UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTITUTO DE BIOLOGIA
Programa de Pós-Graduação em Ecologia, Conservação e Biodiversidade

TESE DE DOUTORADO

Metabolismo Ácido das Crassulaceae e absorção foliar de água: uma análise integrada de determinantes estruturais, fisiológicos e bioquímicos em plantas suculentas

Jéssica Ferreira de Lima

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Tese apresentada à Universidade Federal de Uberlândia como requisito parcial necessário para obtenção do Título de Doutorado em Ecologia, Conservação e Biodiversidade.

Orientadora: Drª. Ana Sílvia Franco Pinheiro Moreira

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SUMÁRIO

RESUMO

As epífitas, incluindo muitas orquídeas e bromélias, desenvolveram estratégias morfológicas e fisiológicas que aumentam a eficiência do uso da água em resposta ao déficit hídrico. Entre essas estratégias, destacam-se a presença de órgãos suculentos, a expressão do Metabolismo Ácido das Crassulaceae (CAM) e a absorção foliar de água (*Foliar water uptake* - FWU). Em alguns casos, essas características estão associadas, como a relação entre a suculência e o metabolismo CAM, bem como a influência da suculência foliar e da composição da parede celular sobre a capacidade de FWU. Diante disso, a hipótese central dessa tese propõe que a FWU esteja relacionada ao ciclo circadiano inerente ao metabolismo CAM. Para isso, a tese está estruturada em 3 capítulos. No primeiro, formulamos como hipótese que o acúmulo de ácidos orgânicos associado à expressão CAM promove uma redução do potencial hídrico foliar ao amanhecer, aumentando as taxas de FWU em *Vanilla phaeantha*. Esperamos ainda que componentes da parede celular associados à retenção e movimentação de água, especialmente pectinas, sejam amplamente marcados nas folhas. No segundo capítulo, levantamos a hipótese de que a restrição hídrica altera o balanço hídrico foliar e a atividade fotossintética, aumentando a expressão CAM (ou seja, promova maior acúmulo de ácidos orgânicos) e, consequentemente, maximizando a FWU. Para testar essa hipótese, condições de estresse hídrico foram simuladas por meio da restrição hídrica em *Cattleya forbesii* e *Cattleya guttata*. No terceiro capítulo, propomos que a FWU aumente a eficiência no uso da água, favoreça a atividade fotossintética e reduza o estresse oxidativo em *Puya chilensis*, uma espécie frequentemente exposta a eventos de neblina. Além disso, levantamos a hipótese de que a frequência da neblina possa modular a intensidade da expressão CAM e influenciar a capacidade de FWU. Para isso, foi conduzido um experimento simulando eventos de neblina artificial e déficit hídrico. Em síntese, nossos resultados contribuem para a compreensão das estratégias de uso da água adotadas por plantas suculentas, como orquídeas epífitas e bromélias, em resposta às condições de flutuação hídrica às quais geralmente crescem expostas.

Palavras-chave: balanço hídrico; bromélias; orquídeas; estresse hídrico; parede celular; plantas CAM.

ABSTRACT

Epiphytes, including many orchids and bromeliads, have evolved morphological and physiological strategies that enhance water-use efficiency in response to water deficit. Among these strategies are the presence of succulent organs, the expression of Crassulacean Acid Metabolism (CAM), and foliar water uptake (FWU). In some cases, these traits are interconnected, such as the relationship between succulence and CAM, as well as the influence of leaf succulence and cell wall composition on FWU capacity. Therefore, the central hypothesis of this thesis is that FWU is related to the circadian cycle inherent to CAM metabolism. To address this hypothesis, the thesis is organized into three chapters. In the first chapter, we hypothesized that the accumulation of organic acids associated with CAM expression reduces leaf water potential at dawn, thereby increasing FWU rates in *Vanilla phaeantha*. We also expected that cell wall components associated with water retention and movement, particularly pectins, would be widely distributed throughout the leaves. In the second chapter, we hypothesized that water restriction alters leaf water balance and photosynthetic activity, increasing CAM expression (i.e., promoting greater accumulation of organic acids) and consequently maximizing FWU. To test this hypothesis, drought conditions were simulated through water restriction in *Cattleya forbesii* and *Cattleya guttata*. In the third chapter, we proposed that FWU enhances water-use efficiency, promotes photosynthetic activity, and reduces oxidative stress in *Puya chilensis*, a species frequently exposed to fog events. Additionally, we hypothesized that fog frequency may modulate the intensity of CAM expression and influence FWU capacity. To test this, an experiment was conducted simulating artificial fog events and water deficit conditions. Overall, our results contribute to a better understanding of the water-use strategies adopted by succulent plants, such as epiphytic orchids and bromeliads, in response to fluctuations in water availability commonly experienced in the environments where they occur.

Keywords: water balance; bromeliads; orchids; water stress; cell wall; CAM plants.

INTRODUÇÃO GERAL

Plantas epífitas crescem como não parasitas sobre outras plantas e obtêm água e nutrientes diretamente de fontes atmosféricas ou do contato com substrato acumulado sobre seus forófitos. Por isso, são mais sensíveis às variações ambientais do que as plantas terrestres, cujo crescimento é influenciado principalmente pelas condições do solo (Benzing, 1990; Taylor et al., 2021). Além disso, desempenham papel importante nos ciclos hidrológicos e de nutrientes e podem representar até 26% das plantas vasculares em regiões tropicais (Gotsch et al., 2016; Taylor et al., 2021). Apesar das limitações impostas pela baixa disponibilidade hídrica no ambiente epifítico, essas plantas desenvolveram características morfológicas e fisiológicas que maximizam a absorção e o armazenamento de água, permitindo sua persistência nesse habitat (Zotz & Hieltz, 2001; Silvera et al., 2009). Entre as principais famílias, destacam-se as Orchidaceae e Bromeliaceae, com aproximadamente 75% e 59% das espécies, respectivamente, vivendo nesta condição (Givnish et al., 2015; Taylor et al., 2021). Entre as principais estratégias morfofisiológicas estão sistemas radiculares especializados (raízes aéreas revestidas pelo velame), folhas e caules suculentos (às vezes com presença de pseudobulbos), cutícula espessa, tricomas foliares especializados, baixa densidade estomática, expressão do Metabolismo Ácido das Crassulaceae (CAM) e capacidade de absorção foliar de água (Foliar Water Uptake, FWU) (Benzing, 1990; Gotsch et al., 2015, 2022).

A suculência foliar, caracterizada pela presença de células do mesofilo com grandes vacúolos, constitui uma adaptação importante ao ambiente epifítico por aumentar a capacidade de armazenamento de água. Além disso, a composição das paredes celulares dessas células confere maior elasticidade, permitindo que se expandam e se retraiam conforme o estado hídrico do tecido (Hermes et al., 2018; Costa et al., 2024). A parede celular é uma estrutura complexa composta por microfibrilas de celulose, pectinas e proteínas estruturais (Cosgrove, 2005). Entre as pectinas predominantes estão os homogalacturonanos (HGs), ramnogalacturonanos I e II, responsáveis por regular a porosidade e o grau de hidratação da parede celular (Willats et al., 2001; Albersheim et al., 2011). Por exemplo, a presença de HGs com elevado grau de metilesterificação nas células do tecido aquífero, foi associada ao aumento da flexibilidade da parede celular nas folhas de *Aechmea distichantha* (Hermes et al., 2018).

Além das modificações estruturais associadas ao armazenamento de água, diversas epífitas combinam múltiplas estratégias de conservação hídrica. A suculência

das folhas, por exemplo, está frequentemente associada à expressão do metabolismo CAM (Silvera et al., 2009; Silvera & Lasso, 2016). Além de manter elevados teores de água nos tecidos e sustentar a atividade fotossintética, a suculência permite armazenamento dos ácidos orgânicos produzidos durante o ciclo CAM (Freschi et al., 2010; Ogburn & Edwards, 2012). O metabolismo CAM representa uma importante adaptação fisiológica ao déficit hídrico, caracterizada pela maior eficiência no uso da água por meio da abertura estomática no período noturno para assimilação do CO_2 atmosférico, reduzindo as perdas de água por transpiração (Moreira et al., 2009, 2013; Silvera et al., 2010). Durante a noite, o CO_2 atmosférico é inicialmente fixado pela fosfoenolpiruvato carboxilase (PEPcase), formando ácidos orgânicos que são armazenados nos vacúolos. Durante o dia, esses compostos são descarboxilados pela enzima NADP-málica, liberando CO_2 para ser fixado pelo ciclo de Calvin por meio da atividade carboxilase da rubisco (RuBisCO) (Cushman, 2001). O acúmulo noturno de ácidos orgânicos pode reduzir o potencial osmótico e, consequentemente, o potencial hídrico celular, intensificando o gradiente favorável à entrada de água na planta (Herrera et al., 2008; Matimati et al., 2012).

Outra estratégia que alivia os efeitos do estresse hídrico é a absorção foliar de água (FWU). Como dependem de fontes atmosféricas de água, as epífitas são particularmente sensíveis a alterações nos regimes de precipitação, neblina e orvalho, sendo frequentemente consideradas bons indicadores de mudanças climáticas (Silvera & Lasso, 2016). Eventos de chuva, neblina ou orvalho promovem a formação de um filme de água na superfície foliar; quando isso ocorre, estabelece-se um gradiente de potencial hídrico favorável ao fluxo de água para o interior dos tecidos, impulsionando a FWU (Burgess & Dawson, 2004). A entrada de água pelas folhas proporciona uma série de vantagens fisiológicas, pois aumenta a hidratação das folhas e seu potencial hídrico, aumenta a eficiência no uso da água, melhora sua capacidade fotossintética e pode reduzir o estresse oxidativo (espécies reativas de oxigênio - ROS) (Berry et al., 2019; Boanares et al., 2020; Pan et al., 2021; Anders et al., 2025). A água pode entrar nos tecidos foliares através de várias vias, incluindo a cutícula, os estômatos e tricomas (Boanares, 2026). Além disso, sua movimentação e retenção podem ser influenciadas pela composição química da parede celular, especialmente pela presença de pectinas específicas e celulose (Boanares et al., 2018, 2024). Dessa forma, a composição da parede celular pode influenciar tanto a quantidade quanto a velocidade de água absorvida pela folha.

Embora plantas epífitas que expressam o metabolismo CAM sejam capazes de absorver água pelas folhas, a suculência pode afetar negativamente a capacidade de FWU (Gotsch et al., 2022; Lima et al., 2023). Diante desse cenário, essa tese teve como principal objetivo investigar o papel da absorção foliar de água em plantas suculentas e sua relação com o ciclo circadiano inerente ao metabolismo CAM. Para alcançar estes objetivos, a tese foi organizada em 3 capítulos. No primeiro, propomos que o ciclo metabólico circadiano decorrente da expressão CAM altera a capacidade de FWU pelo acúmulo de ácidos orgânicos pela manhã. Além disso, investigamos se pectinas especializadas relacionadas à porosidade da parede celular seriam distribuídas de forma a contribuir para a ocorrência desse processo. Como modelo de estudo, utilizamos *Vanilla phaeantha* Rchb.f. (Orchidaceae), uma espécie hemiepífita nativa nas proximidades de Uberlândia. No segundo capítulo, investigamos se condições de déficit hídrico reduzem o grau de hidratação foliar em duas espécies epífitas, favorecendo a expressão CAM e maximizando a absorção de água pelas folhas. Foram utilizadas como modelo de estudo *Cattleya forbesii* Lindl. e *Cattleya guttata* Lindl., duas espécies de orquídeas nativas do Brasil. Por fim, no terceiro capítulo, avaliamos como a absorção de água proveniente da neblina pode contribuir para a manutenção do equilíbrio hídrico foliar, aumentar a eficiência do uso da água, sustentar a atividade fotossintética e reduzir o estresse oxidativo em espécies com folhas suculentas. Além disso, investigamos se o déficit hídrico ou a presença da neblina pode modular a expressão CAM e, consequentemente, influenciar a capacidade de FWU em *Puya chilensis* Molina (Bromeliaceae).

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CAPÍTULO 1

Alterações na absorção foliar de água decorrentes da abertura e do fechamento estomático em *Vanilla phaeantha* Rchb.f. (Orchidaceae)

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Alterações na absorção foliar de água decorrentes da abertura e do fechamento estomático em *Vanilla phaeantha* Rchb.f. (Orchidaceae)

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Resumo

A suculência foliar, o Metabolismo Ácido das Crassuláceas (CAM) e a absorção foliar de água (FWU) são estratégias relevantes para o balanço hídrico em orquídeas. Diante disso, nossa hipótese é que o acúmulo de ácidos orgânicos associado ao metabolismo CAM aumente a FWU. Além disso, propomos que epitopos de pectinas relacionados à porosidade da parede celular sejam amplamente marcados nas folhas. Utilizamos *Vanilla phaeantha* como modelo para verificarmos variações ao longo do dia no conteúdo de saturação hídrica, no conteúdo relativo de água (RWC), no potencial hídrico, na acidez titulável e na capacidade de FWU. Análises imunocitoquímicas foram realizadas para detectar quatro epitopos de pectinas na parede celular. Para verificar a via de entrada de água na folha, usamos o marcador apoplástico Lucifer yellow. Observamos que a abertura e o fechamento dos estômatos influenciam o balanço hídrico foliar ao longo do dia. No período da manhã, quando os estômatos estão fechados, as folhas apresentaram maiores valores de RWC, potencial hídrico e acidez titulável. A capacidade de FWU apresentou uma clara variação temporal, sendo maior no período da manhã, contudo, sem relação com a expressão do metabolismo CAM. As superfícies adaxial e abaxial das folhas são capazes de FWU. A presença de pectinas e de celulose na parede celular pode influenciar a movimentação e retenção de água. Em síntese, a capacidade de FWU pode ajudar a *V. phaeantha* a manter o balanço hídrico foliar ao longo do dia e a enfrentar o déficit hídrico presente no ambiente independentemente do metabolismo CAM.

Palavras-chave: ácidos orgânicos, balanço hídrico, hemiepífita, parede celular, pectinas, plantas CAM.

Introdução

As orquídeas ocupam uma ampla diversidade de habitats e aproximadamente 75% de suas espécies são epífitas (Zotz et al., 2021). Embora o habitat epifítico proporcione vantagens relacionadas ao acesso à luz, viver neste ambiente também impõe uma série de restrições ambientais. Entre elas, a disponibilidade de água é um dos fatores mais limitantes, pois estas plantas dependem de fontes atmosféricas, como chuva, neblina e orvalho (Benzing, 1990; Zotz, 2016). Assim, o sucesso adaptativo das epífitas está associado ao desenvolvimento de estratégias morfológicas e fisiológicas que maximizam o uso da água. Entre estas estratégias, destaca-se o metabolismo ácido das Crassuláceas (CAM), uma via fotossintética amplamente distribuída entre as orquídeas epífitas (Silvera et al., 2010; Zhang et al., 2015; Tay et al., 2019).

Nas plantas que expressam o metabolismo CAM, a captura de CO_2 e sua fixação pelo ciclo de Calvin-Benson ocorrem separadamente no tempo e apresentam quatro fases distintas (Osmond, 1978; Ting, 1985). Na fase I, a assimilação primária de dióxido de carbono (CO_2) ocorre predominantemente durante a noite, quando os estômatos estão abertos e as condições atmosféricas favorecem menores perdas de água por transpiração. O CO_2 é então fixado pela fosfoenolpiruvato carboxilase (PEPcase), formando os ácidos orgânicos, geralmente armazenados na forma de malato dentro dos vacúolos (Winter et al., 2005). No início da manhã, acontece a fase II, quando é observado um declínio na atividade da PEPcase e o aumento da atividade da ribulose-1,5-bisfosfato carboxilase-oxidase (RuBisCO). Os estômatos podem permanecer abertos durante a fase II, podendo capturar o CO_2 por meio das duas enzimas ao mesmo tempo. Iniciando a fase III, o malato é descarboxilado pela enzima NADP-málica, e o CO_2 liberado é utilizado pelo ciclo de Calvin-Benson, através da atividade carboxilase da RuBisCO, quando é incorporado a compostos orgânicos (Cushman, 2001). Na fase IV, ocorre o oposto da fase II: ao final da tarde, a RuBisCO reduz sua atividade, os estômatos começam a se fechar e o CO_2 passa a ser capturado principalmente via PEPcase (Osmond, 1978).

Além de aumentar a eficiência no uso da água, o metabolismo CAM pode estar associado a alterações diárias no estado hídrico dos tecidos, influenciando o armazenamento e a absorção de água. O acúmulo noturno de ácidos orgânicos pode reduzir o potencial osmótico e, consequentemente, o potencial hídrico foliar, favorecendo a retenção e o direcionamento de água para os tecidos (Herrera et al., 2008; Matimati et al., 2012). Durante o dia, com o fechamento dos estômatos e a redução das concentrações de ácidos orgânicos, pode ocorrer a elevação gradual do potencial hídrico (Lüttge, 2004;

Males & Griffiths, 2017). Em *Clusia minor* L. (Clusiaceae), por exemplo, a concentração de ácidos orgânicos obtida na folha ao amanhecer foi positivamente associada à captação de água pelos tecidos, sugerindo que a expressão do metabolismo CAM promoveu uma redução do potencial osmótico e contribuiu para a absorção de água (Herrera et al., 2008). De forma semelhante, em *Senecio medley-woodii* Hutch. (Asteraceae), espécie CAM obrigatória, um aumento no conteúdo de malato foi associado ao aumento da pressão osmótica matinal e relacionado positivamente com a captação de água (Ruess et al., 1988).

Várias espécies de epífitas, incluindo orquídeas, apresentam uma estratégia alternativa para aquisição de água, a absorção foliar de água (em inglês *foliar water uptake* - FWU) (Gotsch et al., 2022; Lima et al., 2023; Anders et al., 2025). Quando uma película de água se forma sobre a superfície foliar, forma-se um gradiente de potencial hídrico entre a atmosfera (cujo potencial hídrico é próximo de 0 Mpa) e os tecidos internos da folha, possibilitando a absorção de água (Burgess & Dawson, 2004). Assim, a intensidade da FWU depende diretamente da magnitude desse gradiente. Considerando que o metabolismo CAM favorece uma redução do potencial hídrico durante o período matutino, é possível que a força motriz para a absorção foliar de água seja maior nesse momento do dia. Essa possibilidade é particularmente relevante em ambientes epifíticos, nos quais a maior disponibilidade de água atmosférica frequentemente ocorre durante as primeiras horas da manhã, associada à presença de orvalho e elevada umidade relativa do ar.

As possíveis vias de entrada de água pela folha incluem os estômatos, a cutícula, os hidatódios e os tricomas (Schreel & Steppe, 2020; Guzmán-Delgado et al., 2021; Boanares et al., 2024). Após atravessar essas estruturas, a água se desloca pela parede celular, outra importante barreira à entrada de água nos tecidos foliares e que influencia a velocidade e a eficiência da absorção. A parede celular é constituída principalmente de celulose, hemiceluloses e pectinas (Albersheim et al., 2011). A celulose é um polissacarídeo composto por unidades (1→4)-β-D-glicose que podem aumentar a porosidade da parede quando associadas a pectinas (Newman, 2004; Albersheim et al., 2010). As pectinas, por sua vez, são polissacarídeos estruturais complexos ricos em ácido galacturônico (GalA). Seus principais domínios incluem os homogalacturonanos (HGs), os ramnogalacturonanos-I (RGI) e os ramnogalacturonanos-II (RGII) (Ridley et al., 2001; Willats et al., 2001; Albersheim et al., 2011), polímeros capazes de formar géis hidratados e modular a porosidade da parede celular (Willats et al., 2001; Cosgrove, 2005; Schreel

et al., 2020). Estudos recentes sugerem que a presença de celulose na parede das células da epiderme aumenta a absorção de água, enquanto as pectinas aumentam a velocidade com que essa água é absorvida (Boanares et al., 2018, 2024).

Além de influenciarem a porosidade e movimentação da água na parede celular, alguns domínios de pectinas estão associados a propriedades mecânicas dos tecidos foliares. Os próprios HGs altamente metilesterificados conferem maior flexibilidade e extensibilidade, enquanto regiões de RGI, particularmente aquelas enriquecidas por cadeias laterais de arabinanos e galactanos, estão associadas ao aumento da elasticidade e da capacidade de deformação reversível das paredes celulares (Hermes et al., 2018; Costa et al., 2024). Essas características podem favorecer alterações no volume celular decorrentes de oscilações hídricas diárias. Em espécies suculentas, característica frequentemente associada ao metabolismo CAM (Cushman, 2001; Moreira et al., 2009, 2013), paredes celulares mais elásticas podem permitir o armazenamento de maiores quantidades de água. Desta forma, a abundância e a distribuição de epítopos específicos de HGs e RGI podem não apenas favorecer a absorção e o transporte apoplástico de água, mas também contribuir para a elevada capacidade de armazenamento hídrico (suculência) característica de folhas CAM.

Vanilla phaeantha Rchb.f. (Orchidaceae) é uma hemiepífita secundária que expressa metabolismo CAM constitutivo e apresenta comprovada capacidade de absorção foliar de água (Buss et al., 2024; Lima et al., 2023), tornando-se um bom modelo para estudo. Propomos que o acúmulo noturno de ácidos orgânicos decorrente da expressão do metabolismo CAM promova uma redução do potencial hídrico foliar ao amanhecer, aumentando o gradiente de potencial hídrico entre a superfície e os tecidos foliares, o que favorece maiores taxas de FWU no período da manhã. Além disso, esperamos que componentes da parede celular associados à retenção e movimentação de água, especialmente pectinas homogalacturonanos (HGs), rhamnogalacturonanos-I (RGI), sejam amplamente distribuídos nas folhas de *V. phaeantha*. Desta forma, especificamente temos como objetivos (1) avaliar a relação entre variações diurnas do potencial hídrico, o acúmulo de ácidos orgânicos e o fechamento estomático nas folhas de *V. phaeantha*; (2) avaliar se há relação entre a taxa de FWU e a redução nos valores de potencial hídrico no período da manhã; (3) identificar estruturas anatômicas potencialmente associadas às vias de captação da água; (4) caracterizar a composição de epítopos de pectinas na parede celular associados à movimentação de água nas folhas de *V. phaeantha*.

Material e métodos

Área de estudo e espécie vegetal

Utilizamos como modelo de estudo a hemiepífita secundária *V. phaeantha* Rchb.f. (n = 10), espécie amplamente distribuída pelas Américas (Karremans et al., 2020). Durante seu desenvolvimento, as sementes germinam no solo e, posteriormente, as plantas estabelecem contato com o forófito, tornando-se epífitas (Alconcero, 1968; Ferreira et al., 2017). Os indivíduos estudados foram encontrados em uma mata de galeria localizada em uma área de Preservação Permanente (APP) no município de Araguari, Minas Gerais, Brasil (763 m de altitude; 18°43'58.5"S, 48°02'58.9"W), na estação chuvosa (janeiro/fevereiro de 2023). As matas de galeria são formações florestais que acompanham pequenos cursos de água, formando corredores de vegetação no Cerrado (Ribeiro & Walter, 1998). O clima da região é classificado como tropical com inverno seco (Aw), segundo a classificação de Köppen (Alvares et al., 2013), apresentando estação seca entre maio e agosto e estação chuvosa entre outubro e março. A umidade relativa do ar e a temperatura foram monitoradas por meio de um registrador de dados (Instrutherm HT-70, Instruther Instrumentos de Medição Ltda., Brasil) durante 24 horas na estação chuvosa (abril de 2018).

