Paula Natália Pereira

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"Prezado Professor,

Sou sobrevivente de um campo de concentração.

Meus olhos viram o que nenhum homem deveria ver. Câmaras de gás construídas por engenheiros formados. Crianças envenenadas por médicos diplomados. Recém-nascidos mortos por enfermeiras treinadas. Mulheres e bebês fuzilados e queimados por graduados de colégios e universidades. Assim, tenho minhas suspeitas sobre a Educação. Meu pedido é: ajude seus alunos a tornarem-se humanos. Seus esforços nunca deverão produzir monstros treinados ou psicopatas hábeis. Ler, escrever e saber aritmética só são importantes para fazer nossas crianças mais humanas."

(Texto encontrado após a 2ª guerra mundial, em um campo de concentração nazista).

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Dedico.

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ÍNDICE GERAL

Introdução Geral

1. O Metabolismo Ácido das Crassuláceas (CAM)

O metabolismo CAM é expresso em aproximadamente 300 gêneros de 24 famílias (Ceusters *et al.*, 2011), sendo encontrado em aproximadamente 50% a 60% das espécies epífitas das famílias Bromeliaceae e Orchidaceae (Larcher, 2006; Silvera & Lasso, 2016). Esse metabolismo auxilia na adaptação dos vegetais a ambientes áridos, já que, comparado ao ciclo de Calvin (C_3), diminui a transpiração em relação à capacidade de assimilação do CO_2 (Kerbauy *et al.*, 2012).

Apesar da vantagem na eficiência do uso da água, plantas CAM apresentam algumas desvantagens. O gasto energético necessário para cada molécula de CO_2 assimilada geralmente é mais elevado em plantas CAM, quando comparado às plantas C_3 (Winter & Smith, 1996). Com relação à fotorrespiração, inicialmente achava-se que as plantas CAM não tinham esse processo. Contudo, recentemente foi descoberto que o efeito da concentração do CO_2 poderia ser contrabalançado pela alta concentração do O_2 produzido pelo ciclo de Calvin no final do período claro (Lüttge, 2011).

O CAM caracteriza-se pelo acúmulo noturno de ácidos orgânicos no vacúolo (Winter & Smith 1996; Lüttge, 2006). O principal ácido acumulado na maioria das espécies CAM é o ácido málico (Lüttge, 2006; Borland *et al.*, 2011). Contudo, em um trabalho realizado com *Tillandsia pohliana*, foi verificado o acúmulo noturno, principalmente, de citrato (Freschi *et al.*, 2010a). O acúmulo desse ácido orgânico é potencialmente mais eficiente do que o de malato, uma vez que a descarboxilação de um mol de citrato produz três moles de CO_2, enquanto a descarboxilação do malato produz apenas um mol de CO_2, por mol de piruvato formado. Dessa forma, o aumento no conteúdo de citrato pode funcionar como um mecanismo suplementar para minimizar a fotorrespiração e a fotoinibição nas plantas CAM

expostas às condições ambientais desfavoráveis (Lüttge, 1988, 2006; Franco *et al.*, 1992, Freschi *et al.*, 2010a).

Muitas plantas podem apresentar ambos os tipos de metabolismo fotossintético (C_3 ou CAM), alternando-os conforme variações nas condições ambientais (Sleslak *et al.*, 2003). A transição entre os tipos de assimilação de CO_2 pode ser induzida por déficit hídrico, aumento no fluxo de fótons fotossinteticamente ativos (FFFA) que atinge as folhas (Maxwell *et al.* 1994; Haslam *et al.*, 2003; Sleslak *et al.*, 2003) ou por variações periódicas de temperatura (Nievola *et al.*, 2005). A mudança para o CAM pode acontecer independentemente entre diferentes regiões da planta. Por exemplo, folhas opostas de um mesmo nó de *Clusia rosea* (Clusiaceae) foram submetidas a diferentes condições de umidade relativa do ar, constatando-se que o CAM foi induzido apenas nas folhas expostas ao ar seco (Schmitt *et al.*, 1998).

Um promissor modelo de estudo para esse tipo de transição metabólica é a espécie *Guzmania monostachia* (Bromeliaceae), uma bromélia epífita que pode alternar, em diferentes condições ambientais, entre os metabolismos C_3 e CAM (Freschi *et al.*, 2010b; Pereira *et al.*, 2013). Resultados recentes obtidos em nosso laboratório mostraram diferenças significativas quanto à acidez noturna, atividade da fosfoenolpiruvato carboxilase (PEPC) e da enzima malato desidrogenase (MDH) nas porções basal, mediana e apical das folhas de *Guzmania monostachia*. As maiores atividades das enzimas PEPC e MDH e de acidez foram observadas na porção apical, seguida da porção mediana das folhas dessa espécie quando submetidas ao déficit hídrico (Freschi *et al.*, 2010b). Esses dados indicaram que, provavelmente, a porção foliar apical dessa bromélia mantida sob deficiência hídrica apresentou maior expressão do CAM, quando comparada com a mesma porção foliar das plantas hidratadas. Por outro lado, a porção basal das plantas mantidas sob déficit hídrico ou hidratadas mostrou atividade fotossintética mais próxima da via C_3, caracterizada pelo pouco acúmulo noturno de ácidos orgânicos e pela reduzida atividade das enzimas PEPC e MDH.

2) Transporte de prótons e acúmulo de ácidos orgânicos no interior do vacúolo

Plantas CAM são caracterizadas pelo acúmulo noturno de ácidos orgânicos, principalmente malato, no interior do vacúolo (Cushman & Borland, 2002). A força próton-motriz para que ocorra o transporte de ácidos orgânicos no interior do vacúolo pode ser gerada por duas enzimas no tonoplasto, ATPase (ATP) e/ou pirofosfatase inorgânica (PPi) que bombeiam prótons (H^+) para o interior do vacúolo (Marin, 1987; Rea & Sanders, 1987). O potencial elétrico negativo no citosol e a diferença de pH transmembrana gerado pelo bombeamento de H^+ no vacúolo tendem a promover o transporte de ânions, como malato, citrato e fumarato, ocasionando o acúmulo de ácidos orgânicos no interior do vacúolo, característico de plantas que realizam a fotossíntese CAM (White & Smith, 1989; Cheffings *et al.*, 1997).

