (i) CAM expression induced by water shortage decreases ROS production and antioxidant enzyme activity in atmospheric plants of *G. monostachia*; and if (ii) these plants recover their photosynthetic activity and ROS metabolism after rehydration. First, plants were kept under daily irrigation (control) and watering suspension for one and eight days. An overnight accumulation of malate was observed in all conditions, which is indicative of CAM expression. However, this metabolic pathway was intensified gradually after one and eight days of drought. After one day of treatment, there was a 50% increase in total ROS content compared to the control, which may be involved in signalling pathways in order to activate acclimatization mechanisms to water loss, such as intensification of CAM. After eight days of drought, there was a decrease in ROS content to similar levels of the control, as well as 38% reduction in antioxidant enzyme activities on average. These results suggest that increased CAM expression may have intensified its prevention feature against ROS production, reducing the demand on the antioxidant system. Afterwards, plants exposed to eight days of drought were irrigated daily for six more days.

These plants were able to recover its water content and CAM intensity to levels similar of the control. However, antioxidant enzyme activities increased and their daily rhythm was altered in comparison to the control. Diurnal production of hydrogen peroxide (H_2O_2) was also changed, which implies that rewatered plants were still under acclimatization process, although they presented rapid modulation of the antioxidant system throughout the day in response to the recent acquisition of water. This study showed that modulation of the CAM pathway in response to leaf water content is closely related to the dynamics between ROS production and the antioxidant system in atmospheric plants of *G. monostachia*. These results may help in creating subsidies for the identification of genes related to CAM modulation and ROS metabolism involved in the drought tolerance of these plants.

Keywords: antioxidant enzymes, Crassulacean acid metabolism, epiphyte, drought, reactive oxygen species, rewatering.

LISTA DE ABREVIATURAS

1WS, 1SR: um dia de suspensão de rega

8WS, 8SR: oito dias de suspensão de rega

ANOVA: análise de variância

APX: ascorbato peroxidase

AsA, ASC: ácido ascórbico/ascorbato

ATP: adenosina trifosfato

C: controle

C_3: ciclo de Calvin-Benson

$Ca(CO)_3$: carbonato de cálcio

CAM: metabolismo ácido das crassuláceas

CAT: catalase

CO_2: dióxido de carbono

CTE: cadeia transportadora de elétrons do cloroplasto

D: tratamento de seca

DCF: diclorofluoresceína

DHA: dehidroascorbato

DHAR: DHA redutase

d.p.: desvio padrão

DW: massa seca

EDTA: ácido etilenodiamino tetra-acético

Fe^{+2}: íon divalente de ferro

Fe^{+3}: íon trivalente de ferro

FW: massa fresca

GC/MS: cromatógrafo a gás acoplado a espectrômetro de massas

GR: glutationa redutase

GSH: glutationa reduzida

GSSG: glutationa oxidada

H_2-DCF: 2′,7′-diidrodiclorofluoresceína

H_2DCF-DA: diacetato de 2′,7′-diidrodiclorofluoresceína

H_2O: água

H_2O_2: peróxido de hidrogênio

H_2SO_4: ácido sulfúrico

LPO: peroxidação lipídica

MDH: malato desidrogenase

MDHA: monodehidroascorbato

MDHAR: MDHA redutase

$MgCl_2$: cloreto de magnésio

MS: massa seca

N_2: nitrogênio

$NADP^+$: nicotinamida adenina dinucleotídeo fosfato oxidado

NADPH: nicotinamida adenina dinucleotídeo fosfato reduzido

NaOH: hidróxido de sódio

NBT: cloreto de nitrotetrazólio azul

NS: não significativo

O_2: oxigênio molecular

$O_2^{\bullet-}$: radical superóxido

OAA: oxaloacetato

$OH^{\bullet}$: radical hidroxila

PEPC: fosfoenolpiruvato carboxilase

PFD: densidade de fluxo fotossintético

R: tratamento de reidratação

ROS: espécies reativas de oxigênio

Rubisco: ribulose-1,5-bisfosfato carboxilase oxigenase

RWC: conteúdo relativo de água

s.d.: desvio padrão

Sm: índice de suculência do mesofilo

SOD: superóxido dismutase

TCA: ácido tricloroacético

Tris-HCl: tris(hidroximetil)aminometano cloridrato

TW: massa túrgida

WC: conteúdo de água

SUMÁRIO

INTRODUÇÃO GERAL

1. Metabolismo ácido das crassuláceas (CAM, sigla do inglês): adaptação fotossintética à seca

A seca é considerada o principal fator limitante no crescimento e desenvolvimento de plantas, de forma que estas evoluíram diversas adaptações que as permitem resistir e sobreviver a períodos de escassez de água (Cruz de Carvalho 2008). Dentre as adaptações metabólicas à seca, a via fotossintética CAM é uma das mais distribuídas dentre o reino das plantas, onde é estimada ser utilizada por cerca de 6% das angiospermas, que incluem principalmente espécies de ambientes mais áridos como cactos e suculentas (Crayn *et al.* 2004, Silvera *et al.* 2010). A vantagem no uso de CAM em ambientes secos está diretamente relacionada à sua alta eficiência no uso da água (razão de CO_2 assimilado: água transpirada) para realizar a fixação de CO_2 em relação a outras vias fotossintéticas, sendo seis vezes maior do que a observada no mecanismo C_3 (Nobel 1996). Isto provém do fato de que, enquanto plantas C_3 abrem seus estômatos durante o dia para fixar o CO_2 atmosférico (Fig. 1A), as plantas CAM realizam este processo durante o período noturno quando as temperaturas são mais amenas e a umidade é maior (Fig. 1B), o que reduz a perda de água pela transpiração (Lüttge 2010, Borland *et al.* 2014).

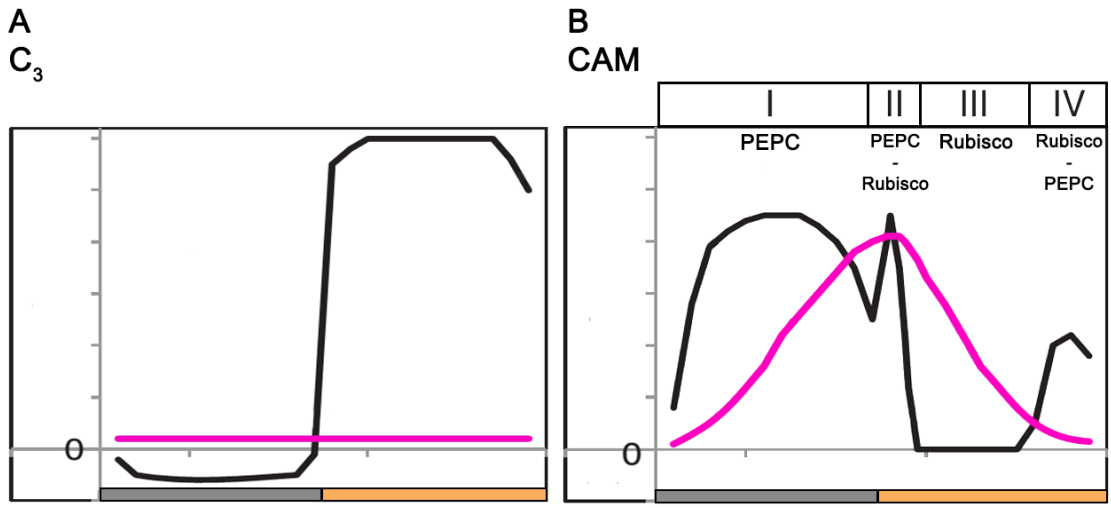

Figura 1. Comparação entre o ciclo diário da fotossíntese C_3 (A) e CAM (B). As barras cinza e laranja na base dos gráficos indicam o período noturno e diurno, respectivamente. A linha preta corresponde à fixação de CO_2 atmosférico e a linha rosa ao conteúdo de ácidos orgânicos. Em (B), os numerais de I a IV indicam as fases de CAM (Osmond, 1978) com as principais enzimas atuantes na fixação de CO_2 no período. PEPC, fosfoenolpiruvato carboxilase; Rubisco, ribulose-1,5-bisfosfato carboxilase oxigenase. Adaptado de Borland *et al.* (2011).

O ciclo diário de CAM é dividido em quatro fases distintas (Fig. 1B), conforme proposto por Osmond (1978). A fase I ocorre durante o período escuro, quando os estômatos se abrem permitindo a entrada de CO_2 atmosférico, que por sua vez é fixado no citosol pela enzima

fosfoenolpiruvato carboxilase (PEPC) em oxaloacetato (OAA). OAA é então convertido em malato pela malato desidrogenase (MDH), além de também poder levar à produção de citrato na mitocôndria (Gawronska & Niewiadomska 2015). Tanto malato quanto citrato são armazenados no vacúolo na forma de ácidos orgânicos, diminuindo o pH da célula à noite. A fase II ocorre no início do dia com os estômatos ainda abertos, quando a fixação de CO_2 está em processo de transição da PEPC para a enzima Rubisco, componente do ciclo de Calvin-Benson (*i.e.* via C_3). A fase III corresponde à grande parte do restante do período claro, quando os estômatos se fecham e os ácidos orgânicos são liberados no citosol para serem descarboxilados, resultando em CO_2. O CO_2 liberado pode então ser refixado através da Rubisco e via C_3 para síntese de carboidratos. A fase IV ocorre no final da tarde, quando o conteúdo de ácidos orgânicos já foi exaurido e os estômatos se reabrem, permitindo novamente a entrada de CO_2 atmosférico e a transição de sua fixação pela Rubisco para a PEPC (Borland *et al.* 2011). Assim, o consumo de principalmente malato durante o dia seguido por seu acúmulo crescente à noite é uma das características fundamentais que diferenciam as plantas CAM daquelas que aplicam a via C_3 de forma exclusiva para fixação de carbono (Winter *et al.* 2015), onde tal flutuação não é observada (Fig. 1).

Atualmente, previsões indicam que as futuras alterações climáticas levarão à ocorrência mais rápida, intensa e frequente de períodos de seca, principalmente em locais de clima mais quente e árido (Trenberth *et al.* 2014). Nestas condições, as plantas serão mais expostas a ciclos de umidade-seca ao longo de seu desenvolvimento. Assim, a sobrevivência dos vegetais dependerá da capacidade destes em realizar ajustes rápidos em seu metabolismo, de forma a adaptá-lo às condições de seca e recuperá-lo quando o fornecimento de água é restabelecido (*i.e.* condições de pré-estresse), para que possam retomar seu crescimento e desenvolvimento (Xu *et al.* 2010). Portanto, supõe-se que espécies com metabolismo plástico estarão inerentemente mais aptas à sobrevivência em vista da intensificação da seca prevista para o futuro.

Em particular, espécies que utilizam a via fotossintética do CAM naturalmente apresentam grande plasticidade metabólica, pois são capazes de modular a magnitude de expressão e duração de cada fase do ciclo diário deste mecanismo de acordo com as condições ambientais (Dodd *et al.* 2002, Borland *et al.* 2011). Esta modulação de CAM em resposta ao ambiente ocorre em espécies classificadas como C_3-CAM facultativas, onde são capazes de alternar a fixação de carbono pela via C_3 para CAM e modular sua intensidade de acordo com a severidade dos fatores abióticos (Borland *et al.* 2011). Espécies facultativas estão presentes principalmente dentre Aizoaceae, Crassulaceae, Portulaceae e Vitaceae (Smith & Winter 1996). No entanto, a mais estudada é a aizoácea *Mesembryanthemum crystallinum* L., uma halófita

anual que alterna entre C_3 e CAM de acordo com o nível de salinidade do solo, que induz a perda de água pelas células (Winter & Holtum 2014). De fato, a troca entre C_3 e CAM nestas espécies é coordenada principalmente pela variação na disponibilidade hídrica e conteúdo de água nos tecidos, onde as plantas mantém o uso da via C_3 quando em condições favoráveis de umidade a fim de otimizar seu desenvolvimento, e alternam para CAM quando expostas a condições de seca como um mecanismo de resistência (Cushman & Borland 2002). Assim, espécies C_3-CAM facultativas podem ser consideradas importantes modelos de estudo para avaliar os mecanismos fisiológicos envolvidos na resiliência das plantas à exposição aos ciclos de umidade-seca do ambiente.

1.1. CAM como adaptação à seca em bromélias epífitas

Plantas do hábito epifítico são aquelas que se sustentam sobre outros indivíduos, principalmente em árvores. Portanto, plantas epífitas são dependentes de água originada exclusivamente da atmosfera através da chuva, orvalho e/ou neblina, o que as sujeitam a frequentes períodos de escassez hídrica (Kerbauy *et al.* 2012). A via CAM é descrita como uma importante adaptação destas espécies à seca sazonal de seu ambiente, permitindo que estas mantenham altas taxas fotossintéticas mesmo em momentos de baixa disponibilidade de água (Silvera *et al.* 2010, Crayn *et al.* 2015). O grupo Bromeliaceae apresenta o segundo maior número de epífitas após Orchidaceae (Givnish *et al.* 2014), sendo que a presença de CAM já foi descrita para diversas bromélias deste hábito (Martin 1994, Crayn *et al.* 2015). Além disso, a importância deste metabolismo para a tolerância à seca de bromélias epífitas pode ser averiguada pelo fato de que a incidência de CAM aumenta dentre espécies de florestas tropicais mais secas (Lüttge 2004).

Além da adaptação metabólica do CAM, bromélias epífitas apresentam diversas outras adaptações morfológicas e anatômicas que permitem sua sobrevivência aos constantes períodos de seca e otimizam a absorção e estocagem de água durante os breves momentos de maior umidade (Matiz *et al.* 2013). Dentre estas, podemos destacar a estrutura do tanque ou *phytotelmata*, formada por folhas sobrepostas que permite o armazenamento de água atmosférica para uso em momentos de seca; e os tricomas, que são células epidérmicas das folhas que absorvem água do ar e/ou daquela armazenada no tanque (Benzing 2000). A ocorrência destas características define o tipo morfológico da planta, onde bromélias com *phytotelmata* são do tipo tanque, enquanto aquelas cujas folhas são finas e lanceoladas que não formam esta estrutura são do tipo atmosférico (Benzing 2000). A ausência do tanque para estocagem de água em bromélias atmosféricas as tornam mais suscetíveis ao déficit hídrico. Entretanto, estas plantas possuem folhas cobertas por um maior número de tricomas do que

indivíduos tanque, permitindo rápida reidratação durante os períodos de maior umidade (Schmidt & Zotz 2001). Assim, é provável que bromélias atmosféricas tenham estratégias primariamente metabólicas para resistirem aos períodos de seca e se recuperarem frente à rápida absorção de água pelos tricomas após serem reidratadas. Este tipo de resistência à seca realizada sobretudo através de ajustes metabólicos é denominado de tolerância (Verslues *et al.* 2014), que de fato já foi descrito em diversas bromélias atmosféricas (Zotz & Hietz 2001, Graham & Andrade 2004, Bader *et al.* 2009, Reyes-García *et al.* 2012). De forma interessante, existem diversas bromélias que podem apresentar ambos os tipos morfológicos ao longo de sua ontogenia, onde as plantas da fase juvenil são atmosféricas, e as da fase adulta são do tipo tanque (Meisner & Zotz 2012, Meisner *et al.* 2013). Neste caso, as plantas juvenis devem ajustar seu metabolismo rapidamente de forma a tolerarem a perda de água causada pelos períodos de seca para garantirem sua sobrevivência até se desenvolverem em indivíduos adultos que, por sua vez, possuem estratégias de adaptação que visam principalmente à manutenção do conteúdo de água nos tecidos.

A bromélia epífita *Guzmania monostachia* (L.) Rusby ex Mez var. *monostachia* é uma espécie com ontogenia diferenciada entre o estágio atmosférico juvenil e tanque adulto, com ampla distribuição a partir da Flórida (EUA) até o oeste da América do Sul e nordeste do Brasil (Govaerts *et al.* 2016). Particularmente no território brasileiro, esta espécie é encontrada em regiões altamente fragmentadas da caatinga e é sujeita ao extrativismo ilegal, sendo classificada como vulnerável (Forzza *et al.* 2010). *G. monostachia* também apresenta a característica distinta de ser a única espécie de Bromeliaceae descrita como C_3-CAM facultativa (Winter & Holtum 2014), onde é capaz de alternar da fotossíntese C_3 para CAM quando em condições de seca. Isto foi relatado tanto para plantas na fase atmosférica (Fig. 2A) (Beltrán *et al.* 2013) como na fase tanque (Fig. 2B) (Medina *et al.* 1977), sendo que indivíduos de ambos estágios ontogenéticos apresentam um controle altamente plástico deste metabolismo. Por exemplo, estudos com plantas tanque mostram que a ativação de CAM em resposta a seca é intensificada em direção à região apical da folha (Freschi *et al.* 2010), além de mostrarem com sucesso a reversão à via C_3 quando reidratadas após a falta de água (Medina *et al.* 1977). No caso das plantas atmosféricas, Beltrán *et al.* (2013) verificaram que a transição de C_3 para CAM ocorre após um dia de exposição à seca, de forma mais rápida que em plantas tanque. Esta rapidez na modulação de CAM pode ser considerada essencial para a tolerância à seca das plantas juvenis. No entanto, ainda não foram realizados estudos sobre a capacidade das plantas atmosféricas de *G. monostachia* em recuperarem o conteúdo hídrico e a atividade fotossintética após serem reidratadas, o que é importante para avaliar suas estratégias de sobrevivência aos ciclos de umidade-seca do ambiente, principalmente considerando as futuras alterações climáticas.