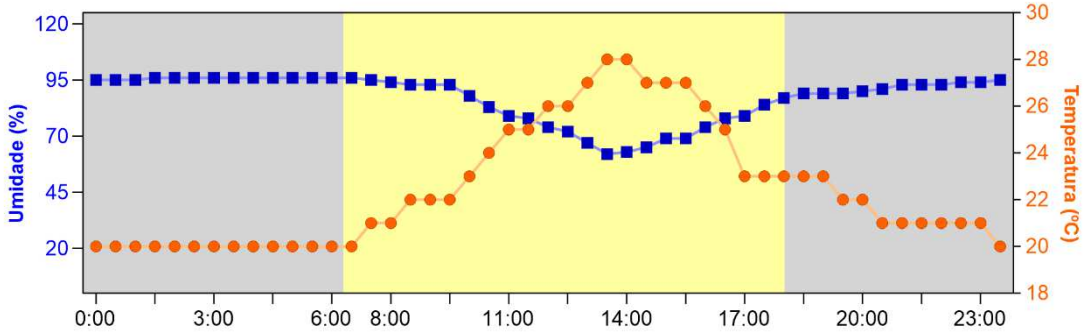


Figura 1: Registros de umidade relativa (%) e temperatura do ar (°C) monitorados durante 24 horas na estação chuvosa em uma mata de galeria no Cerrado (Araguari), utilizando o Instrutherm HT-70.

Os horários utilizados para as avaliações da absorção foliar de água, expressão do metabolismo CAM e do status hídrico foliar (SWC, RWC e potencial hídrico) foram definidos com base em dados de condutância estomática obtidos ao longo de 24 horas, com medições realizadas em intervalos de uma hora utilizando um analisador portátil de

gases por infravermelho (IRGA, Li-Cor 6400 USA) conforme obtido por Buss et al., (2024). A partir do padrão diário de condutância estomática, foram selecionados quatro horários representativos do ciclo CAM: 4:00 e 8:00, correspondentes aos períodos antes e após o fechamento estomático, respectivamente, e 17:00 e 20:00, correspondendo aos períodos antes e após a abertura estomática.

Avaliação do status hídrico foliar

Para as análises do conteúdo de saturação hídrica (SWC) e do conteúdo relativo de água (RWC), foi coletada uma folha de sete indivíduos (n=7) de *V. phaeantha* em cada horário de amostragem (4:00, 8:00, 17:00 e 20:00 h). O SWC foi utilizado como indicador de suculência foliar e calculado a partir da equação $SWC = (MT - MS)/MS$, na qual *MT* corresponde à massa túrgida após hidratação por 24 horas e *MS* à massa seca, determinada após secagem do material em estufa a 60 °C até atingir peso constante (Ogburn & Edwards, 2012). O estado hídrico das folhas no momento da coleta foi avaliado por meio do RWC, calculado a partir da equação $RWC = [(MF-MS)/(MT-MS)]*100$, na qual *MF* corresponde à massa fresca da amostra (Turner, 1981). As determinações de massa fresca, túrgida e seca foram realizadas em uma balança analítica (Shimadzu AUY 220, Japão).

O potencial hídrico foliar (Ψ_w) foi determinado nos mesmos horários de amostragem (4:00, 8:00, 17:00 e 20:00 h) utilizando uma câmera de pressão de Scholander (Soilmoisture Equipment Corp., USA). Para cada horário, foi coletada uma folha de sete indivíduos (n=7), sendo as medições realizadas imediatamente após a excisão da folha (Rodriguez-Dominguez et al., 2022).

Determinação da expressão do metabolismo CAM

A expressão do metabolismo CAM foi avaliada por meio da variação diurna da acidez titulável (ΔH^+). Com base no padrão diário de condutância estomática, folhas de dez indivíduos (n=10) de *V. phaeantha* foram coletadas antes e após o fechamento estomático (4:00 e 8:00) e antes e após sua abertura ao final da tarde (17:00 e 20:00). A acidez titulável do tecido foliar foi determinada em amostras de 200 mg pesadas em balança digital (Shimadzu AUY 220, Japão). As amostras foram submetidas à fervura em 5 ml de água destilada por cinco minutos e, posteriormente, 2,5 ml dos extratos obtidos foram titulados com NaOH 0,01 N (Hartsock & Nobel, 1976). Os resultados foram expressos pela acidez do tecido em cada horário mensurado ($\mu eq\ H^+\ g^{-1}$ MF), sendo a

intensidade da expressão CAM sugerida pela diferença entre os horários de maior e menor acidez do tecido (ΔH^+).

Determinação da absorção foliar de água (FWU)

A capacidade de absorção foliar de água foi avaliada em ramos de *V. phaeantha* coletados no campo, tendo suas extremidades imediatamente seladas com vaselina em pasta para evitar perdas de água durante o transporte ao laboratório. Em seguida, uma folha intacta e madura de cinco indivíduos (n=5) foi amostrada às 4:00, 8:00, 17:00 e 20:00 h, sendo a base de cada folha selada com vaselina em pasta e parafina. As folhas foram inicialmente pesadas em uma balança analítica (Shimadzu AUY 220, Japão) para obtenção da massa fresca inicial. Posteriormente, as folhas foram completamente imersas em água destilada e submetidas a pesagens sucessivas para a construção das curvas de absorção de água. As medições foram realizadas a cada 15 minutos durante as primeiras duas horas, depois a cada 30 minutos nas duas horas subsequentes e, por fim, após uma hora adicional, totalizando cinco horas de avaliação (Liang et al., 2009). Antes de cada pesagem, as folhas foram cuidadosamente secas com toalhas de microfibra absorventes para remover a água da superfície. Ao final do experimento, a área foliar de cada amostra foi determinada utilizando o software ImageJ (Schindelin et al., 2012).

A absorção de água foliar (FWU) foi calculada com base na variação entre a massa fresca e a massa final ao longo do tempo por unidade de área foliar, a partir da equação FWU = (massa fresca – massa final)/área foliar. A taxa de captação de água pelas folhas (WC) com base na área foliar (g cm^{-2} de área foliar) foi calculada utilizando uma equação diferencial $WC = (C_{max} - C_i) \times (1 - e^{-kt}) + C_i$), onde C_{max} é a absorção máxima de água foliar, C_i é o conteúdo inicial de água foliar, *k* é a velocidade de absorção de água pela área foliar (coeficiente exponencial ou número de curva da função de absorção) e *t* é o tempo de absorção. Essa equação diferencial fornece dados relativos à absorção foliar de água (FWU), à absorção máxima de água foliar (C*max*) e à velocidade de absorção (k) (Liang et al., 2009, modificado; Pan et al., 2021).

Marcador apoplástico fluorescente

Para verificar possíveis vias de entrada de água nas folhas, três folhas maduras de cinco indivíduos de *V. phaeantha* (n=5) foram analisadas. No laboratório, a base de cada folha foi selada com vaselina em pasta. Em seguida, foram aplicados 100 µL de solução aquosa a 1% de Lucifer Yellow (LY; sal dilithium CH, Sigma) na superfície adaxial de

uma folha e na superfície abaxial de outra folha de cada indivíduo. Como controle, foi aplicada água destilada em ambas as superfícies de uma terceira folha. As soluções foram aplicadas na região mediana da lâmina foliar, visando melhor representar as características da folha (Oparka & Read, 1994). Após a aplicação do marcador fluorescente, as folhas foram colocadas em placas de Petri contendo papel-filtro umedecido com água destilada. Em seguida, as placas foram envolvidas em papel-alumínio, formando uma câmara escura, na qual permaneceram por 5 h (Widholzer, 2005; Eller et al., 2016; Boanares et al., 2018). Após esse intervalo, as folhas foram lavadas em água destilada para retirar o excesso de marcador e cuidadosamente secas em papel-filtro. Posteriormente, foram obtidas seções transversais à mão livre nas regiões onde a solução de LY foi aplicada.

As seções foram montadas em glicerina 50% e observadas em microscópio óptico com sistema de fluorescência (Leica® DM4000 B LED, Leica, Alemanha) acoplado a uma câmera digital (Leica® DFC 3000 G, Leica, Alemanha) e utilizando o software de análise LAS X 1.1.0.12420. As observações foram realizadas sob intensa excitação verde (450-490 nm, filtro FITC) e filtro de barreira de 515 nm (Oparka & Read, 1994, modificado).

Análises histoquímica

Para análises histoquímicas foram coletados fragmentos foliares de cinco indivíduos de *V. phaeantha* (n=5). As amostras foram fixadas em FAA50 (formaldeído, ácido acético, álcool etílico 50%, 1:1:18 v/v) por 48 horas e depois armazenadas em álcool etílico 70% (Johansen, 1940). Seções transversais foram realizadas manualmente com lâminas de barbear e tratadas com Sudan III para detecção de lipídios totais (Johansen, 1940). Para detecção de pectinas, as seções foram tratadas com vermelho de rutênio (CAS 11103-72-3; Sigma-Aldrich), lavadas em ácido acético a 0,5% e posteriormente em água destilada (Kraus & Arduin, 1997; Johansen, 1940). As lâminas temporárias foram montadas em água e imediatamente observadas em microscópio óptico (DM1000 LED, Leica Microsystems, Alemanha).

A distribuição de celulose (β-glucana) nas paredes celulares foliares foi investigada em seções transversais obtidas manualmente e tratadas com Calcofluor White em água destilada (1:1, v/v; Sigma-Aldrich) por 30 minutos no escuro (Herth & Schnepf, 1980). As lâminas foram montadas em água destilada e analisadas em um microscópio com sistema de fluorescência (Leica® DM4000 B LED, Leica, Alemanha), acoplado a

uma câmera digital (Leica® DFC 3000 G, Leica, Alemanha) com um filtro de emissão DAPI (4′,6-diamidino-2-phenylindole) (espectro de excitação 515 nm).

Análises imunocitoquímicas

Folhas de três indivíduos de *V. phaeantha* (n=3) foram coletadas e levadas ao laboratório para análises imunocitoquímicas. Fragmentos foliares foram fixados em solução de Karnovsky (paraformaldeído 4%, glutaraldeído 0,01 M e tampão fosfato 0,2 M, pH 7,2, 5:3:2, v/v) por 48 horas (Karnovsky, 1965, modificado por Kraus & Ardiun, 1997) e posteriormente armazenados em etanol 70% (Johansen, 1940). As amostras foram desidratadas em série etanólica e incluídas em 2-hidroxietil metacrilato (Historesin, Leica Microsystems, Alemanha). Em seguida, foram obtidas seções transversais e longitudinais com 8 µm de espessura utilizando micrótomo rotativo (YD315, ANCAP, Brasil) e navalhas de aço inoxidável de alto perfil. Os cortes foram fixados nas lâminas histológicas e submetidos às análises de imunocitoquímica.

Para detecção de epítopos de pectinas, foram utilizados anticorpos monoclonais JIM5, JIM7, LM5 e LM6 (Centre for Plant Sciences, University of Leeds, UK), conforme especificado na Tabela 1. Os cortes foram inicialmente incubados por 30 min em solução bloqueadora de leite em pó (Molico®) em tampão fosfato-salino (PBS). Posteriormente, as seções foram incubadas por duas horas com anticorpos primários diluídos em solução de leite e PBS (1:10, v/v). Após lavagem em PBS, os cortes foram incubados por mais duas horas e na ausência de luz, com anticorpo secundário conjugado ao fluorocromo FITC (1:100 em 3% de leite/PBS). Como controle, foram preparadas secções submetidas ao mesmo protocolo, porém sem incubação com os anticorpos primários. As lâminas foram montadas em glicerina 50% e observadas no microscópio de fluorescência (DM4000 LED, Leica Microsystems, Alemanha) acoplado a uma câmera monocromática HD (DFC3000 G). Foram utilizados os comprimentos de onda de excitação de 450–490 nm e o filtro de barreira de 515 nm. A autofluorescência dos tecidos foi marcada com o filtro DAPI (Kuster et al., 2026), em função da sobreposição no software do microscópio (LAS X Version: 1.1.0.12420, Leica), para melhorar a visualização dos resultados de anticorpos monoclonais. De modo que os resultados positivos (+) foram marcados com a coloração verde, enquanto os resultados negativos (-) foram marcados com a coloração azul.

Tabela 1: Anticorpos monoclonais primários e epítopos de pectinas utilizados na análise imunocitoquímica das folhas de *Vanilla phaeantha* (Orchidaceae).

Monoclonal antibodies	Epitopes	References
Homogalacturonan		
JIM5	Partially (40%) Methylesterified / no ester	Clausen et al. (2004)
JIM7	Partially (15-80%) Methylesterified	Clausen et al. (2004)
Rhamnogalacturonan		
LM6	(1→5)-α-L-arabinan	Willats et al. (1998), Verhertbruggen et al. (2009)
Rhamnogalacturonan-I / Galactan +		
LM5	(1→4)-β-D-galactan	Jones et al. (1997), Andersen et al. (2016)

Análises dos dados

A diferença no conteúdo relativo de água (RWC) ao longo do dia foi avaliada através de uma ANOVA de medidas repetidas utilizando a função "ezANOVA" do pacote ez (Bakeman, 2005). Os dados de ANOVA foram submetidos ao teste de normalidade de Shapiro-Wilk e à esfericidade dos dados pelo teste de Mauchly, ambos com nível de significância de 5%. Caso os dados não sejam esféricos, foram usadas as correções de Greenhouse-Geisser e Huynh-Feldt. Os dados não paramétricos foram analisados utilizando o teste de Friedman, ou seja, para avaliar se havia diferença no conteúdo de saturação hídrica (SWC), no potencial hídrico, na acidez titulável, na absorção máxima de água nas folhas (C*max*) e na velocidade de captação de água (*k*) ao longo do dia. Diferenças nas características foram significativas considerando a probabilidade de 5%. As médias foram comparadas pelo teste de Dunn-Bonferroni. Todas as análises foram realizadas no software R (i386 4.4.0; R Development Core Team, 2024).

Resultados

Variações no balanço hídrico foliar e expressão do metabolismo CAM ao longo do dia

O SWC das folhas de *V. phaeantha* não variou ao longo do dia (Fig. 2A). Em contraste, o RWC diferiu entre os horários avaliados, apresentando maiores valores às 8:00 h e menores às 4:00 h (Fig. 2B). O potencial hídrico das folhas também variou ao longo do dia, com maiores valores às 8:00 h e menores às 20:00 h (Fig. 2C).

A variação diurna dos ácidos orgânicos (ΔH^+=12.90 $\mu eqH^+g^{-1}MF$) nas folhas de *V. phaeantha* indica a expressão do metabolismo CAM. A maior acidez do tecido foi observada no período da madrugada e no início da manhã (4:00 e 8:00 h), com médias de

22.7 e 20.4 μeqH^+g^{-1}MF, respectivamente, enquanto a menor acidez foi observada às 20:00 h (média de 13.7 μeqH^+g^{-1}MF; Fig. 1D).

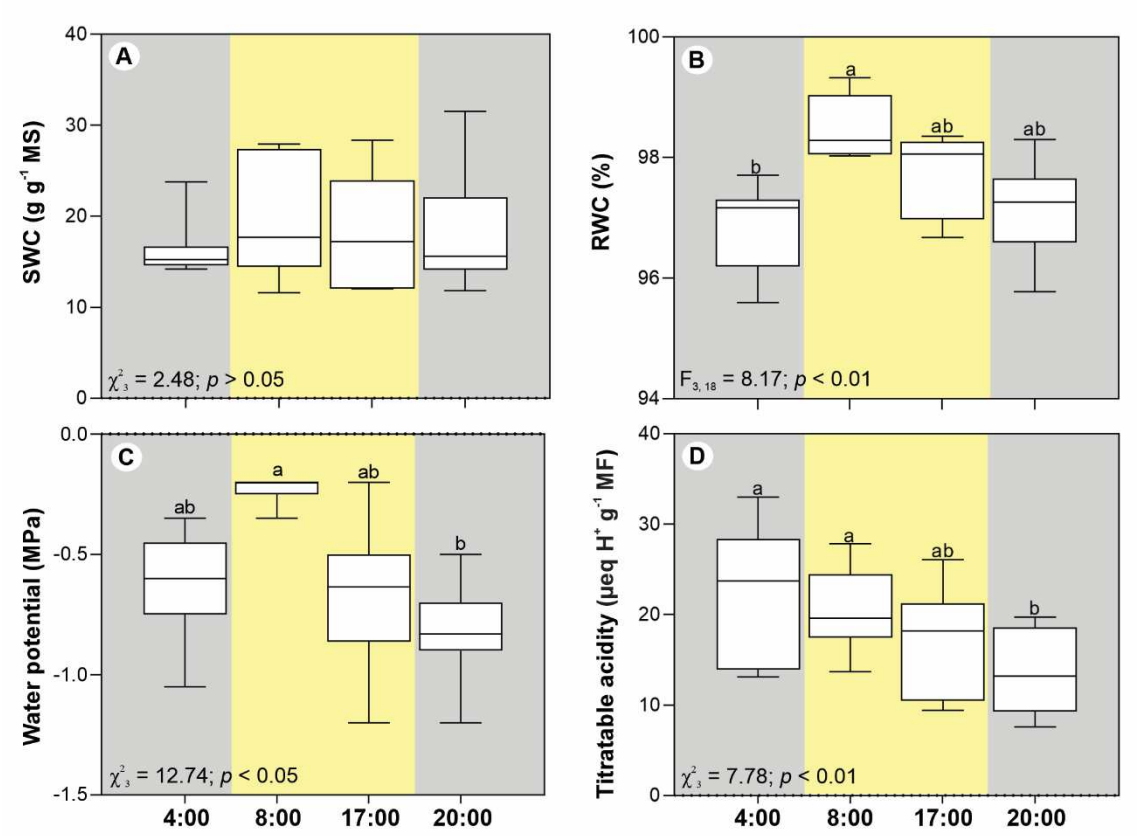
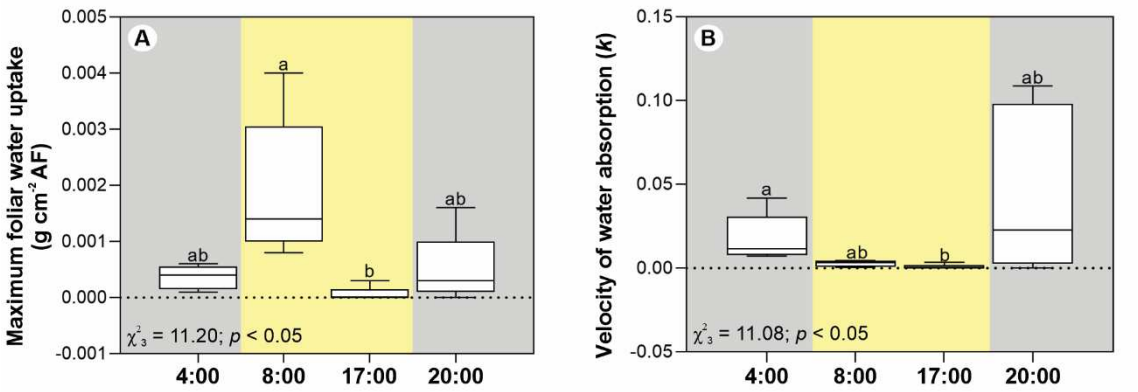


Figura 2: Variações no balanço hídrico e na acidez titulável nas folhas de *V. phaeantha* (Orchidaceae) ao longo de um ciclo diário. (A) Conteúdo de saturação hídrica (SWC; n=7), (B) conteúdo relativo de água (RWC; n=7), (C) potencial hídrico (n=7) e (D) acidez titulável (n=10), indicando a expressão do metabolismo CAM. Letras diferentes indicam diferenças entre os horários, de acordo com ANOVA de medidas repetidas ou o Teste de Friedman. Foram atribuídas diferenças quando encontrados valores de p≤0.05 e as médias foram comparadas pelo teste de Dunn-Bonferroni.

Alterações na FWU ao longo do dia e marcador fluorescente apoplástico

A maior taxa de absorção de água foliar (C*max*) foi observada às 8:00 horas, enquanto os menores valores foram encontrados ao final da tarde, às 17:00 h (Fig. 3A). A velocidade de absorção foliar de água (k) foi maior às 4:00 h, com menores valores encontrados às 17:00 h (Fig. 3B).



343 **Figura 3:** Variações na capacidade de absorção foliar de água em *V. phaeantha*
344 (Orchidaceae; n = 5) ao longo do dia. (A) Absorção máxima de água (C*max*) e (B)
345 velocidade de absorção de água (k). Letras diferentes indicam diferenças entre os
346 horários, de acordo com o teste de Friedman. As médias foram comparadas pelo teste de
347 Dunn-Bonferroni. Para ambos os testes, diferenças foram atribuídas considerando
348 $p \leq 0.05$.
349
350 O marcador fluorescente LY atravessou a cutícula tanto da face adaxial quanto da
351 face abaxial das folhas da *V. phaeantha* (Fig. 4). Na face adaxial, a fluorescência foi
352 observada na cutícula e espalhada pelas células da epiderme e do mesofilo de forma
353 menos intensa (Fig. 4B). Na face abaxial, intensa fluorescência do marcador LY foi
354 observada na cutícula, nos espessamentos das células epidérmicas e no estômato, se
355 espalhando pelo tecido mesofílico (Fig. 4C).

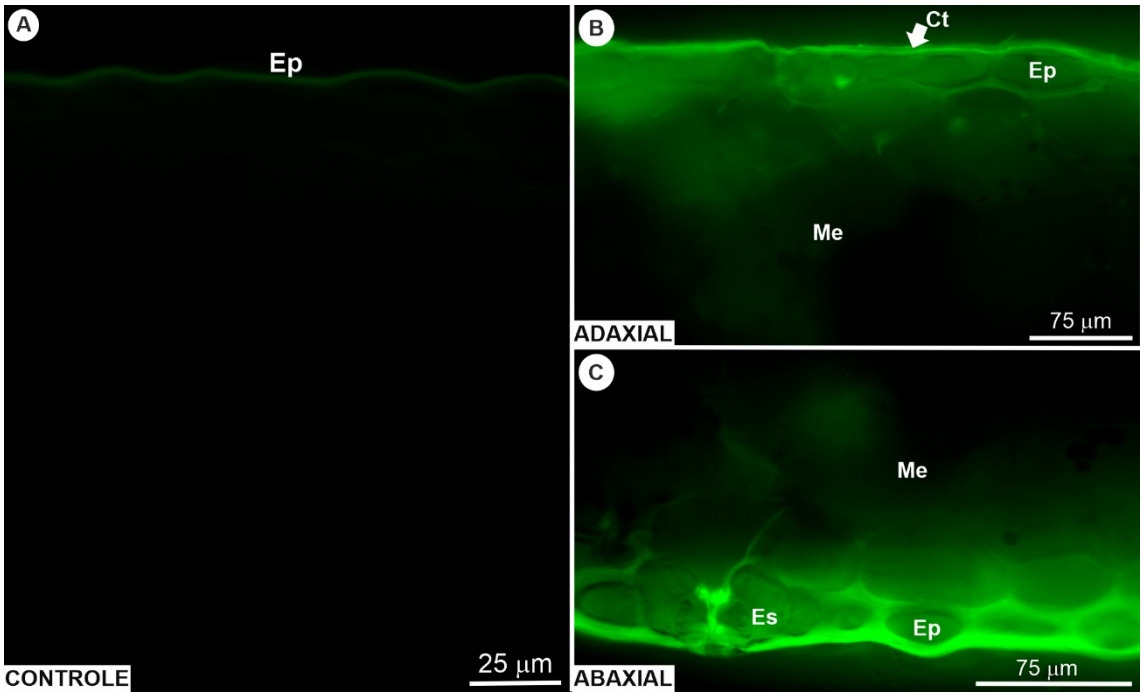


Figura 4: Detecção de via apoplástica usando solução aquosa de Lucifer yellow 1% (dilithium salt) (controle; A) em secção transversal de folha de *Vanilla phaeantha* (Orchidaceae) na superfície adaxial (B) e abaxial (C).