Estudos realizados com plantas de *Kalanchoë daigremontiana* (Crassulaceae), uma espécie CAM constitutiva, mostraram as maiores taxas de transporte de prótons através do tonoplasto na presença de malato (White & Smith, 1989). Nesse mesmo estudo, foi verificado que não havia uma preferência no transporte de H^+ pelo ATP ou PPi. Apesar de muitos estudos verificarem o transporte de prótons dependente de ATP ou PPi em espécies de *Kalanchoë*, apenas um trabalho mostrou o transporte de açucares e prótons em *Ananas comosus* (McRae *et al.*, 2002) e nenhum trabalho tem mostrado o transporte de ácidos orgânicos e prótons em bromélias. Será que assim como observado em *Kalanchoë*, bromélias CAM não apresentariam uma preferência por ATP ou PPi (**Capítulo 1**)? Será que o transporte de prótons no vacúolo estaria associado com o maior acúmulo de malato e consequentemente a maior expressão do CAM (**Capítulo 1**)? Em um trabalho desenvolvido com plantas de *Mesembryanthemum crystallinum* foi observado um aumento de três vezes nas taxas de transporte de prótons dependente de ATP quando as plantas foram submetidas ao estresse salino (induzidas ao CAM), comparado com as plantas C_3 (Lüttge *et*

al., 2000). O transporte de H^+ dependente de ATP também foi verificado em plantas de *Nicotiana tabacum* (Solanaceae), uma espécie C_3, cultivadas sob diferentes fontes de nitrogênio inorgânico (NH_4^+ ou NO_3^-). Maiores taxas de transporte de prótons e malato no interior do vacúolo e na presença de ATP foram verificadas nas plantas mantidas na presença de nitrato comparado àquelas cultivadas em amônio (Lüttge *et al.*, 2000).

Apesar de inúmeros trabalhos na literatura descreverem os transportes de prótons e ácidos orgânicos no interior do vacúolo em plantas CAM e C_3, nenhum trabalho demonstrou ainda a influência de diferentes fontes de nitrogênio no transporte de H^+ e ácidos orgânicos através do tonoplasto em plantas CAM constitutivas e facultativas. Será que o transporte de prótons no vacúolo e a expressão de transportadores vacuolar de malato e fumarato (ALMT9) seria influenciada por diferentes fontes inorgânicas de nitrogênio e pela concentração dessas fontes(**Capítulos 2 e 3**)? Como seria o transporte de prótons e ácidos orgânicos no vacúolo e a preferência por ATP ou PPi em plantas CAM constitutivas e facultativas das famílias Bromeliaceae e Crassulaceae (**Capítulos 2 e 3**)?

3) Nutrição e indução ao CAM

Apesar de muitos trabalhos terem mostrado a indução do CAM por diferentes condições ambientais como, disponibilidade de água, intensidade luminosa e fotoperíodo (Maxwell *et al.*, 1994; Nievola *et al.*, 2005; Pereira *et al.*, 2013), poucos estudos têm mostrado a influência da deficiência nutricional na expressão desse metabolismo fotossintético (Ota 1988; Winter &Holtum, 2011; Rodrigues *et al.*, 2014). Winter & Holtum (2011) verificaram que o fornecimento de nutrientes afeta o balanço entre as vias fotossínteticas C_3 e CAM em *Calandrinia polyandra* (Montiaceae). Quando mantida sob reduzida disponibilidade de água ou nutrientes, foi observada a mudança na fixação diurna do CO_2 para a fixação noturna nessa herbácea. A reversão da fotossíntese CAM para C_3 foi

possível após a reidratação ou adição de solução nutritiva no solo dessa espécie (Winter & Holtum, 2011).

Ota (1988) verificou que plantas de *Kalanchoe blossfeldiana* (Crassulaceae) mantidas em solução nutritiva com presença de nitrato (1 mM) apresentaram um aumento no conteúdo de malato e no acúmulo noturno de acidez, quando comparadas com plantas mantidas em solução com presença de amônio (1 mM). Esses resultados sugerem que a ausência do nitrato tem maior influência na indução ao CAM nessa espécie do que a ausência de amônio. Será que outras espécies CAM constitutivas dessa família teriam esse metabolismo fotossintético influenciado por diferentes concentrações dessas fontes inorgânicas de nitrogênio (**Capítulo 2**)?

Embora alguns trabalhos tenham estudado a correlação entre CAM e nutrição, apenas um estudo conduzido em folhas destacadas da bromélia epífita com tanque, *Guzmania monostachia*, mostrou a influência da deficiência de cada macronutriente (N, P ou K) na indução do CAM (Rodrigues *et al.*, 2014). Nesse estudo foi verificado que a ausência de nitrogênio promoveu um aumento de duas vezes na atividade das enzimas PEPC e MDH na porção apical das folhas de *Guzmania monostachia* comparado à ausência de P ou K (Rodrigues *et al.*, 2014). Em relação aos estudos mais recentes realizados com a bromélia epífita *G. monostachia*, ainda não se sabe como as fontes de nitrogênio regulam a expressão do CAM. A regulação desse metabolismo em *G. monostachia* seria bioquímica e molecular (**Capítulo 3**)?

4) Aquaporinas e CAM

As aquaporinas (AQPs) são proteínas responsáveis pelo transporte de água, pequenos solutos e gases através das membranas (Maurel, 2007). Elas pertencem à família das proteínas intrínsecas de membrana (MIPs) e se subdividem em cinco subfamílias,

denominadas PIPs (Plasma membrane Intrinsic Proteins), TIPs (Tonoplast Intrinsic Proteins), NIPs (Nodulin26-like Intrinsic Proteins), SIPs (Small basic Intrinsic Proteins) e XIPs (X Intrinsic Proteins) (Johanson *et al.,* 2001; Danielson & Johanson, 2008).

Variações de condutividade hidráulica em raízes (Lpr) têm sido associadas às alterações de expressão de genes de aquaporinas nesse órgão. Variações diuturnas de condutividade são acompanhadas por variações no nível de transcritos de aquaporinas do tipo PIP em raízes de milho e ervilha (Lopez *et al.,* 2003, Beaudette *et al.,* 2007). Nessas espécies, a expressão de aquaporinas se mostrou dependente do tipo de raiz (primária ou secundária) (Beaudette *et al.,* 2007) e do estágio de desenvolvimento das mesmas, sendo observado em geral um aumento de expressão na zona de alongamento celular e nas regiões mais maduras da raiz primária (Hachez *et al.,* 2006).