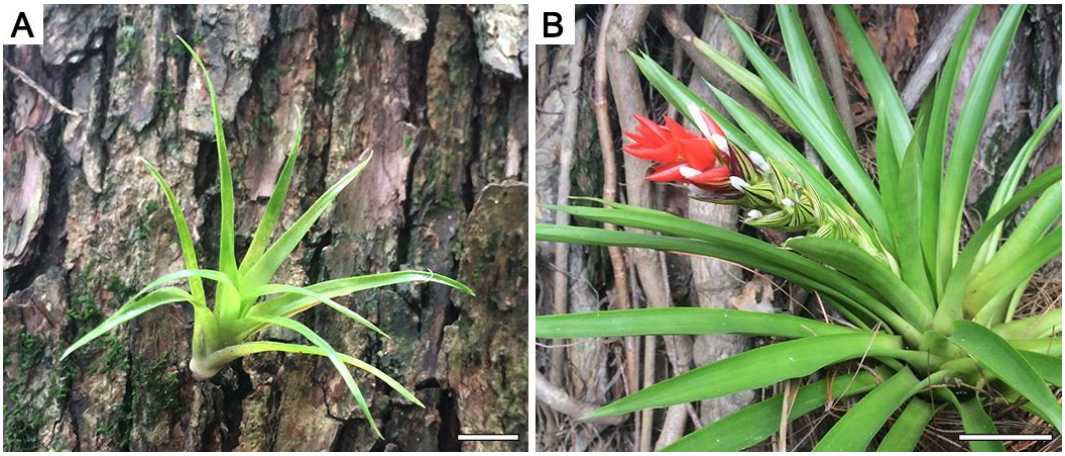

Figura 2. Aspecto geral de plantas de *Guzmania monostachia* (L.) Rusby ex Mez var. *monostachia*. A) Indivíduo jovem na forma atmosférica, B) indivíduo adulto na forma tanque. Barras: A) 1 cm, B) 4 cm. Fotos: Antônio A. C. Neto.

2. Efeitos da seca na produção de espécies reativas de oxigênio (ROS, sigla do inglês) em plantas CAM

A exposição à seca em que plantas epífitas como *G. monostachia* estão sujeitas pode levar a efeitos consequenciais à perda de água dos tecidos, como o aumento excessivo na produção de ROS (Cruz de Carvalho 2008, Hasanuzzaman *et al.* 2013). As ROS podem danificar estruturas celulares como as membranas lipídicas e levar à morte celular quando estão em quantidades altas nas células, causando o estresse oxidativo (Mittler 2002). Em plantas, a produção de ROS ocorre principalmente nos cloroplastos, iniciando-se pela incorporação de elétrons em excesso da cadeia transportadora ao oxigênio molecular (O_2), gerando o radical superóxido ($O_2^{\bullet-}$). A presença deste radical pode levar à produção de outras ROS como o peróxido de hidrogênio (H_2O_2) e a hidroxila ($OH^{\bullet}$). O H_2O_2 é formado pela redução espontânea de $O_2^{\bullet-}$ ou através da enzima superóxido dismutase (SOD); e os radicais $OH^{\bullet}$ são gerados a partir de uma reação entre H_2O_2 e íons de ferro ou outros metais de transição (Hajiboland 2014). Além dos cloroplastos, esta sequência de produção de ROS ocorre em outras organelas com alto metabolismo, como as mitocôndrias e peroxissomos (Gill & Tuteja 2010). Particularmente nos peroxissomos, há grande formação de H_2O_2 através do mecanismo da fotorrespiração, iniciado pela função de oxigenase da Rubisco (Noctor *et al.* 2002).

De modo a evitar o efeito tóxico das ROS e sua produção excessiva, as células vegetais têm um sistema antioxidante presente na maioria dos seus compartimentos para a eliminação destas moléculas, que inclui enzimas como a SOD, catalase (CAT), ascorbato peroxidase (APX) e glutationa redutase (GR) (Asada 1999). Em suma, a SOD é uma das primeiras respostas defensivas contra o aumento das ROS, onde realiza a remoção de $O_2^{\bullet-}$, liberando H_2O_2

e O_2. Em seguida, as moléculas de H_2O_2 produzidas são removidas pela CAT e APX (Fig. 3A). Esta última enzima também atua dentro do ciclo ascorbato-glutationa juntamente com a GR e diversos outros componentes enzimáticos (Fig. 3B), onde são responsáveis por regenerarem o ascorbato (ou ácido ascórbico) e a glutationa reduzida (Gill & Tuteja 2010, Groß *et al.* 2013). Estes metabólitos não enzimáticos têm importante função na eliminação dos radicais $OH^{\bullet}$, considerados as mais reativas e danosas formas das ROS (Foyer & Noctor 2011). Os pigmentos fotossintéticos carotenoides também são antioxidantes não enzimáticos cruciais para eliminar as principais ROS precursoras da peroxidação lipídica, que pode danificar as membranas na região dos cloroplastos (Smirnoff 2007).

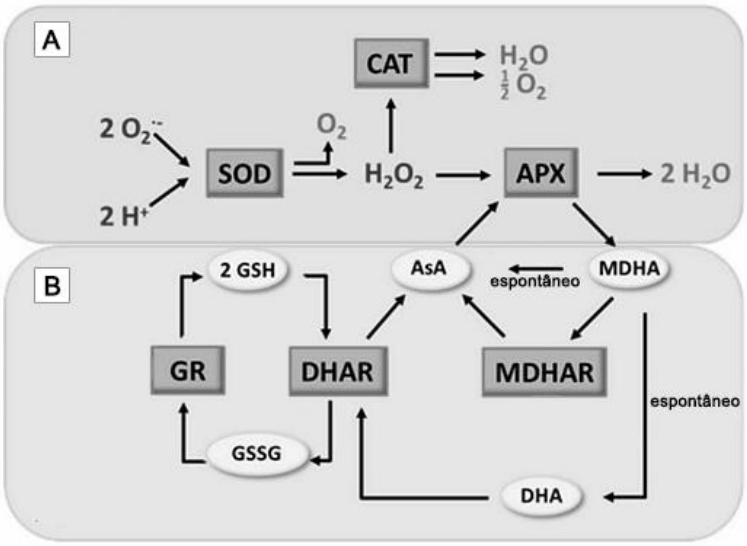

Figura 3. Principais componentes enzimáticos do sistema antioxidante em plantas. A) Etapas de detoxificação do radical superóxido ($O_2^{\bullet-}$) e peróxido de hidrogênio (H_2O_2), B) componentes do ciclo ascorbato-glutationa. SOD, superóxido dismutase; O_2, oxigênio molecular; CAT, catalase; H_2O, água; APX, ascorbato peroxidase; AsA, ácido ascórbico/ascorbato; MDHA, monodehidroascorbato; MDHAR, MDHA redutase; DHA, dehidroascorbato; DHAR, DHA redutase; GR, glutationa redutase; GSH glutationa reduzida; GSSG, glutationa oxidada. Adaptado de Groß *et al.* (2013).

O mecanismo em que a seca leva à maior produção de ROS foi amplamente estudado em plantas C_3, e atualmente sabe-se que esta ocorre devido à restrição na disponibilidade de CO_2 nas células ocasionada pelo fechamento de estômatos, induzido pela falta de água, que por fim leva ao acúmulo de elétrons nos cloroplastos que podem desencadear as reações de formação de ROS (Miller *et al.* 2010). Ainda não há estudos conclusivos sobre o funcionamento do metabolismo de ROS em plantas expressando o CAM, no entanto, existem duas principais hipóteses sobre como esta via fotossintética pode afetar o conteúdo oxidativo das células, conforme revisado por Niewiadomska e Borland (2008). Primeiramente, foi proposto que a ocorrência da cadeia transportadora de elétrons (CTE) dos cloroplastos sob estômatos fechados durante o dia (fase III, Fig. 1B) poderia elevar a concentração de O_2 interna, aumentando o

potencial oxidativo da célula (Spalding *et al.* 1979). De forma oposta, a outra hipótese diz que o acúmulo de CO_2 durante o mesmo período do ciclo de CAM (fase III, Fig. 1B) impediria o acúmulo de elétrons na CTE, evitando a formação excessiva de ROS (Griffiths *et al.* 1989). De fato, estudos com a espécie C_3-CAM facultativa *M. crystallinum* mostraram o potencial da ativação do CAM no alívio do estresse oxidativo após exposição ao estresse salino (Borland *et al.* 2006, Sunagawa *et al.* 2010). Ainda assim, são necessários mais estudos de forma a definir como ocorre a resposta oxidativa em diferentes espécies que expressam a fotossíntese CAM.

2.1. Função sinalizadora de ROS na indução ao CAM em espécies facultativas

Além do caráter deletério do aumento de ROS em resposta à seca, sabe-se que um breve aumento destas substâncias pode ter um efeito benéfico para as células, pois este pode estar envolvido em vias de ativação de mecanismos de aclimatação ao estresse (Cruz de Carvalho 2008, Mittler *et al.* 2011, Noctor *et al.* 2014). Neste caso, o sistema antioxidante funcionaria como um mecanismo de controle sobre os sinais emitidos pelas ROS, enquanto as mantém em níveis suportáveis pelas células (Munné-Bosch *et al.* 2013). Sabendo que o CAM pode ser induzido em espécies C_3-CAM facultativas como forma de adaptação à seca, podemos supor que ROS possam estar envolvidas na sinalização para ativar este metabolismo quando as plantas são expostas à falta de água. De fato, alguns estudos com espécies C_3-CAM facultativas sugeriram uma possível função de H_2O_2 como sinalizador da indução de CAM (Ślesak *et al.* 2007, 2008), inclusive em folhas destacadas de indivíduos tanque da bromélia *G. monostachia* (Mioto & Mercier 2013).

3. Justificativa e hipóteses

O uso de espécies C_3-CAM facultativas é recorrente na literatura para avaliar a influência de fatores ambientais sobre a modulação da fotossíntese e outras respostas fisiológicas, pois estas apresentam plasticidade e rapidez metabólica (Winter & Holtum 2014). Portanto, plantas atmosféricas de *G. monostachia* são adequadas para estudar o metabolismo de ROS quanto a ativação do CAM em resposta à seca e recuperação da atividade fotossintética após a reidratação. Esta bromélia é utilizada pelo grupo do Laboratório de Fisiologia Vegetal (LFV) do Instituto de Biociências da Universidade de São Paulo (IB-USP) em diversos estudos sobre os mecanismos indutores de CAM, que fazem parte de um projeto financiado pela FAPESP coordenado pela Dra. Helenice Mercier (processo 11/50637-0). O presente trabalho de mestrado foi desenvolvido como parte deste projeto temático, do qual a orientadora Dra. Catarina Carvalho Nievola participa como pesquisadora associada.

Este trabalho se baseia nas hipóteses de que a ativação de CAM em resposta à seca em plantas atmosféricas de *G. monostachia* funciona como um mecanismo de defesa não só contra a perda de água, mas também contra a produção excessiva de ROS; e de que estas plantas conseguem se recuperar às condições de pré-estresse em termos de atividade fotossintética e produção/eliminação de ROS quando reidratadas após a seca. Com isto, visamos responder as seguintes questões:

(a) A expressão de CAM induzida pela deficiência hídrica diminui a produção de ROS e a atividade enzimática antioxidante em plantas atmosféricas de *G. monostachia*?

(b) A reidratação de plantas atmosféricas de *G. monostachia* permite sua recuperação às condições de pré-estresse quanto à atividade fotossintética e metabolismo de ROS?

Os resultados obtidos neste trabalho, juntamente com aqueles gerados pelo projeto temático em que está inserido, visam formar subsídios para a identificação de genes envolvidos na modulação da via CAM, o que pode auxiliar na potencialização do uso agronômico de plantas com este metabolismo em ambientes áridos (Borland *et al.* 2009). Adicionalmente, estes estudos também podem auxiliar no desenvolvimento de projetos de conservação de espécies C_3-CAM facultativas quanto à seca mais intensa prevista para o futuro.

4. Objetivos e experimental

O objetivo do presente estudo foi avaliar a expressão de CAM, atividade de enzimas antioxidantes e produção de ROS em plantas atmosféricas da bromélia *G. monostachia* expostas a déficit hídrico e reidratadas após o estresse.

O experimental foi realizado nas dependências do LFV do IB-USP, com autorização da coordenadora Dra. Helenice Mercier. O material vegetal foi obtido de uma coleção *in vitro* pré-estabelecida neste laboratório (Fig. 4A), onde as plantas foram transferidas para aclimatização em câmara de crescimento com condições ambientais controladas (Fig. 4B).

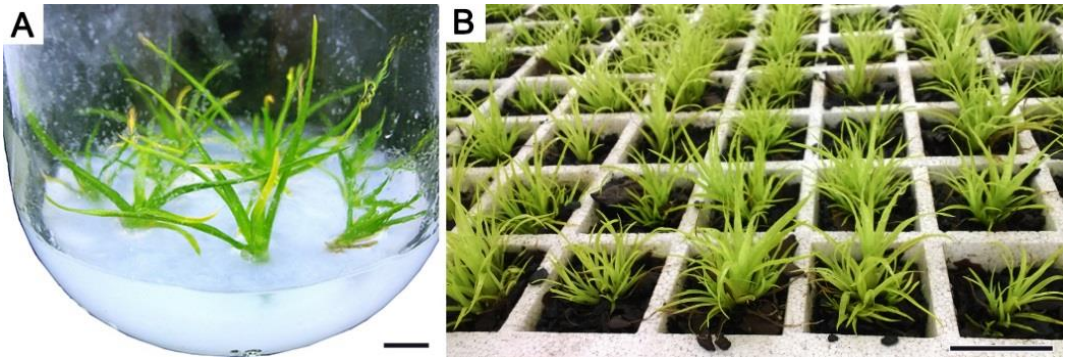

Figura 4. Cultivo de plantas atmosféricas de *Guzmania monostachia* (L.) Rusby ex Mez var. *monostachia*. A) Plantas em cultivo *in vitro*, B) plantas obtidas do cultivo *in vitro* em fase de aclimatização em substrato. Barras: A) 1 cm, B) 2 cm. Fotos: A) Antônio A. C. Neto, B) V. de Carvalho.

Após este período, parte das plantas foi mantida sob irrigação diária (controle) e outra submetida ao tratamento de suspensão de rega por oito dias de forma a induzir o déficit hídrico. Posteriormente, parte das plantas expostas a oito dias de seca foi utilizada para o tratamento de reidratação, onde a irrigação diária foi retomada por mais seis dias (Fig. 5). Plantas expostas à seca foram coletadas para análises após um e oito dias de tratamento, pois sabe-se que a expressão de CAM em plantas atmosféricas de *G. monostachia* é iniciada após um dia de déficit hídrico (Beltrán *et al.* 2013) e que esta via fotossintética está em seu maior nível de ativação na espécie após oito dias de seca (Freschi *et al.* 2010). Para as análises, foram utilizadas amostras compostas de folhas obtidas de 10 plantas por ponto de coleta e tratamento, de forma a verificar a tendência nas respostas fisiológicas das mesmas aos tratamentos.