Abreviações: Ct = cutícula; Ep = epiderme; Me = mesófilo; Es = estômato.

Composição da parede celular e histoquímica

Substâncias lipídicas foram observadas na cutícula da face abaxial e adaxial das folhas de *V. phaeantha* (Fig. 5A e B) perpassando na forma de uma camada delgada pelos estômatos (Fig. 5B). Forte impregnação péctica foi observada tanto nas células da epiderme de ambas as faces da folha quanto no mesofilo (Fig. 5C e D).

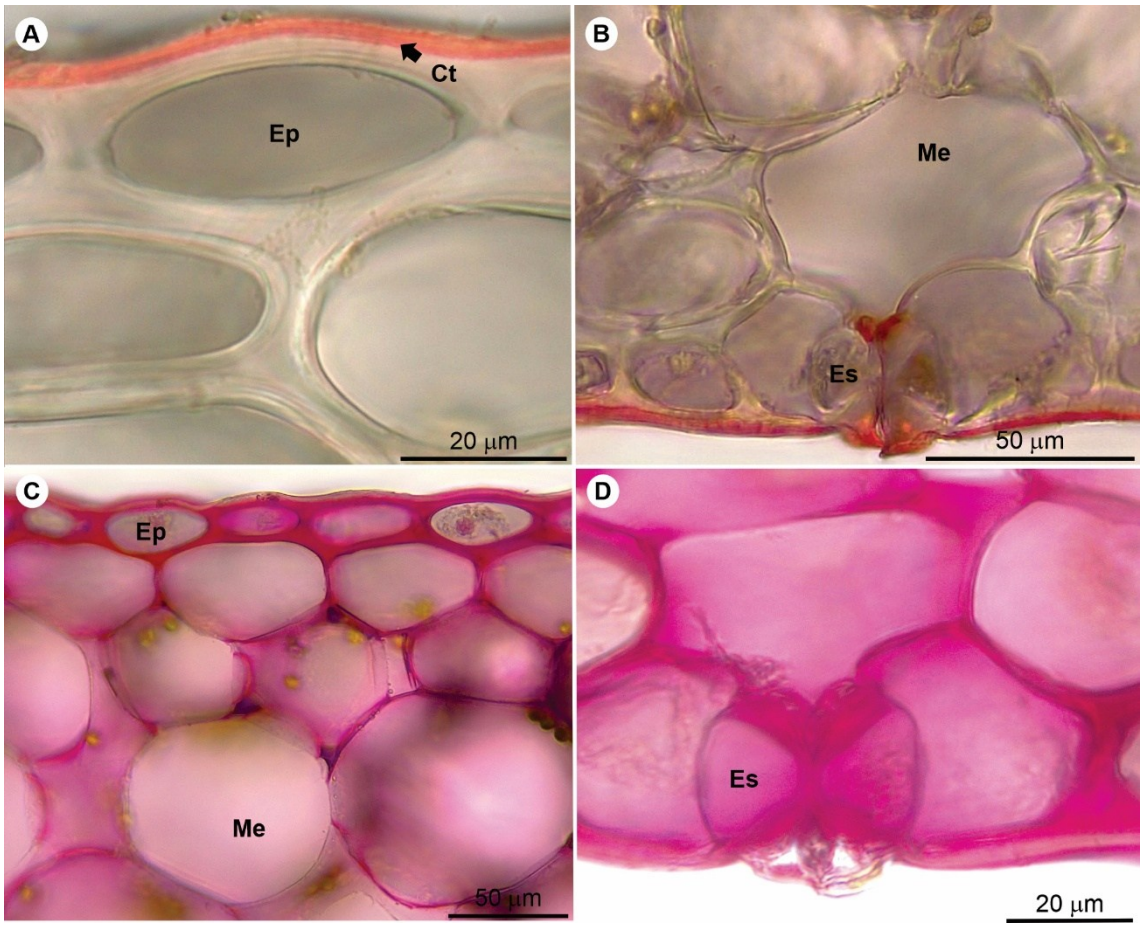


Figura 5: Secções transversais mostrando a presença de lipídios totais e pectinas nas folhas de *Vanilla phaeantha* (Orchidaceae). A impregnação lipídica foi detectada utilizando-se de Sudan III, sendo mais evidente na cutícula da face adaxial (A) e da face abaxial (B). A presença de pectinas na parede celular das células foi verificada pela intensa coloração rosa quando em contato com vermelho de rutênio. Intensa marcação foi observada nas células epidérmicas da face adaxial (C) e da face abaxial (D) e nas células do mesofilo. *Abreviações:* Ct = cutícula; Ep = epiderme; Me = mesofilo; Es = estômato.

Os epítopos de HGs e RGI foram identificados nos tecidos foliares de *V. phaeantha* (Tabela 2; Fig. 6). Os epítopos de HG com baixa e alta metilesterificação, reconhecidos por JIM5 e JIM7, respectivamente, foram marcados na epiderme e nas células do mesofilo (Fig. 6B-F). Os epítopos de (1→4)-β-D-galactanos, identificados por LM5, foram detectados na epiderme, no mesofilo e no floema (Fig. 6G-I). Os epítopos de (1→5)-α-L-arabinanos (reconhecidos por LM6) não foram marcados em nenhum dos tecidos. Observamos a presença de celulose na parede das células do mesofilo e do floema (Fig. 6J e K).

Tabela 2: Resultados dos anticorpos primários monoclonais e do Calcofluor White nas folhas de *Vanilla phaeantha* (Orchidaceae). Resultado positivo (+) e negativo (-).

Vanilla phaeantha	Plant tissues	Monoclonal antibodies				Calcofluor
		JIM5	JIM7	LM5	LM6	
Leaf	Epidermis	+	+	+	-	-
	Mesophyll	+	+	+	-	+
	Phloema	-	-	+	-	+
	Xylem	-	-	-	-	-

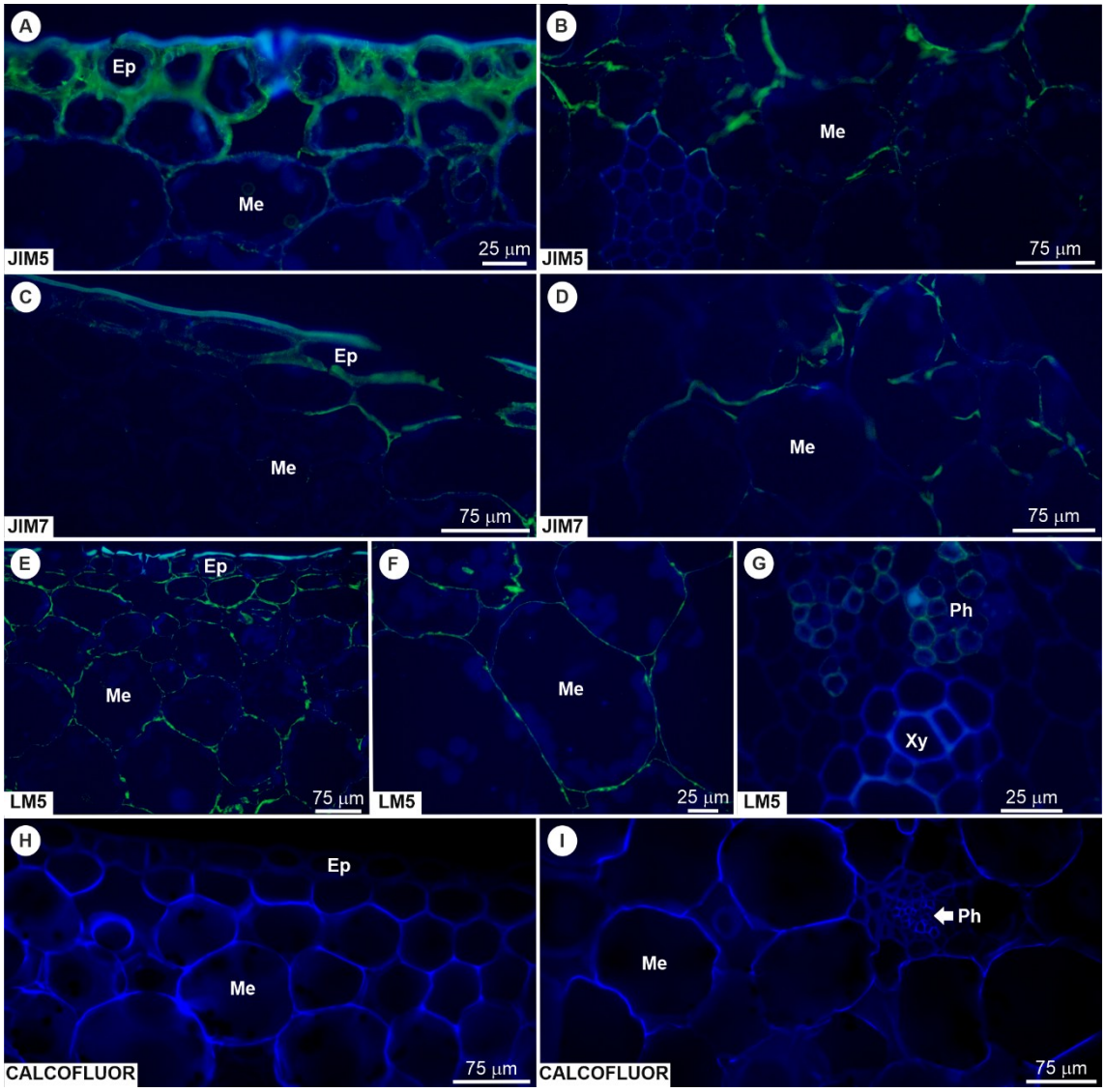


Figura 6: Distribuição de epítopos de pectinas e celulose em folhas de *Vanilla phaeantha* (Orchidaceae). A e B: HG com baixa metil-esterificação reconhecidos por JIM5 nas paredes celulares das células da epiderme e do mesofilo. C e D: HG com alta metil-esterificação reconhecidos por JIM7 nas paredes celulares das células da epiderme e do mesofilo. E - G: Epitopos de RG com galactanos reconhecidos por LM5 nas paredes

celulares das células do mesofilo e do floema. H e I: Calcofluor White, marcando celulose na parede celular nas células do mesofilo e do floema.

Abreviações: Ep = Epiderme; Me = Mesofilo; Ph = Floema; Xy = Xilema.

Discussão

Neste trabalho, observamos que o status hídrico foliar (avaliado por meio do RWC e do potencial hídrico), a acidez titulável do tecido e a taxa de absorção foliar de água de *V. phaeantha* variam ao longo do ciclo diário, acompanhando as mudanças fisiológicas associadas à expressão do metabolismo CAM. Durante o período da manhã (8:00 h), após o fechamento dos estômatos, as folhas apresentaram maiores valores de RWC e potencial hídrico, coincidindo também com uma elevada acidez titulável (às 04:00 e 08:00 h). Ao longo do dia, uma redução gradual nos valores destes parâmetros foi observada, acompanhando a descarboxilação dos ácidos orgânicos durante as fases subsequentes do ciclo CAM. Entretanto, a maior taxa de absorção de água nas folhas (C*max*) também foi registrada às 08:00, com menores valores ao final da tarde (17:00). Este padrão foi diferente do esperado, porém também evidenciou uma variação temporal na capacidade de absorção de água pelas folhas. O marcador apoplástico LY revelou que a FWU ocorre tanto na superfície adaxial quanto na abaxial das folhas de *V. phaeantha*, e a presença de epítopos específicos de pectinas (homogalacturonanos e rhamnogalacturonanos-I) e de celulose pode desempenhar papel importante na movimentação e retenção de água nos tecidos foliares.

Relação entre a expressão do metabolismo CAM e a dinâmica de água nas folhas ao longo do ciclo diário

A disponibilidade de água é um dos principais fatores ambientais que limitam a sobrevivência, o crescimento e a distribuição das epífitas vasculares (Zotz et al., 2010; Zotz, 2016; Eller et al., 2020). Como resposta a essa restrição, diversas espécies desenvolveram estratégias morfológicas e fisiológicas que favorecem a conservação e o armazenamento de água, como a suculência foliar e o metabolismo ácido das crassuláceas (CAM) (Silvera et al., 2009; Zotz et al., 2010; Kerbauy et al., 2012; Zhang et al., 2018). Em *V. phaeantha*, a amplitude da variação diurna da acidez titulável observada neste estudo confirma a expressão constitutiva do metabolismo CAM, corroborando os valores de $\delta^{13}C$ (-16.42‰; Lima et al., 2023) e os resultados obtidos por Buss et al. (2024) para indivíduos avaliados durante a estação chuvosa. Conforme o esperado para plantas que

expressam CAM, o padrão de abertura estomática noturno observado por Buss et al. (2024) permite a fixação do CO_2 atmosférico, culminando no acúmulo de ácidos orgânicos durante a madrugada e o início da manhã e na posterior redução gradual ao longo do dia, em decorrência da descarboxilação dos ácidos orgânicos durante as fases subsequentes do ciclo CAM. Nestas plantas, a abertura noturna dos estômatos para a captura de CO_2 reduz a perda de água, conferindo maior eficiência do uso da água (Kluge & Ting, 1978; Winter et al., 2005; Silvera et al., 2009).

O status hídrico foliar acompanhou as alterações observadas na acidez titulável ao longo de um ciclo diário. Os maiores valores de conteúdo relativo de água e de potencial hídrico foram registrados às 8:00 h, período imediatamente posterior ao fechamento estomático. Segundo Buss et al. (2024), os estômatos de *V. phaeantha* já se encontram fechados por volta das 6:00 h durante a estação chuvosa, de forma que a recuperação do status hídrico foliar ocorre rapidamente nas primeiras horas da manhã. Esse padrão sugere que, apesar da abertura estomática ao longo da noite, a elevada umidade relativa do ar, o menor déficit de pressão de vapor e a maior disponibilidade de água durante a noite não favorecem grandes perdas por transpiração, mas favorecem uma rápida recuperação quando os estômatos se fecham nas primeiras horas da manhã. Assim, embora o acúmulo noturno de ácidos orgânicos possa contribuir para reduzir o potencial osmótico celular, este efeito é superado pelo aumento do conteúdo de água nos tecidos, que parece ser responsável por direcionar o potencial hídrico foliar nas primeiras horas da manhã nesta espécie. Entretanto, é importante lembrar que como o potencial hídrico nas folhas de *V. phaeantha* está relacionado com características ambientais, essa relação pode se alterar, por exemplo, durante a estação seca. De acordo com Buss et al. (2024), esta espécie apresenta uma redução de até 2 h no tempo de abertura estomática quando comparada com o período chuvoso, com taxas de transpiração mais elevadas, o que indica que alterações no conteúdo de água nas folhas podem ser esperadas.

O controle da abertura e fechamento estomático é importante para o balanço hídrico, e vários fatores podem influenciar esse processo, como concentração de CO_2, luz e disponibilidade de água (Andrade-Souza et al., 2009). Ao longo de um gradiente vertical no sub-bosque, a *V. phaeantha* apresenta variações fisiológicas em resposta à disponibilidade hídrica e à umidade do ar de acordo com a sazonalidade hídrica neste bioma (Buss et al., 2024). Por exemplo, a estação seca influencia o horário de abertura e fechamento dos estômatos, reduzindo a duração da abertura estomática em aproximadamente 2 h ao longo do ciclo diurno de 24 horas do metabolismo CAM entre

as estações (Buss et al., 2024). Em nosso estudo, observamos que a abertura estomática em momentos de alta umidade do ar pode não afetar o status hídrico das folhas, confirmado pelas baixas taxas de transpiração registradas por Buss et al. (2024). Por outro lado, mesmo com os estômatos fechados, foi observada uma queda gradual no RWC durante o dia, culminando nos menores valores de potencial hídrico às 20:00 h (seguem as variações diurnas de umidade do ar ao longo do dia).

Nossos resultados indicam que a capacidade estrutural de armazenamento de água das folhas não varia ao longo do dia, sendo as alterações observadas no status hídrico decorrentes principalmente de mudanças fisiológicas associadas ao balanço hídrico e à expressão do metabolismo CAM. Altos valores de SWC indicam suculência foliar, uma característica frequentemente associada ao metabolismo CAM por permitir o armazenamento de água e de ácidos orgânicos em grandes vacúolos (Silvera et al., 2009; Zhang et al., 2015; Yang et al., 2016). Apesar da suculência foliar ser geralmente associada ao metabolismo CAM (Cushman, 2001; Moreira et al., 2013; Gotsch et al., 2022), a alta capacidade de armazenamento de água em folhas costuma ser associada negativamente com a capacidade de FWU (Gotsch et al., 2022).

Em *V. phaeantha*, a capacidade de absorção foliar variou ao longo do ciclo diário, com maiores valores de absorção máxima de água (*Cmax*) observados às 8:00 h e menores valores ao final da tarde. Da mesma forma, a velocidade de absorção foi maior durante a madrugada, indicando que a FWU apresenta um padrão temporal bem definido. Inicialmente, esperava-se que o acúmulo noturno de ácidos orgânicos reduzisse o potencial hídrico foliar e aumentasse a força motriz para a entrada de água nas folhas. Entretanto, embora a maior capacidade de absorção tenha coincidido com elevados valores de acidez titulável, o potencial hídrico mais negativo foi registrado ao final do dia. Dessa forma, os resultados não corroboram a hipótese de uma relação direta entre a expressão do metabolismo CAM e a magnitude da FWU, mas reafirmam a necessidade de mais estudos. Os valores relativamente baixos de *Cmax* observados em *V. phaeantha* são consistentes com o elevado grau de suculência das folhas, uma característica que tem sido associada à menor dependência da absorção foliar de água devido à grande capacidade de armazenamento hídrico dos tecidos (Lima et al., 2023). No entanto, durante a estação seca do Cerrado, quando a demanda evaporativa é maior e a disponibilidade de água no ambiente é reduzida, mesmo pequenas quantidades de água absorvidas pelas folhas podem contribuir para a manutenção do status hídrico e do funcionamento

fisiológico das plantas. Dessa forma, a importância ecológica da FWU para *V. phaeantha* durante os períodos de déficit hídrico merece ser investigada em estudos futuros.

Vias de entrada de água e composição da parede celular associadas ao balanço hídrico foliar

O marcador apoplástico Lucifer Yellow revelou que *V. phaeantha* é capaz de absorver água tanto pela superfície adaxial quanto pela abaxial das folhas. Em ambas as superfícies, observamos uma cutícula relativamente fina contendo impregnação lipídica. Embora a cutícula seja tradicionalmente associada à redução da perda de água, estudos recentes demonstram que sua composição química pode aumentar sua permeabilidade, permitindo a absorção de água (Eller et al., 2013; Fernández et al., 2017; Matos et al., 2022; Boanares et al., 2018, 2024). Em geral, a difusão pela cutícula e pelos estômatos tem sido reportada como a principal via de entrada da água na folha (Berry et al., 2019; Guzmán-Delgado et al., 2021), locais onde o marcador também foi mais evidente em *V. phaeantha*. Embora a maior FWU tenha sido observada após o fechamento estomático (para horário de fechamento estomático, ver Buss et al., 2024), a marcação obtida com LY indica que tanto a cutícula quanto a região estomática podem atuar como vias potenciais de entrada de água. Assim, os dados sugerem uma contribuição importante da via cuticular, mas não permitem excluir a participação dos estômatos.

Além da permeabilidade da superfície foliar, a composição da parede celular parece desempenhar papel importante na movimentação da água absorvida. As paredes celulares das folhas de *V. phaeantha* apresentaram forte marcação para HGs (detectados por JIM5 e JIM7) na epiderme, principalmente pouco metilesterificados, e por ramnogalacturonanos-I (LM5) nas células do mesofilo, compondo um grupo de pectinas associadas à retenção de água e ao controle da porosidade da matriz celular (Cosgrove, 2005; Willats et al., 2001). De um modo geral, as HGs formam géis altamente hidratados que facilitam o deslocamento da água pelo apoplasto e aumentam a capacidade de retenção hídrica (Cosgrove, 2005; Willats et al., 2001). Neste contexto, as HGs pouco metil-estereificados têm sido mais frequentemente associadas ao aumento na capacidade de retenção de água, uma vez que nelas há maior disponibilidade de grupos carboxila carregados negativamente e que permitem a formação de pontes com Ca^{2+} (formação de estrutura “egg-box”) (Cosgrove, 2005; Willats et al., 2001). Além disso, o aumento da porosidade promovido por esses polissacarídeos tem sido associado ao incremento da velocidade de absorção foliar de água em espécies sujeitas à deposição frequente de

neblina ou orvalho (Boanares et al., 2018, 2024), padrão compatível com os resultados observados para *V. phaeantha*.

Por outro lado, a presença de ramnogalacturonanos-I ricos em galactanos (detectada pelo anticorpo LM5) sugere uma parede celular com elevada capacidade de hidratação e flexibilidade mecânica. Esses polissacarídeos têm sido associados à elasticidade da parede celular e à capacidade de suportar ciclos reversíveis no volume celular (McCartney et al., 2003; Moore et al., 2008), características particularmente importantes em tecidos suculentos e em espécies com metabolismo CAM. Em *V. phaeantha*, embora o conteúdo de saturação hídrica tenha permanecido constante ao longo do ciclo diário, indicando elevada capacidade de armazenamento de água, observamos variações significativas no conteúdo relativo de água e no potencial hídrico das folhas. Assim, além de poder favorecer a absorção foliar de água, a composição da parede celular pode contribuir para a manutenção da hidratação dos tecidos durante as oscilações diárias. Nesse contexto, os homogalacturonanos e os ramnogalacturonanos-I podem desempenhar um papel adicional ao conferir maior elasticidade à parede celular, permitindo variações reversíveis no volume celular sem perda da integridade estrutural. Essa propriedade é frequentemente associada a tecidos suculentos, nos quais a capacidade de armazenar e liberar água ao longo do dia constitui um componente fundamental da estratégia de conservação hídrica.

A presença de celulose nas paredes celulares das células do mesofilo pode complementar esse processo. Além de fornecer suporte mecânico, as microfibrilas de celulose apresentam elevada afinidade por moléculas de água que pode contribuir para a manutenção da hidratação da parede celular, formando géis capazes de aumentar a quantidade de água absorvida (Boanares et al. 2018, 2024). Dessa forma, a combinação entre pectinas e celulose parece constituir uma matriz capaz de promover tanto a absorção quanto a retenção de água nos tecidos.