Também em folhas, a expressão de diferentes aquaporinas obedece a um padrão de regulação temporal e espacial. Enquanto algumas isoformas são expressas nas folhas jovens em expansão, outras são expressas preferencialmente em folhas completamente expandidas, sendo que as aquaporinas expressas nas folhas maduras estão provavelmente envolvidas em processos fisiológicos como o carregamento do floema, perda de água do xilema, abertura e fechamento estomático, transporte de CO_2 na fotossíntese e movimento foliar (Heinen *et al.,* 2009).Na leguminosa *Samanea saman* (Mimosaceae), o acúmulo de transcritos do gene *Ss*AQP2 (PIP) no pulvino foi maior no início da fase iluminada, coincidente com o aumento na permeabilidade da água em células motoras (Moshelion *et al.*, 2002).

Em *Mesembryanthemum crystallinum*, mudanças na permeabilidade à água de protoplastos isolados de folhas e raízes durante o ciclo diuturno coincidiram com flutuações na abundância de transcritos de três PIPs: *Mc*PIP1;4, *Mc*PIP1;5 e *Mc*PIP2;1 e uma TIP: *Mc*TIP1;2 em plantas CAM adultas (Vera-Estrella *et al.* 2012). No entanto, variações similares não foram observadas em plantas C_3 jovens. De forma interessante, oscilações

diuturnas na atividade de PEPC, no acúmulo de pinitol e na osmolaridade da seiva foram observadas somente nas plantas CAM sendo que esses parâmetros permaneceram inalterados nas plantas C_3. Essas mudanças, em períodos distintos do ciclo CAM, ressaltam a necessidade do controle do potencial osmótico em compartimentos celulares específicos concomitante com a regulação metabólica e do fluxo de água na planta. (Vera-Estrella *et al.*, 2012). De forma similar, não foram verificadas variações evidentes nos níveis de transcritos de *VgPIP1,5* e *VgTIP2* em função do regime luminoso, no ápice e na base das folhas de *Vriesea gigantea*, uma bromélia com metabolismo C_3. A ausência de um padrão nítido de expressão dia/noite sugere que esses genes sejam expressos de forma quase contínua, principalmente nas bases foliares, maximizando a absorção de nutrientes, cuja disponibilidade é imprevisível e sujeita à rápida degradação e/ou utilização por outros organismos habitantes do tanque (Cambuí, 2009).

Recentemente foi feito o transcriptoma da bromélia epífita com tanque, *G. monostachia*, que abriu uma ampla gama de possibilidades para o estudo da regulação gênica nessa espécie (dados não publicados). Nesse sentido, será que os genes que codificam aquaporinas nessa espécie seriam regulados pelo horário do dia (início e final do dia) (**Capítulo 4**)? Será que haveria uma regulação desses genes pelo horário do dia, estresse hídrico e fontes inorgânicas de nitrogênio nas porções basal, mediana e apical das folhas de *G. monostachia* que expressam diferentes graus de intensidade do CAM (**Capítulo 4**)?

Objetivos Gerais

Investigar o transporte de prótons e ácidos orgânicos e acúmulo noturno de malato e citrato no interior do vacúolo nas folhas de alguns representantes CAM das famílias Bromeliaceae e Crassulaceae submetidos a diferentes fontes inorgânicas de nitrogênio associadas ou não ao déficit hídrico.

Avaliar a expressão de genes que codificam aquaporinas nas porções basal e apical de folhas de *Guzmania monostachia* submetidas a diferentes fontes de nitrogênio associadas ou não ao déficit hídrico.

1. Objetivos específicos de cada capítulo

Capítulo 1:

Investigar o transporte de prótons e ânions, como malato, fumarato e citrato, dependente de ATP ou PPi no interior do vacúolo e o acúmulo noturno de malato e citrato em seis espécies de bromélias CAM constitutivas e uma C_3.

Capítulo 2:

Determinar o acúmulo noturno de ácidos orgânicos, malato, fumarato e citrato e o transporte de H^+ e ânions, malato, citrato e fumarato, no interior do vacúolo dependente de ATP ou PPi em duas espécies de *Kalanchoë*, *Kalanchoë tubiflora* e *Kalanchoë laxiflora*, mantidas sob diferentes fontes de nitrogênio inorgânico (NH_4^+ e/ou NO_3^-) e diferentes concentrações dessas fontes (2.5 ou 5.0 mM).

Capítulo 3:

Investigar a influência da presença e/ou ausência de fontes inorgânicas de nitrogênio (NH_4^+ e/ou NO_3^-) associada ou não à deficiência hídrica (PEG 6000 30%), sobre a expressão do CAM, transporte de prótons e ânions no interior do vacúolo, expressão do gene ALMT9 que codifica o transportador de malato dependente de alumínio 9, atividade das principais enzimas antioxidantes (catalase, ascorbato peroxidase, superóxido dismutase e glutationa redutase) e quantificação de ácucares solúveis, glicose, frutose e

sacarose, na porção apical das folhas pertencentes ao 8-12^{o} nós de *Guzmania monostachia*.

Capítulo 4:

Verificar a expressão de alguns genes que codificam aquaporinas (PIPs, TIPs, NIPs e SIPs) nas porções basal, mediana e apical de folhas pertencentes ao 8-12^{o} nós de *Guzmania monostachia* mantidas sob diferentes fontes de nitrogênio (NH_4^+ ou NO_3^-) associadas ou não à deficiência hídrica (PEG 6000 30%).

2. Estratégias de estudo

1) Avaliar o transporte de prótons dependente de ATP ou PPi e o acúmulo noturno de malato e citrato em folhas de bromélias classificadas como CAM constitutivas, CAM facultativas ou C_3 e em folhas de duas espécies CAM constitutivas de *Kalanchoë*.

2) Analisar o grau de expressão do CAM por meio de ensaio da atividade das enzimas PEPC e MDH e da dosagem da variação de acidez (acúmulo noturno de ácido málico e cítrico) na porção apical de folhas de *G. monostachia* após o tratamento de deficiências nutricionais (NH_4^+ e/ou NO_3^-) associadas ou não à deficiência hídrica por sete dias.

3) Determinar a atividade das enzimas antioxidantes e o acúmulo de açucares solúveis na porção apical de folhas de *Guzmania monostachia* submetidas à deficiência de diferentes fontes de nitrogênio inorgânico (NH_4^+ e/ou NO_3^-) associada ou não à deficiência hídrica por sete dias.

4) Analisar os níveis de transcritos do ALMT9 na porção apical das folhas de *G. monostachia* após o tratamento de deficiências nutricionais (NH_4^+ e/ou NO_3^-) associadas ou não à deficiência hídrica por sete dias.