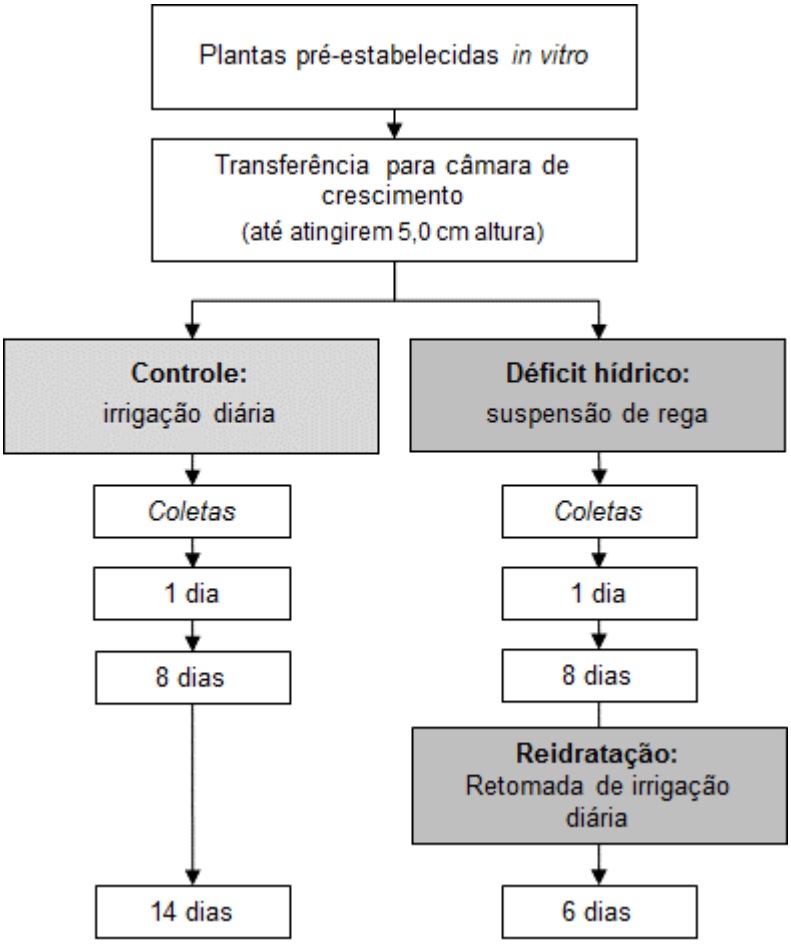

Figura 5. Fluxograma experimental do trabalho.

O texto desta dissertação divulga os resultados em dois capítulos formatados na forma de artigos científicos para publicação em periódicos internacionais. Assim, os capítulos 1 e 2 correspondem aos resultados e discussão que respondem a primeira e segunda questão levantada no projeto, respectivamente. O artigo do capítulo 1 já foi revisado quanto à língua inglesa e foi elaborado visando à submissão ao periódico *Functional Plant Biology*. O segundo

artigo do capítulo 2 foi elaborado para submissão ao periódico *Plant Physiology and Biochemistry*, cuja língua inglesa será propriamente revisada e corrigida. Todos os coautores que colaboraram à elaboração dos artigos estão indicados em ambos os capítulos.

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CAPÍTULO 1

Artigo para submissão ao periódico *Functional Plant Biology*

CAM intensification may prevent increased ROS production in atmospheric bromeliad *Guzmania monostachia* plants in response to drought

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ABSTRACT

Juvenile atmospheric plants of *Guzmania monostachia* (L.) Rusby ex Mez var. *monostachia* are constantly subjected to drought due to the epiphytic habitat. A shift from C_3 to Crassulacean acid metabolism (CAM) is one adaptation among several of this species to tolerate water loss. Therefore, atmospheric *G. monostachia* represents a suitable model for studying the influence of drought and CAM up-regulation on reactive oxygen species (ROS) metabolism. In the present study, we investigated how ROS production and scavenging are affected by CAM up-regulation during water suspension in atmospheric plants of *G. monostachia*. Plants tolerated drought, although water losses of 14% and 36% were observed after one and eight days of treatment, respectively. Nocturnal malate accumulation increased *ca.* 42% in plants after eight days of drought, indicating CAM intensification. Total ROS content that increased by 50% after one day of water suppression decreased to similar levels of the control plants after eight days of treatment. No significant lipid peroxidation (*i.e.* membrane damage) or chlorophyll degradation was observed in plants submitted to drought, but carotenoids increased at the end of treatment, suggesting successful plant acclimation. Increased ROS after one day of total water restriction may have signalled acclimation responses to drought and oxidative stress, including CAM intensification – which is known to prevent excessive ROS generation. Hence, the reduced activities of antioxidant enzymes and ROS after eight days of drought in *G. monostachia* could be related to the enhancement of CAM photosynthesis.

Keywords: antioxidant enzymes, C_3-CAM intermediate, epiphyte, metabolic plasticity, organic acids, oxidative stress

1. INTRODUCTION

Guzmania monostachia (L.) Rusby ex Mez var. *monostachia* is an epiphytic bromeliad with a wide distribution throughout the Neotropics, ranging from Florida (USA) to western South America and northeastern Brazil (Govaerts *et al.* 2016). Due to its epiphytic habit, this bromeliad has to cope with drought stress constantly due to scarce environmental water availability (Cushman and Borland 2002). Adult plants of *G. monostachia* develop a tank, a structure formed by overlapping broad leaves that allows water storage from rain. Tank plants avoid tissue water loss and may sustain their hydric status during drier seasons. In contrast, juvenile plants of *G. monostachia* are atmospheric, *i.e.* they have narrow, lanceolate leaves that do not form a tank but possess numerous water-absorbing trichomes, allowing quick rehydration during brief humid periods (Meisner *et al.* 2013). However, juvenile plants are naturally more prone to water losses due to reduced surface-to-volume ratio (Schmidt and Zotz 2001; Zotz and Hietz 2001); therefore, it has likely evolved metabolic strategies to tolerate the dehydration stress corresponding to the water regime of its respective habitat. Indeed, drought tolerance, defined as the ability to cope with reduced water potential of the plant tissue due to metabolic adjustments (Verslues *et al.* 2014), has been described in diverse atmospheric bromeliads (Zotz and Hietz 2001; Graham and Andrade 2004; Bader *et al.* 2009; Reyes-García *et al.* 2012).

G. monostachia is classified as C_3-CAM intermediate because it can utilize C_3 photosynthesis under favourable conditions to enhance growth and development and shift to CAM photosynthesis under drought as a resistance mechanism (Freschi and Mercier 2012). It has been also reported that both atmospheric and tank plants of *G. monostachia* can express CAM in well-watered conditions, hence presenting an intensification of this mechanism during exposure to drought (Freschi *et al.* 2010b; Beltrán *et al.* 2013).

CAM pathway initiates with CO_2 uptake during the dark period when stomata are open, followed by fixation through phosphoenolpyruvate carboxylase (PEPC) producing oxaloacetate (OAA), which is then converted into malate by malate dehydrogenase (MDH). OAA may also lead to citrate production in the mitochondria (Gawronska and Niewiadomska 2015). Both malate and citrate are stored in the vacuole as acids. During the subsequent light period, when stomata are closed, the organic acids are released into the cytosol and decarboxylated into CO_2 so it can be refixated by the C_3 cycle (Winter *et al.* 2015). Interestingly, Beltrán *et al.* (2013) have reported that atmospheric *G. monostachia* plants significantly increase nocturnal CO_2 uptake 24 hours after drought onset, indicating rapid and effective metabolic modulation, which could be considered essential for a drought-tolerant species that has to withstand drastic water

loss (Verslues *et al.* 2014).

Exposure to drought is inherent to the epiphytic habitat and not only can cause direct effects on the cells, such as reduction in water content but also inflict secondary responses, like oxidative stress (Chamoli and Verma 2014). Indeed, it is widely reported that drought can lead to an increase in reactive oxygen species (ROS) production (Cruz de Carvalho 2008; Noctor *et al.* 2014). When in high concentrations, ROS can oxidize and damage several cellular components, most importantly the membranes through lipid peroxidation (Gill and Tuteja 2010). However, the initial increase in ROS due to exposure to abiotic stresses can also have beneficial effects on the cells. If kept under tight control, ROS production acts as a signal to activate acclimation and defence pathways to the new stressful environmental conditions (Miller *et al.* 2010). Control of the ROS production occurs through a scavenging system composed of a wide range of antioxidant metabolites, including several enzymes, such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and glutathione reductase (GR). Carotenoids are also important antioxidants because they are able to quench several ROS, especially singlet oxygen, which is one of the main precursors of lipid peroxidation (Gill and Tuteja 2010; Smirnoff 2007).

As atmospheric bromeliads are subject to variable water availability, they have to adjust their metabolism rapidly to tolerate such conditions. Therefore, it is reasonable to speculate that ROS could play an important role in the plant's acclimation to drought in these species. Consequently, they must also require an effective antioxidant mechanism for protection against augmented cell ROS generation. Indeed, González-Salvatierra *et al.* (2010) demonstrated that the total antioxidant capacity of adult *Tillandsia brachycaulos* Schltdl. plants, an atmospheric CAM bromeliad, increased during the dry season so as to reduce oxidative damage derived from water shortage.

It has been proposed that CAM expression could be an adaptive mechanism to curtail oxidative burst in plants due to its carbon-concentrating feature, which helps prevent over-reduction of the electron transport chain in chloroplasts during the day (Griffiths 1989; Niewiadomska and Borland 2008). In fact, some studies have indicated that CAM expression helps to reduce oxidative burst in the C_3-CAM intermediate *Mesembryanthemun crystallinum* L. (Borland *et al.* 2006; Sunagawa *et al.* 2010). Therefore, ROS could also play a role in signalling CAM expression when plants of *G. monostachia* are exposed to water deficit, as suggested for *M. crystallinum* (Ślesak *et al.* 2007, 2008). More studies are needed to define the involvement of these substances in the CAM induction and intensification in the atmospheric bromeliad *G. monostachia*.

Reports detailing the influence of drought on ROS generation and antioxidant responses

in epiphytic bromeliads that express CAM are scant. Atmospheric *G. monostachia* plants are C_3-CAM intermediates; therefore, they can be considered a suitable model to study the influence of both drought and CAM on ROS metabolism since they up-regulate CAM when exposed to water suspension (Beltrán *et al.* 2013). Thus, studies on how these plants cope with reduced water content and oxidative stress can provide important information on drought-tolerance mechanisms in C_3-CAM intermediate plants. In the present study, we investigated how the ROS production/scavenging balance is affected by CAM induction in atmospheric *G. monostachia* plants subjected to drought.

2. MATERIALS AND METHODS

2.1. Plant material and growth conditions

Atmospheric plants of *Guzmania monostachia* (L.) Rusby ex Mez var. *monostachia* were obtained from a previously established pool of *in vitro* plantlets. Plantlets were cultivated in flasks containing 100 mL of growth medium composed of Knudson (1946) macronutrients, Murashige and Skoog (1962) micronutrients, 0.5% (w/v) $Ca(CO)_3$, 2% (w/v) sucrose, 0.2% (w/v) Phytagel™ (Sigma-Aldrich) and a pH of 5.8. Flasks with plantlets were kept in a growth room under the following conditions: 25 ± 2ºC, 12-h photoperiod, and a light intensity of 50 μmol photons m^{-2} s^{-1} for six months. Afterwards, plantlets were transferred to trays containing commercial organic substrate (Tropstrato HT - Vida Verde®) and ground *Pinus* bark and then maintained in a controlled environment chamber (Fig. 1A) under the following conditions: 25/20°C (light/dark), relative humidity of 60/70% (light/dark), 12-hour photoperiod, and photosynthetic flux density (PFD) of about 200 μmol photons m^{-2} s^{-1}. PFD inside the growth chamber was monitored with an LI-190 quantum sensor connected to an LI-250 A meter (LI-COR Instruments). All plants were watered daily with tap water and fertilized with a nutritive solution with half the concentration of Knudson (1946) macronutrients and Murashige and Skoog (1962) micronutrients every 14 days until developing into atmospheric plants with an average of 13 ± 2 leaves, 5.0 ± 0.5 cm in height (Fig. 1B), 0.29 ± 0.1 g and 0.029 ± 0.009 g of fresh and dry weight, respectively (values are means ± s.d., n=14). Plants were maintained for three weeks without fertilization but normally irrigated with distilled water prior to the water withholding treatment.

Figure 1. Pool of atmospheric *Guzmania monostachia* plants utilized in the present study. A) Plants in tray with substrate in a controlled environment chamber, B) average size of atmospheric individual of *G. monostachia*. Scale bar: 1 cm.

2.2. Drought treatment and leaf sampling

Plants of *G. monostachia* were submitted to well-watered (control) or drought treatment in the controlled environment chamber, as cited in item 2.1. Well-watered plants were irrigated daily with distilled water (control), while drought-treated plants had watering suspended. After one and eight days of treatment, survival rate of control and treated plants was assessed, followed by harvesting of leaves for determination of relative water content and biochemical analyses, as described below. At each harvesting point, a pool of 10 plants was sampled.

2.3. Water content measurement

To assess the relative water content (RWC) of plants, their leaves were immediately weighed (fresh weight, FW), followed by rehydration in distilled water for 24 h in the dark (turgid weight, TW), and transferred to an oven at 60°C until reaching a constant weight (dry weight, DW). The RWC was expressed according to the formula (Barrs and Weatherley 1962):

$$RWC = (FW - DW)/(TW - DW) * 100.$$

2.4. Organic acid quantification

Malate and citrate levels were determined in leaves harvested every 4 hours during a diel cycle and analysed by gas chromatography coupled to mass spectrometry (GC/MS), as described Freschi *et al.* (2010a), with modifications. Briefly, leaf tissue frozen at -80°C was ground with liquid N_2, and 100 mg were homogenized in 500 µL of methanol:chloroform:water (12:5:1, v/v/v), containing 500 µg of benzoic acid ^{13}C as internal standard. The mixture was kept at 60°C for 30 min. Then, 500 µL of water were added and the mixture centrifuged at 16000 *g*, 4°C for 10 min. An aliquot of 100 µL of the extract was transferred to vials and vacuum dried. The remaining residue was dissolved in 25 µL of pyridine and derivatized at 92°C for 60 min with *N*-tert-butyldimethylsilyl-*N*-methyltrifluoroacetamide (Sigma-Aldrich). One µL

samples of the derivatized extracts were analysed in a GC/MS (Shimadzu QP2010SE). The initial running condition was 100°C for 6 min, followed by a gradient up to 300°C at 6°C min^{-1}. The column used for separation was an Agilent DB-5MS (30 m, I.D. 0.25 mm, 0.50 µm) with helium as the carrier gas at a flow rate of 1 mL min^{-1}. The endogenous metabolite concentration was obtained by comparing the peak areas of the chromatograms with commercial standards (Sigma-Aldrich). The results were expressed as mg organic acid g DW^{-1}.

2.5. Total ROS quantification

Total ROS was quantified using a hydrolysed probe 2′,7′-dihydrodichlorofluorescein (H_2-DCF), which is oxidized by intracellular ROS into the highly fluorescent compound 2′,7′-dichlorofluorescein (DCF), whose fluorescence is directly proportional to the ROS content in the tissue. Probe hydrolysis was undertaken following the protocol of Yadav and Bhatla (2011), with modifications. An aliquot of 2.5 µL of 100 mM 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA, Sigma-Aldrich) was added to 200 µL of 0.01 N NaOH and kept in the dark for 30 min at room temperature, obtaining H_2-DCF. ROS extraction from leaf samples and reactions with H_2-DCF were performed according to Jambunathan (2010), with some modifications. Leaf tissue frozen at -80°C sampled from treated and control plants harvested at 8 h (light period) was ground with liquid N_2, and 100 mg were homogenized in 1 mL of 10 mM Tris-HCl (pH 7.2), followed by centrifugation at 13000 *g*, 4°C for 30 min. Next, 50 µL of the extracts were mixed with 950 µL of 10 mM Tris-HCl (pH 7.2) and 10 µL of the hydrolysed probe previously diluted 50x. Reaction mixtures were incubated in the dark for 10 min at room temperature, and resultant fluorescence values were measured using a fluorometer adjusted to an excitation wavelength of 485 nm and emission of 535 nm. Total protein in the extracts was quantified with the Bradford reagent (Sigma-Aldrich) and bovine serum albumin as a standard. Fluorescence values were normalized according to the protein content and expressed as fluorescence units μg^{-1} protein.

2.6. Lipid peroxidation (LPO)

The occurrence of lipid peroxidation in tissues indicates membrane damage (Gill and Tuteja 2010). In the study, it was quantified following the ferrous oxidation-xylenol orange assay described by Jiang *et al.* (1991), with modifications. This method quantifies LPO at initial stages and is based on the oxidation of Fe^{+2} by lipid hydroperoxides at acid pH in the presence of the Fe^{+3}-complexing dye, xylenol orange. Leaf tissue frozen at -80°C sampled from treated and control plants harvested at 8 h (light period) was ground with liquid N_2, and 100 mg were homogenized in 2 mL of 80% (v/v) ethanol, followed by centrifugation at 3000 *g*, 25°C for 10 min. Subsequently, 200 µL of the supernatants were added to 800 µL of reaction mixture

containing 100% (v/v) methanol, 0.1 mM xylenol orange (Sigma-Aldrich), 25 mM H_2SO_4, 4 mM butylated hydroxytoluene, and 0.25 mM ammonium iron(II) sulphate. Resulting solutions were incubated at room temperature for 20 min and mixed by inversion every 3 min. Absorbances were read at 560 nm in a spectrophotometer and LPO levels expressed as cumene hydroperoxide equivalents (mmol cumene g^{-1} DW).