Conclusão

Em síntese, nossos dados demonstram que a expressão do metabolismo CAM, e consequentemente a abertura e o fechamento dos estômatos, influencia diretamente o balanço hídrico nas folhas de *V. phaeantha* ao longo do dia. A maior acidez titulável entre 04 e 08:00 h confirma a expressão do metabolismo CAM nas folhas de *V. phaeantha*, refletindo o acúmulo noturno de ácidos orgânicos decorrente da fixação de CO_2. Após o fechamento estomático, as folhas apresentam maiores valores de RWC e potencial

hídrico, indicando uma rápida recuperação do status hídrico foliar. Embora a hipótese de que o acúmulo de ácidos orgânicos aumentaria diretamente a absorção foliar de água não tenha sido corroborada, a capacidade de FWU apresentou clara variação temporal, com maiores valores no período da manhã. Esse padrão sugere que *V. phaeantha* pode aproveitar períodos de maior disponibilidade hídrica atmosférica, como aqueles associados à ocorrência de orvalho e elevada umidade relativa do ar, para complementar seu balanço hídrico. Além disso, a permeabilidade de ambas as superfícies foliares à água e a presença de HGs na epiderme e de ramnogalacturonanos-I principalmente no mesofilo indicam que a composição da parede celular pode favorecer não apenas a movimentação e a retenção de água nos tecidos (essencial em folhas suculentas), mas também a elasticidade da parede durante as variações diárias do RWC foliar.

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CAPÍTULO 2

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Changes in foliar water uptake caused by water stress in *Cattleya* Lindl. (Orchidaceae)

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Abstract

In response to water restrictions imposed by the epiphytic environment, orchids have developed strategies to increase water-use efficiency, such as the expression of Crassulacean Acid Metabolism (CAM) and the foliar water uptake (FWU). In a climate change scenario, FWU may emerge as a strategy to reduce water stress during drought. In this study, we propose that, in *Cattleya forbesii* Lindl. and *Cattleya guttata* Lindl., drought alters the leaf water balance, increases CAM expression, and may consequently increase the FWU rate. To test this hypothesis, drought stress was induced by restricting water for 30 days in *C. forbesii* (n = 4) and *C. guttata* (n = 6). After that, we evaluated the relative water content (RWC), overnight organic acid accumulation (ΔH^+), and FWU capacity. To verify the pathway of water entry into the leaves, we used the apoplastic marker Lucifer Yellow. We observed that in both *Cattleya* species, drought impacted leaf water status, photosynthetic activity, and FWU capacity. After water restriction, the leaf water status decreased, impairing photosynthetic activity in both species (lower RWC, potential quantum yield of PSII, and quantum efficiency under light-adapted conditions), whereas nonphotochemical quenching increased. Despite minor changes in ΔH^+ values, FWU increased following drought, partially supporting our hypothesis. In both *Cattleya* species, LY solution diffused through the epidermis and reached the mesophyll, however, in *C. guttata*, this occurred only through the abaxial epidermis. These results demonstrate that FWU may serve as a strategy for *Cattleya* species to cope with water scarcity intensified by drought periods.

Keywords: organic acids, epiphytes, adaptive strategies, water status, apoplastic pathway.

Introduction

The epiphytic environment imposes a series of restrictions on plants, including greater fluctuations in water availability, nutrient availability, and light incidence (Benzing, 1990). One of the most concerning factors is water scarcity, since the hydration of epiphytes depends exclusively on atmospheric sources (Benzing, 1990; Zotz et al., 2010; Zotz, 2016; Zhang et al., 2016; Rosado-Calderón et al., 2020). Orchids are found in various types of habitats, but approximately 75% of the species are epiphytes (Zotz et al., 2021). The success of this group in tree canopy is due to the morphological and physiological strategies of their vegetative organs, which favor greater water use efficiency (Benzing, 1990; Zotz & Hietz, 2001; Zhang et al., 2018). Succulent leaves and the expression of Crassulacean Acid Metabolism (CAM) are examples of these strategies (Gibson, 1982; Cushman, 2001; Zhang et al., 2015; Chukina et al., 2024). The succulence of the leaves increases water storage in the tissue and functions as a form of escape from water stress (Ogburn & Edwards, 2012). Moreover, it has a direct relationship with CAM metabolism, since the organic acids produced at night can be stored in the vacuoles of mesophyll cells (Cushman, 2001; Moreira et al., 2009, 2013).

The CAM pathway is a photosynthetic process whose water use efficiency is greater than that of other photosynthetic pathways (C3 or C4), since atmospheric carbon dioxide (CO_2) fixation occurs at night, when water loss through transpiration is minimal (Winter et al., 2005; Freschi et al., 2007; Castillo et al., 2016). In general, CAM plants separate CO_2 capture and fixation by the Calvin–Benson cycle in time (Osmond, 1978; Ting, 1985). During the night, the stomata open, and atmospheric CO_2 is fixed via phosphoenolpyruvate carboxylase (PEPcase) and stored in vacuoles, usually in the form of organic acids (mainly malate). In the morning, malate is decarboxylated by the NADP-malic enzyme, and the released CO_2 is supplied to the Calvin–Benson cycle through the carboxylase activity of ribulose 1,5-bisphosphate carboxylase oxidase (RuBisCO), where it is incorporated into organic compounds (Cushman, 2001).

Water scarcity can affect the water status and photosynthetic efficiency of epiphytes, often assessed by the reduction in relative leaf water content (RWC) (Anjum et al., 2011) and by changes in parameters related to chlorophyll *a* fluorescence (Stancato et al., 2001; de la Rosa-Manzano et al., 2015, 2017; Tay et al., 2015; Sharma et al., 2020). For example, six species of epiphytic orchids under water stress conditions were previously studied by Tay et al. (2015), who reported that changes in RWC were accompanied by a decrease in photosystem II (PSII) quantum efficiency. Photochemical

performance in response to water deficit has been analyzed through chlorophyll *a* fluorescence, with studies addressing light-adapted PSII quantum efficiency (ϕ_{PSII}), PSII potential quantum yield (F_v/F_m), nonphotochemical quenching (NPQ), and the fluorescence decay rate (R_{fd}) (Genty et al., 1989; Horton & Ruban, 1992; Lichtenthaler & Miehe, 1997; Stancato et al., 2001; Baker, 2008; Oxborough, 2004; Masrahi et al., 2015; Ceusters et al., 2019). The light-adapted PSII quantum efficiency (ϕ_{PSII}) and F_v/F_m indicate the photochemical efficiency in light and the degree of damage to the PSII reaction center, respectively (Kitajima & Butler, 1975; Björkmam & Demmig, 1987; Maxwell & Johnson, 2000). Nonphotochemical quenching functions as a photoprotective mechanism, preventing damage to the photosynthetic apparatus through the thermal dissipation of excess light energy absorbed by PSII (Nobel, 2009; Takahashi & Badger, 2011; Demmig-Adams et al., 2006, 2012). The fluorescence decline ratio, also known as the vitality index, is related to CO_2 assimilation, and a decrease in this value may indicate damage to the photosynthetic apparatus (Babani & Lichtenthaler, 1996; Lichtenthaler et al., 2005; Rao et al., 2021).

A complementary process that helps vascular epiphytes to better cope with water deficits is foliar water uptake (FWU) (Gotsch et al., 2015; Pan et al., 2021). FWU has already been demonstrated in several species distributed across various ecosystems (Limm et al., 2009; Eller et al., 2013, 2016; Goldsmith, 2018; Steppe et al., 2018; Bryant et al., 2021), including epiphytic orchids (Pan et al., 2021; Lima et al., 2023). Foliar water uptake occurs in response to a water potential gradient caused by the difference in vapor pressure between the atmosphere and the interior of the leaf, causing water to pass through the leaf surface and promote hydration of leaf tissues (Burgess & Dawson, 2004). Water enters leaves through different pathways, including the cuticle (Ritpitakphong et al., 2016; Fernández et al., 2017; Gui et al., 2021), trichomes (Schreel et al., 2020; Gui et al., 2021), hydathodes (Martin & von Willert, 2000; Boanares et al., 2019; Fradera-Soler et al., 2024), and stomata (Berry et al., 2014; Binks et al., 2020; Guzman-Delgado et al., 2021). Most of the pathways used for FWU are still unknown, as they vary from species to species (Eller et al., 2016; Schreel & Steppe, 2020; Liu et al., 2021; Gui et al., 2021). However, FWU has many advantages in terms of leaf physiology, as it increases hydration (water potential), reduces stomatal conductance, increases water use efficiency, and consequently, can influence the photosynthetic capacity of plants in different ways (Limm et al., 2009; Eller et al., 2016; Vesala et al., 2017; Berry et al., 2019).

In a climate change scenario in which drought events will be more frequent and prolonged (Schreel & Steppe, 2020), FWU appears to be an interesting strategy for alleviating water stress, especially for plants that suffer from this condition daily, such as epiphytes. Given that epiphytic orchids capable of expressing CAM metabolism can absorb water through their leaves (Lima et al., 2023), and that FWU is influenced by leaf water balance, we experimentally tested whether water deficit reduces leaf water content, thereby increasing the water potential gradient between the leaf and the external environmental and favoring water entry. In addition, we evaluated whether increased CAM expression, associated with nocturnal accumulation of organic acids, could further contribute to increase FWU rates. We used two epiphytes capable of expressing CAM metabolism*, Cattleya forbesii* and *Cattleya guttata*, as study models. We hypothesize that both species, after being subjected to water deficit conditions for 30 days, will show a reduction in leaf water status and photosynthetic activity, while their leaf water absorption capacity (FWU) will be maximized. We also expect an increase in CAM expression (i.e., higher accumulation of organic acids) under drought conditions. Thus, we proposed to: (1) evaluate changes in the water status, and photosynthetic activity of the leaves of *C. forbesii* and *C. guttata* subjected to drought conditions; (2) test whether FWU rates incresae after drought; and (3) investigate the apoplastic pathway of water uptake in both species.

Materials and methods

Plant species and experimental design

Cattleya forbesii Lindl. and *C. guttata* Lindl. are epiphytic species endemic to Brazil, are found in the Atlantic Forest (van den Berg, 2020), and express CAM metabolism (Lima et al., 2023). Orchids are widely used as ornamental plants and have high commercial value; among the cultivated genera, *Cattleya* is very popular (Teixeira da Silva et al., 2014; Colombo et al., 2017). According to the Ministry of the Environment (Ordinance No. 443, issued on December 17, 2004), several Brazilian orchids are at risk of extinction, including *C. guttata*. Adult samples of both species were available at the Institute of Biology's greenhouse on the Umuarama campus of the Federal University of Uberlândia (UFU), Uberlândia, Minas Gerais. The greenhouse is maintained under natural temperature and humidity conditions, with the region's climate characterized by a dry winter (April--September) and a rainy summer from October--March, as classified by the Köppen system (Alvares et al., 2013). In the year prior to the experiment (May 2022

- April 2023), the annual average temperature was 24.38°C, with the maximum temperature recorded being 35.4°C and the minimum temperature being 7.9°C (INMET).

In May 2023, seven individuals of *C. forbesii* and eleven of *C. guttata* underwent acclimatization in the greenhouse for one year and were irrigated with water three times a week during the dry season. The experiment was subsequently conducted at the Laboratório de Anatomia, Desenvolvimento Vegetal e Interações (LADEVI) of the Federal University of Uberlândia, in a growth room under controlled light (29.27 mol m^{-2} s^{-1}) and temperature conditions (24°C). Some individuals were kept as controls, and others were subjected to drought (treatment). For the control individuals of *C. forbesii* (n = 3) and *C. guttata* (n = 5), irrigation was maintained three times a week. To induce drought (treatment), irrigation of *C. forbesii* (n = 4) and *C. guttata* (n = 6) individuals was suspended until signs of water stress were detected. In this way, the onset of water stress was determined by a decrease in the potential quantum yield (F_v/F_m); photosynthetic performance was assessed weekly using chlorophyll *a* fluorescence with an imaging fluorometer (Handy FluorCam PSI, Czech Republic). The experiment was concluded after 30 days, when the plants exhibited signs of significant water stress, as indicated by a decrease in the potential quantum yield (F_v/F_m) values compared to those in the control group (Fig. 1).

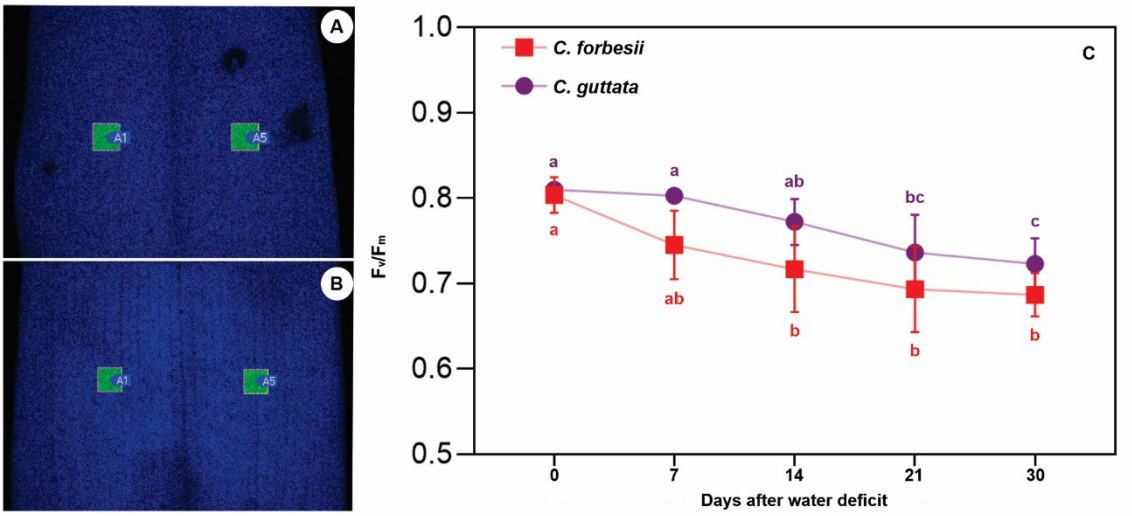


Figure 1: Evaluation of chlorophyll *a* fluorescence in the leaves of *Cattleya forbesii* (A) and *C. guttata* (B) subjected to drought. The analyses were conducted weekly until a significant decrease in the potential quantum yield of PSII (F_v/F_m) was observed (C). The analyses used the *quenching* protocol available in the FluorCam 7.0 software (Handy FluorCam, PSI). Lowercase letters represent significant differences in F_v/F_m between the

times (0, 7, 14, 21, and 30 days) analyzed in the leaves of *C. forbesii* (χ^2 = 40.11, Df = 4, $p < 0.0001$, $R_m^2 = 0.65$ and $R_c^2 = 0.77$; lowercase letters in pink) and *C. guttata* leaves (χ^2 = 51.43, Df = 4, $p < 0.0001$, $R_m^2 = 0.68$ and $R_c^2 = 0.68$; lowercase letters in purple) via Mixed Linear Models (LMMs) ($p < 0.05$), and we compared the means using Tukey's adjusted paired comparisons ("emmeans" package; $p < 0.05$).

Evaluation of structural parameters and leaf water balance

Fragments from the middle region of leaves of *C. forbesii* and *C. guttata* were collected for anatomical description, fixed in FAA_{70} (formaldehyde, acetic acid, 70% ethyl alcohol, 1:1:18 v/v) for 48 h, and then stored in 70% ethanol (Johansen 1940). To prepare the histological slides, transverse and longitudinal sections were prepared manually using razor blades. The fragments were clarified in 50% sodium hypochlorite, washed in distilled water, and then stained with 0.5% Alcian Blue and 0.5% safranin (9:1 v/v) (Kraus and Arduin 1997). The slides were mounted with 50% glycerin and photographed with an ICC50 HD digital camera attached to a DM500 microscope (Leica, Germany).

To determine relative water content (RWC), water saturation content (SWC), and leaf mass per area (LMA), 1 cm^2 leaf fragments were collected from each individual of *C. forbesii* and *C. guttata*, including both control plants and those subjected to 30 days of drought. The samples were collected between 7 and 8 h. The RWC was used to assess the water status of the leaves, calculated using the equation $RWC = [FM - DM]/[TM - DM] * 100$, where FM is the leaf fresh mass, DM is the dry mass after drying the material in an oven at 50°C until it reaches a constant weight, and TM is the turgid mass after hydration for 24 hours (Turner, 1981). As an indicator of leaf succulence, we used the water saturation content (SWC), which was determined by the formula $SWC = (TM - DM)/DM$ (Ogburn & Edwards, 2012). Leaf mass per area (LMA) was used to assess tissue density, obtained by the formula $LMA = DM/A$, where A corresponds to the fragment area, according to Witkowski and Lamont (1991).

Chlorophyll a fluorescence

To obtain the fluorescence parameters of chlorophyll *a* after 30 days of experimentation, an image-based fluorescence meter (Handy FluorCam, Photo Systems Instrument, Czech Republic) was used to calculate the Kautsky effect according to the Quenching protocol (FluorCam 7.0 software, Photo Systems Instrument), with saturating

196 light at 1.350 µmol m^{-2} s^{-1} and actinic light at 350 µmol m^{-2} s^{-1}. The leaves from the
197 control and treated plants were kept in the dark for 30 minutes to open the photosynthetic
198 electron transport chain before analysis. For the chlorophyll *a* fluorescence parameters,
199 we used the potential quantum yield of PSII - F_v/F_m (Oxborough, 2004), the light-adapted
200 quantum efficiency of PSII - ϕ_{PSII} (Genty et al., 1989), the nonphotochemical quenching
201 in the steady state - NPQ (Horton & Ruban, 1992), and the rate of fluorescence decline
202 in steady-state - R_{fd} (Lichtenthaler & Miehé, 1997). Under water deficit conditions,
203 reductions in F_v/F_m, ϕ_{PSII}, and R_{fd} values may indicate a decline in photochemical
204 efficiency in light and damage to the PSII reaction center (Maxwell & Johnson, 2000).
205 Meanwhile, the increase in NPQ values reflects the increase in the dissipation of excess
206 light energy absorbed by PSII in the form of heat to protect the photosynthetic apparatus
207 (Lichtenthaler et al., 2005; Demmig-Adams et al., 2006, 2012).
208
209 *Measurement of FWU*
210 To assess foliar water uptake, one intact and mature leaf was collected from each
211 *C. forbesii* and *C. guttata* individuals in both the control and treatment groups at 8 a.m.
212 The leaf bases were immediately sealed with Vaseline paste for weighing. The leaves
213 were then taken to the laboratory and weighed (fresh weight in g) using an analytical
214 balance (Shimadzu AUY 220, Japan). After that, they were immersed in distilled water
215 and weighed every 15 min for 2 h, every 30 min for 2 h, and then again after one more
216 additional hour to obtain the foliar water uptake curve – FWU (final weight in g per unit
217 of time) (Liang et al., 2009). Before weighing each, the leaves were gently dried with
218 absorbent microfiber towels to remove surface water. At the end of the experiment, leaf
219 areas were measured using ImageJ software (AxionVision 1.54g). Foliar water uptake
220 curve (FWU) was calculated from the differences between fresh weight and final weight
221 by leaf area using the equations: *FWU* = (fresh weight - final weight)/(leaf area). The rate
222 of water capture by the leaves (WC) based on leaf area, was calculated using the
223 equations: $WC = (Cmax - Ci) * (1 - e^{-kt}) + Ci$, where *Cmax* is the maximum foliar
224 water uptake, *Ci* is the initial foliar water content, k is the absorption exponential
225 coefficient of the leaf (or the number of the absorption function curve), and t is the
226 absorption time. Water capture by the leaves (WC) provides data considering the
227 maximum foliar water uptake (C*max*; g cm^{-2} of leaf area) and the velocity of water
228 absorption (k) (Liang et al., 2009, modified; Pan et al., 2021).
229

Determination of the diurnal variation in titratable acidity

To evaluate changes in CAM metabolism expression in the leaves of both species, both in control plants and those subjected to drought after 30 days, we used the diurnal variation in titratable acidity (ΔH^+). The diurnal variation in organic acids (ΔH^+) was obtained from 200 mg of leaves collected at 6 a.m. and 6 p.m., and the difference in acidity accumulated during these two moments was represented by ΔH^+. The samples were then weighed on a digital scale and boiled in 5 mL of distilled water for five minutes. The extracts (2.5 mL) were titrated with 0.01 M NaOH (pH 7.0) according to the protocol proposed by Hartsock and Nobel (1976). The results were expressed in μeq of H^+ per g of fresh mass (FM).

Fluorescent markers for apoplastic water entry

To verify the apoplastic pathway of water entry into the leaves of *C. forbesii* and *C. guttata*, both from control plants and plants subjected to drought for 30 days, three mature leaves were collected from two individuals of each species in each treatment (n = 3). In the laboratory, the leaf bases were sealed with Vaseline paste, and 100 μL of a 1% aqueous solution of Lucifer Yellow (LY; lithium salt, Sigma) was applied using a Pasteur pipette to the adaxial and abaxial sides of each leaf. As a control, distilled water was applied to both surfaces of a third leaf (Suppl. 1). The solutions were applied to the middle third of the leaf, as described by Oparka and Read (1994). After LY was applied, the leaves were placed in gearbox boxes lined with filter paper moistened with distilled water. The boxes were then wrapped in aluminum foil, creating a dark chamber in which they remained for five hours (Widholzer, 2005; Eller et al., 2016). The excess marker on the leaves was subsequently washed off with distilled water, and freehand sections were obtained from the material where the marker had been applied. The slides were mounted in glycerin 50%, observed, and photomicrographed under an optical microscope with a fluorescence (Leica® DM4000 B LED, Leica, Germany) system coupled to a camera (Leica® DFC 3000 G), using LAS X 1.1.0.12420 analysis software. Samples were excited with intense blue light (450-490 nm, FITC filter) with a 515 nm barrier filter (Oparka & Read, 1994, modified).

Data analysis

Mixed linear models (LMMs) were constructed to verify the difference in the potential quantum yield of PSII (F_v/F_m) of *C. forbesii* and *C. guttata* leaves between the

analyzed times (0 - control, 7, 14, 21, and 30 days), considering time (temporal replication) and species as fixed factors and individuals as random effects (using the *lmerTest* package: the *lmer* function; Kuznetsova et al., 2017). To determine whether there was a difference in the leaf structural parameters (LMA and SWC), leaf water balance (RWC), nonphotochemical quenching (NPQ), and rate of fluorescence decline in the steady state (R_{fd}) between the control and treatment (water stress) and between each species (*C. forbesii* and *C. guttata*), as well as the interaction between treatments and species, a factorial ANOVA for independent samples was performed. These parameters met the assumptions of factorial ANOVA, presenting normality and homogeneity of variance. Considering the positive values of F_v/F_m and ϕ_{PSII} (nonparametric data), Generalized linear models (GLMs) with a Gamma error family and inverse link function were constructed to verify differences between treatments and species (Akaike Information Criterion - AIC: F_v/F_m = -57.04; ϕ_{PSII} = -48.64). The diurnal variation in organic acids between species and treatments was verified using GLM adjusting a ziGamma distribution (using the glmmTMB package; AIC: 76.70), given the presence of zero values and positive values (Brooks et al., 2023). To verify the variations in foliar water uptake (C*max* and *k*) between the control and treatment of the species, GLMs were constructed with a quasi-Poisson distribution.

The GLMs were selected after verifying the homogeneity and normality of the model residues via the *DHARMa* package (Hartig, 2022). To simplify the models, the interaction between species and treatments was removed from the parameter models (LMA, SWC, F_v/F_m, NPQ, C*max*, and *k*) when there was no significant effect. The means were compared using Tukey-adjusted paired contrasts between treatments and species using the R package "emmeans" (Lenth, 2023). For LMM, we calculated the marginal and conditional pseudo-R^2 (Nakagawa et al. 2017) using the r.squaredGLMM function from the MuMIn package. The analyses were performed using R software (i386 4.5.0; R Core Team, 2024), and the significance level was set at 5%.