5) Analisar os níveis de transcritos de AQPs (PIP, TIP, NIP e SIP) nas diferentes porções foliares (basal e apical) de *G. monostachia* após o tratamento com diferentes fontes de nitrogênio (NH_4^+ ou NO_3^-) associadas ou não à deficiência hídrica por sete dias.

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Proton transport across the tonoplast vesicles in six bromeliad species

Abstract

Crassulacean acid metabolism (CAM) is one of the key innovations in the family Bromeliaceae. Numerous studies have been performed to check the induction of this photosynthesis based on nocturnal organic acids accumulation into the vacuole. However, the interrelationship among CAM, proton and organic acids transport in tonoplast vesicles has not been studied in any member from this family. The present study has verified the ATP- and PPi-dependent proton transport rates in the presence of three anions, fumarate, malate and citrate in six CAM and one C_3 bromeliad species used for comparison. The greater activity of the ATP-driven H^+ pump at the tonoplast compared with the PPi-driven H^+ pump is a consistent feature of vacuoles in the different species of bromeliads tested. The average of ATP- and PPi-dependent proton transport in the presence of fumarate across the six CAM species was 2040 and 414% min^{-1} mg $protein^{-1}$, respectively. For all six CAM species studied, the ATP-dependent proton transport presented the following order of effectiveness: fumarate > malate > citrate. The average ratio of nocturnal malate accumulation to citrate levels was 5 times higher, which correlates with the quinacrine fluorescence-quenching which showed that for all CAM bromeliad species that the permeability of the tonoplast membrane for malate appears to be much higher than its permeability for citrate. Finally, the results showed the highest degree of CAM photosynthesis in *B. pyramidalis* while *T. usneoides* presented the lowest compared with the other CAM bromeliad species used in this study.

Keywords: bromeliad, crassulacean acid metabolism, organic acids, proton transport, tonoplast.

Abbreviations: ATP, adenosine 5′-triphosphate; CAM, crassulacean acid metabolism; DW, dry weight; PEPC, phospho*enol*pyruvate carboxylase; PPi, inorganic pyrophosphate.

Introduction

The family Bromeliaceae represents a highly diverse group of vascular plants containing over 3000 species. Members of this family exhibit some innovations associated with their wide variety of habitats (Benzing 1980, 2000; Smith *et al.* 1986; Smith 1989; Crayn *et al.* 2015). One key innovation is the epidermal trichomes which are responsible for absorbing water and nutrients through the leaf surface in epiphytic species (Mez 1904; Smith and Till 1998). Another is the presence of a 'tank' structure (phytotelm) formed by the overlapping basal portions of the rosulate leaves. This structure is important because it collects the detritus and water that is ultimately absorbed by the epidermal trichomes, which are present at higher density towards the basal compared with apical portion of the leaves, and which in the more extreme 'atmospheric' epiphytic forms in this family cover almost the entire shoot surface. The epidermal trichome and the tank may have been two of the key adaptations that aided the evolution of the epiphytic life-forms in Bromeliaceae (Benzing 1980, 2000; Smith 1989; Givnish *et al.* 1997). CAM photosynthesis is another physiological innovation found in this family (Coutinho 1963; Medina 1974; Crayn *et al.* 2004). This photosynthetic metabolism is characterized by nocturnal CO_2 assimilation via the enzyme phospho*enol*pyruvate carboxylase (PEPC), which represents a water-conserving photosynthetic pathway since it allows stomata to remain closed for much of the daytime (Kluge and Ting 1978; Winter and Smith 1996a; Silvera and Lasso 2016). In a recent survey of the Bromeliaceae encompassing nearly two-thirds of the family, Crayn *et al.* (2015) found that 43% of the total species sampled showed carbon-isotope ratios indicative of obligate CAM photosynthesis, and discussed the distribution of CAM among the eight constituent subfamilies. From 792 sampled species in subfamily Tillandsioideae, only 28% showed $\delta^{13}C$ values indicative of obligate CAM, all in the genus *Tillandsia*, with the remaining genera, including *Vriesea*, *Werauhia*, *Catopsis*, *Alcantarea* and *Racinaea*, showing C_3

photosynthesis. In contrast, in subfamily Bromelioideae, the majority of genera (including 90% of the 499 sampled species) exhibited the CAM pathway, with only a few genera, namely *Fernseea*, *Fascicularia*, *Greigia*, *Lapanthus* and *Ochagavia*, showing C_3 photosynthesis (Crayn *et al.* 2015).

Over the years, numerous physiological and biochemical studies have been conducted on the terrestrial CAM bromeliad *Ananas comosus*, the pineapple, which is one of the best-known species in the family, because of its economic importance (Neales *et al.* 1968; Martin 1994; Ming *et al.* 2015). ATP-dependent transport of soluble sugars in tonoplast vesicles in *Ananas comosus* is well understood, however little has been studied about organic acids ATP- or PPi-dependent transport across the vacuolar membrane in this species or other members from the family Bromeliaceae (McRae *et al.* 2002). White and Smith (1989) found in *Kalanchoë daigremontiana*, a constitutive CAM plant, that the highest rates of ATP- or PPi-dependent H^+ transport across the vacuolar membrane could be observed in the presence of malate and certain other four-carbon dicarboxylate anions. This was subsequently shown to be attributable to a distinctive inward-rectifying anion channel (Hafke *et al.* 2003), which seems to be an inherent feature of the vacuolar membranes of CAM plants. Lüttge *et al.* (2000) studied the inducible CAM plant *Mesembryanthemum crystallinum* and showed that rates of ATP-dependent H^+ transport at the vacuolar membrane were 3 times higher than in plants in the CAM mode compared with the C_3 mode. In another study on *M. crystallinum*, CAM induction by salt stress appeared to be associated with increased permeability of the tonoplast membrane to malate (Struve and Lüttge 1987).

Although studies have been done on proton transport and the permeability of vacuolar membranes by malate and fumarate in CAM constitutive plants, no studies have been conducted to check this transport within different CAM species from the same family or even from the same subfamily. In addition, no studies have been performed to evaluate the

difference in the vacuolar transport between CAM plants kept in the same growth conditions. Based on this scenario, this study aims to evaluate the ATP-driven H^+ pump at the tonoplast compared with the PPi-driven H^+ pump, and measure the organic acids, malate and citrate, accumulated during the night in six bromeliad species, all of which perform CAM, from the subfamilies Bromelioideae or Tillandsioideae (Fig. 1). All results were obtained through a combination of biochemical approaches. This study also examines the possible correlation between malate and citrate's nocturnal accumulation, their transport across the tonoplast and the degree of CAM expression among bromeliad species kept in the same environmental conditions. Finally, this study evaluates each bromeliad species' preference for either ATPase or PPiase to the proton transport into their respective vesicles, as well as the order of effectiveness of fumarate, malate and citrate in all species.