2.7. Antioxidant enzyme assays

Fresh leaf samples of 200 mg obtained from treated and control plants harvested at 8 h (light period) were ground with 2 mL of extraction solution described by Souza *et al.* (2013). Homogenates were centrifuged at 11000 *g*, 4°C for 30 min and supernatants frozen at -80°C until analysis. Total protein in the extracts was quantified with the Bradford reagent (Sigma-Aldrich) and bovine serum albumin as a standard for calculating specific activities of the enzymes. All samples were analysed in triplicate in a spectrophotometer.

SOD (EC 1.15.1.1) activity was determined according to Beauchamp and Fridovich (1971) with modifications by Balen *et al.* (2009). Reaction mixture contained 50 mM potassium phosphate (pH 7.8), 0.1 mM EDTA, 0.075 mM nitrotetrazolium blue chloride (NBT, Sigma-Aldrich), 13 mM methionine, and 0.002 mM riboflavin. One mL of the reaction mixture was mixed with 40 µL of extract and placed under 23 watt fluorescent light (Phillips®) for 5 min. SOD activity was determined at 560 nm and expressed as units mg^{-1} protein. One unit of SOD activity was defined as the amount of enzyme required to inhibit the reduction of NBT by 50% per minute.

GR (EC 1.6.4.2) activity was determined following Schaedle and Bassham (1977). Reaction mixture contained 50 mM Tris-HCl (pH 7.5), 0.5 mM oxidized glutathione (GSSG), 0.15 mM NADPH and 3 mM $MgCl_2$. Reaction was started by adding 100 µL of leaf extract in a total volume of 1 mL and monitored at 340 nm. Activity was calculated with extinction coefficient of 6.2 mM^{-1} cm^{-1} and expressed as nmol $NADP^+$ min^{-1} mg^{-1} protein.

APX (EC1.11.1.11) activity was assayed using the method described by Nakano and Asada (1981), modified by Weng *et al.* (2007). Reaction mixture contained 100 mM potassium phosphate (pH 7.0), EDTA 0.1 mM, 0.5 mM ascorbate and 0.2 mM H_2O_2. Reaction was started by adding 40 µL of leaf extract in a total volume of 1 mL and monitored at 290 nm. Activity was calculated with extinction coefficient of 2.8 mM^{-1} cm^{-1} and expressed as nmol ascorbate min^{-1} mg^{-1} protein.

CAT (EC 1.11.1.6) activity was quantified according to Luck (1974), with some modifications. Reaction solution contained 100 mM potassium phosphate (pH 7.5) and 15 mM H_2O_2. Reaction was started by adding 100 µL of leaf extract in a total volume of 1 mL and

monitored at 240 nm. CAT activity was calculated with extinction coefficient of 0.4 mM^{-1} cm^{-1} and expressed as µmol H_2O_2 min^{-1} mg^{-1} protein.

2.8. Photosynthetic pigment quantification

Chlorophyll *a*, *b* and carotenoids were extracted from leaves following Munné-Bosch and Lalueza (2007), with modifications. Leaf tissue frozen at -80°C sampled from control and treated plants harvested at 8 h (light period) was ground with liquid N_2, and 100 mg were homogenized in 1 mL of 100% (v/v) acetone. Homogenates were kept in an ultrasonic bath for 30 min at 4°C, followed by centrifugation at 8000 rpm, 4°C for 20 min. Supernatants were cooled, and the remaining precipitates underwent a second extraction and ultrasonic bath procedure. After centrifugation, supernatants of both extractions were combined. Pigment contents were calculated using equations described by Lichtenthaler and Buschmann (2001) and expressed as mg pigment g^{-1} DW.

2.9. Statistical analyses

Experiments were conducted in a completely randomised design. Data were submitted to analysis of variance (ANOVA) with variation among means compared by using Tukey's test at $P < 0.05$. Means of the control (well-watered plants) indicated in the results section for all the parameters were calculated with values obtained from plants harvested on both points of treatment (one and eight days), considering they were in the same conditions of irrigation.

3. RESULTS

3.1. CAM expression characterisation

A survival rate of 100% was observed in well-watered plants (control) as well as in those submitted to water suppression. However, plants kept under one or eight days of water suspension underwent a significant decrease of *ca*. 14% and 36% of the RWC, respectively, when compared to the well-watered control (Fig. 2).

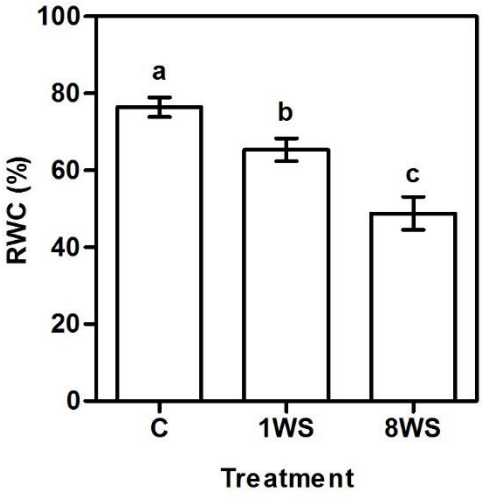

Figure 2. Relative water content (RWC) in atmospheric *Guzmania monostachia* plants under well-watered (control) and drought treatment. C, control (well-watered plants); 1WS, one day of water suspension; 8WS, eight days of water suspension. Values are means ± s.d. (n=5). Means of the control were calculated with values obtained from plants harvested at both points of treatment (one and eight days). Each replicate contains pooled leaves from 10 plants. Letters compare treatments by Tukey test ($P < 0.05$).

Organic acid quantification indicated that malate and citrate were depleted throughout the day and resynthesized during the night period in control and drought treatment, indicating the occurrence of CAM photosynthesis (Fig. 3). The fluctuation observed for these organic acids are in accordance to previous results obtained for detached leaves of adult *G. monostachia* plants by our research group (Pereira *et al.* 2013), assuring the replication value of the results obtained in this study. Furthermore, nocturnal production of malate was higher than citrate in atmospheric *G. monostachia*, indicating that the former is the main carbon storage compound in these plants. Malate and citrate accumulation during the dark period was also noted in plants under well-hydrated conditions, although at reduced levels compared to those kept without irrigation (Fig. 3). Therefore, well-watered plants from the control were performing CAM at a lower degree when compared to drought treatments. Regarding citrate diel cycle in plants exposed to water suspension, a rapid increase in nocturnal production was observed after one day, followed by stabilization to lower values after eight days of drought (Fig. 3B). However, the amount of citrate accumulated at 8 h was statistically similar between plants kept for one or eight days under water suspension, being *ca*. 29% higher than in control (Fig. 3B, Table 1). A similar response was observed for malate synthesis, in which after one day of drought there was a significant increase in the acid production during the dark period in comparison to the control, peaking at 4 h (Fig. 3A, Table 1). At this point, malate production was 31% higher in plants subjected to one day of drought in comparison to those under eight days of water shortage. Nevertheless, the highest accumulation of malate was exhibited by plants under eight days of

water suspension at the onset of the light period (8 h), which was *ca*. 42% higher than in control and one day of drought, on average (Fig. 3A, Table 1). These results indicate that CAM activity, in terms of malate accumulation, increased throughout the drought treatment.

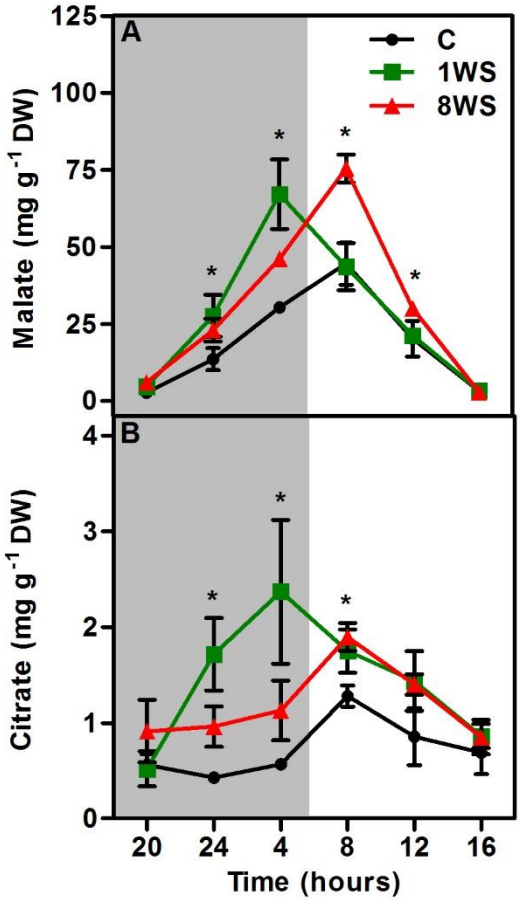

Figure 3. Daily variation in the leaf content of malate (A) and citrate (B) in atmospheric *Guzmania monostachia* plants under well-watered (control) and drought treatment. The grey region indicates the dark period. DW, dry weight; C, control (well-watered plants); 1WS, one day of water suspension; 8WS, eight days of water suspension. Values are means ± s.d. (n=3). Means of the control were calculated with values obtained from plants harvested at both points of treatment (one and eight days). Each replicate contains pooled leaves from 10 different plants. Asterisks indicate statistical difference among treatments by ANOVA ($P < 0.05$). Mean comparison is detailed in Table 1.

Table 1. Statistical analysis for the daily variation in malate and citrate content in atmospheric *Guzmania monostachia* plants under well-watered (control) and drought treatment. DW, dry weight; C, control (well-watered plants); 1WS, one day of water suspension; 8WS, eight days of water suspension. Means of the control were calculated with values obtained from plants harvested at both points of treatment (one and eight days). Values are means ± s.d. (n=3). Each replicate contains pooled leaves from 10 different plants. Letters on rows compare treatments per time by Tukey test ($P < 0.05$).

Organic acids	Time (hours)	Treatment											
		C				1WS				8WS			
Malate	20	2.74	±	0.57		4.72	±	2.07		6.01	±	2.33	
(mg g^{-1} DW)	24	13.59	±	3.61	b	27.66	±	6.82	a	23.00	±	3.73	ab
	04	30.35	±	1.20	b	67.11	±	11.25	a	46.10	±	1.08	b
	08	44.58	±	6.80	b	43.55	±	7.69	b	75.47	±	4.56	a
	12	20.26	±	5.77	b	21.31	±	2.02	ab	30.03	±	2.00	a
	16	3.21	±	0.25		3.44	±	0.10		2.89	±	0.64	
Citrate	20	0.56	±	0.11		0.52	±	0.18		0.91	±	0.33	
(mg g^{-1} DW)	24	0.43	±	0.05	b	1.72	±	0.38	a	0.96	±	0.21	b
	04	0.57	±	0.03	b	2.37	±	0.53	a	1.13	±	0.31	b
	08	1.28	±	0.11	b	1.75	±	0.23	a	1.90	±	0.14	a
	12	0.86	±	0.30		1.44	±	0.31		1.40	±	0.10	
	16	0.69	±	0.22		0.86	±	0.13		0.85	±	0.18	

3.2. ROS metabolism in response to drought

Total ROS production was altered after one day of water suspension with a 50% increase compared to well-watered plants (Fig. 4A). However, after eight days of water suspension, ROS generation decreased to levels similar to the control. Moreover, there was no significant difference in LPO between treatment and control, which indicates that water suspension or increased ROS did not lead to augmented LPO chain reactions (Fig. 4B).

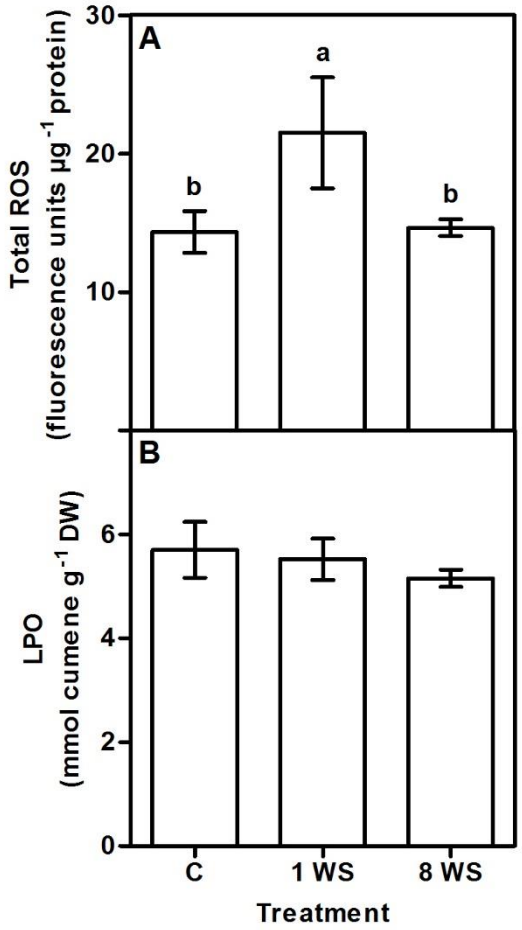

Figure 4. Total reactive oxygen species (ROS) production (A) and lipid peroxidation (LPO) (B) in atmospheric *Guzmania monostachia* plants under well-watered (control) and drought treatment. DW, dry weight; C, control (well-watered plants); 1WS, one day of water suspension; 8WS, eight days of water suspension. Values are means ± s.d. (n=3). Means of the control were calculated with values obtained from plants harvested at both points of treatment (one and eight days). Each replicate contains pooled leaves from 10 different plants. Letters compare treatments by Tukey test ($P < 0.05$).

In general, antioxidant enzyme activities were not positively affected by the imposed stress. After one day of drought, the activities of most enzymes remained unchanged compared to control plants (Fig 5A-C) with the exception of GR, which decreased by *ca*. 20% (Fig. 5D). However, when plants were kept for eight days under water shortage, the activities of SOD, CAT and APX decreased significantly when compared to the other conditions (Fig 5A-C). On average, CAT showed the most prominent decrease (48%), followed by SOD and APX (29%). Meanwhile, GR remained statistically similar to plants exposed to one day of water suspension (Fig. 5D).

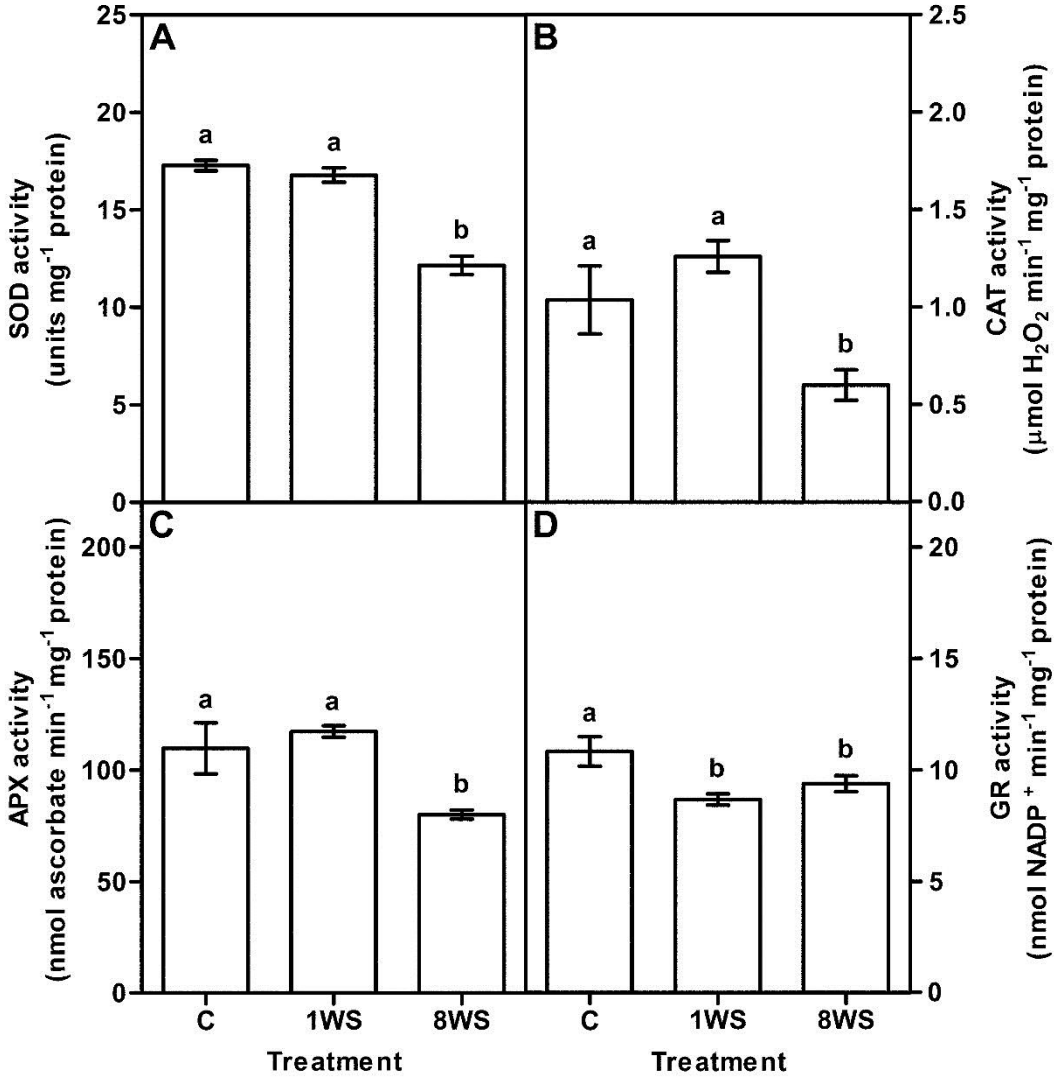

Figure 5. Antioxidant enzyme activities in atmospheric *Guzmania monostachia* plants under well-watered (control) and drought treatment. A) Superoxide dismutase (SOD), B) catalase (CAT), C) ascorbate peroxidase (APX), D) glutathione reductase (GR). DW, dry weight; C, control (well-watered plants); 1WS, one day of water suspension; 8WS, eight days of water suspension; SOD enzyme unit is the amount of enzyme required to inhibit the reduction of NBT by 50%. Values are means ± s.d. (n=3). Means of the control were calculated with values obtained from plants harvested at both points of treatment (one and eight days). Each replicate contains pooled leaves from 10 different plants. Letters compare treatments by Tukey test ($P < 0.05$).