To identify patterns of influence on the physiological parameters and leaf characteristics of *C. forbesii* and *C. guttata* in each treatment (control and drought), we performed a principal component analysis (PCA) using R software and the *factoextra* package (Kassambara & Mundt, 2020). In PCA, all structural and physiological parameters were used as response variables, whereas species and treatments were considered complementary variables. The variables used in PCA were centered and scaled due to the different units.

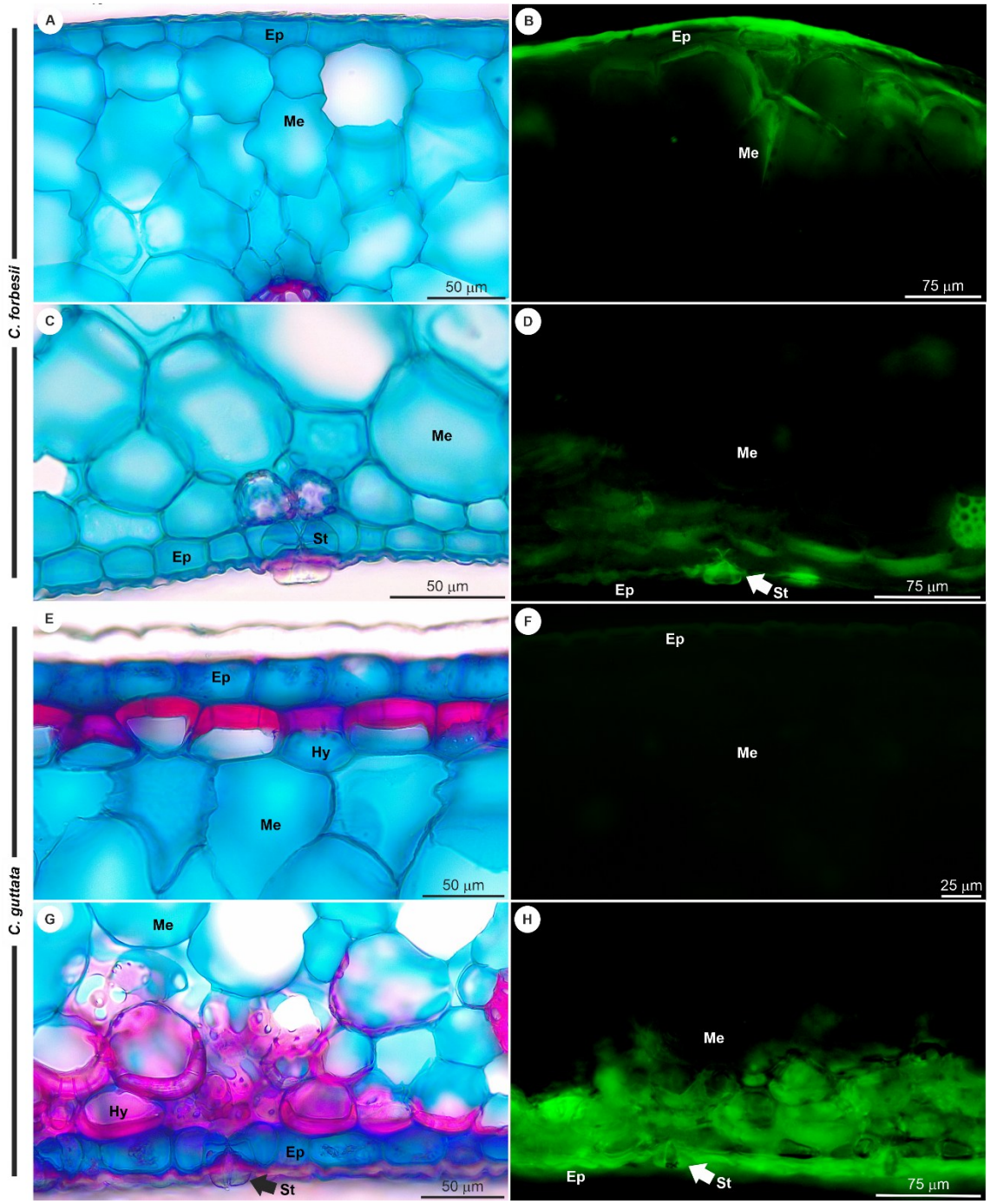
298

299 **Results**

300 The leaves of both *Cattleya* species were hypostomatic (Fig. 2). In *C. forbesii*, the
301 leaves were covered by a thick cuticle and had a uniseriate epidermis, composed of thin-
302 walled cells with on the adaxial and abaxial surfaces (Fig. 2A and C). The stomata were
303 internally followed by two cells exhibiting reticulate wall thickenings. The mesophyll
304 was homogeneous, consisting of large cells with small intercellular spaces (Fig. 2A and
305 C). In *C. guttata*, the adaxial surface was covered by a thick cuticle, and both surfaces
306 exhibited a uniseriate epidermis. On the adaxial surface, the epidermis was followed by
307 a hypodermis with thickened outer periclinal walls extending to the anticlinal walls (Fig.
308 2E and G). Near the abaxial surface, the mesophyll comprised two to three cell layers
309 with conspicuous wall thickenings (Fig. 2G), remaining mesophyll cells were large and
310 arranged with small intercellular spaces (Fig. 2E and G).

311 Individuals of *C. forbesii* and *C. guttata* maintained under control conditions and
312 subjected to drought for 30 days presented distinct responses in terms of leaf water
313 content, foliar water uptake rate, and chlorophyll *a* fluorescence.

314



315 **Figure 2:** Leaf anatomy and water uptake assessed using Lucifer Yellow to trace the
316 apoplastic pathway in *C. guttata* and *C. forbesii*. Internal structure (A, C) and the
317 apoplastic pathway visualized with the fluorescent marker (B, D) in *C. forbesii*, and the
318 same sequence in *C. guttata* (E-H). *Acronyms*: Ep: epidermis; Hy: hypodermis; Me:
319 mesophyll; and St: stomata (arrows).
320

Variations in leaf structure and water balance

No significant changes were observed in leaf mass per area (LMA) or water saturation content (SWC) when the plants were subjected to drought (Fig. 3A and B). In general, *C. guttata* has leaves with significantly higher leaf mass (LMA) compared to *C. forbesii* (Fig. 3A). However, there was no difference in SWC between the species (Fig. 3B). There was a significant difference in relative water content (RWC) in relation to species and treatment (control and drought) (Fig. 3C). After being subjected to water stress, the plants in the treatment presented a reduction of approximately 44% in the relative water content (RWC) in *C. forbesii* and 56% in *C. guttata* (Fig. 3C).

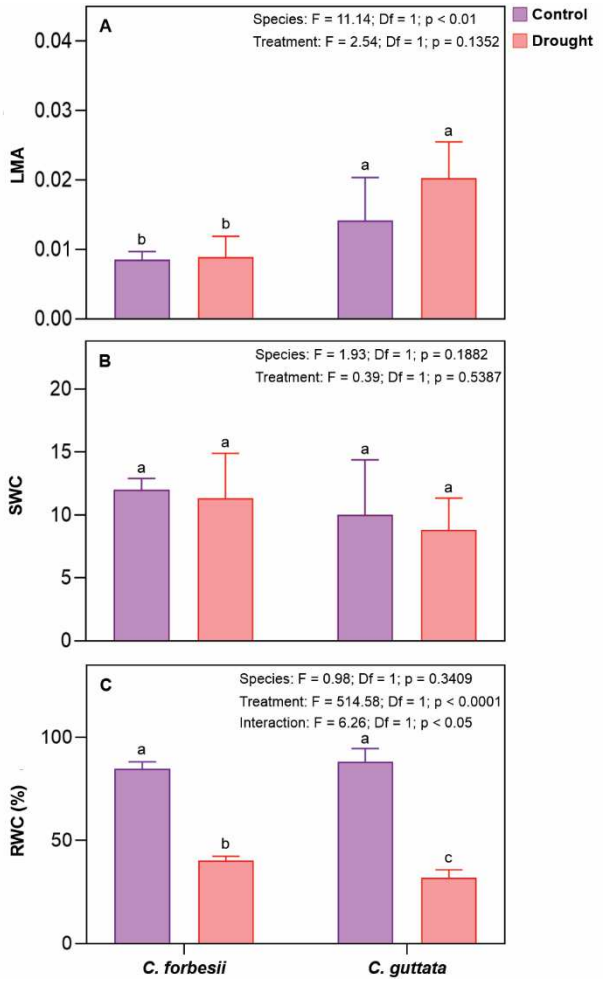
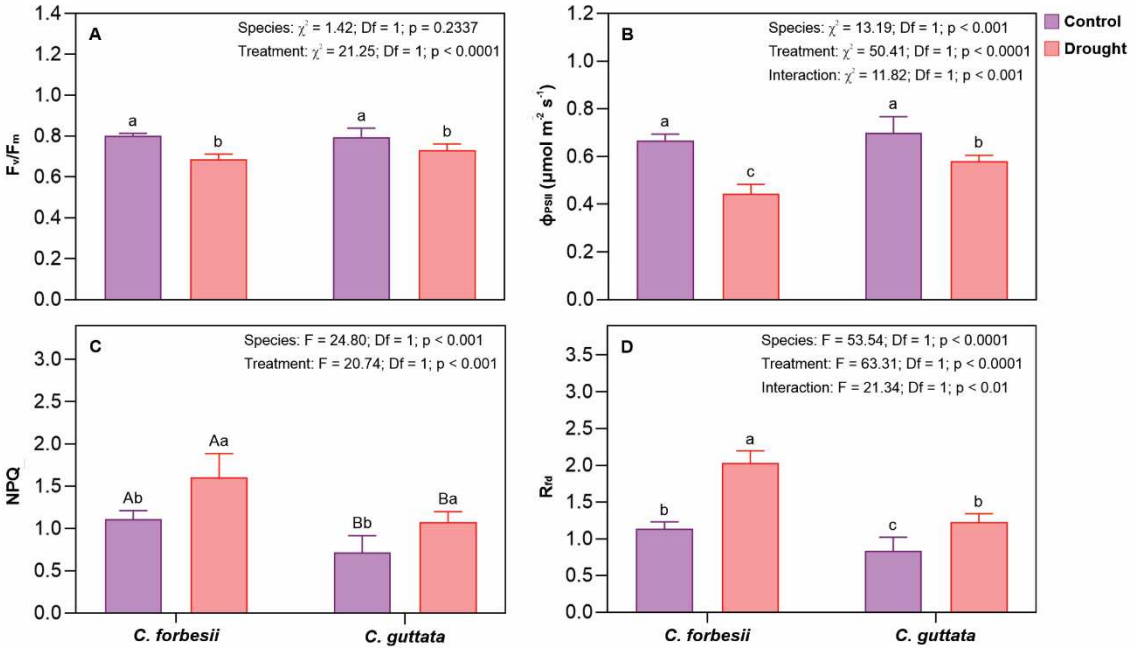


Figure 3: Variations in the leaf mass per area and leaf water balance of individuals of *C. forbesii* and *C. guttata* under control conditions (with irrigation three times a week; purple bars) and treatment conditions (with drought induced for 30 days; pink bars). (A) LMA - leaf mass per area; (B) SWC - saturated water content; and (C) RWC - relative water content. Lowercase letters represent significant differences between predictor variables, without interaction between them, using Factorial Analysis of Variance ($p < 0.05$), and we compare the means using Tukey's adjusted paired comparisons ("emmeans" package; $p < 0.05$).

Chlorophyll a fluorescence

The highest F_v/F_m and ϕ_{PSII} values were observed in individuals from the control group of both species, with a reduction when subjected to drought for 30 days (Fig. 4A and B). Compared with *C. guttata*, *C. forbesii* presented greater decreases in ϕ_{PSII} values. In general, NPQ values were greater in *C. forbesii*. In both species, drought promoted an

355 increase in NPQ values (Fig. 4C). The highest R_{fd} values were detected in *C. forbesii*
356 individuals in the treatment group, and the lowest R_{fd} values were detected in *C. guttata*
357 control individuals (Fig. 4D). In both species, the induction of drought significantly
358 increased the R_{fd} values.
359



360 **Figure 4:** Chlorophyll *a* fluorescence was measured in individuals of *C. forbesii* and *C.*
361 *guttata* under control conditions (irrigated three times a week) and treatment conditions
362 (induced drought for 30 days). (A) Potential quantum yield of PSII (F_v/F_m); (B) light-
363 adapted quantum yield of PSII (ϕ_{PSII}). (C) Nonphotochemical quenching (NPQ); (D) rate
364 of fluorescence decline in steady-state (R_{fd}). Individuals in the control group are
365 represented by purple bars, and individuals subjected to water stress are represented by
366 pink bars. Changes in letters indicate significant differences between predictor variables
367 (species, uppercase letters; treatments, lowercase letters) when the GLM with gamma
368 distribution (F_v/F_m and ϕ_{PSII}) and factorial ANOVA (NPQ and R_{fd}) ($p < 0.05$) were used,
369 and in both cases, the means were compared using Tukey's adjusted paired comparisons
370 ("emmeans" package; $p < 0.05$).
371
372 *Diurnal variation in titratable acidity*
373 Compared with *C. guttata*, which had an average of 6.16 µeq H^+ g^{-1} FM, *C.*
374 *forbesii* individuals showed a significantly greater diurnal variation in organic acids
375 (ΔH^+), with an average of 24.42 µeq H^+ g^{-1} FM (Species: $\chi^2 = 12.72$; Df = 1; $p < 0.001$).
376 However, neither species showed a significant difference in diurnal variation in leaf

acidity between individuals in the control group and those subjected to drought for 30 days (Treatment: $\chi^2 = 1.37$; Df = 1; p = 0.2410).

Foliar water uptake and fluorescent apoplastic marker

Significantly higher rates of maximum foliar water uptake (higher C*max* values) and velocity of water absorption (higher *k* values) were observed in individuals of *C. guttata* and *C. forbesii* after 30 days of drought (Fig. 5A and B). The apoplastic fluorescent marker Lucifer Yellow stained the cuticle, epidermal cell walls, and some mesophyll cells of the adaxial and abaxial surfaces of *C. forbesii* leaves (Fig. 2B and D), whereas in *C. guttata,* LY was detected only on the abaxial surface (Fig. 2F and H).

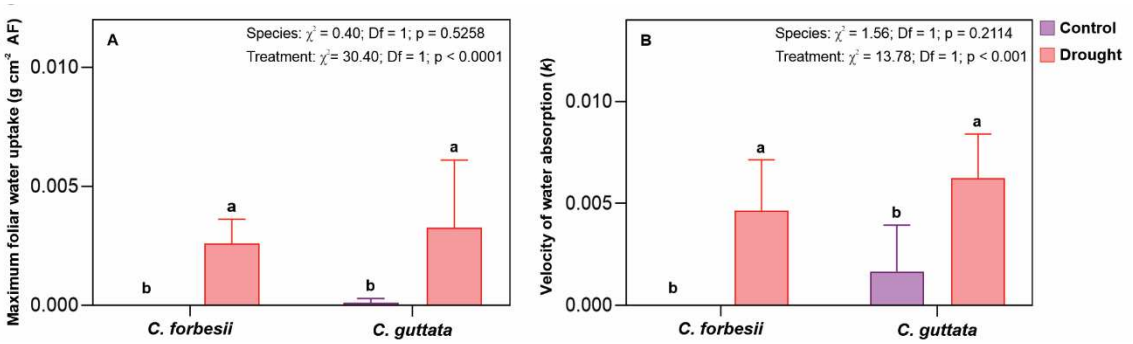


Figure 5: Variations in the parameters of foliar water uptake of hydrated individuals of *C. forbesii* and *C. guttata* (control; purple bars) and those that experienced 30 days of drought (treatment; pink bars). (A) The bar graphs represent changes in the maximum foliar water uptake (C*max*) and (B) velocity of water absorption (*k*) in the control and treatment groups of *C. forbesii* and *C. guttata*. Different letters represent significant differences between species (capital letters) and treatments (lowercase letters), as determined by GLM with a quasi-Poisson distribution, and we compared the means using Tukey's adjusted paired comparisons ("emmeans" package; $p < 0.05$).

Principal component analysis (PCA) revealed that the first two components accounted for approximately 77.5% of the total variance in the data, indicating that the variables included contributed strongly to discrimination between control plants and those subjected to 30 days of drought (Fig. 6). We observed that the control individuals of *C. forbesii* and *C. guttata* were differentiated from those subjected to drought (Dim. 1 - 50.7% explanation), which is related to the RWC, photochemical parameters (F_v/F_m, ϕ_{PSII}, NPQ, and R_{fd}), and FWU values (C*max* and *k*). In Dim. 2 (27%), *C. forbesii* and *C. guttata*

individuals subjected to drought for 30 days were separated. *C. forbesii* was influenced by NPQ and R_{fd} values, whereas *C. guttata* was influenced by LMA values and parameters related to the FWU.

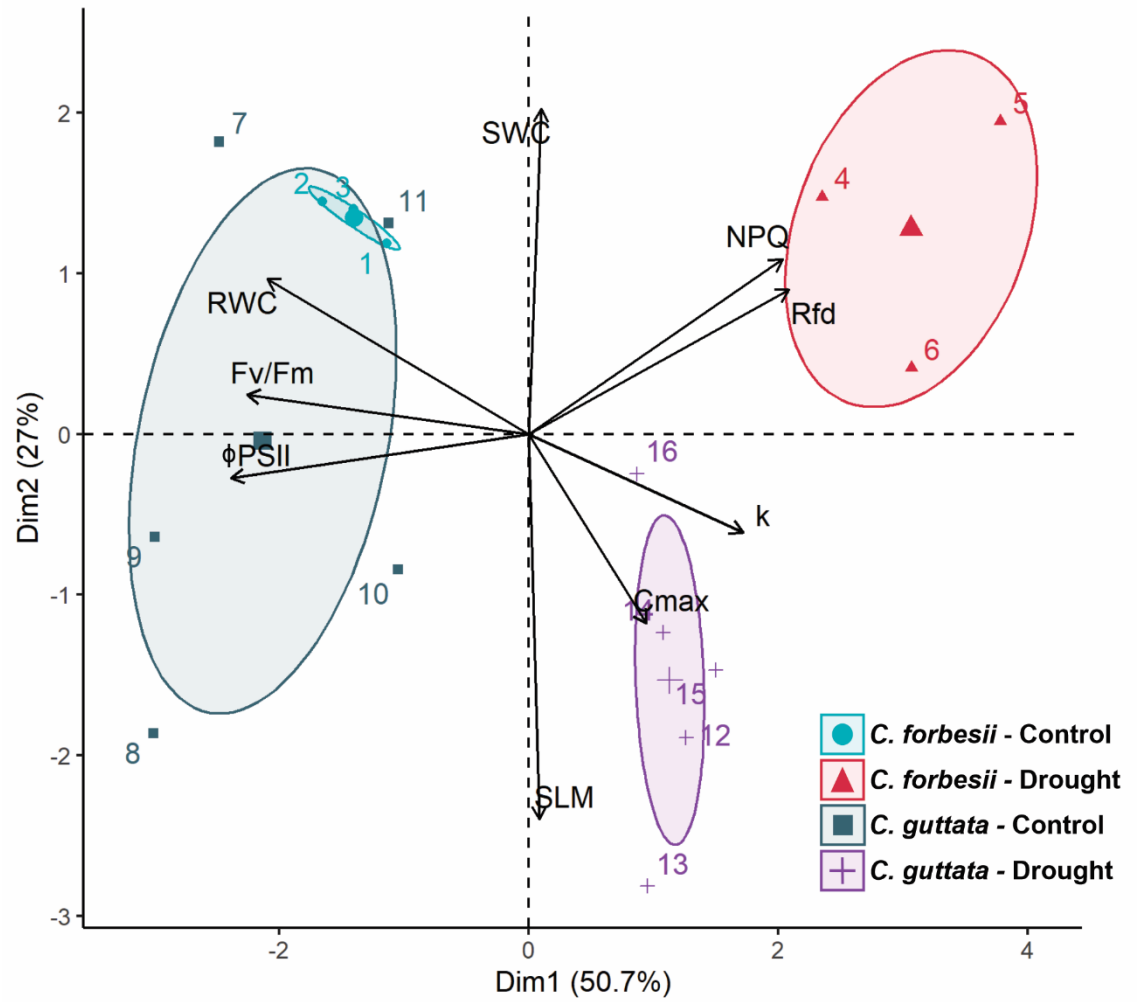


Figure 6: Principal component analysis (PCA) was performed to verify the relationships among chlorophyll *a* fluorescence, leaf water status, structural characteristics, and foliar water uptake, as well as species (*C. forbesii* and *C. guttata*) and treatments (control and drought). The variables included LMA (leaf mass per area), SWC (saturated water content), RWC (relative water content in leaves), F_v/F_m (potential quantum yield of PSII), ϕ_{PSII} (light-adapted quantum yield of PSII), NPQ (nonphotochemical quenching), R_{fd} (the rate of fluorescence decline in steady-state), C*max* (maximum foliar water uptake), and *k* (velocity of water absorption).

Discussion

The induction of drought altered the leaf water status, photosynthetic activity, and water absorption capacity of the leaves of both *Cattleya* species. After 30 days of drought, the leaf water status and photosynthetic activity of both species decreased (with lower RWC, F_v/F_m, and ϕ_{PSII} values), but nonphotochemical quenching (NPQ) and index values indicating photosystem vitality (R_{fd}) increased. Despite low C*max* values and no variation in organic acid accumulation, the rate of foliar water uptake increased after drought, partially corroborating our hypothesis. Regarding the species studied, we observed that *C. guttata* showed higher LMA compared to *C. forbesii*. Non-photochemical quenching (NPQ), one of the most important photoprotective mechanisms, was greater in *C. forbesii* compared to *C. guttata*, indicating an investment in increased dissipation of excess light energy. FWU may be a strategy for the *Cattleya* species studied during periods of water deficit. Both species showed a significant reduction in RWC at the end of the experiment, which may have consequently boosted their FWU capacity.

Effect of water stress on the leaf water balance

As an alternative to constant fluctuations in water availability, several epiphytic species, including orchids, can store water in their tissues, a phenomenon known as leaf succulence (Oliveira & Sajo, 1999; Pires et al., 2012; Moreira et al., 2013; Guevara Pérez et al., 2019). In our study, the leaf succulence of *Cattleya* species was not affected by water stress, maintaining SWC values even after 30 days of water restriction. High SWC values can be associated with a cellular and tissue-level strategy to escape water-deficient conditions (Ogburn & Edwards, 2012). This is because cellular activities are maintained by the presence of water in leaf cells, even under environmental conditions of water restriction (Ogburn & Edwards, 2012). The structural characteristics associated with leaf succulence include a mesophyll composed of several cell layers, whose cells are large, with thin walls and vacuoles, and have few cell spaces (Ogburn & Edwards, 2012; Zhang et al., 2018; Lamont & Lamont, 2025). In addition, come cell wall arrangements may increase wall plasticity and favor succulence (Fradera-Soler et al., 2022). In particular, the elasticity of succulent plant walls is associated with a high degree of methyl esterification of homogalacturonans (HGs) and a high abundance of type I rhamnogalacturonans (RG-I), which confer dynamic properties to the cell wall. The degree of leaf succulence varies between species and depends on the relative composition and arrangement of dry tissue mass, water, and air (Lamont & Lamont, 2025). In general,

tropical orchid leaves have a high-water content (Perez-Lopez et al., 2023) and may be classified as semi-succulent (or semi-succophyll, according to Lamont & Lamont, 2025). Because epiphytic plants depend on the atmosphere as a source of water and nutrients, they may be subject to severe variations in water availability at certain times of the year or even during the day (Zotz & Bader, 2009; Zhang et al., 2016). Succulence can reduce the rate of water deficit in tissues during the day and protect against daily fluctuations and seasonal variations in water loss (Perez-Lopez et al., 2023). Even with leaves formed by tissues that favor water storage, some epiphytic species show a reduction in the relative water content and water potential of their leaves during periods of water deficit (de la Rosa-Manzano et al., 2017; Chávez-Sahagún et al., 2019; Tay et al., 2015, 2019), as we observed in both *Cattleya* species used in this study. In other words, water restriction impacts the amount of water present in leaf tissues of the species (reduction in RWC), compromising basic metabolic processes (F_v/F_m and ϕ_{PSII}).