Material and Methods

Plant material and growth conditions

Adult plants of *Aechmea nudicaulis* (L.) Griseb., *Ananas comosus* (L.) Merr. var. *ananassoides*, *Billbergia pyramidalis* (Sims) Lindl., *Nidularium billbergioides* (Schult. & Schult.f.) L.B.Sm., *Tillandsia pohliana* Mez, *Tillandsia usneoides* (L.) L. and *Vriesea sucrei* L.B.Sm & Read (used for comparison as a species that performs C_3 photosynthesis) were collected in nature, and were then transferred to a controlled environment growth chamber, under a photosynthetic flux density (PFD) of about 200 µmol m^{-2} s^{-1} of photosynthetic active radiation to the top leaf surfaces of the bromeliad species, a 12 h photoperiod, a day/night air temperature of 25/20 ºC, and a day/night relative humidity of 60/70%. After all plants were collected from the Institute of Botany of São Paulo, they were then cultivated in pots containing fine sand, with one plant per pot. Over 10 days of acclimation, all plants were watered with distilled water on a daily basis. After this period, about 8 to 12 fully developed

leaves, or stem internodes in the case of *Tillandsia usneoides*, were collected for the biochemical assays.

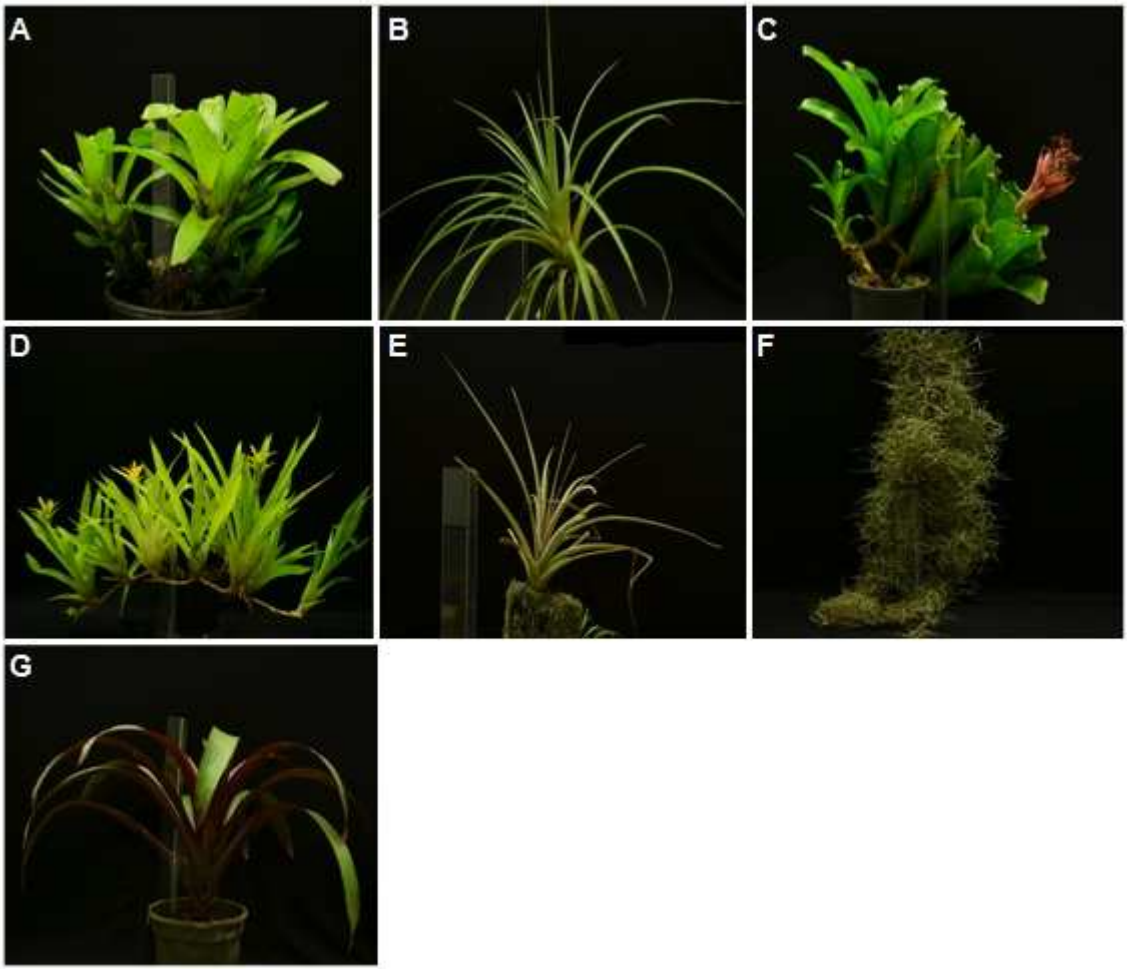

Fig. 1 The seven bromeliad species used in the experiments. (A) *Aechmea nudicaulis*, (B) *Ananas comosus*, (C) *Billbergia pyramidalis*, (D) *Nidularium billbergioides*, (E) *Tillandsia pohliana*, (F) *Tillandsia usneoides*, (G) *Vriesea sucrei*.

Tonoplast Isolation

Tissue was harvested from plants in the controlled environment chambers 1.0 to 1.5 h after commencement of the light period. Tonoplast fractions from the mesophyll tissue were isolated according to the method of White & Smith (1989) and McRae *et al.* (2002) with minor modifications. The leaf tips and margins were removed using a razor blade and sections of leaf lamina or stem totaling approximately 80 g fresh mass were suspended in 250

mL of ice-cold extraction buffer containing the following: 450 mM mannitol, 3.0 mM $MgSO_4$, 2.0 mM ethylenediaminetetraacetic acid disodium salt (EDTA), 10 mM DL-dithiothreitol (DTT), 1.0% (w/v) polyvinylpyrrolidone (PVP-40), 0.5% (w/v) bovine serum albumin, 100 mM tris(hydroxymethyl)aminomethane (Trizma® base, adjusted to pH 8.0 with HCl), 1mM phenylmethysulphonyl fluoride, 1.1 M glycerol, 0.5 mM 3,5-di-*tert*-4-butylhydroxytoluene, 25.19 mM potassium disulfite and 1.0 mM benzamidine hydrochloride. After precooling, the tissue was homogenized in a commercial blender and the homogenate filtered through two layers of cheesecloth and then centrifuged at 18000 × *g* for 20 min. The resulting supernatant was centrifuged at 80000 × *g* for 60 min. The resulting pellet was layered over a 25% (w/v) sucrose cushion containing 1.1 M glycerol, 1.0 mM EDTA, 10 mM Tricine (*N*-[tris(hydroxymethyl)methyl]glycine), adjusted to pH 8.0 with BTP (1,3-bis[tris(hydroxymethyl)methylamino]propane), and 2.0 mM DTT. The gradients were centrifuged at 100 000 × *g* for 70 min, after which tonoplast vesicles were removed from the interface using a Pasteur pipette. Vesicles were then pelleted at 100 000 × *g* for 50 min and finally resuspended in the same buffer as the first pellet. All steps were performed at 4°C. Preparations were stored at −80°C until required.