3.3. Photosynthetic pigment production in response to drought

Water loss in plants under drought did not lead to significant changes in total chlorophyll production or the chlorophyll *a: b* ratio (Fig. 6A, B). However, carotenoid contents decreased when plants were subjected to water suspension for one day, then increased by 27% after eight days to values significantly higher than those observed in control (Fig. 6C). This also reflected positively in the carotenoids: total chlorophyll ratio of plants under eight days of drought, which was 9% higher than in control and one day of water suppression, on average (Fig. 6D).

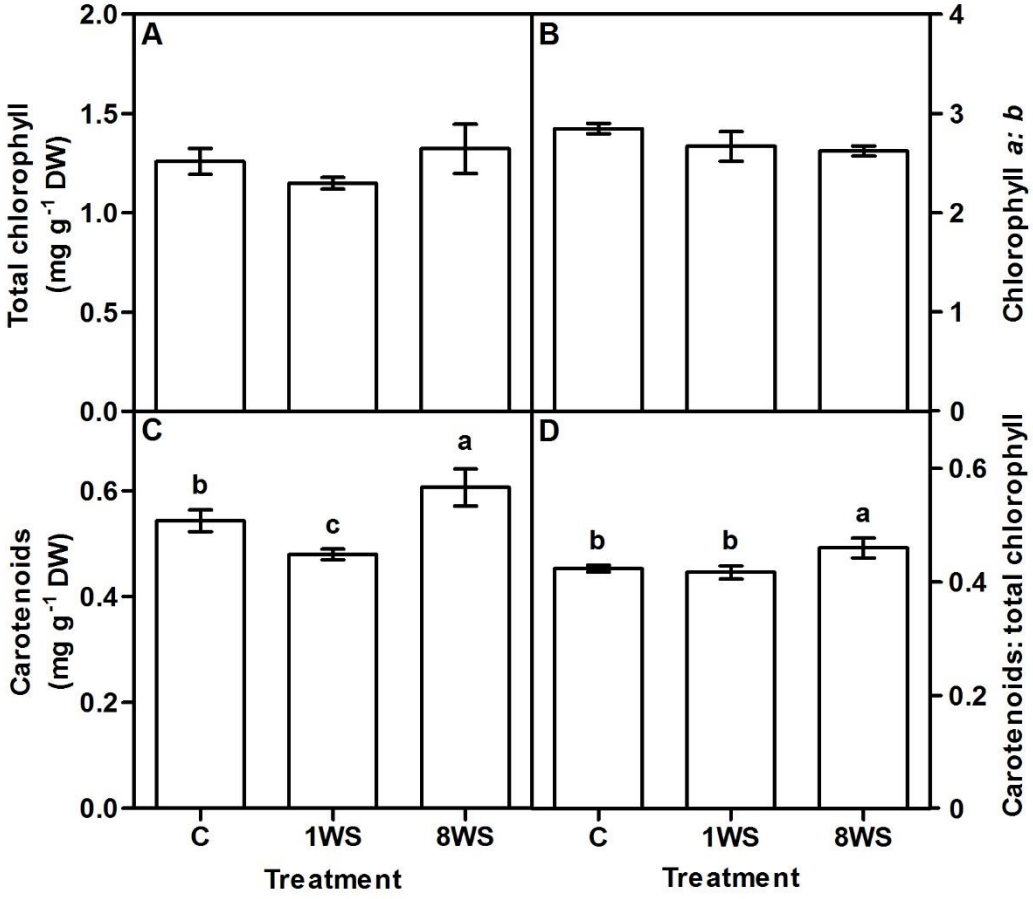

Figure 6. Content of photosynthetic pigments in atmospheric *Guzmania monostachia* plants under well-watered (control) and drought treatment. A) Total chlorophyll, B) chlorophyll *a: b* ratio, C) carotenoids, D) carotenoids: total chlorophyll ratio. DW, dry weight; C, control (well-watered plants); 1WS, one day of water suspension; 8WS, eight days of water suspension. Values are means ± s.d. (n=3). Means of the control were calculated with values obtained from plants harvested at both points of treatment (one and eight days). Each replicate contains pooled leaves from 10 different plants. Letters compare treatments by Tukey test ($P < 0.05$).

4. DISCUSSION

Atmospheric *G. monostachia* plants submitted to drought for eight days were capable of resisting the imposed stress, even though they underwent a progressive water loss during the treatment (Fig. 2). The decrease in leaf water content of atmospheric plants proved to be higher than tank plants of *G. monostachia* exposed to a similar period of drought. Middle and apical leaf parts of tank plants showed a 25% reduction and no water loss, respectively (Freschi *et al.* 2010b). Schmidt and Zotz (2001) verified that *Vriesea sanguinolenta* Cogn. & Marchal adult tank plants possess higher stomatal control than juvenile atmospheric individuals, allowing avoidance of water loss in the former. Furthermore, the same authors state that the smaller size

of atmospheric plants predisposes them to higher water deficits due to reduced surface-to-volume ratio.

The resistance of atmospheric plants of *G. monostachia* to the progressive water loss could be partly due to CAM intensification, as indicated by increased malate and citrate content during the dark-light transition since the first day of water shortage (Fig. 3). Such results are similar to those reported by Beltrán *et al.* (2013), who observed that atmospheric *G. monostachia* plants enhanced nocturnal CO_2 uptake after one day of water suspension. In addition, our findings also indicate that progression of water deficit led to shifts in the dynamics of acid accumulation in atmospheric plants, as observed after one day of drought, in which the decarboxylation phase of CAM started earlier compared with plants under eight days of water suspension (Fig. 3). In fact, the ability to alter the magnitude of CAM expression and the duration of each pathway step in response to changing environmental conditions has been reported in CAM plants already, crucial to maintain high, efficient photosynthetic rates (Borland *et al.* 2011). Furthermore, previous studies with atmospheric and tank individuals of *G. monostachia* have also shown that even under well-watered regimes there is some nocturnal acid accumulation, which is then intensified during the drought treatment (Beltrán *et al.* 2013; Freschi *et al.* 2010b).

When *G. monostachia* plants were exposed for one day to water suspension, ROS generation in the cells increased (Fig. 4A), probably due to the onset of drought and tissue water loss (Cruz de Carvalho 2008). This punctual ROS increase at the initial period of drought may have acted as a signal to trigger acclimation responses so the plant could adapt to a water-deficient environment (Cruz de Carvalho 2008; Miller *et al.* 2010). Furthermore, by the eighth day of water suspension, ROS production decreased to levels similar to the well-watered plants. Ślesak *et al.* (2008) have verified a similar response in H_2O_2 production during CAM induction by salinity (stress that leads to osmotic stress, *i.e.* water deficit) in *M. crystallinum* plants, in which a peak of H_2O_2 content was detected in leaves prior to CAM activation, followed by a decrease after full expression of this photosynthetic mode. Thus, these authors considered that such behaviour could be involved in signalling pathways of salinity defence mechanisms, including CAM expression. This being the case, it is reasonable to suppose that atmospheric *G. monostachia* plants reached a steady-state metabolism adapted to the new water-deficient environment after eight days, reducing ROS production (Fig. 4A). Indeed, the increase in ROS after the onset of drought did not cause significant membrane damage during the water suspension treatment (verified from LPO analysis), suggesting successful plant acclimation (Fig. 4B). The shift towards ROS production is directly influenced by the intensity and duration of the imposed stress (Apel and Hirt 2004); thus, ROS-derived damage, such as LPO, may only

occur when the plant's antioxidant capacity has been outdone by the oxidative burden in the cells. Such an effect has been noticeably demonstrated for the CAM succulent *Rosularia elymaitica* (Boiss. & Hausskn.) A. Berger, in which LPO was not observed until 20 days of water suppression (Habibi and Hajiboland 2011). Similarly, in epiphyllous buds of another CAM succulent *Kalanchoe tubiflora* Raym.-Hamet, membrane damage increased during the drought period and higher ROS production (Luo *et al.* 2015). Therefore, we could expect that longer drought exposure over atmospheric *G. monostachia* plants would eventually lead to accumulation of ROS and higher LPO occurrence.

Accordingly, chlorophyll content was also unaffected during drought treatment (Fig. 6A, B). However, the increased ROS in plants submitted to water suspension for one day may have degraded carotenoids (Fig. 6C), considering the fact that such pigments are highly susceptible to oxidative destruction (Farooq *et al.* 2009). Nevertheless, the carotenoids: total chlorophyll ratio remained unchanged (Fig. 6D), suggesting that the imposed stress did not cause alterations in the proportion of pigments of the antenna complex, maintaining its light harvesting capacity. Meanwhile, plants under eight days of water suspension not only maintained a stable chlorophyll content but were also capable of increasing carotenoid production, which led to an augmented carotenoids: total chlorophyll ratio (Fig. 6C, D). Such accessory pigments are well-known antioxidants because they can react with singlet oxygen and/or directly with lipid peroxyl radicals, both of which are precursors of LPO chain reactions (Smirnoff 2007), therefore helping to maintain membrane integrity (Gill and Tuteja 2010).

The control of the signals emitted through ROS is made by the antioxidant system, which keeps their production under tolerable levels by the cells, preventing eventual damage (Munné-Bosch *et al.* 2013). In *G. monostachia* plants exposed to one day of water suspension, which had higher ROS production, the activities of antioxidant enzymes SOD, CAT and APX remained unchanged (Fig. 5A-C), while GR decreased compared to the well-watered control (Fig. 5D). This may have contributed, in part, to maintain high ROS generation and consequently its signal emission while also preventing cell damage. By the eighth day of drought exposure, there was a significant reduction in activities of all antioxidant enzymes compared to the well-watered control (Fig. 5). The influence of drought over the enzymatic scavenging system is highly variable between species (Noctor *et al.* 2014), even among CAM plants. In the C_3-CAM intermediate *Sedum album* L., SOD and CAT activities decreased under severe drought stress and maximal CAM expression (Castillo 1996), similarly to *G. monostachia*, although GR and APX activities were enhanced in the same period in *S. album*. Plants of the halophyte C_3-CAM intermediate *M. crystallinum* showed increased SOD but lower CAT activity when fully expressing CAM in response to salt stress (Ślesak *et al.* 2002;

Niewiadomska and Miszalski 2008). Reports on ROS production and scavenging system in epiphytic bromeliads exposed to drought are scant. González-Salvatierra *et al.* (2010) have assessed the total antioxidant capacity in adult plants of an epiphytic (*T. brachycaulos*) and a terrestrial bromeliad (*Bromelia karatas* L.) in the field, both CAM species. They demonstrated that antioxidant activity increased in both species during the dry season, probably related to water deficit and higher light conditions.

Considering the fact that in the present study plants of *G. monostachia* were able to adapt to the water-deficient environment after eight days of drought, the reduction of antioxidant enzyme activities could also be due to the activation of alternative protective mechanisms. Among those mechanisms, the intensification of CAM expression *per se* may be a candidate. In fact, CAM has been considered an adaptive mechanism to avoid oxidative stress because the concentration of CO_2 in chloroplasts during the day may aid in preventing over-reduction of the electron transport chain (Griffiths 1989; Niewiadomska and Borland 2008). Studies have demonstrated that *M. crystallinum* CAM-deficient mutants have higher ROS production and antioxidant enzymatic activity compared to wild-type individuals expressing CAM, clearly indicating that the latter have lower oxidative burden (Borland *et al.* 2006; Sunagawa *et al.* 2010). Therefore, we can speculate that ROS production in atmospheric *G. monostachia* plants subjected to longer drought exposure was kept, in part, under the same levels of well-watered plants due to the intensification of the decarboxylation phase of CAM during the day. In fact, Niewiadomska and Borland (2008) comment that CAM plants must rely on diverse mechanisms that aid in curtailing increased ROS production, such as the water-water cycle, cyclic electron flow, and chlororespiration. These chloroplastic processes assist in regulating the ATP: NADPH ratio by utilizing excessive electrons from the electron transport chain, given that the daily requirement of ATP and NADPH in CAM plants is highly flexible (Winter and Smith 1996). Furthermore, *G. monostachia* plants under eight days of drought would presumably rely less on the enzymatic antioxidant system to balance ROS generation due to intensified CAM expression and associated electron-consuming mechanisms, hence the lower antioxidant enzyme activities in comparison to plants performing weaker CAM (well-watered control and one day of water suspension) (Fig. 5).

In summary, our data indicated that atmospheric *G. monostachia* plants were able to reach a steady-state metabolism adapted to the water-deficient environment, as observed from high CAM expression, decreased ROS/LPO and sustained photosynthetic pigment content. Also, an increase in ROS at the onset of drought could be involved in signalling pathways in order to activate acclimation responses to dehydration, such as enhancement in CAM and carotenoids production. Finally, these results illustrate how plants with flexible CAM

expression, such as atmospheric *G. monostachia*, rely on rapid modulation of diverse mechanisms involved in drought tolerance, which is an important feature for the adaptation of such species to the adverse habitats in which they reside.

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CAPÍTULO 2

Artigo para submissão ao periódico *Plant Physiology and Biochemistry*

Drought recovery in CAM atmospheric bromeliad *Guzmania monostachia* involves altered daily H_2O_2 production and increased antioxidant enzyme activities

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ABSTRACT

Young atmospheric plants of the epiphytic bromeliad *Guzmania monostachia* rely on plastic metabolic responses to endure shifts between humid and dry periods, such as modulation of Crassulacean acid metabolism (CAM) intensity. In terms of antioxidant responses, our group verified that H_2O_2-scavenging enzymes activities reduce when these plants are exposed to drought and expressing strong CAM. Considering atmospheric *G. monostachia* plants have to adjust their metabolism rapidly when water availability is restored, the question arises if they are able to fully reverse the daily rhythm of CAM expression and H_2O_2 production/scavenging balance when they are rewatered after drought, reaching pre-stress conditions. Hence, atmospheric *G. monostachia* plants under pre-stress (daily irrigation control), drought and rewatering conditions were sampled at the end of the light and dark period for analyses. Eight days of water suspension decreased water content, mesophyll succulence and increased CAM expression, which was associated with reduced daytime H_2O_2 production and decreased activities of superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and glutathione reductase (GR) on at least one period of the day when compared to control. After six days of rewatering, plants recovered water content, CAM intensity and daytime H_2O_2 production to similar levels of the control. However, rewatering altered daily variation of most ROS parameters and increased enzyme activities on distinct periods. We concluded that rewatered plants restored CAM activity to pre-stress conditions, but were still recovering H_2O_2 metabolism. These results provide guidance for research on how plants modulate their photosynthetic and ROS metabolism to endure humid-dry cycles, which may be intensified in the future.