Lamont and Lamont (2025) reported that leaf area per area (SLA—area per unit mass) is not directly related to leaf succulence but can serve as an index of drought tolerance because of its relationship with leaf thickness, i.e., with the volume and number of cells present in the mesophyll. LMA values, in turn, are inversely related to SLA values and are generally greater in epiphytic orchids than in terrestrial orchids, as is the case for water saturation content, which corroborates a positive relationship between these attributes, as proposed by Zhang et al. (2015, 2018). Despite similar SWC values among the species studied, the highest LMA values were observed in the leaves of *C. guttata*. We speculate that, in this species, water storage occurs in a larger number of cells rather than in larger cells.

Effect of water stress on photochemical efficiency

Low water availability in the environment can affect the water availability of leaves (reducing their relative water content) of epiphytic orchids and, consequently, their photosynthetic efficiency (Tay et al., 2015). Despite the low accumulation of organic acids during the night observed in our work, the *C. guttata* and *C. forbesii* individuals used in this study were the same as those used by Lima et al. (2023), presented carbon isotopic signature values (δ^{13}) of 16.49 and -14.95‰, respectively, and were classified as CAM. Many orchids use the CAM pathway as a strategy to cope with fluctuations in water availability in the epiphytic environment (Cushman, 2001; Silvera et al., 2009; Moreira et al., 2013; Kerbauy et al., 2012; Zhang et al., 2018), with the intensity of the

CAM pathway expression varying between different plant organs or even depending on water availability (Rodrigues et al., 2013). Although water restriction for 30 days affected the water status (RWC) in the leaves of *C. frobesii* and *C. guttata*, it was not able to intensify the expression of CAM metabolism (diurnal variation in organic acids - ΔH^+).

Although no changes were observed in the expression of CAM metabolism when the plants were subjected to drought, *Cattleya* leaves experienced a reduction in photosynthetic efficiency. A reduction in photochemical efficiency during a period of drought has previously been reported in other epiphytic orchids (Stancato et al., 2001; Lüttge, 2010; Tay et al., 2015; de la Rosa-Manzano et al., 2017), and chlorophyll *a* fluorescence has been established as a sensitive tool for diagnosing water stress, even in CAM plants. Our results suggest that the photosynthetic apparatus of both species was compromised after drought (lower F_v/F_m and ϕ_{PSII} values). Monitoring chlorophyll fluorescence parameters in CAM plants under water stress conditions has revealed a reduction in the electron transport rate and ϕ_{PSII} (de Mattos et al., 1999; Zheng et al., 2019; Ceusters et al., 2019). For example, the Fv/Fm values of species such as *Kalanchoë daigremontiana* and *Opuntia ficus-indica* decrease after dehydration (Borland et al., 2014), reaching levels correlated with the occurrence of photoinhibition (Maxwell & Johnson, 2000).

To prevent damage to the photosynthetic apparatus, plants employ various photoprotective strategies, such as the thermal dissipation of light energy (Pieters et al., 2003; Demmig-Adams et al., 2006; Sharma et al., 2020). Heat dissipation prevents the formation of reactive oxygen species (ROS) and is measured from the perspective of nonphotochemical quenching (NPQ) of PSII chlorophyll fluorescence (Niyogi, 2000; Demmig-Adams et al., 2006). Under stress conditions, chlorophyll de-excitation through the xanthophyll cycle results in nonphotochemical energy dissipation, which increases NPQ (Adams & Demmig-Adams, 1996; Holt et al., 2005; Tay et al., 2019), as we observed in both species under water restriction (high NPQ values). In addition, dimension 1 of the PCA revealed that *C. forbesii* and *C. guttata* individuals subjected to water stress were related to NPQ and the rate of fluorescence decline in steady-state (R_{fd}), indicating the relevance of these parameters under drought. Primarily for *C. forbesii*, as represented in dimension 2 of the PCA, we observed an influence of nonphotochemical extinction and rate of fluorescence decline in steady-state ($R_{fd)}$ in individuals subjected to water restriction. Similarly, in other epiphytic orchids, the dissipation of excess energy absorbed by PSII increases because of seasonal drought, whereas the maximum quantum

519 efficiency of PSII decreases (de la Rosa-Manzano et al., 2015, 2017). As in these orchids,
520 the nonphotochemical energy dissipation strategy may help *Cattleya* species cope with
521 water stress during the dry season, functioning as an efficient photoprotective mechanism.
522
523 *Effect of water stress on foliar water uptake (FWU)*
524 The induction of water stress compromised the water status of both *Cattleya*
525 species (low RWC values), possibly contributing to an increase in the FWU (higher C*max*
526 values). However, the variation in organic acid accumulation (ΔH^+) apparently does not
527 influence FWU capacity. Foliar water uptake phenomenon occurs when atmospheric
528 water exceeds the leaf surface in response to a water potential gradient, promoting tissue
529 hydration (Burgess & Dawson, 2004; Dawson & Goldsmith, 2018; Steppe et al., 2018).
530 In epiphytes, the ability to absorb water through leaves is an alternative uptake strategy
531 that helps species cope with water scarcity (Gotsch et al., 2015; Pan et al., 2021; Lima et
532 al., 2023). When atmospheric humidity increases, such as in the early morning, FWU
533 helps maintain water status and can improve stomatal conductance and photosynthetic
534 activity (Limm et al., 2009; Eller et al., 2013; Berry et al., 2014, 2019; Guzmán-Delgado
535 et al., 2018).
536 In a climate change scenario, which predicts an increase in drought events and a
537 reduction in the leaf water potential (the driving force of FWU; Eller et al., 2016; Dawson
538 & Goldsmith, 2018; Schreel et al., 2020), FWU may represent an alternative pathway for
539 water acquisition and to alleviate water stress in *C. forbesii* and *C. guttata*. These species
540 occur in Atlantic Forest and, particularly in montane areas, the fog events are frequent
541 (Rosado et al. 2010). Although there was no difference in foliar water uptake (FWU)
542 between species, considering individuals subjected to drought, dimension 2 of the PCA
543 indicated a relationship between C*max* and *k* in individuals of *C. guttata*. The Ministry of
544 the Environment (Ordinance No. 443, dated December 17, 2004) includes *C. guttata* on
545 the list of endangered species. Our results indicate that, under water-restricted conditions,
546 the leaf water absorption capacity of this species may help it cope with the constant
547 fluctuations in water availability in the epiphytic environment and its adaptation to
548 environmental changes. However, future experiments are needed to effectively test the
549 effect of FWU on plant performance and survival during drought, as well as the
550 contribution of exposure to events that promote leaf hydration, such as fog.
551 Several leaf traits may favor FWU, including more negative leaf water potentials,
552 low dry matter content, elastic cell walls, and high stomatal and cuticular conductance

(Matos et al. 2024). Foliar water uptake can occur through stomata, trichomes, hydathodes, or the cuticle (Eller et al., 2016; Fernández et al., 2017; Losada et al., 2021; Gui et al., 2021; Guzmán-Delgado et al., 2021; Fradera-Soler et al., 2024). Because cuticular permeability depends on its structure and chemical composition, differences between leaf surfaces may influence FWU patterns. Cuticle permeability is generally affected by lipids, such as waxes and cutin (hydrophobic compounds), as well as polysaccharides (hydrophilic compounds) composition (Fernández et al., 2016, 2017; Guzmán-Delgado et al., 2016). In our study, the apoplastic fluorescent marker LY indicated that both *Cattleya* species were capable of absorbing water through the leaf surface, although distinct patterns were observed between species. *Cattleya forbesii* absorbed water through both the adaxial and abaxial surfaces, whereas *C. guttata* absorbed water only through the abaxial surface, which may indicate differences in cuticle composition and permeability, since the marker was not detected on the adaxial surface. Despite the presence of a hypodermis with highly lignified outer periclinal walls in *C. guttata*, this structure does not appear to function as a barrier to water movement. In contrast, the stronger fluorescence observed in mesophyll cells near the abaxial surface may indicate that their lignified cell walls contribute to apoplastic water flow within the leaf tissues.

Conclusion

In this study, we observed how water restriction can influence water status, photosynthetic performance, and leaf water absorption capacity in two epiphytic orchids: *Cattleya forbesii* and *Cattleya guttata*. In hydrated plants, the relative water content in the leaves and photosynthetic activity remained high, but the FWU rate was low. After water restriction, both *Cattleya* species showed a reduction in leaf water status accompanied by an increase in FWU (C*max* values). Despite this increase in FWU, photosynthetic activity was still negatively affected. Our results indicate that the greater amount of water in the leaf tissue (semi-succulent leaves) of both species may limit the FWU, whereas water scarcity (dry period) may increase this capacity. The apoplastic fluorescent marker Lucifer Yellow (LY) was predominantly detected in the cuticle of both species; however, water uptake occurred through both leaf surfaces in *C. forbesii*, whereas in *C. guttata,* absorption was restricted to the abaxial surface. Although our results indicate that FWU can occur in both species, experimental approaches combining drought and leaf-wetting events are necessary to determine whether this pathway

effectively mitigates water stress and enhances plant performance under natural conditions.

Author contributions

Jéssica Ferreira de Lima contributed to experimental design, data collection, data analysis, statistical processing, and manuscript writing.

Gisely Souza dos Santos contributed to the data collection, reviewing, and editing of the manuscript.

Denis Coelho de Oliveira assisted in data collection and analysis, as well as in the review and editing of the manuscript.

Ana Silvia Franco Pinheiro Moreira, as supervising author, contributed to the development of the concept and the review and editing of the manuscript.

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Data availability

The data will be made available upon request.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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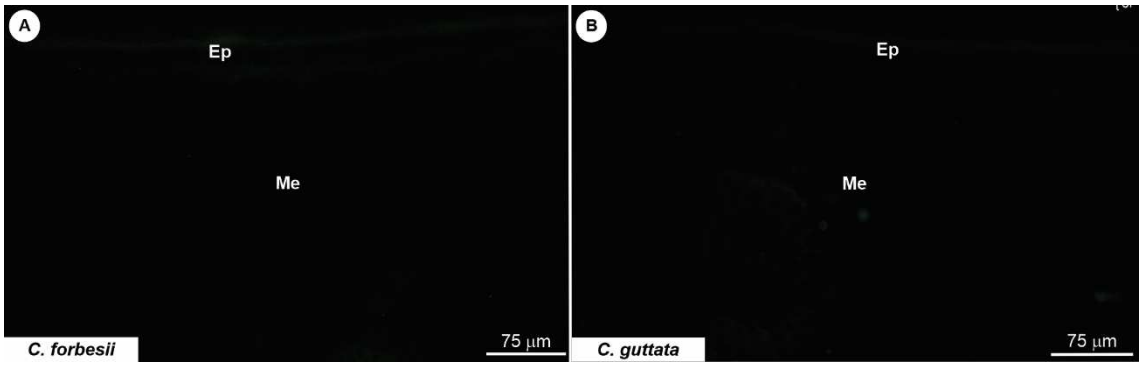
974 **Supporting information**

975



976 **Suppl. 1:** Control leaves, with distilled water applied to both surfaces, for analysis of the
977 apoplastic pathway visualized with the fluorescent marker (Lucifer Yellow) in *C. forbesii*
978 (A), and in *C. guttata* (B). *Acronyms*: Ep: epidermis and Me: mesophyll.

CAPÍTULO 3

(Artigo submetido à revista *Functional Plant Biology*)

Water deficit modulates foliar water uptake in *Puya chilensis* Molina (Bromeliaceae): physiological and biochemical adjustments

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Water deficit modulates foliar water uptake in *Puya chilensis* Molina (Bromeliaceae): physiological and biochemical adjustments

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Abstract

In response to water scarcity, plants develop adaptive strategies that enhance water-use efficiency, such as foliar water uptake (FWU). We hypothesize that FWU may increase water use efficiency, support photosynthetic activity, and reduce oxidative stress in *Puya chilensis*, which frequently experiences fog events. We proposed that fog frequency may modulate CAM intensity and influence FWU capacity. To this end, we subjected *P. chilensis* individuals to an experiment simulating artificial "fog" events and water deficit. Variations in relative water content, quantum efficiency of photosystem II, oxidative stress, overnight organic acid accumulation, and FWU capacity were assessed. Our findings indicate that water deficit negatively affects leaf water status and PSII efficiency and induces oxidative stress (increased accumulation of H_2O_2 and superoxide anions). Artificial fog partially helped maintain the leaf water balance and reduced physiological stress but did not alter CAM expression. The absence of changes in lipid peroxidation in plants exposed to artificial fog and water deficit indicates that antioxidant mechanisms control membrane damage. Foliar water uptake capacity increased under water stress, suggesting an important adaptive response to dehydration. Our results highlight the ecological importance of fog as an alternative water source for bromeliads inhabiting Mediterranean environments, increasingly threatened by climate change.

Keywords: Bromeliads, Crassulacean Acid Metabolism, CAM plants, enzymatic activity, oxidative stress, photosynthesis, water relations, water stress.

Introduction

The vegetative organs of plants exhibit a wide range of adaptive morphological and physiological strategies to cope with biotic and abiotic stresses, such as water scarcity (Anjum *et al*. 2011; Farooq *et al*. 2012; Mignolet-Spruyt *et al*. 2016). Among the mechanisms that contribute to water deficit tolerance, foliar water uptake (FWU) has attracted increasing attention (Gotsch *et al*. 2015; Pan *et al*. 2021). Foliar water uptake has been documented in numerous species across a variety of ecosystems, particularly in environments where atmospheric water inputs from dew and fog represent important supplementary water sources (Eller *et al*. 2013, 2016; Bryant *et al*. 2021; Pan *et al*. 2021). This process occurs in response to a water potential gradient established by differences in vapor pressure between the atmosphere and the leaf interior, allowing water to cross the leaf surface and contribute to tissue rehydration (Burgess and Dawson 2004). Water entry through leaves can confer several physiological benefits, such as improving water use efficiency, increasing leaf water content and water potential, modulating stomatal conductance, and consequently maintaining or enhancing photosynthetic capacity (Limm *et al*. 2009; Eller *et al*. 2016; Vesala *et al*. 2017; Berry *et al*. 2019). Although the pathways involved in FWU remain under investigation, evidence suggests that water may penetrate through the cuticle (Ritpitakphong *et al*. 2016; Gui *et al*. 2021; Boanares *et al*. 2024), trichomes (Boanares *et al*. 2018, 2024; Schreel *et al*. 2020; Gui *et al*. 2021), hydathodes (Martin and Von Willert 2000; Fradera-Soler *et al*. 2024), and even stomata (Guzmán-Delgado *et al*. 2021).

During drought periods, FWU can alleviate both water stress and oxidative stress (Eller *et al*. 2016; Boanares *et al*. 2020; Schreel *et al*. 2020; Gui *et al*. 2021; Liu *et al*. 2021). Several biological processes in plants, including responses to abiotic stress, are mediated by reactive oxygen species (ROS) (Mittler *et al*. 2011; Baxter *et al*. 2014). Reactive oxygen species, such as superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (•OH), are primarily generated in mitochondria, chloroplasts, and peroxisomes (Apel and Hirt 2004; Das *et al*. 2015). However, overproduction of ROS can impair cellular metabolism, making antioxidant defense systems essential for plant survival. These systems include enzymatic antioxidants such as superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione peroxidase (GPX), and catalase (CAT) (Apel and Hirt 2004; Foyer and Noctor 2013). Superoxide dismutase constitutes the first line of defense in ROS detoxification by catalyzing the dismutation of O_2^- into H_2O_2, which is subsequently scavenged through the coordinated action of APX, GPX, and CAT (Apel and Hirt 2004). The balance between ROS production and antioxidant enzyme activity plays a crucial role in cellular signaling and acclimation to

oxidative stress (Moller *et al*. 2007; Shi *et al*. 2013; Golldack *et al*. 2014). In rupicolous species with high FWU capacity, for instance, reduced H_2O_2 levels have been observed alongside increased antioxidant enzyme activities, highlighting the relationship between FWU and oxidative stress modulation (Boanares *et al*. 2020). Thus, in these species, FWU helps mitigate oxidative damage associated with limited water availability during the dry season (Boanares *et al*. 2020).

Other adaptive strategies associated with increased water use efficiency include leaf succulence and crassulacean acid metabolism (CAM) (Gibson 1982; Griffiths *et al*. 1986; Rundel and Dillon 1998; Zhang *et al*. 2015). Leaf succulence not only increases water storage capacity within tissues but is also closely linked to CAM metabolism, as organic acids produced during the night accumulate in cell vacuoles (Griffiths *et al*. 1986; Cushman 2001; Moreira *et al*. 2009; 2013). Compared with other photosynthetic pathways, CAM is associated with greater water use efficiency because carbon dioxide (CO_2) fixation occurs at night, when water loss by transpiration is reduced (Winter *et al*. 2005; Castillo *et al*. 2016). However, CAM expression is highly plastic and can be modulated or induced by different environmental conditions, such as water stress and salinity (Osmond 1978; Ting 1985). Epiphytic *Tillandsia* species (Bromeliaceae) occurring in the coastal region of the Chilean desert, for example, exhibit either full or partial CAM expression (Rundel and Dillon 1998). Although CAM expression is often associated with leaf succulence, the nocturnal accumulation of organic acids in vacuoles can reduce osmotic potential and, consequently, leaf water potential (Herrera *et al*. 2008; Matimati *et al*. 2012). In this context, CAM expression may increase the FWU during the early morning hours, when fog events are more frequent and when the water potential gradient between the leaf surface and internal tissues becomes more pronounced.

In Mediterranean and semiarid regions, particularly during dry summers and prolonged drought periods, fog can represent an important water source for plants (Barbosa *et al*. 2010). Chile is a Mediterranean region recognized as a biodiversity hotspot, harboring numerous endemic species (Myers *et al*. 2000; Mittermeier *et al*. 2004, 2011). However, this region is among the most vulnerable to climate change, mainly because of reduced precipitation, prolonged droughts, and rising mean temperatures (Garreaud *et al*. 2017; del Pozo *et al*. 2019, 2023). Considering that *Puya chilensis* (Bromeliaceae) is native to Chile and grows in environments frequently exposed to fog events, we propose this species as a model to investigate the relationship between FWU and CAM expression in succulents. We hypothesize that, even in species with succulent leaves, FWU may contribute to maintaining the leaf water balance, increasing water use efficiency, supporting photosynthetic activity, and reducing

oxidative stress. Furthermore, given that CAM expression can modify leaf water potential, we propose the additional hypothesis that variations in fog frequency may modulate CAM intensity and, consequently, influence FWU capacity. To test these hypotheses, we conducted an experiment using artificial "fog" events to evaluate how variation in fog frequency affects CAM expression, FWU, and associated morphophysiological adjustments in *P. chilensis*. Specifically, we sought to (1) investigate whether changes in fog frequency alter water status (relative water content), (2) assess whether these changes affect the quantum efficiency of photosystem II (PSII) and oxidative stress markers, (3) determine the expression pattern of CAM metabolism and its possible variation in response to fog modulation, and (4) evaluate the FWU capacity of *P. chilensis* and analyze whether this process is influenced by changes in CAM expression resulting from variations in fog availability.

Materials and methods

Plant material and experimental design

Twenty-four *Puya chilensis* Molina individuals, approximately 30 cm in height, were obtained from commercial nurseries and subjected to a four-month acclimation period in a growth chamber. For this period, the growth chamber was maintained at 20 ± 2 °C and 65% relative humidity, with a 16 to 8 h light/dark photoperiod and a light intensity of 50 μmol m^{-2} s^{-1}. Throughout the acclimation period, individuals were irrigated twice a week with approximately 100 mL of water. The entire experiment was conducted in the growth chamber of the Laboratory of Semiochemistry (LSqA) at the University of Concepción, Biobío Region, Chile, where *P. chilensis* naturally occurs.

After the acclimation period, two water availability conditions were simulated inside the growth chamber: artificial fog and water deficit. For the control treatment, eight randomly selected individuals of *P. chilensis* (n = 8) were irrigated three times per week with approximately 100 mL of distilled water throughout the experimental period. To simulate artificial fog, eight randomly selected individuals of *P. chilensis* (n = 8) were exposed to fog conditions at night (from 11:00 p.m. to 7:00 a.m.), corresponding to the time of highest natural fog occurrence during the winter in the Biobío Region, Chile. Artificial fog was generated using a 3 L air humidifier placed inside a wooden chamber covered with plastic to maintain high nighttime humidity. During the day (after 7:00 a.m.), the chamber door was opened to allow ventilation. In this treatment, to prevent the artificial mist from coming into contact with the root system, the plant pots were completely sealed with plastic wrap. To induce water deficit, irrigation was withheld from eight randomly selected individuals (n = 8) throughout the

experimental period. The 30-day experimental period was defined on the basis of the detection of physiological signs of water stress, specifically a significant reduction in the maximum quantum yield of photosystem II (PSII) (F_v/F_m). The F_v/F_m ratio was measured systematically using a portable chlorophyll fluorometer (OS30P+, Opti-Sciences, Inc., Hudson, NH, USA) to detect any decrease.

Assessment of the leaf water balance

The leaf water status was assessed by measuring the relative water content (RWC). For this purpose, at the end of the experiment (after 30 days), one leaf from a control or treated individual of *P. chilensis* (n = 8) was collected at 8:30 p.m. before the light in the growth chamber was turned off. Relative water content was calculated using the following equation: RWC = [(FW − DW)/(TW − DW)] × 100, where FW corresponds to fresh weight, DW to dry weight after the material was oven-dried (NovaTécnica-Termostato 70) at 50 °C until constant weight was reached, and TW to turgid weight after 24 h of rehydration (Turner 1981).