Measurement of vesicle acidification

Rates of intravesicular acidification on energization of the tonoplast H^+-ATPase or H^+-PPiase were determined according to the method described by White & Smith (1989) with minor modification. Initial rates of H^+ transport at 25ºC were determined from the initial rates of fluorescence quenching upon the addition of 3.0 mM Tris-ATP or 500 mM Na_4PPi to the reaction medium. For assays of ATP-dependent H^+ transport, the reaction medium contained approx. 2–9 µg protein, 3.0 µM quinacrine (6-chloro-9-{[4-(diethylamino)-1-methylbutyl]amino}-2-methoxyacridine dihydrochloride), 6.0 mM $MgSO_4$, 0.3 mM EDTA,

150 mM mannitol and 25 mM BTP buffered to pH 8.0 with Mes (2-(*N*-morpholino)ethansulphonic acid). For measurements of PPi-dependent H^+ transport, the reaction medium was identical except that the $MgSO_4$ concentration was increased to 7.5 mM and the medium also contained 100 mM K-Mes, permeant anions to be tested were present at 50 mM (supplied as fumaric acid, malic acid, or citric acid, and buffered to pH 8.0 with BTP). Inhibitors tested were 50 mM potassium nitrate (inhibitor of vacuolar H^+-ATPase), 100 µM sodium orthovanadate (inhibitor of plasma membrane H^+-ATPase), 100 µM sodium azide (inhibitor of mitochondrial ATP synthase), and 2.0 mM ammonium sulphate (uncoupler of transmembrane pH gradients). Fluorescence quenching was measured using a model LS-55 luminescence spectrometer (Llantrisant, UK) with excitation at 422 nm and emission at 495 nm, both with a slit width of 5 nm.

Protein Determination

Protein concentration was measured according to Bradford (1976), using bovine serum albumin as the standard.

Organic Acid Quantification

Malate and citrate in tissue extracts were determined by HPLC in a chromatographic system (Hewlett-Packard®, series 110, Waldbronn, Germany) equipped with a Supelcogel C-610H (30 cm × 7.8 mm) column (at 30ºC) and a diode-array detector (210 nm, for acid analysis) according to the method described by Amorós *et al.* (2003) and Pereira *et al.* (2013).

Statistical Analysis

All data are presented as mean values ± standard deviation (SD). Significant differences among the distinct bromeliad species or treatments were determined using the Tukey–Kramer test as $P < 0.05$. Differences between treatments with two inhibitors in the same species were evaluated by using Student's *t*-test at $P < 0.05$.

Results

Proton transport rates in the presence of inhibitor of vacuolar or non-vacuolar membranes

Initially, the proton transport rates were checked for their % quinacrine fluorescence-quenching (F) min^{-1} mg $protein^{-1}$ in the presence of a specific inhibitor of non-vacuolar membranes (non V-ATPases) (NaN_3 plus Na_3VO_4) or in the presence of a specific inhibitor of vacuolar membranes (V-ATPases) (KNO_3), in order to determine the relative contribution of both types of membranes to the ATP-dependent H^+ transport rates in the seven bromeliad species. As we can see in table 1, all of the species showed a significantly higher inhibition in the presence of KNO_3 rather than in the presence of NaN_3 plus Na_3VO_4. *A. comosus*, *T. usneoides* and *V.sucrei* showed about 3 times higher inhibition by KNO_3 than by NaN_3+Na_3VO_4. *A. nudicaulis*, *N. billbergioides* and *T. pohliana* displayed a decrease in proton transport rates by KNO_3, about twice that of NaN_3+Na_3VO_4, and in *B. pyramidalis* this inhibition was 10 times higher (Table 1).

Table 1 ATP-dependent proton transport in the presence of fumarate and a specific inhibitor of vacuolar membranes (KNO_3) or non-vacuolar membranes (NaN_3 plus Na_3VO_4) or no-inhibitors (control), measured as initial rates of quinacrine fluorescence-quenching, in the tonoplast vesicles of *Aechmea nudicaulis, Ananas comosus, Billbergia pyramidalis, Nidularium billbergioides, Tillandsia pohliana, Tillandsia usneoides* and *Vriesea sucrei*. Results are expressed as the mean (±SD) for three preparations. The rates of proton transport

were measured in % H^+ min^{-1} mg $protein^{-1}$ and % of inhibition. Different capital letters indicate an average that is significantly different among the treatments in the same species (Tukey–Kramer test; $P < 0.05$). Asterisks indicate significant differences between inhibitors in the same species by Student's t-test at 5% significance.

	Specific activity (% min^{-1} mg $protein^{-1}$) (inhibition relative to control)		
Species	**Control**	**+ KNO_3**	**NaN_3 + Na_4VO_3**
Aechmea nudicaulis	1074 ± 2 A	604 ± 8 C (44 %) *	829 ± 9 B (23 %)
Ananas comosus	627 ± 6 A	140 ± 7 C (78 %) *	463 ± 9 B (26 %)
Billbergia pyramidalis	1200 ± 1 A	675 ± 4 C (44%) *	1146 ± 3 B (5%)
Nidularium billbergioides	546 ± 5 A	291 ±7 C (47%) *	431 ± 9 B (21%)
Tillandsia pohliana	1045 ± 9 A	612 ± 3 C (42%) *	788 ± 3 B (25%)
Tillandsia usneoides	432 ± 1 A	114 ± 3 C (74%) *	310 ± 3 B (28%)
Vriesea sucrei	927 ± 6 A	116 ± 16 C (88%) *	681 ± 8 B (27%)

ATP-dependent proton transport rates

After checking for the presence of mostly V-ATPases in the extracted vesicles, more experiments were conducted in order to verify the degree to which these bromeliad species exhibited ATP- and PPi-dependent proton transport into the vacuole.