Keywords: C_3-CAM intermediate, epiphyte, metabolic plasticity, oxidative stress, rehydration, ROS, water deficit

1. INTRODUCTION

The global climatic setting is facing a shift towards heating that could result in quicker setting, more intense and longer periods of drought (Trenberth et al., 2014). In this scenario, plants will inevitably be more exposed to humid-dry cycles along its lifetime (Xu et al., 2010). Therefore, plants will have to recover rapidly and effectively during the intermittent periods of higher water availability in order to boost their growth and development, ensuring the completion of its life cycle. Hence, it is important to evaluate how plants metabolism respond to rewatering after drought exposure, in face of future environmental changes. As reviewed by Xu et al. (2010), studies regarding physiological responses to drought and subsequent recovery are scant when compared to those concerning drought responses alone. However, more attention is being paid to the matter in the recent years for diverse species (Maevskaya and Nikolaeva, 2013; Ji et al., 2014; Jin et al., 2015).

Considering that humid-dry cycles become more frequent and intense, species from water-limiting environments may be under threat of survival. Among them, epiphytic bromeliads are considered highly vulnerable to such climatic changes due to their dependency on water derived exclusively from rainfall, dew and/or fog in order to survive (Cach-Pérez et al., 2014). Nevertheless, these bromeliads present plastic and fast metabolic responses to environmental conditions (Haslam et al., 2002; Reyes-García et al., 2012; Chaves et al., 2015), which could aid in their resistance in face of future climatic changes. The photosynthetic mode Crassulacean acid metabolism (CAM) is an important metabolic feature for many epiphytic bromeliads to withstand water shortage periods (Crayn et al., 2015) because it improves water conservation through nocturnal CO_2 uptake when transpiration is reduced (Winter et al., 2015). Such metabolism is well-known for its high plasticity, where it is possible for plants to alter the magnitude of CAM expression and length of each pathway step within a 24-hour day (Borland et al., 2011). Modulation of CAM expression as an acclimation response to changing environmental conditions is observed in species characterized as C_3-CAM intermediates. For instance, these species can up-regulate CAM and modulate its intensity when under drought to optimize water usage (Cushman and Borland, 2002).

Among the Bromeliaceae, *Guzmania monostachia* (L.) Rusby ex Mez var. *monostachia* is the only species described as C_3-CAM intermediate (Winter and Holtum, 2014). The C_3-CAM shift is observed in both juvenile and adult stages of this epiphytic bromeliad (Freschi et al., 2010b; Beltrán et al., 2013), which are morphologically distinct. The adult plants possess the leaf-formed tank structure for water storage, while young plants are atmospheric, with long and lanceolate leaves that do not form the tank. Nevertheless, the latter have numerous

trichomes along their leaves, which allows quick rehydration when exposed to humid periods (Meisner et al., 2013). Hence, the younger atmospheric plants of *G. monostachia* have to show faster metabolic adjustments to endure the shifts between dry and humid periods in their habitat, considering they cannot store water in tanks but absorb water rapidly through trichomes. Indeed, Beltrán et al. (2013) verified that atmospheric *G. monostachia* plants perform complete induction of nocturnal CO_2 uptake after one day of drought exposure, while adults do it more gradually during ca. four days of treatment. Such results are in accordance to a previous report by our group, in which intensification of CAM in atmospheric *G. monostachia* plants was observed after one day of drought (Carvalho et al., 2016[1]). However, no reports on how these plants respond to rewatering in terms of photosynthetic modulation have been performed in the literature.

Metabolic plasticity of epiphytic bromeliads like *G. monostachia* may also provide them important adaptation value to consequential effects of dehydration, like increased reactive oxygen species (ROS) generation (Cruz de Carvalho, 2008). High ROS levels can lead to excessive oxidation and therefore, damages on diverse molecules and cell structures, resulting in oxidative stress (Noctor et al., 2014). Formation of ROS occurs especially in the chloroplasts through a sequence of reduction reactions of O_2, in which the first product is the superoxide radical ($O_2^{\bullet-}$), that can be dismutated to hydrogen peroxide (H_2O_2) by the enzyme superoxide dismutase (SOD). Plant cells have diverse mechanisms of scavenging H_2O_2, including enzymes like ascorbate peroxidase (APX) and catalase (CAT). APX utilizes ascorbate as reductant, which is then regenerated through another set of enzymatic reactions, including glutathione reduction by glutathione reductase (GR) (Asada, 1999). There is also H_2O_2 generation in the peroxisomes through photorespiration, which is the pathway initiated by the oxygenase function of Rubisco that includes CAT as the main peroxide scavenger (Noctor et al., 2002).

H_2O_2 in itself has moderate reactivity, but can cause damage to enzymes when under high concentrations. In addition, H_2O_2 can react with transition metals such as Fe^{+2} and generate hydroxyl radicals, which are the most reactive and destructive ROS to the cells (Hajiboland, 2014). However, H_2O_2 may also function as a signalling molecule to up-regulate acclimation responses to abiotic stresses due to its long half-life (Mittler et al., 2011). Hence, H_2O_2 production have to be kept under tight and swift control, so it can be adjusted within hours of the day in order to prevent oxidative stress, especially considering that stressful environmental conditions can be set at any moment. Indeed, Ślesak et al. (2002) have shown that antioxidant enzyme activities are diurnally regulated in plants of the C_3-CAM intermediate species

[1] Correspondente ao artigo apresentado no capítulo 1.

Mesembryanthemum crystallinum L. when under saline stress, which induces water loss and CAM expression. Accordingly, Sunagawa et al. (2010) verified that H_2O_2 levels shift throughout the day when plants of the same species are expressing CAM. Our group showed that high CAM expression induced by drought in atmospheric *G. monostachia* plants was associated with reduced activities of H_2O_2-scavenging enzymes (Carvalho et al., 2016[2]), although more studies are required to verify the influence of day/night shifts over these enzymes activities, and if they are related to the diurnal CAM cycle.

It is still inconclusive how drought and subsequent recovery affects oxidative stress in plants expressing flexible CAM photosynthesis. Nevertheless, successful recovery to post-drought rewatering in terms of photosynthetic activity and ROS metabolism have already been verified in species like *Portulaca oleracea* L., a C_4-CAM intermediate (Jin et al., 2015); and in *Sedum album* L., a C_3-CAM intermediate succulent (Castillo, 1996). Nevertheless, recovery to drought strongly depends on species, water shortage intensity and duration (Xu et al., 2010). Hence, further research with species that have plastic CAM expression would be necessary to evaluate their physiological strategies to endure humid-dry cycles.

Considering atmospheric *G. monostachia* plants perform fast photosynthetic modulation and water absorption according to the level of humidity in the environment, the question arises on how such plants would respond to drought and subsequent rewatering in terms of CAM expression and H_2O_2 metabolism within hours of the day. Therefore, in this study, we aimed to verify if rewatering atmospheric *G. monostachia* plants after drought exposure allows the recovery of the day/night variation of malate concentration (indicative of CAM up-regulation); H_2O_2 production and enzymatic scavenging to pre-stress conditions. Such results could further aid in uncovering drought tolerance and recovery mechanisms of metabolically fast species like C_3-CAM intermediates and epiphytes, providing insight on how they may respond to future environmental changes.

2. MATERIALS AND METHODS

2.1. Plant material and growth conditions

Atmospheric plants of *Guzmania monostachia* (L.) Rusby ex Mez var. *monostachia* were obtained from a previously established pool of *in vitro* individuals, grown in medium composed of Knudson (1946) macronutrients, Murashige and Skoog (1962) micronutrients, 0.5% (w/v) $Ca(CO)_3$, 2% (w/v) sucrose, 0.2% (w/v) Phytagel™ (Sigma-Aldrich) and a pH of 5.8. Plants

[2] Correspondente ao artigo apresentado no capítulo 1.

were kept in a growth room under the following conditions: 25 ± 2ºC, 12-h photoperiod, and a light intensity of 50 μmol photons m^{-2} s^{-1} for six months. Afterwards, plants were transferred to trays containing commercial organic substrate (Tropstrato HT - Vida Verde®) and ground *Pinus* bark and then maintained in a controlled environment chamber under the following conditions: 25/20°C (light/dark), relative humidity of 60/70% (light/dark), 12-hour photoperiod, and photosynthetic flux density (PFD) of about 200 μmol photons m^{-2} s^{-1}. PFD inside the growth chamber was monitored with an LI-190 quantum sensor connected to an LI-250 A meter (LI-COR Instruments). All plants were watered daily with tap water and fertilized with a nutritive solution with half the concentration of Knudson (1946) macronutrients and Murashige and Skoog (1962) micronutrients every 14 days for seven months. Subsequently, plants were maintained for three weeks without fertilization but normally irrigated with distilled water prior to treatment.

2.2. Drought and rewatering treatments

Plants of *G. monostachia* were submitted to treatment in the controlled environment chamber cited in item 2.1. To induce drought, plants had watering suspended for eight days. After the water suspension period, daily irrigation with distilled water was resumed and maintained for six more days. Control plants were irrigated daily with distilled water throughout the 14 days of experiment (i.e. plants under pre-stress conditions). Sampling of control and treated plants was performed at the eighth day of water suspension and sixth day of rewatering. At each harvesting point, a pool of 10 plants was harvested.

2.3. Water content analysis

To assess the water content (WC) of plants, their leaves were immediately weighed (fresh weight, FW), and transferred to an oven at 60°C until reaching constant weight (dry weight, DW). The WC was expressed in a DW basis, according to the formula:

$$WC = (FW-DW)/DW*100$$

2.4. Mesophyll succulence index (Sm)

CAM plants present large vacuoles to store water and malic acid. Hence, the Sm index gives an estimate of how much water is in the vacuole at a given moment, suggesting if it is being utilized mostly for water or malic acid storage, according to the level of CAM expression. Mesophyll succulence was measured as the ratio between the quantity of water in leaves per plant ($FW - DW$) and chlorophyll concentration (mg pigment g^{-1} FW) (Kluge and Ting, 1978). Chlorophyll extraction was done according to the described by Munné-Bosch and Lalueza (2007), with modifications. Leaf tissue frozen at -80°C was ground with liquid N_2, and 100 mg

were homogenized in 1 mL of 100% (v/v) acetone, followed by ultrasonic extraction. This procedure was repeated a second time, and resultant extracts were assayed in a spectrophotometer as described by Lichtenthaler and Buschmann (2001).

2.5. Malate quantification

CAM expression is characterized by nocturnal increase in malate production (Borland et al., 2011). In the present study, CAM was determined as the difference between malate concentration in the dark and light period (Δmalate). Hence, malate levels were determined in leaves harvested at 16 h (day: 2 h prior to dark period) and 4 h (night: 2 h prior to light period) and analysed by gas chromatography coupled to mass spectrometry (GC/MS) as described Freschi et al. (2010a), with modifications. Leaf tissue frozen at -80°C was ground with liquid N_2, and 100 mg were homogenized in 500 µL of methanol:chloroform:water (12:5:1, v/v/v), containing 500 µg of benzoic acid ^{13}C as internal standard. The mixture was kept at 60°C for 30 min. Then, 500 µL of water were added and the mixture centrifuged at 16000 *g*, 4°C for 10 min. An aliquot of 100 µL of the extract was transferred to vials and vacuum dried. Residues were dissolved in 25 µL of pyridine and derivatized at 92°C for 60 min with *N*-tert-butyldimethylsilyl-*N*-methyltrifluoroacetamide (Sigma-Aldrich). Derivatized extracts were analyzed in a GC/MS (Shimadzu QP2010SE) with Agilent DB-5MS column (30 m, I.D. 0.25 mm, 0.50 µm) and helium as the carrier gas at a flow rate of 1 mL min^{-1}. The initial running condition was 100°C for 6 min, followed by a gradient up to 300°C at 6°C min^{-1}. Malate concentration in extracts was obtained by comparing the peak areas of the chromatograms with a commercial standard (Sigma-Aldrich). Δmalate was assessed by subtracting the acid content obtained at 4 h from that at 16 h, with results expressed as mg malate g^{-1} DW.

2.6. H_2O_2 quantification

Daily production of H_2O_2 was determined in leaves harvested at 16 h and 4 h, following the described by Junglee et al. (2014) with modifications. Leaf tissue frozen at -80°C was ground with liquid N_2, and 100 mg were homogenized in 1 mL of 0.1% (w/v) trichloroacetic acid (TCA). Homogenates were centrifuged at 12000 *g*, 4°C for 20 min and 100 µL of the supernatant were added to solution containing 250 µL of 10 mM potassium phosphate buffer (pH 7.0), 500 µL of 1 M potassium iodide and 150 µL of 0.1% (w/v) TCA. Resulting solutions were kept in ice protected from light for 30 min, with absorbances assayed in a spectrophotometer. Values are expressed as µmol H_2O_2 g DW^{-1}.

2.7. Antioxidant enzyme assays

Fresh leaf samples of 200 mg obtained from treated and control plants harvested at 16 h and 4 h were ground with 2 mL of extraction solution described by Souza et al. (2013).

Homogenates were centrifuged at 11000 *g*, 4°C for 30 min and supernatants frozen at -80°C until analysis. Total protein in the extracts was quantified with the Bradford reagent (Sigma-Aldrich) and bovine serum albumin as a standard for calculating specific activities of the enzymes. All samples were analysed in triplicate in a spectrophotometer. SOD (EC 1.15.1.1) activity was determined according to Beauchamp and Fridovich (1971) with modifications by Balen et al. (2009). An aliquot of 40 µL from leaf extracts were used for reactions, which were performed under 23 watt fluorescent light (Phillips®) for 5 min. SOD activity is expressed as units mg^{-1} protein, in which one unit corresponds to the amount of enzyme required to inhibit the reduction of nitrotetrazolium blue chloride by 50% per minute. APX (EC1.11.1.11) activity was assayed with the method described by Nakano and Asada (1981), modified by Weng et al. (2007). Reaction was done with 40 µL of leaf extract, and activity expressed as nmol ascorbate min^{-1} mg^{-1} protein. CAT (EC 1.11.1.6) activity was quantified according to Luck (1974), with some modifications. Reaction solution contained 100 mM potassium phosphate (pH 7.5) and 15 mM H_2O_2. Reaction was started by adding 100 µL of leaf extract, in a total volume of 1 mL. CAT activity is expressed as µmol H_2O_2 min^{-1} mg^{-1} protein. GR (EC 1.6.4.2) activity was determined following Shaedle and Bassham (1977), in which reaction was done with 100 µL of leaf extract. GR activity is expressed as nmol $NADP^+$ min^{-1} mg^{-1} protein.

2.8. Statistical analyses

Experiments were conducted in a completely randomised design. WC, Sm and Δmalate data were submitted to one-way analysis of variance (ANOVA) with variation among means compared by using Tukey's test at $P < 0.05$. For H_2O_2 content and antioxidant enzyme activities, data were submitted to two-way ANOVA with period of harvesting (day and night) and watering treatment (control, drought, rewatering) as factors. Variation among means of the period factor was compared by using *t* tests ($P < 0.05$) and for means of the watering treatment factor by using Tukey tests ($P < 0.05$). Means of the control (plants under daily irrigation) indicated in the results section for all the parameters were calculated with values obtained from plants harvested on both points of treatment (eight days of drought and six days of rewatering), considering they were in the same conditions of irrigation.

3. RESULTS

3.1. Tissue water content and CAM expression

G. monostachia plants exposed to eight days of water suspension underwent a significant decrease by 33% of WC when compared to pre-stress plants (control, Fig. 1A). Such

results are in accordance to the Sm index, which showed more severe reduction of ca. 57% due to drought exposure (Fig. 1B). After plants were rewatered for six days, WC was recovered to statistically similar levels of pre-stress individuals (Fig. 1A). Although mesophyll succulence increased in rehydrated plants, mean values remained significantly smaller than in control plants by 19% (Fig. 1B).