Assessment of foliar water uptake

To evaluate FWU, one intact and fully expanded leaf was collected from five control and treated individuals of *P. chilensis* (n = 5) at the end of the experiment. After collection, the bases of the leaves were immediately sealed with petroleum jelly and weighed using an analytical balance (BA2204B; BIOBASE Biodustry Co., China). The leaves were subsequently immersed in distilled water and weighed every 15 min for 2 h and then every 30 min for an additional 2 h. The leaves were subsequently weighed after 1 h to complete the experiment, after which the maximum Foliar water uptake curve (FWU) was obtained (Liang *et al*. 2009). Before being weighed, each leaf was gently dried with absorbent microfiber towels to remove surface water. To obtain dry weight, leaves were oven-dried at 60 °C (VENTICELL® 111, MMM Medcenter, Germany) until they reached a constant weight. The leaf area was determined from previously scanned leaf images (n = 5) using ImageJ software (Schindelin *et al*. 2012). The dynamic leaf water content (*C*) was calculated as the difference between the fresh weight measured at each time interval and the final weight, normalized by the leaf area, according to Equation (1):

$$FWU = \frac{fresh\ weight - final\ weight}{leaf\ area} \qquad (1)$$

The rate of water capture by the leaves (WC) was calculated using a differential equation based on the leaf area (Liang *et al*. 2009 modified; Pan *et al*. 2021), providing the maximum

foliar water uptake (*Cmax*) and the velocity of water absorption (*k*) (g cm^{-2} leaf area), according to Equation (2):

$$WC = (Cmax - Ci) * (1 - e^{-kt}) + Ci \quad (2)$$

where *Cmax* is the dynamic leaf water content at time *t*, *Ci* is the initial leaf water content, *k* is the water absorption rate per unit leaf area (exponential coefficient or curve parameter of the absorption function), and *t* is the time the leaf remained submerged in water.

Chlorophyll a fluorescence

The effects of treatments (control, artificial fog, and water deficit) on chlorophyll *a* fluorescence were evaluated in leaves from five individuals of *P. chilensis* (in the median region of the rosette) after 30 days of experimentation using a portable chlorophyll fluorometer (OS30P+, Opti-Sciences, Inc., Hudson, NH, USA). The leaves were dark-adapted for 30 min before the measurements. The following parameters were recorded: the maximum quantum yield of PSII (F_v/F_m) and the maximum efficiency of PSII photochemical processes (F_v/F_0) (Kitajima and Butler 1975; Maxwell and Johnson 2000).

Photosynthetic pigment content

Photosynthetic pigment contents, including chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), and carotenoids, were quantified, and the chlorophyll *a/b* and total chlorophyll (Chl *a*+*b*)-to-carotenoid ratios were calculated. For this purpose, 8 mm² leaf samples were collected from one leaf of each control individual (n = 8) and from individuals subjected to artificial fog (n = 8) and water deficit (n = 8) for 30 days. Leaf samples were collected in the afternoon to avoid interference from tissue acidity, weighed, and immersed in 5 mL of 80% acetone for 48 h at 4 °C. The material was then macerated and centrifuged, after which 250 µL of the extract supernatant was dispensed in triplicate into 96-well microplates. The absorbance was subsequently measured at 470, 646, and 663 nm using a microplate reader (ELX800, Bio-Tek). Pigment contents were calculated according to the equations proposed by Lichtenthaler and Wellburn (1983) and were expressed as mg per gram of fresh weight (FW).

Diurnal variation in titratable acidity

The diurnal variation in organic acids (ΔH^+) in *P. chilensis* leaves was assessed to determine the expression of either the C3 or CAM photosynthetic pathway after 30 days of experimentation. For this purpose, 200 mg of leaves were collected from control plants (n = 8) and from plants subjected to artificial fog (n = 8) and water deficit (n = 8) at 4:00 a.m. and 8:00

p.m. The samples were weighed using a digital balance and boiled in distilled water (5 mL) for 5 min. The extracts (2.5 mL) were titrated with 0.01 N NaOH to pH 7.0, following the method proposed by Hartsock and Nobel (1976). The results are expressed in μeq of H^+ per g of fresh mass (FM).

Quantification of hydrogen peroxide and malondialdehyde levels

For the quantification of hydrogen peroxide (H_2O_2) and malondialdehyde (MDA), leaf fragments from control *P. chilensis* plants (n = 5) and from plants subjected to artificial fog (n = 5) and water deficit (n = 5) for 30 days were collected, immediately frozen in liquid nitrogen, and subsequently stored in an ultrafreezer at −80 °C until analysis.

For MDA quantification, 0.100 g of sample was ground in an electric mill with liquid nitrogen supplemented with 10 mg of polyvinylpolypyrrolidone (PVPP) (Hodges *et al*. 1999). Afterward, 1.5 mL of 20% trichloroacetic acid (TCA) was added, and the samples were centrifuged at 12,000 × g for 15 min at 4 °C. An aliquot (250 μL) of the supernatant was collected and mixed with 1 mL of 0.5% thiobarbituric acid (TBA) solution and 1 mL of 20% TCA. An additional 250 μL of the extraction medium was used as a blank. The samples and blanks were incubated in a water bath at 90 °C for 20 min, immediately cooled on ice for 10 min to stop the reaction, and centrifuged at 3,000 × g for 4 min. Spectrophotometric readings were performed using a microplate reader (ELX800, Bio-Tek) at wavelengths of 532, 440, and 600 nm. The amount of the MDA–TBA complex was calculated according to Heath and Packer (1968), and the results were expressed as nmol g^{-1} fresh weight.

The hydrogen peroxide (H_2O_2) content was determined as described by Junglee *et al*. (2014). The leaf samples were ground in an electric mill with liquid nitrogen, and 0.100 g of tissue was weighed for homogenization with the reaction mixture. The reaction mixture consisted of 0.5 mL of 0.1% TCA, 0.7 mL of 100 mM potassium phosphate buffer (pH 7.0), and 1 mL of 1 M potassium iodide (KI). The reaction was then incubated in the dark for 1 h. A 0.1% TCA solution without sample extract was used as a blank. The samples were subsequently centrifuged at 12,000 × g for 15 min at 4 °C. The absorbance was measured at 390 nm using a microplate reader (ELX800, Bio-Tek). The hydrogen peroxide content was calculated on the basis of a calibration curve prepared with a 0.1% H_2O_2 standard solution and expressed as mM g^{-1} fresh weight.

Histochemical localization of $O_2^{\cdot -}$

Fragments from the median region of leaves of three *P. chilensis* individuals were collected from control plants and from those subjected to artificial fog and water deficit (n = 3) for 30 days. To detect superoxide anion ($O_2 \cdot^-$), the leaf fragments were incubated in a 0.5% p-nitroblue tetrazolium (NBT) solution for 24 h in the dark and then hand-sectioned (Kumar *et al*. 2014). All slides from the histochemical reactions, along with their respective blanks, were analyzed and photographed using a light microscope (Leica DM2500, Germany) equipped with a digital camera (Leica ICC50W, Germany), and the superoxide anions were indicated by blue pigmentation.

Determination of antioxidant enzyme activity

For the analysis of antioxidant enzyme activity, leaf samples were collected from five control individuals of *P. chilensis* (n = 5), as well as from plants subjected to artificial fog (n = 5) and water deficit (n = 5) for 30 days. The leaves were frozen in liquid nitrogen and subsequently stored at -80 °C in an ultrafreezer. The samples were ground in an electric mill with liquid nitrogen, and protein extracts were obtained from 0.1 g of each sample using 50 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM EDTA, 10 mM mercaptoethanol, 2.27 mM ascorbate, and 20% polyvinylpolypyrrolidone (PVPP) (Palma et al., 2014). The extracts were subsequently centrifuged at 15,000 × g for 20 min at 4 °C. The supernatant was collected for subsequent determination of the activities of superoxide dismutase (SOD, EC 1.15.1.1), ascorbate peroxidase (APX, EC 1.11.1.11), glutathione reductase (GR, EC 1.8.7), dehydroascorbate reductase (DHAR, EC 1.8.5.1), and peroxidase (POX, EC 1.11.7).

The protein content was determined by mixing 150 µL of the protein extract with 300 µL of Bradford reagent and incubating at room temperature for 5 min (Bradford 1976). Immediately after incubation, the absorbance was measured at 595 nm using a microplate reader (ELX800, Bio-Tek). Bovine serum albumin (BSA) solutions ranging from 0 to 0.5 mg mL^{-1} were used to construct the calibration curve. The total soluble protein content was expressed as mg g^{-1} fresh weight.

To measure SOD activity, a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, 75 µM NBT, 0.1 mM EDTA, and 50 µL of protein extract was used to inhibit the reduction of nitro blue tetrazolium (NBT). The reaction was initiated by adding 2 µM riboflavin and exposing the mixture to fluorescent light for 15 min. The reaction was stopped by placing the mixture in the dark, and the absorbance was measured at 560 nm (Beyer and Fridovich 1987), which was expressed in U mg^{-1} protein. We use two controls: one that has not been exposed to light and another that has been exposed to light.

To extract the CAT, a reaction mixture consisting of 50 mM potassium phosphate buffer (pH 6.8) and 20 mM H_2O_2 was used in a total volume of 2 mL. H_2O_2 consumption at 240 nm over 3 min at 25 °C was used to determine catalase activity (Cakmak and Marschner 1992). Catalase activity was determined using a molar extinction coefficient of 36 M^{-1} cm^{-1} (Anderson *et al*. 1995), which was expressed in micromoles per minute per milligram of protein (mmol min^{-1} mg^{-1} protein).

Ascorbate peroxidase activity was measured by monitoring the decrease in absorbance at 290 nm as ascorbate was oxidized. The reaction mixture contained 50 mM Tris–HCl buffer (pH 7.8), 0.4 mM ascorbate, 0.3 mM H_2O_2, and 50 µL of protein extract (Hossain and Asada 1984). These values are expressed in µmol H_2O_2 mg^{-1} prot min^{-1}.

For POX activity, a reaction mixture containing 200 mM sodium phosphate buffer (pH 5.8), 7.2 mM guaiacol, 11.8 mM H_2O_2, and 50 µL of protein extract was used (Kato and Shimizu 1987). Enzyme activity was determined by monitoring guaiacol oxidation at 470 nm, which was expressed in µmol H_2O_2 mg^{-1} prot min^{-1}.

To assess DHAR activity, a reaction mixture containing 50 mM potassium phosphate buffer (pH 6.5), 0.1 mM EDTA, 0.5 mM reduced glutathione (GSH), and 0.5 mM dehydroascorbate (DHA), along with 50 µL of protein extract, was used (Dalton *et al*. 1993), which was expressed in mM Asc mg^{-1} prot h^{-1}.

For GR activity, the reaction mixture consisted of 0.1 mM HEPES–NaOH buffer (pH 7.8), 3 mM $MgCl_2$, 0.25 mM oxidized glutathione (GSSG), 0.2 mM NADPH, 1 mM EDTA, and 50 µL of protein extract (Edwards *et al*. 1990). Glutathione reductase activity was determined by monitoring NADPH oxidation through the decrease in absorbance at 340 nm, which was expressed in µmol NADP mg^{-1} min^{-1}.

Quantification of flavonoids and phenolics

Extract preparation

For the quantification of phenolics and flavonoids, leaves from five control individuals of *P. chilensis* (n = 5), as well as from plants subjected to artificial fog (n = 5) and water deficit (n = 5) for 30 days, were collected and dried in an oven at 40 °C. To prepare the extracts, the samples were ground and extracted three times every 24 h with 80% aqueous methanol (0.2 g mL^{-1}). The extracts were pooled and filtered using syringe filters.

Total flavonoid content

To determine the total flavonoid content, 135 µL of each extract was mixed with 7.5 µL of 5% $NaNO_2$, homogenized, and allowed to react for 5 min. Then, 30 µL of 2.5% $AlCl_3$ was

added, the mixture was homogenized, and the reaction was allowed to continue for an additional 6 min. Subsequently, 50 µL of 1 M NaOH and 50 µL of water were added, the mixture was homogenized, and the reaction was allowed to proceed for 5 more min (Kim *et al*. 2003). The absorbance was measured at 510 nm in triplicate using a microplate reader (ELX800, Bio-Tek), with water used as a blank. The total flavonoid content was calculated based on a quercetin (QE; 1 mg mL^{-1}) calibration curve and expressed as mg per gram of dry weight (mg QE g^{-1} DW).

Total phenol content

For phenolic quantification, 10 µL of methanolic extract from each sample was mixed with 20 µL of Folin–Ciocalteu reagent, 200 µL of ultrapure water, and 100 µL of 15% sodium carbonate (Na_2CO_3) (Mongkolsilp *et al*. 2004). The mixture was maintained at room temperature for 1 h, and the absorbance was measured in triplicate at 759 nm using a microplate reader. Methanol containing all the reagents except the sample extract was used as the blank. Gallic acid standard solutions were used to construct the calibration curve (1 mg mL^{-1}). The total phenolic content was expressed as milligrams of gallic acid equivalents (GAE) per gram of dry weight (mg GAE g^{-1} DW).

Data analysis

Generalized linear models (GLMs) with a Gaussian family were constructed to test for differences in parameters related to relative water content (RWC), chloroplast pigment content (chlorophyll *a* content, total chlorophyll content, carotenoids, chlorophyll *a*/*b* ratio, and total chlorophyll/carotenoid ratio), chlorophyll *a* fluorescence parameters (F_v/F_m, F_v/F_0), FWU capacity (*Cmax* and *k*), oxidative stress (H_2O_2 and MDA), antioxidant enzyme activities (APX, SOD, and POX), and phenolic and flavonoid compounds between control plants and those subjected to artificial fog and water deficit for 30 days. GLMs with a gamma error distribution were used to evaluate whether the chlorophyll *b* content and activity of antioxidant enzymes (GR, DHAR, and catalase) differed. To evaluate differences in the diurnal variation in organic acids across treatments, generalized linear models (GLMs) with a zero-inflated Gamma error distribution were constructed because of the number of zeros observed in the samples (using the glmmTMB package; Brooks *et al.* 2023). Model selection was based on the normality and homogeneity of variance of the model residuals and was assessed using the DHARMa package (Hartig 2022). The means were compared using Tukey's adjusted paired comparisons across treatments (control, artificial fog, and water deficit) using the emmeans package (Lenth 2023). Statistical analyses were performed using R (i386 4.5.2; R Development Core Team 2024),

with a significance level of 5% ($p \leq 0.05$). For statistical analyses, each plant was treated as an independent biological replicate.

Results

Variations in water status and foliar water uptake (FWU)

Despite the decline in RWC values after 30 days of the experiment in *P. chilensis* leaves, the individuals exposed to artificial fog maintained higher RWC values than those subjected to water deficit conditions (Fig. 1a). In contrast, compared with plants exposed to artificial fog, *P. chilensis* individuals under water deficit for 30 days presented the highest foliar water uptake capacity (*Cmax*) (Fig. 1b). No significant differences were observed in the foliar velocity of water uptake (*k*) among the treatments (Fig. 1c).

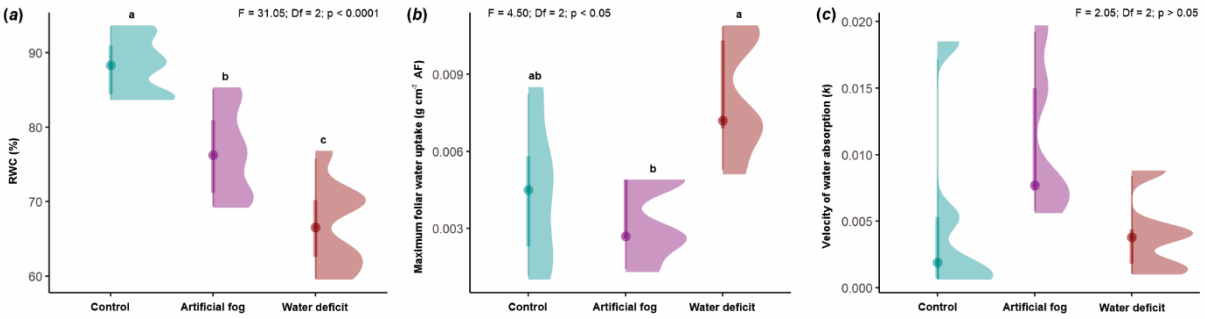

Fig. 1: Variations in the leaf water balance and foliar water uptake (FWU) parameters of *Puya chilensis* under hydrated conditions—the control (n = 8), artificial fog (n = 8), and water deficit (n = 8)—after 30 days. (*a*) Relative water content (RWC), (*b*) maximum leaf water uptake capacity (*Cmax*), and (*c*) velocity of water uptake (*k*). Different letters indicate differences among treatments based on GLMs with Gaussian or Gamma family distributions (for both, $p \leq 0.05$ was considered significant).

Chlorophyll a fluorescence and photosynthetic pigment content

Compared with plants in which irrigation was suspended for 30 days, control plants and those subjected to artificial fog presented significantly greater potential quantum yields of PSII (F_v/F_m) and greater PSII activity (F_v/F_0) (Fig. 2).

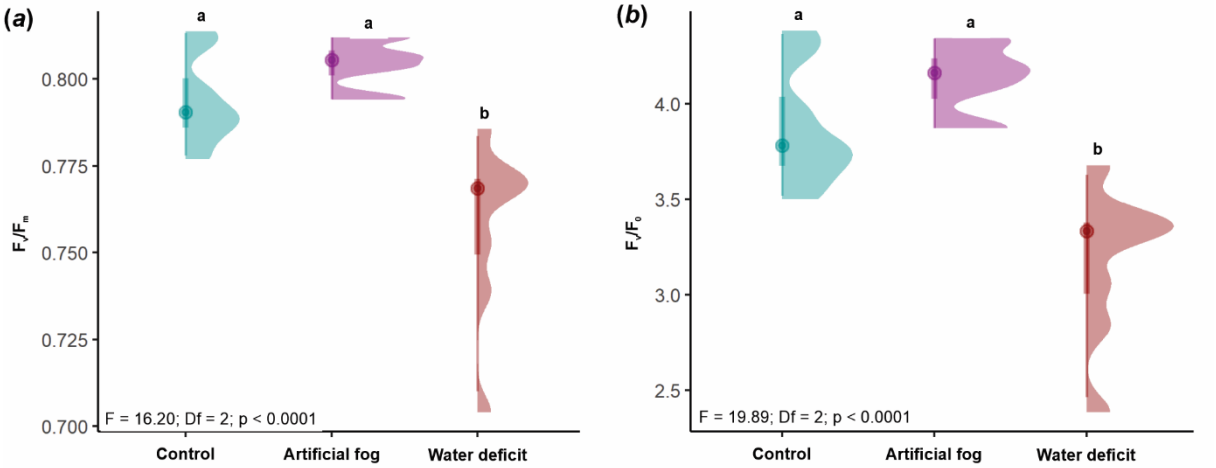

Fig. 2: Variations in chlorophyll *a* fluorescence parameters among control (hydrated; n = 8), artificially fog-treated (n = 8), and water deficit-treated (n = 8) *Puya chilensis* individuals after 30 days. (*a*) Potential quantum yield of PSII (F_v/F_m) and (*b*) PSII activity (F_v/F_0). The data correspond to statistical analyses performed using GLMs with a Gaussian family distribution, considering a significance level of 5% ($p \leq 0.05$).

The ratio between total chlorophyll and carotenoid contents was significantly greater in hydrated plants (control) than in plants subjected to water stress for 30 days, regardless of the presence of artificial fog (Table 1). No significant variations were observed in the chlorophyll and carotenoid contents or in the chlorophyll a/b ratio among the treatments (Table 1).

Table 1: Variations in chloroplastic pigment content (mg g^{-1} FW) in leaves of *Puya chilensis* from control individuals (n = 8), individuals subjected to artificial fog (n = 8), and water deficit (n = 8) after 30 days.

Parameters	Control	Artificial fog	Water deficit	Df	F value	p value
Chlorophyll *a*	0.49 ± 0.10	0.46 ± 0.07	0.49 ± 0.09	2	0.31	> 0.05
Chlorophyll *b*	0.15 ± 0.04	0.17 ± 0.05	0.16 ± 0.02	2	0.29	> 0.05
Total Chl (*a* + *b*)	0.65 ± 0.13	0.67 ± 0.13	0.65 ± 0.11	2	0.06	> 0.05
Carotenoids	0.09 ± 0.01	0.09 ± 0.01	0.10 ± 0.02	2	0.86	> 0.05
Chlorophyll *a*/*b*	3.24 ± 0.41	3.07 ± 0.46	3.13 ± 0.23	2	0.44	> 0.05
Chl (*a* + *b*)/carot	7.00 ± 0.57a	6.43 ± 0.30b	6.26 ± 0.20b	2	7.88	< 0.01

Data are presented as means ± standard deviations. Statistical analyses were performed using Generalized Linear Models (GLMs) with Gaussian or Gamma family distributions, considering a significance level of 5% ($p \leq 0.05$).

Diurnal variation in titratable acidity

There was no significant difference in the diurnal variation in leaf acidity (ΔH^+; H^+ g^{-1} FM) among individuals of *P. chilensis* in the control (medium-stratum 1.50 ± 2.26), artificial

fog (medium-stratum 0.04 ± 0.06), or water deficit (medium-stratum 0.02 ± 0.04) groups (χ^2 = 5.58; Df = 2; p > 0.05).

Variations in hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) concentrations

The H_2O_2 concentration in the leaves of *P. chilensis* subjected to water deficit for 30 days was significantly greater than that in the leaves of control plants (Fig. 3a). However, no significant changes in the MDA concentration were detected among the treatments (Fig. 3b).

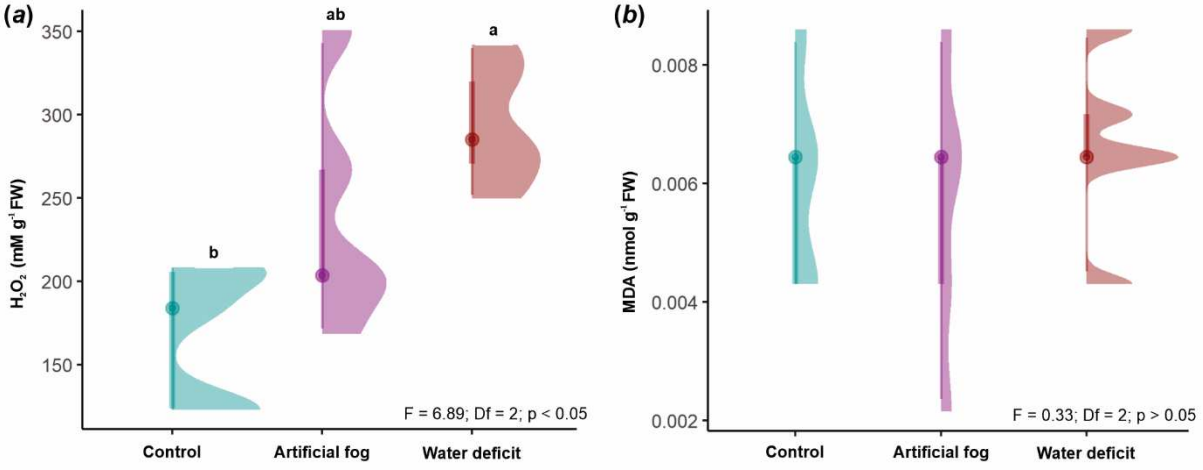

Fig. 3: Hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) concentrations in leaves of *Puya chilensis* under control conditions (n = 5) and under artificial fog (n = 5) and water deficit (n = 5) conditions for 30 days. (*a*) Hydrogen peroxide (H_2O_2) and (*b*) malondialdehyde (MDA). Different letters indicate significant differences among treatments based on GLMs with Gaussian family distributions, considering a significance level of 5% (p ≤ 0.05).

Histolocalization of superoxide anion

In control leaves of *P. chilensis* and those exposed to artificial fog (Fig. 4a–c), superoxide anions were detected mainly in the xylem and were less evident in the phloem, whereas leaves subjected to water deficit (Fig. 4d) showed pronounced staining in both xylem and phloem tissues. In addition, superoxide anions were detected in the chlorenchyma cells of leaves exposed to artificial fog and water deficit for 30 days (Fig. 4).