For all of the species, the highest %F in the presence of three anions ($fumarate^{-2}$, $malate^{-2}$ and $citrate^{-3}$) and ATP or PPi was calculated based on the proton and anion transport rates (Supplementary Figures 1 and 2). The recovery of quinacrine fluorescence-quenching to the level observed before the addition of ATP occurred after adding 2mM NH_4^+ ions (after 400-800 s, depending on the species) into the cuvette, proving the integrity of the tonoplast vesicles extracted in this study, this successfully restored the pH to the same level it was at before the addition of ATP (Supplementary Figure 1).

In all of the experiments, fumarate supported the highest rates of vesicle acidification in the CAM bromeliads (Table 2). *B. pyramidalis* presented the highest % H^+ transported while in the presence of fumarate (about 3000% min^{-1} mg $protein^{-1}$, subtracting the control), followed *by A. comosus, A. nudicaulis*, *N. billbergioides, T. pohliana* and *T. usneoides* (Table 2). For *B. pyramidalis*, the ATP-dependent proton transport rates in the presence of fumarate were about 13 times higher than that of *T. usneoides* (Table 2). In the presence of malate, the highest proton transport rates were observed in *B. pyramidalis*; while the lowest rates, about 10 times lower than *B. pyramidalis*, were seen in *T. usneoides* (Table 2). Thereby, the order of effectiveness (not considering the control) was fumarate > malate > citrate in the six CAM species (Table 2).

Table 2 ATP-dependent proton transport, measured as initial rates of quinacrine fluorescence-quenching in the control (no anion), presence of fumarate, malate and citrate (present as their BTP-salts at 50 mM) in the tonoplast vesicles extracted from leaves of *Aechmea nudicaulis*, *Ananas comosus*, *Billbergia pyramidalis*, *Nidularium billbergioides*, *Tillandsia pohliana*, *Tillandsia usneoides* and *Vriesea sucrei*. Results are expressed as the mean (±SD) and are relative to rates of proton transport measured in % H^+ min^{-1} mg $protein^{-1}$. Different capital letters indicate averages that are significantly different among species that use the same anion (Tukey–Kramer test; $P < 0.05$). Different lower case letters indicate averages that are significantly different among anions in the same species (Tukey–Kramer test; $P < 0.05$).

	Specific activity (% min^{-1} mg $protein^{-1}$)			
Species	**Fumarate**	**Malate**	**Citrate**	**Control**
Aechmea nudicaulis	2235 ± 19 C a	512 ± 19 B b	116 ± 10 B c	465 ±15 B b
Ananas comosus	2573 ± 33 B a	503 ± 3 B b	37 ± 1 C d	112 ± 1 E c
Billbergia pyramidalis	3542 ± 1 A a	632 ± 33 A b	208 ± 17 A d	577 ± 4 A c
Nidularium billbergioides	1983 ± 9 D a	339 ± 8 D b	44 ± 8 C d	266 ± 9 D c
Tillandsia pohliana	1635 ± 22 E a	444 ± 6 C b	114 ± 12 B d	336 ± 27 C c
Tillandsia usneoides	271 ± 10 F a	65 ± 3 E b	33 ± 2 C c	54 ± 9 F bc
Vriesea sucrei	89 ± 4 G a	25 ± 3 F c	46 ± 4 C b	27 ± 2 F c

PPi-dependent proton transport rates

Different from ATP-dependent proton transport which exhibited the same pattern of effectiveness for all of the CAM bromeliad species, PPi-dependent H^+ transport did not present any noticeable pattern. For *B. pyramidalis*, the highest rates of PPi-dependent vesicle acidification was observed in the presence of fumarate (Table 3). *A. nudicaulis and N. billbergioides* presented the highest rates of proton transport while in the presence of either malate or fumarate (Table 3). *T. usneoides* showed a higher rate of vacuole acidification in the presence of citrate, when compared with the other two anions (Table 3). Thereby, the order of effectiveness was fumarate = malate > citrate for *A. nudicaulis* and *N. billbergioides*, fumarate > malate>citrate for *A. comosus* and *B. pyramidalis*, fumarate > citrate > malate for *T. pohliana* and citrate > fumarate = malate for *T. usneoides*. Despite the fact that fumarate supported the highest rates of vesicle acidification in most of the CAM bromeliad species studied, these rates were higher in ATP-dependent rather than in PPi-dependent proton transport (Tables 2 and 3).

Table 3 PPi-dependent proton transport, measured as initial rates of quinacrine fluorescence-quenching in the control (no anion), presence of fumarate, malate and citrate (present as their

BTP-salts at 50 mM) in the tonoplast vesicles extracted from leaves of *Aechmea nudicaulis*, *Ananas comosus*, *Billbergia pyramidalis*, *Nidularium billbergioides*, *Tillandsia pohliana*, *Tillandsia usneoides* and *Vriesea sucrei*. Results are expressed as the mean (±SD) and are relative to rates of proton transport measured in % H^+ min^{-1} mg $protein^{-1}$. Different capital letters indicate averages that are significantly different among species that use the same anion (Tukey–Kramer test; $P < 0.05$). Different lower case letters indicate averages that are significantly different among anions in the same species (Tukey–Kramer test; $P < 0.05$).

	Specific activity (% min^{-1} mg $protein^{-1}$)			
Species	**Fumarate**	**Malate**	**Citrate**	**Control**
Aechmea nudicaulis	627 ± 4 B a	668 ± 38 B a	99 ± 6 A c	194 ± 20 B b
Ananas comosus	266 ± 0.5 D a	197 ± 1 D b	37 ± 2 C d	49 ± 1 C c
Billbergia pyramidalis	848 ± 19 A a	729 ± 10 A b	3 ± 0.2 D d	327 ±18 A c
Nidularium billbergioides	383 ± 8 C a	407 ± 8 C a	1 ± 0.2 D c	194 ± 8 B b
Tillandsia pohliana	345 ± 14 C a	48 ± 5 EF bc	73 ± 4 B b	39 ± 3 C c
Tillandsia usneoides	13 ± 1 E c	13 ± 2 F c	31 ± 3 C b	49 ± 2 C a
Vriesea sucrei	25 ± 3 E b	77 ± 6 E a	26 ± 1 C b	21 ± 3 C b

Nocturnal malate and citrate accumulation

Levels of malate and citrate, accumulated during the night, were measured in the leaves (and also internodes of *T.usneoides*) of these seven bromeliad species (Fig. 2). All of the species used in this study showed a higher accumulation of malate compared with citrate, and higher levels of ATP-dependent proton transport in the presence of malate rather than the citrate anion (Fig. 2 and Table 2).