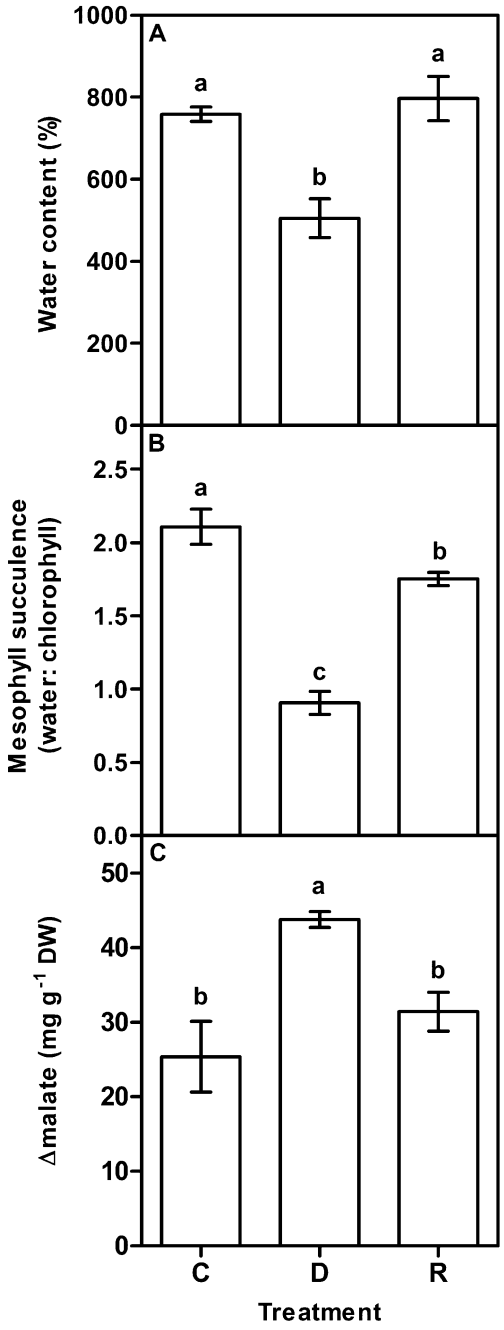

Figure 1. Hydric status and CAM activity in *Guzmania monostachia* plants exposed to drought and rewatering. A) Water content, B) mesophyll succulence, C) daily variation of malate. DW, dry weight; C, control (daily irrigation, i.e. pre-stress conditions); D, eight days of water suspension (drought); R, six days of rewatering post-drought. Values are means ± s.d. (n=3). Means of the control were calculated with values obtained from plants harvested at both points of treatment (drought and rewatering). Letters compare treatments by Tukey test ($P < 0.05$).

The Δmalate showed positive values in control, drought and rewatered plants, indicating CAM up-regulation occurred in all conditions (Fig. 1C). Nevertheless, mean values of such parameter almost doubled after eight days of water suspension when compared to control plants, indicating that CAM expression intensified due to increased water loss. After irrigation was resumed for six days, levels of Δmalate reduced to statistically similar values of control plants (Fig. 1C), showing that plants exposed to water suspension were capable of restoring basal levels of CAM when WC and Sm were recovered.

3.2. H_2O_2 production and antioxidant activity

Significant interactions between period of harvest and watering regime indicated by two-way ANOVA showed that most oxidative stress markers analysed had distinct diurnal patterns according to the plant's hydric status (significant difference indicated by asterisks in Fig. 2 and 3). In control plants, H_2O_2 production (Fig. 2) and antioxidant enzyme activities (Fig. 3) were statistically similar between light and dark periods. However, water loss after drought exposure led to alterations in daily rhythm of most parameters. Nocturnal H_2O_2 increased by 51% in comparison to the light period (Fig. 2). Interestingly, such increase was associated with reductions in SOD and GR activities by 9% and 19%, respectively (Fig. 3A, D).

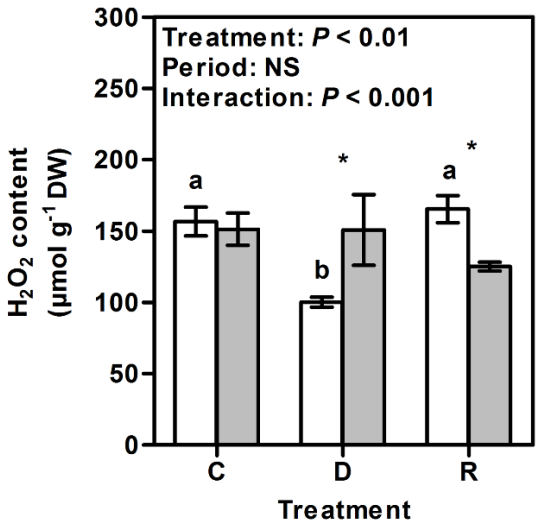

Figure 2. Daily variation of hydrogen peroxide content in *Guzmania monostachia* plants exposed to drought and rewatering. DW, dry weight; NS, not significant; C, control (daily irrigation, i.e. pre-stress conditions); D, eight days of water suspension (drought); R, six days of rewatering post-drought. White and grey-shaded bars indicate day and night, respectively. Values are means ± s.d. (n=3). Means of the control were calculated with values obtained from plants harvested at both points of treatment (drought and rewatering). Significant differences between groups were tested by two-way analysis of variance (ANOVA), with asterisks comparing periods of the day per treatment by *t* tests ($P < 0.05$) and letters comparing treatments per period by Tukey test ($P < 0.05$). Significance of each factor and interaction calculated by two-way ANOVA is indicated by the *P* value.

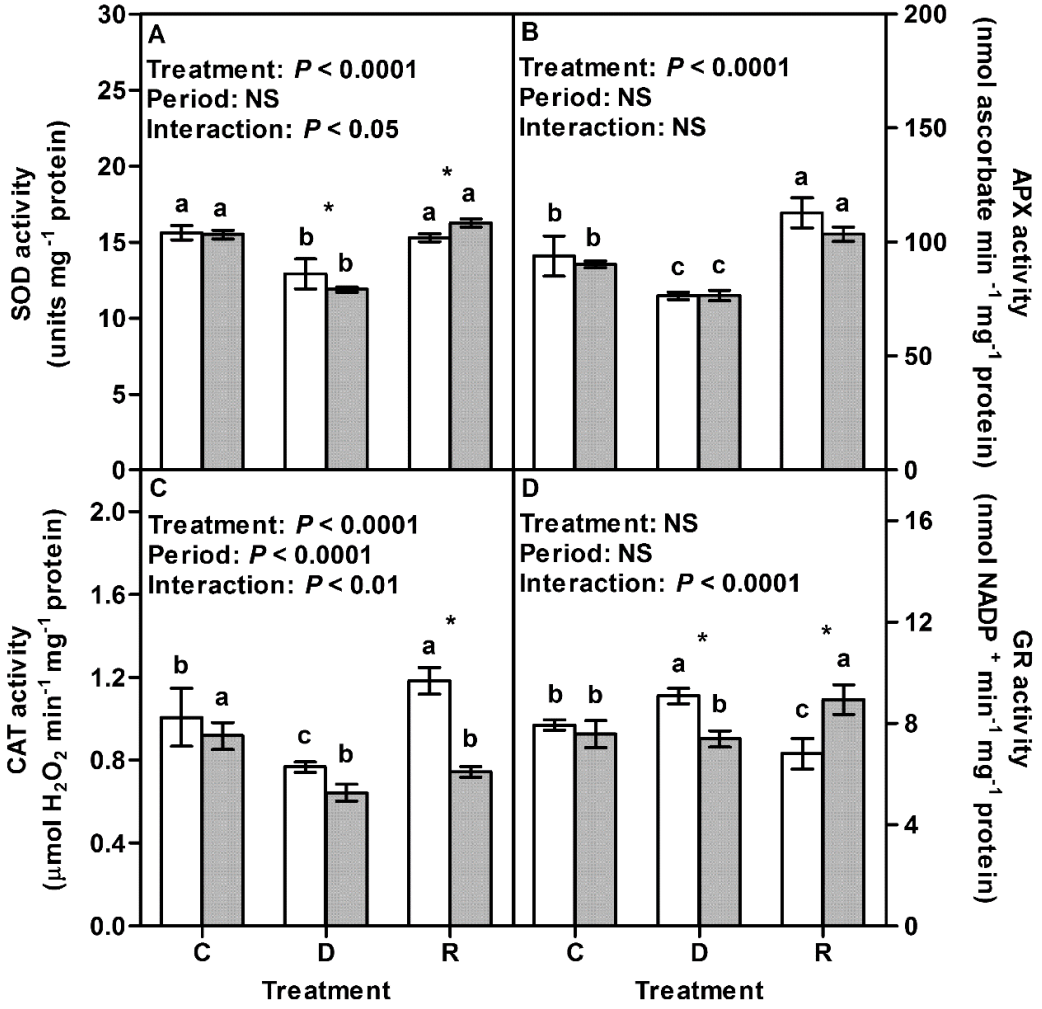

Figure 3. Antioxidant enzyme specific activities in *Guzmania monostachia* plants exposed to drought and rewatering. A) Superoxide dismutase (SOD), B) ascorbate peroxidase (APX), C) catalase (CAT), D) glutathione reductase (GR). NS, not significant; C, control (daily irrigation, i.e. pre-stress conditions); D, eight days of water suspension (drought); R, six days of rewatering post-drought. White and grey-shaded bars indicate day and night, respectively. Values are means ± s.d. (n=3). Means of the control were calculated with values obtained from plants harvested at both points of treatment (drought and rewatering). SOD unit is the amount of enzyme required to inhibit the reduction of NBT by 50%. Significant differences between groups were tested by two-way analysis of variance (ANOVA), with asterisks comparing periods of the day per treatment by *t* tests ($P < 0.05$) and letters comparing treatments per period by Tukey test ($P < 0.05$). Significance of each factor and interaction calculated by two-way ANOVA is indicated by the *P* value.

When drought-treated plants underwent rewatering for six days, more alterations in daily H_2O_2 production occurred, leading to a distinct pattern from the observed in control plants (Fig. 2). Rewatered plants showed higher H_2O_2 production at daytime in comparison to the dark period, when it reduced by 24%. Accordingly, daily variation of SOD, CAT and GR activities in rewatered plants were also different from control, although APX maintained the same diurnal pattern (Fig. 3). Specifically for SOD and GR activities, significant nocturnal increases of 7%

and 31% occurred in comparison to the light period, respectively (Fig. 3A, D), while CAT activity was reduced by 37% at the same period (Fig. 3C).

Watering regimes also led to significant alterations in oxidative stress markers among each period of the day (differences indicated by letters in Fig. 2 and 3). Interestingly, daytime H_2O_2 levels in drought-treated plants were significantly smaller than in well-watered plants (control and rewatered), although nocturnal production did not differ among treatments according to Tukey test (Fig. 2). In terms of the antioxidant enzymes, SOD activity significantly reduced after drought exposure in both periods, followed by recovery to similar levels of the control after rewatering (Fig. 3A). APX activity also decreased after drought throughout the day, but then increased by 41% to higher levels than control after rewatering (Fig. 3B). A similar response was observed for CAT at the light period, although nocturnal activity in drought-treated and rewatered plants were statistically smaller than control (Fig. 3C). In contrast, GR daytime activity was the highest among treatments after drought exposure, but then reduced to lower values than control after rewatering (Fig. 3D). However, nocturnal GR activity was 1.2 times higher in rehydrated plants than in remaining treatments, in average. The influence of day/night period and treatment over antioxidant enzyme activities are summarized in Fig. 4.

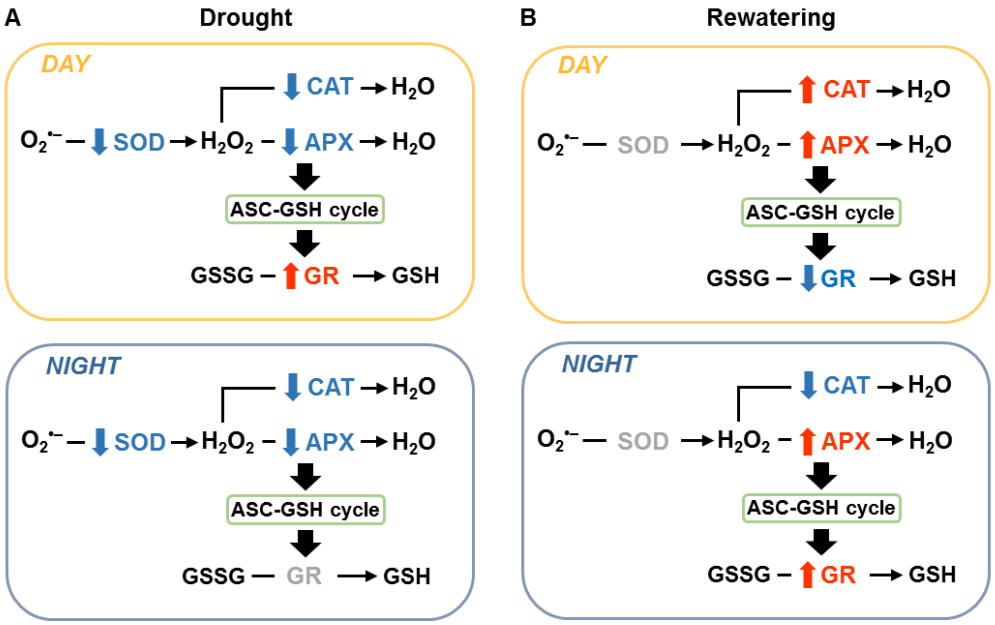

Figure 4. Antioxidant enzymes responses in *Guzmania monostachia* plants exposed to drought (A) and rewatering (B) at day and night-time. Blue up and red down arrows indicate increased and decreased activity in comparison to pre-stress plants (daily irrigated control), respectively. Enzymes written in grey had unchanged activity in comparison to control. APX, ascorbate peroxidase; ASC, ascorbate; CAT, catalase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; H_2O_2, hydrogen peroxide; $O_2^{\bullet-}$, superoxide radical; SOD, superoxide dismutase.

4. DISCUSSION

Atmospheric *G. monostachia* plants underwent high water loss after drought exposure (Fig. 1), which was followed by increased CAM expression when compared to control plants that presented CAM activity despite being well-hydrated. Similar results were reported previously by our group (Carvalho et al., 2016[3]) and by Beltrán et al. (2013), which verified nocturnal increase in acidity in well-watered atmospheric *G. monostachia* plants. Sm also decreased after drought, leading us to assume that vacuoles of photosynthetic cells under water deficit focused on malate storage. A similar tendency was reported for the terrestrial bromeliad *Puya floccose* (Linden) E. Morren ex. Mez, where 14 days of drought reduced Sm by 61%, which also corresponded to high CAM expression (Herrera et al., 2010). After rewatering, atmospheric *G. monostachia* plants were capable of restoring tissue water content and reducing CAM intensity (Fig. 1), as similar to observed by Freschi et al. (2010b) for adult individuals of this bromeliad. Effective and fast recovery after post-drought irrigation has been reported to other C_3-CAM intermediate species, like *Aptenia cordifolia* (L.f.) Schwantes plants, which completely recovered water status and CO_2 assimilation after four days of rewatering in comparison to pre-stress plants (Fleta-Soriano et al., 2015).

The highest CAM activity in drought-treated plants was associated with reduced daytime H_2O_2 in comparison to control (Fig. 2), which could be due to low SOD activity at the same period (Fig. 4A). In addition, reduced APX and CAT activities at the light period indicate smaller necessity for H_2O_2 scavenging in drought-treated plants (Fig. 4A). Such results are in accordance to previous reports by our group, which have also showed that drought-exposed atmospheric *G. monostachia* plants with high CAM expression had reduced antioxidant enzyme activities in comparison to well-watered plants with lower CAM levels (Carvalho et al., 2016[3]). CAT in particular is involved in the photorespiration pathway, which occurs in the peroxisomes (Ogren, 1984). This mechanism is said to be reduced in CAM plants because the high CO_2 concentration in the chloroplasts during the day avoids its oxygenation by Rubisco (Niewiadomska and Borland, 2008). Considering that a great portion of H_2O_2 is produced by photorespiration (Noctor et al., 2002), we hypothesize that decreased H_2O_2 and CAT activity in drought-treated *G. monostachia* plants at the light period was, at least in part, contributed by increased suppression of photorespiration due to high CAM levels. Nevertheless, it is still debated whether photorespiration is inhibited or enhanced in CAM plants, because such mechanism is highly influenced by the $[O_2]$: $[CO_2]$ proportion in the cells along the 24-hour

[3] Correspondente ao artigo apresentado no capítulo 1.

CAM cycle (Lüttge, 2011). Moreover, in the present study, GR may have aided in reducing H_2O_2 levels because it was the most activated enzyme during the light period in drought-treated plants (Fig. 3D, 4A). GR is a component of the ascorbate-glutathione cycle, therefore it is important for maintaining the restoration of such antioxidant metabolites in order to prevent excessive H_2O_2 generation (Foyer and Noctor, 2011).

Interestingly, increased H_2O_2 at the dark period in atmospheric *G. monostachia* plants under water suspension was not followed by increased SOD activity or reduced activities of H_2O_2-catabolizing enzymes CAT and APX (Fig. 4A). Such results led us to presume that high nocturnal malate synthesis in such plants could be related to some extent to H_2O_2 production at the same period. Sunagawa et al. (2010) have reported a similar pattern of nocturnal H_2O_2 synthesis for C_3-CAM intermediate *M. crystallinum* plants expressing high rates of CAM, due to water loss induced by salt stress. Considering that the mitochondria is a major source of ROS at night-time (Rhoads et al., 2006) and that it provides great part of ATP for malate transport to the vacuoles in CAM plants (Smith et al., 1982), we can hypothesize that intense CAM activity could lead to increased nocturnal H_2O_2 generation through the mitochondrial electron transport chain. However, more research is necessary to uncover details accounting malate synthesis and ROS generation in CAM plants.