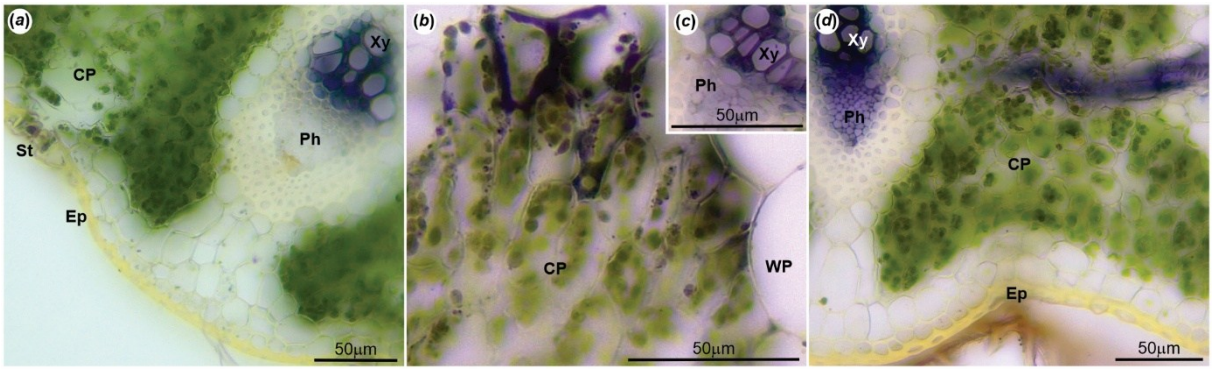

Fig. 4: Transverse sections of *Puya chilensis* leaves indicating the presence of superoxide anions detected by the blue NBT reaction. (*a*) Leaves of control *P. chilensis* plants; (*b* and *c*) leaves of *P. chilensis* subjected to artificial fog for 30 days; and (*d*) leaves of *P. chilensis* subjected to water deficit for 30 days. (*a*) In control leaves and (*b* and *c*) leaves subjected to artificial fog, the presence of superoxide anions was evident in the chlorenchyma and xylem, with weak staining in the phloem. (*d*) In plants subjected to water deficit, superoxide anions were evident in the chlorenchyma as well as in both the xylem and phloem tissues. CP: chlorenchyma, WP: water-storage parenchyma, Ep: epidermis, St: stomata, Xy: xylem, Ph: phloem.

Antioxidant enzyme activities

In *P. chilensis* individuals exposed to artificial fog, the protein content was significantly lower than that in control plants that were irrigated throughout the 30-day experimental period (Table 2). No significant changes in SOD, CAT, or APX activity were detected among the *P. chilensis* treatments (Table 2). In contrast, compared with control plants, plants subjected to artificial fog and water deficit for 30 days presented higher POX activity (Table 2). DHAR activity was not affected by the treatments; however, compared with those of the control plants, the leaves of *P. chilensis* plants maintained under artificial fog presented significantly greater GR activity (Table 2).

Table 2: Protein and enzymatic activity in leaves of control *Puya chilensis* plants (n = 5) and subjected to artificial fog (n = 5) and water deficit (n = 5) for 30 days. Protein and the activity of the antioxidant enzymes superoxide dismutase (SOD), dehydroascorbate reductase (DHAR), ascorbate peroxidase (APX), glutathione reductase (GR), peroxidase (POX), and catalase were measured. Results are expressed as µmol min^{-1} mg^{-1} protein.

Enzymes	Control	Artificial fog	Water deficit	Df	F value	p value
Protein	0.14 ± 0.01a	0.11 ± 0.01b	0.12 ± 0.02ab	2	6.21	< 0.05
SOD (U mg^{-1} prot)	0.55 ± 0.08	0.67 ± 0.10	0.51 ± 0.10	2	3.86	> 0.05
CAT (mmol min^{-1} mg^{-1} prot)	5.50 ± 1.67	5.93 ± 2.45	4.18 ± 2.07	2	0.96	> 0.05
APX (µmol H_2O_2 mg^{-1} prot min^{-1})	0.81 ± 0.41	0.84 ± 0.27	1.27 ± 0.39	2	2.51	> 0.05
POX (µmol H_2O_2 mg^{-1} prot min^{-1})	0.43 ± 0.12b	0.74 ± 0.15a	0.65 ± 0.08a	2	8.65	< 0.01
DHAR (mM Asc mg^{-1} prot h^{-1})	0.09 ± 0.04	0.10 ± 0.03	0.10 ± 0.04	2	0.18	> 0.05
GR (µmol NADP mg^{-1} min^{-1})	1.15 ± 0.05b	2.51 ± 0.70a	1.70 ± 0.45ab	2	15.07	< 0.001

Data are presented as means ± standard deviations. Different letters indicate significant differences based on GLMs with Gaussian or Gamma family distributions, both considering a significance level of 5% ($p \leq 0.05$).

Secondary metabolite contents (phenolic compounds and flavonoids)

The total phenolic content of *Puya chilensis* leaves maintained under artificial fog conditions significantly increased (Fig. 5a). However, the flavonoid content remained unchanged among the treatments (Fig. 5b).

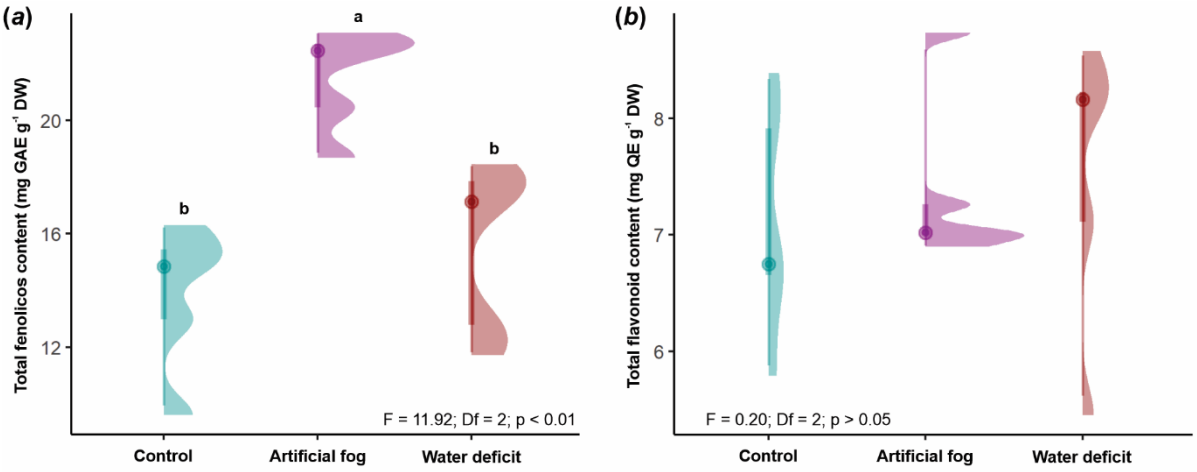

Fig. 5: Secondary metabolite contents (phenolic compounds and flavonoids) in the leaves of *Puya chilensis* under control conditions, artificial fog, and water deficit conditions (n = 5). (*a*) Total phenolics and (*b*) total flavonols. Different letters indicate significant differences based on generalized linear models (GLMs) with a Gaussian family distribution, considering a significance level of 5% ($p \leq 0.05$).

Discussion

Water deficit negatively affected the leaf water status of *P. chilensis*, whereas the presence of artificial nocturnal fog partially contributed to the maintenance of the leaf water balance. Under water deficit conditions, plants also exhibited reduced photosynthetic performance, as evidenced by lower values of the maximum quantum efficiency of PSII (F_v/F_m)

and PSII activity (F_v/F_0), suggesting impairment of the photosynthetic apparatus. In addition, water deficit promoted oxidative stress, leading to increased accumulation of hydrogen peroxide and superoxide anions in leaf tissues. However, no changes in lipid peroxidation were observed, suggesting that oxidative damage was effectively controlled by antioxidant mechanisms, particularly through increased peroxidase activity. In parallel, the foliar water uptake capacity increased under water deficit conditions, highlighting its importance as an adaptive response to drought stress. Nevertheless, contrasting water conditions appeared to induce different antioxidant responses. While peroxidase activity increased in leaves from both treatments, glutathione reductase activity and total phenol content increased only in plants exposed to artificial nocturnal fog.

Foliar water uptake as a mechanism for the partial maintenance of leaf water status and photosynthetic integrity in Puya chilensis

Puya chilensis is an endemic Chilean species that commonly grows on rocky soils and has a broad latitudinal distribution, extending from semiarid regions to Mediterranean and temperate oceanic climates (Zizka *et al*. 2009; Echeverria-Echeverria *et al*. 2022; Ugas-Bravo *et al*. 2025). In xeric Mediterranean-oceanic regions, precipitation patterns are highly seasonal and characterized by prolonged drought periods (Luebert and Pliscoff 2006; Armesto *et al.* 2007), exposing plants to recurrent water limitations and promoting the evolution of adaptive mechanisms associated with water acquisition and conservation.

Relative water content is widely recognized as a reliable indicator of plant water status because it directly reflects tissue hydration and is closely associated with metabolic activity, transpiration rates, and drought intensity (Anjum *et al*. 2011; Leroy *et al*. 2019; Takahashi and Mercier 2024). In the present study, compared with well-watered plants, *P. chilensis* individuals subjected to water deficit presented an approximately 21% reduction in leaf water content, indicating substantial dehydration. Similar reductions in RWC under drought conditions have also been reported for the epiphytic bromeliad *Guzmania monostachia* (Abreu *et al*. 2018), suggesting that reduced tissue hydration may be a common physiological response among bromeliads exposed to water restriction.

Plants subjected to drought also exhibited higher foliar water uptake capacity (C*max*), which was likely associated with their lower leaf water content. This increase in FWU capacity under dehydration suggests a physiological adjustment that enhances the potential for water absorption when external water sources become available. However, the presence of artificial nocturnal fog partially alleviated the effects of water restriction by promoting leaf hydration

494 and helping maintain the leaf water balance in *P. chilensis*. Compared with control plants, plants
495 exposed to artificial fog presented only an approximately 11% reduction in RWC, and
496 compared with plants under water deficit alone, plants under artificial fog maintained
497 substantially higher leaf hydration levels. Fog events are common along the Chilean coast,
498 where several *Puya* species naturally occur (Zizka *et al*. 2009), and may represent important
499 supplementary water sources in environments subjected to seasonal drought.

500 External water inputs such as fog and dew can provide favorable conditions for foliar
501 water uptake through the formation of a water film on the leaf surface with higher water
502 potential than the leaf interior, thereby generating a water potential gradient that drives water
503 movement into the tissues (Burgess and Dawson 2004; Limm *et al*. 2009; Eller *et al*. 2013;
504 Berry and Smith 2013; Schreel and Steppe 2020). Several studies involving conifers, ferns,
505 shrubs, and epiphytic bromeliads have demonstrated that fog-derived FWU contributes to
506 improvements in leaf water balance, water potential, stomatal conductance, photosynthesis, and
507 plant growth (Burgess and Dawson 2004; Limm *et al*. 2009; Steppe *et al*. 2018; Berry *et al*.
508 2019; Chávez-Sahagún *et al*. 2019; Chin *et al*. 2023). For instance, Limm *et al*. (2009)
509 demonstrated that in different taxa, including redwood conifers, understory ferns, and shrubs,
510 RWC increased by 2–11% after fog exposure, reinforcing the importance of fog-derived water
511 in maintaining tissue hydration. A similar pattern was observed in *P. chilensis*, highlighting the
512 ecological relevance of FWU as an alternative water acquisition pathway under drought
513 conditions.

514 The reduction in the impact on the RWC values under artificial fog conditions was
515 accompanied by the preservation of photosynthetic integrity, as indicated by the higher F_v/F_m
516 and F_v/F_0 values in these plants than in drought-stressed plants. In addition, the stability of the
517 chlorophyll contents suggests that the photosynthetic apparatus remained structurally
518 preserved, even under restricted soil water availability. Together, these findings indicate that
519 nocturnal fog simulation contributed not only to the partial maintenance of leaf hydration
520 (~75% RWC even with soil water restriction) but also to the preservation of photosynthetic
521 functionality and a reduction in drought-induced physiological stress. Therefore, our results
522 support the hypothesis that FWU represents an important adaptive mechanism allowing *P.*
523 *chilensis* to mitigate the effects of seasonal drought and maintain metabolic activity under
524 water-limited conditions.

Oxidative stress and antioxidant responses in P. chilensis under water deficit

Puya chilensis has been described as a facultative CAM species, with CAM expression becoming more pronounced under drought conditions in natural environments (Quezada *et al*. 2014, 2017). However, in the present study, during the 30-day water restriction period, no evidence of CAM induction was detected through diel acid fluctuation (ΔH^+), indicating the maintenance of C3 metabolism. Although *P. chilensis* leaves exhibit drought-associated traits, such as a certain degree of succulence, the shallow and rocky soils where this species naturally occurs are highly susceptible to rapid drying during short rainless periods (Smith and Downs 1974; Quezada *et al*. 2014, 2017). Under these conditions, prolonged water limitation may strongly affect photosynthetic performance and cellular redox homeostasis, which remains to be demonstrated in *P. chilensis*.

Drought-induced impairment of photosynthetic performance is commonly associated with increased production of ROS, resulting from excess energy excitation and disruption of electron transport in PSII (Miller *et al*. 2010; Sharma *et al*. 2012; Mattos and Moretti 2015). Reactive oxygen species such as superoxide radicals, hydroxyl radicals, and hydrogen peroxide are essential signaling molecules involved in plant metabolism and stress responses (Sharma *et al*. 2012; Baxter *et al*. 2014). However, excessive ROS accumulation can trigger oxidative stress and damage lipids, proteins, chlorophylls, and nucleic acids (Noctor 2006; Sharma *et al*. 2012; Chugh *et al*. 2013; Lal *et al*. 2019). Consistent with this pattern, *P. chilensis* plants subjected to water deficit presented increased accumulation of H_2O_2 and superoxide anions in their leaf tissues. Histolocalization analyses further demonstrated the presence of superoxide anions in the chlorenchyma, xylem, and phloem, particularly under drought conditions, indicating that oxidative stress occurred in both photosynthetic and vascular tissues. Similar increases in ROS accumulation under water deficit conditions have been reported in several plant species, including the epiphytic bromeliad *G. monostachia*, which exhibited elevated H_2O_2 levels in all leaf portions during drought stress (Abreu *et al*. 2018).

Although drought promoted oxidative stress, no increase in the MDA content was detected, indicating that lipid peroxidation and membrane damage were effectively controlled. Malondialdehyde is widely used as a biomarker of oxidative membrane damage, and lower or unchanged MDA levels are generally associated with efficient antioxidant protection (Dhanda *et al*. 2004; Ozkur *et al*. 2009; Chugh *et al*. 2013; Talbi *et al*. 2015). Therefore, the maintenance of stable MDA concentrations despite increased ROS accumulation suggests that *P. chilensis*

activated effective antioxidant mechanisms capable of preventing severe oxidative damage during drought.

The antioxidant response observed in *P. chilensis* involved both enzymatic and nonenzymatic components. Plants possess a complex antioxidant network composed of enzymes and metabolites that detoxify ROS and maintain cellular redox balance (Noctor and Foyer 1998; Mittler *et al*. 2004; Sharma *et al*. 2012, 2020). Within the enzymatic system, SOD catalyzes the conversion of $O_2^{\bullet -}$ into H_2O_2, whereas enzymes such as APX, DHAR, GR, CAT, and POX are involved in H_2O_2 detoxification and the removal of lipid peroxides (Noctor and Foyer 1998; Foyer and Shigeoka 2011).

The increased accumulation of hydrogen peroxide, together with the increased activity of POX, suggests the activation of antioxidant mechanisms involved in the detoxification and redox regulation of H_2O_2 in both artificial fog and drought treatments. These findings suggest that the POX enzyme represents a major component of the antioxidant response in *P. chilensis*. In addition to its role in H_2O_2 scavenging, POX is involved in several physiological processes related to abiotic stress tolerance, including cell wall remodeling, regulation of cell expansion, and modulation of ROS signaling (Csiszár *et al*. 2012). Under drought conditions, POX activity has also been associated with the maintenance of tissue hydration and increased relative water content (Mercado *et al*. 2004; Khanna-Chopra and Selote 2007; Harb *et al*. 2010; Noctor *et al*. 2014), which may help explain its increased activity in both treatments involving atmospheric water exposure and water restriction.

Among the antioxidant enzymes evaluated, GR activity increased in plants exposed to artificial fog, highlighting the importance of the ascorbate–glutathione cycle in maintaining redox homeostasis under improved hydration conditions. Glutathione reductase plays a central role in regenerating reduced glutathione from oxidized glutathione (GSSG) using NADPH, thereby sustaining the antioxidant capacity of the cell (Noctor and Foyer 1998; Sharma *et al*. 2012). The enhanced GR activity under artificial fog conditions suggests that even moderate ROS production was sufficient to activate protective redox mechanisms, contributing to the maintenance of lower oxidative stress levels and the preservation of photosynthetic performance. In contrast, the absence of increased GR activity under strict water deficit suggests that antioxidant responses were restricted mainly to immediate hydrogen peroxide detoxification through peroxidase activity. This finding indicates that the improved leaf hydration promoted by artificial fog may support a more integrated antioxidant response involving both glutathione recycling and nonenzymatic antioxidant defenses, as also evidenced by the higher total phenol contents observed under this treatment.

Phenolic compounds are important nonenzymatic antioxidants and may contribute to photoprotection, membrane stabilization, and the scavenging of ROS, thereby protecting cellular structures under stressful conditions (Harborne and Williams 2000; Sánchez-Rodríguez *et al.* 2010; Isah *et al.* 2019; Ali *et al*. 2020; Jabeen *et al*. 2022; Rao and Zheng 2025). Interestingly, the increase in phenolics was accompanied by reduced protein content under artificial fog conditions, which may reflect metabolic adjustments associated with the synthesis of protective secondary metabolites. This response suggests that the improved hydration promoted by artificial fog supported the activation of antioxidant and redox-regulatory mechanisms without leading to severe oxidative damage.

Conclusion

In conclusion, our results demonstrate that artificial fog events partially maintained the leaf water balance in *P. chilensis* through foliar water uptake, contributing to the preservation of photosynthetic integrity and the mitigation of oxidative stress under water deficit conditions. Although drought reduced the relative water content and impaired PSII efficiency, plants exposed to nocturnal fog maintained higher tissue hydration and experienced lower physiological stress. Water restriction also increased foliar water uptake capacity, suggesting that FWU represents an important adaptive response under dehydration conditions. In addition, drought promoted oxidative stress through increased accumulation of H_2O_2 and superoxide anions; however, the absence of changes in lipid peroxidation indicates that antioxidant mechanisms, particularly the activities of GR and POX and the accumulation of phenolic compounds, were effective in preventing severe oxidative damage. Contrary to our expectations, variation in fog frequency did not induce changes in CAM expression, since *P. chilensis* maintained C3 metabolism throughout the experimental period. Therefore, our findings indicate that, even without shifts in CAM metabolism, FWU plays a central role in maintaining the physiological performance of *P. chilensis*, highlighting the ecological importance of fog as an alternative water source for bromeliads inhabiting Mediterranean and semiarid environments that are increasingly threatened by climate change.

Author contributions

Jéssica Ferreira de Lima: Writing—original draft, Writing—review & editing, Methodology, Investigation, Formal analysis, Data curation, and Conceptualization. **Lubia M. Guedes:** Writing – review & editing, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, and Conceptualization. **Leticia Ponticell Nobrega:** Writing –

review & editing, Visualization, Methodology, and Formal analysis. **José Ortiz:** Writing – review & editing, Validation, Methodology, and Formal analysis. **Lorena Rodríguez-Cerda:** Writing – review & editing, and Methodology. **Narciso Aguilera:** Writing – review & editing, Funding Acquisition, Visualization, Validation, Supervision, Methodology, Investigation, and Conceptualization. **Ana Silvia Franco Pinheiro Moreira:** Writing – review & editing, Supervision, Funding Acquisition, Visualization, Validation, Methodology, Investigation, Data curation, and Conceptualization.

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Data availability

The data will be made available upon request.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Declaration of generative AI and AI-assisted technologies in the manuscript preparation process

During the preparation of this work, the authors used American Journal Experts (AJE Rubriq 2024) for English translation. The authors reviewed and edited the output as needed and take full responsibility for the content of the published article.

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CONCLUSÃO GERAL

Ao longo desta tese, demonstramos que a absorção foliar desempenha um papel importante na manutenção do balanço hídrico mesmo em plantas suculentas, embora sua relação com a expressão do metabolismo CAM seja mais complexa do que inicialmente proposta. Em *V. phaeantha,* a capacidade de FWU apresentou uma variação ao longo do dia, mas não esteve diretamente relacionada com o acúmulo noturno de ácidos orgânicos decorrente da expressão do metabolismo CAM. As maiores taxas de FWU foram observadas no período da manhã, quando as folhas apresentavam estômatos fechados e maior conteúdo hídrico, sugerindo que condições atmosféricas favoráveis, como orvalho e elevada umidade relativa do ar, são os fatores determinantes. Além disso, a presença de homogalacturonanos e ramnogalacturonanos-I na parede celular dos tecidos foliares pode favorecer tanto a movimentação e a retenção de água nos tecidos (essenciais em folhas suculentas) quanto a elasticidade da parede durante as variações diárias do balanço hídrico.

Os resultados obtidos para as espécies de *Cattleya* reforçam como o déficit hídrico reduz a hidratação de folhas suculentas e compromete sua eficiência fotossintética. Em ambas as espécies, a restrição hídrica promoveu aumento na capacidade de FWU, indicando que a absorção foliar de água pode atuar como um mecanismo complementar para atenuar os efeitos da seca. Entretanto, mesmo registrando taxas de FWU, nossos resultados reforçam a ideia de que elevados níveis de suculência limitam este processo. Neste trabalho, foi possível também verificar que mesmo espécies congenéricas apresentam diferente permeabilidade da superfície foliar: em *C. forbesii*, a absorção de água ocorreu por ambas as superfícies foliares, enquanto em *C. guttata* a absorção foi restrita à superfície abaxial.

Em *Puya chilensis*, nossos resultados mostram que o déficit hídrico compromete o conteúdo hídrico foliar, a atividade fotossintética e promove o estresse oxidativo. Entretanto, aumentou a capacidade de FWU, reforçando o papel dessa estratégia em condições de baixa disponibilidade hídrica. Os eventos de neblina contribuíram para a manutenção do balanço hídrico e ajudaram a sustentar a eficiência fotossintética e a reduzir os danos oxidativos causados pelo déficit hídrico. Dessa forma, a neblina constitui uma importante fonte alternativa de água para bromélias que habitam ambientes mediterrâneos, onde sofrem fortes oscilações na disponibilidade de água.

Em síntese, os resultados desta tese demonstram que a absorção foliar de água representa uma estratégia relevante para a manutenção do balanço hídrico e do desempenho fisiológico de plantas suculentas, mesmo que o grau de suculência ofereça resistência a este processo. Embora a FWU não tenha se mostrado diretamente dependente da expressão do metabolismo CAM, sua intensidade é influenciada pelo estado hídrico dos tecidos, pelas características estruturais das folhas e pelas condições ambientais. Assim, esta tese amplia a compreensão dos mecanismos envolvidos no uso da água por orquídeas epífitas e bromélias, destacando a importância da FWU como uma estratégia complementar para enfrentar a elevada variabilidade hídrica característica dos ambientes onde essas espécies ocorrem.