Rehydration drastically altered daily variation of H_2O_2 in comparison to control and drought-treated plants, having peaked at the light period and decreased by night-time (Fig. 2). Accordingly, APX and CAT daytime activities increased to higher mean values than in other treatments (Fig. 3B, C; 4B), which suggests that rewatered plants required more intense control of H_2O_2 levels during the light period. It is hypothesized that APX functions as a fine regulator of signals emitted by H_2O_2 due to its wide distribution in organelles and high affinity to peroxide, while CAT is responsible for the excess removal of H_2O_2 due to its lower affinity to its substrate (Mittler, 2002). Hence, in the present study, APX could be acting to regulate H_2O_2 signals involved in the up-regulation of acclimation responses to recent water recovery, and CAT to remove potentially damaging content of H_2O_2 in the cells of atmospheric *G. monostachia* plants. The reduced levels of nocturnal H_2O_2 in rewatered plants (Fig. 2) suggests that daytime peroxide generation was successfully controlled. CAT activity also reduced at night-time, but APX scavenging remained high (Fig. 4B), which supports the idea that CAT functions as a remover of excess ROS and APX as a regulator of lower H_2O_2 levels.

Furthermore, GR nocturnal activity in rewatered plants increased in comparison to remaining treatments (Fig. 3D, 4B). A similar response occurred in drought-treated plants at daytime (Fig. 3D, 4A), which reinforces the notion that GR enhances peroxide scavenging through glutathione recovery in atmospheric *G. monostachia*. Besides increased activities of

CAT, APX and GR, rewatered plants had increased chlorophyll synthesis in comparison to the control as indicated by lower Sm levels (Fig. 1B), which could be considered an acclimation response to water absorption. Such results suggest that rewatered plants were still in recovery process from previous drought conditions.

Despite alterations in daily rhythm of peroxide production and antioxidant enzyme activities, rewatering generally enabled their recovery to similar levels of the control on at least one period of the day (Fig. 4B). Recovery from oxidative stress after rehydration has been reported for other species with flexible CAM expression, as the C_4-CAM *P. oleracea*, in which rewatering reduced $O_2^{\bullet-}$ levels and SOD activity to similar values of pre-drought plants (Jin et al., 2015). Interestingly, APX, CAT and GR activities of *G. monostachia* rewatered plants were higher than in control on at least one period of the day (Fig. 4B), indicating higher demand of enzymatic antioxidant activity to restrain ROS levels. Similar responses were observed for *Poa pratensis* L. grasses, that had higher antioxidant enzyme activities after 1 day of rewatering post 5 days of drought, in which the authors concluded it was due to enhanced need for ROS removal after rehydration (Bian and Jiang, 2009).

In summary, post-drought rewatering allowed for water content and CAM intensity recovery in atmospheric *G. monostachia* plants. However, increased APX, CAT and GR activities in distinct periods of the day associated with higher chlorophyll synthesis suggest rewatered plants were still undergoing metabolic recovery. This was also supported by the fact that rehydration altered day/night variation in H_2O_2 production and scavenging. Nevertheless, such results indicate how plants were able to adjust the H_2O_2 scavenging system in a few hours in order to cope with recent water reacquisition and CAM intensity modulation, which is an important feature for juvenile atmospheric plants to endure the adverse conditions of the epiphytic niche. These results provide guidance for future research on how C_3-CAM intermediates and epiphytic plants such as atmospheric *G. monostachia* modulate their metabolism in response to repeated exposure to humid-dry cycles, as predicted to be the future environmental setting.

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CONSIDERAÇÕES FINAIS E PERSPECTIVAS

A alternância entre as vias C_3 e CAM de acordo com o nível de umidade do ambiente já foi estudada por diversos autores em indivíduos adultos tanque de *G. monostachia* (Medina *et al.* 1977, Maxwell *et al.* 1994, Pereira *et al.* 2013). No entanto, apenas recentemente esta avaliação foi realizada para indivíduos jovens atmosféricos de *G. monostachia* por Beltrán *et al.* (2013), onde foi verificado que estes também são capazes de ativar o CAM em resposta à seca, corroborando a importância deste mecanismo como estratégia de sobrevivência da espécie. Com o presente trabalho, novos detalhes sobre a modulação e expressão de CAM em plantas atmosféricas de *G. monostachia* foram descritos, como o fato de que estas também acumulam citrato além de malato no período noturno, assim como indivíduos adultos (Pereira *et al.* 2013). Curiosamente, também verificamos que mesmo em condições bem hidratadas (conteúdo hídrico relativo em *ca*. de 76%) estas plantas estavam fixando carbono via CAM ao invés de C_3, fossem elas do controle (plantas irrigadas diariamente) (Fig. 1A) ou reidratadas após o déficit hídrico. Com isto, podemos supor que as condições de umidade da câmara de crescimento (60/70%) possam ter ocasionado uma queda no conteúdo hídrico que, apesar de baixa, foi suficiente para desencadear a transição de C_3 para CAM. Adicionalmente, constatamos a capacidade destas plantas em modular a duração da fase de fixação de CO_2 atmosférico em ácidos (fase noturna) e a intensidade de CAM ao longo da exposição à seca, além de serem capazes de recuperarem os níveis de expressão deste metabolismo a valores similares ao de plantas do controle após serem reidratadas. Considerando estas observações, podemos concluir que de fato indivíduos atmosféricos de *G. monostachia* apresentam elevada plasticidade na expressão de CAM, o que apoia a ideia de que estas plantas são altamente dependentes de seu metabolismo para suportarem as variações na disponibilidade hídrica.

Além disso, os resultados deste trabalho mostraram que a plasticidade na expressão de CAM quanto às condições de déficit hídrico e reidratação em plantas atmosféricas de *G. monostachia* foi diretamente associada a alterações no sistema antioxidante e produção de ROS, que possibilitaram a obtenção de respostas satisfatórias às hipóteses levantadas inicialmente. No entanto, esta investigação também surtiu em novas perguntas sobre como ocorre o envolvimento entre os metabolismos de CAM e ROS nestas plantas. Assim, as principais conclusões e perguntas geradas a partir da discussão das questões dos capítulos 1 e 2 estão detalhadas a seguir:

(a) A expressão de CAM induzida pela deficiência hídrica diminui a produção de ROS e a atividade enzimática antioxidante em plantas atmosféricas de *G. monostachia*?

Os resultados apresentados no capítulo 1 mostraram que a maior expressão de CAM em plantas expostas a oito dias de seca se relacionou com a manutenção de ROS a níveis similares ao de plantas bem hidratadas (controle, Fig. 1B), que tiveram CAM reduzido em 42% em comparação às plantas em déficit hídrico (Fig. 1A). Além disso, plantas com maior expressão de CAM tiveram atividades enzimáticas antioxidantes reduzidas em comparação ao controle (exemplificado pela atividade de SOD na Fig. 1C), porém esta redução não levou a danos de membrana significativos. Estes resultados sugerem que o aumento na expressão de CAM diminuiu a demanda sobre o sistema antioxidante, provavelmente por tal aumento ter intensificado o caráter preventivo de CAM à produção de ROS em condições de seca, o que confirma a hipótese formulada. Existem relatos com conclusões similares sobre a influência de CAM na produção de ROS para a espécie C_3-CAM facultativa *M. crystallinum*, onde mostram que a expressão de CAM diminui a carga oxidativa nas plantas (Borland *et al.* 2006, Sunagawa *et al.* 2010). Assim, o presente trabalho afirma a ideia de que CAM pode ser uma estratégia de adaptação não só à queda no conteúdo hídrico do tecido, mas também quanto ao estresse oxidativo causado por esta perda de água em espécies C_3-CAM facultativas (Niewiadomska & Borland 2008).

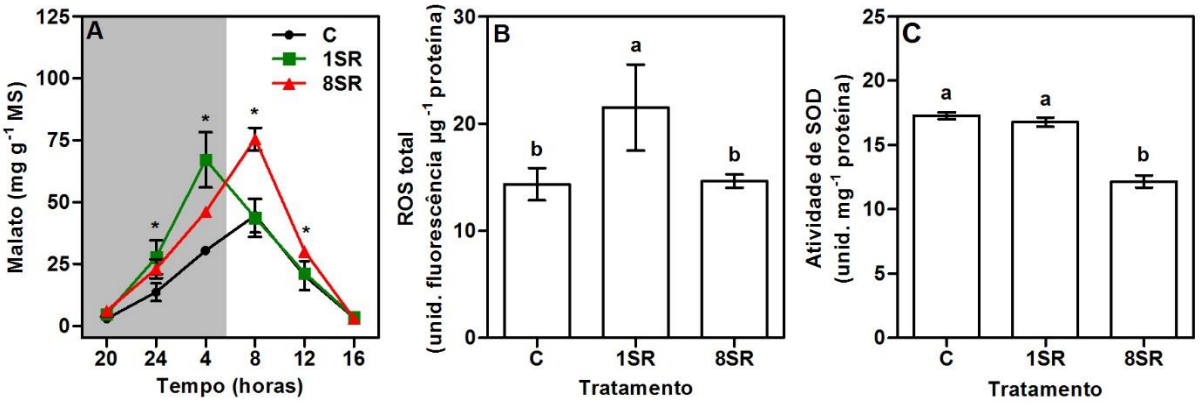

Figura 1. Parâmetros de expressão de CAM e metabolismo de ROS em plantas atmosféricas de *Guzmania monostachia* expostas à suspensão de rega por um e oito dias. A) Variação diária de malato, B) conteúdo total de ROS, C) atividade de SOD. MS, massa seca; C, controle (plantas irrigadas diariamente); 1SR, plantas em um dia de suspensão de rega; 8SR, plantas em oito dias de suspensão de rega. A região cinza em (A) indica o período escuro. Uma unidade de SOD corresponde à quantidade de enzima necessária para inibir a redução de cloreto de nitrotetrazólio azul (NBT) em 50%. Valores são médias ± s.d. (n=3). Asteriscos em (A) indicam diferença significativa de acordo com ANOVA ($P < 0.05$). Letras em (B) e (C) comparam tratamentos por teste de Tukey ($P < 0.05$).

Adicionalmente, foi observado um aumento de 50% na produção de ROS após 24 horas do início do tratamento de suspensão de rega em plantas de *G. monostachia* (Fig. 1B). Com isto, supomos que ROS têm um envolvimento em vias de sinalização para ativar mecanismos

de aclimatação à seca, inclusive a intensificação de CAM e produção aumentada de carotenoides, conforme notado nas plantas mantidas por oito dias sem irrigação. De fato, a função de ROS como sinalizadores para mecanismos de defesa contra diferentes tipos de estresse vem sendo intensamente estudada nos últimos anos (Baxter *et al.* 2014, Xia *et al.* 2015, Sewelam *et al.* 2016).

(b) A reidratação de plantas atmosféricas de *G. monostachia* permite sua recuperação às condições de pré-estresse quanto à atividade fotossintética e metabolismo de ROS?

Os dados apresentados no capítulo 2 demonstraram que, após a retomada da irrigação diária por seis dias, as plantas previamente expostas a oito dias de seca foram capazes de recuperar seu conteúdo hídrico e intensidade na expressão de CAM a níveis similares ao de plantas não expostas ao estresse e irrigadas diariamente (controle). No entanto, um aumento nas atividades de principalmente CAT e APX nas plantas reidratadas indica maior requerimento na eliminação das ROS de forma a mantê-las em níveis toleráveis pelas plantas (Fig. 2A, B). Além disso, o ritmo na produção de H_2O_2 ao longo do dia se alterou nas plantas reidratadas (Fig. 2C), o que pode estar relacionado ao novo padrão de atividade diária de CAT e o aumento em atividade de APX nestas plantas em comparação ao controle (Fig. 2A, B). Estes resultados implicam que as plantas reidratadas ainda mostravam-se em processo de aclimatação à recente aquisição de água nos tecidos quando as análises foram realizadas, pois os parâmetros de ROS não foram totalmente recuperados às condições de pré-estresse (controle). De qualquer forma, as alterações de CAT e APX associadas à produção de dia/noite de H_2O_2 mostram como as plantas atmosféricas de *G. monostachia* foram capazes de realizar ajustes rápidos em seu sistema antioxidante de forma a promover a regulação da produção de ROS, provavelmente como parte da estratégia de aclimatação às novas condições de umidade. Esta característica pode ser considerada de suma importância para indivíduos juvenis de espécies epífitas como *G. monostachia*, devido às constantes variações nas condições abióticas de seu habitat (Cach-Pérez *et al.* 2014).

De forma interessante, através da discussão dos dados no capítulo 2, notou-se também que o acúmulo noturno intensificado de malato em plantas expostas a oito dias de déficit hídrico foi acompanhado por um aumento de H_2O_2 no mesmo período (Fig. 2C), sugerindo que o ritmo de CAM esteja de alguma forma envolvido com a geração do peróxido ao longo do dia, o que ainda não havia sido reportado em detalhes para espécies C_3-CAM facultativas. Estes resultados abrem novos caminhos para pesquisa sobre os ritmos diurnos do metabolismo de ROS em plantas CAM.

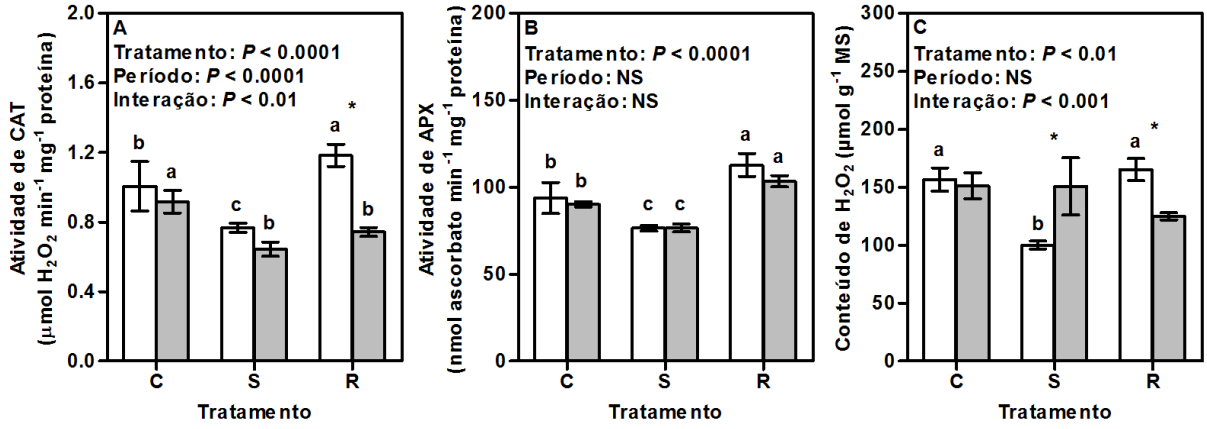

Figura 2. Atividade enzimática antioxidante e produção de H_2O_2 em plantas atmosféricas de *Guzmania monostachia* expostas à suspensão de rega por oito dias e reidratadas por seis dias após o estresse. A) Variação dia/noite de atividade de CAT, B) APX, C) e conteúdo de H_2O_2. MS, massa seca; NS, não significativo; C, plantas irrigadas diariamente (condições de pré-estresse; controle); S, plantas em oito dias de suspensão de rega; R, plantas reidratadas por seis dias após a suspensão de rega. As barras claras e cinzas indicam o período claro (16 h) e escuro (4 h), respectivamente. Valores são médias ± s.d. (n=3). Diferenças significativas dentre grupos foram testadas por ANOVA dois fatores, com asteriscos comparando períodos do dia por tratamento através de testes *t* ($P < 0.05$) e letras comparando tratamentos por período através de testes Tukey ($P < 0.05$). A significância dos fatores e interação calculada pela ANOVA dois fatores está indicada pelo *P* valor.

Por fim, os resultados discutidos em ambos os capítulos indicam que a modulação da via fotossintética CAM feita de acordo com o nível de hidratação nos tecidos está estreitamente relacionada com a dinâmica entre a produção de ROS e o sistema antioxidante em plantas atmosféricas de *G. monostachia*. A partir da discussão envolvida nestes capítulos, também foram obtidas diversas informações relevantes e inovadoras que enriquecem o conhecimento sobre o metabolismo de ROS em plantas CAM, além de gerar subsídios para novos estudos visando o detalhamento desta relação, inclusive sobre a participação de ROS como sinalizadores para a intensificação de CAM; os mecanismos em que o ritmo diurno da via CAM afeta a produção de ROS ao longo do dia; a função do sistema antioxidante não enzimático nestas plantas, como o ascorbato e glutationa; e principalmente para a identificação de genes de enzimas da via CAM e sistema antioxidante envolvidos na tolerância destas plantas à seca.

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