As shown in Fig. 2A, malate was the main organic acid accumulated during the nocturnal period for all CAM bromeliads species. *B. pyramidalis* showed the highest nocturnal malate content followed by *A. nudicaulis*, *A. comosus* and *N. billbergioides* (Fig

2A). *A. comosus* and *N. billbergioides*, followed by *B. pyramidalis* were the species that showed the highest citrate accumulation during the dark period (Fig. 2B). As expected, *V. sucrei*, a C_3 species, did not accumulate any citrate and a small amount of diurnal malate storage was observed (Fig. 2).

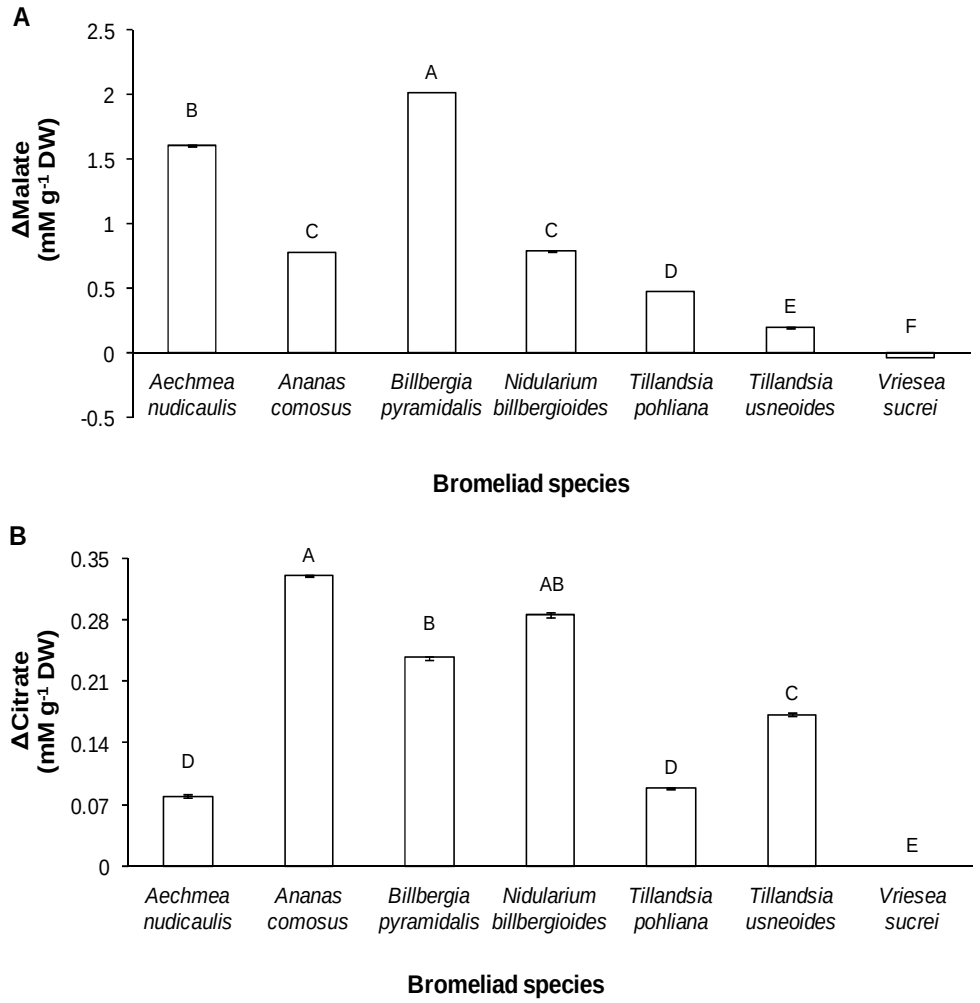

Fig.2 Nocturnal malate (A) and citrate (B) accumulation in the leaves of seven bromeliad species. Data are expressed as the mean (±SD) (n = 3; each biological replicate is a pool of three individual plants). Δmalate and Δcitrate values were obtained from the dawn minus dusk values. Different letters indicate averages that are significantly different among bromeliad species (Tukey–Kramer test; $P < 0.05$).

Discussion

Numerous works in scientific literature have presented methods that can be used to evaluate CAM expression in bromeliad plants. Recently, Crayn *et al.* (2015) revealed the photosynthetic pathways in 1893 bromeliad species using carbon isotope ratios. PEPC activity, combined with other methods, was also used to verify CAM induction in the bromeliad *Guzmania monostachia* (Freschi *et al.* 2010; Pereira *et al.* 2013). Although there have been many studies about ATP- and PPi-dependent proton (H^+) transport in tonoplast vesicle of CAM species, only a few have shown the probable relationship between CAM photosynthesis and H^+ transport rates (White and Smith 1989; Barkla *et al.* 1995; Mc Rae *et al.* 2002). Moreover, only one study has verified proton and sucrose transport rates across the tonoplast vesicles in a bromeliad species (McRae *et al.* 2002). For the first time, in this study, we compared ATP- and PPi-dependent H^+ transport in tonoplast vesicles, as well as the order of effectiveness of fumarate, malate and citrate in six CAM bromeliads (*A. nudicaulis*, *A. comosus*, *B. pyramidalis*, *N. billbergioides*, *T. pohliana*, *T. usneoides*) and one C_3 (*Vriesea sucrei*) that was employed as a control, from two subfamilies, Bromelioideae and Tillandsioideae, tested in controlled environmental conditions.

The proton transport rates checked in the presence of a specific inhibitor of non-vacuolar membranes (non V-ATPases) (NaN_3 plus Na_3VO_4) or in the presence of a specific inhibitor of vacuolar membranes (V-ATPases) (KNO_3), showed that a higher inhibition in the presence of KNO_3rather than in the presence of NaN_3 + Na_3VO_4 for all of the seven bromeliad species used in this study. This is evidence that the bromeliad vesicles, extracted from the bromeliads used in this study, are mostly from the tonoplast. Mc Rae *et al.* (2000) observed in the species *Ananas comosus* about a 96% inhibition of V-ATPase activity in the presence of KNO_3, while in the presence of NaN_3 plus Na_3VO_4 this percentage was only 7%. The difference between the results presented by Mc Rae *et al.* (2000) and our results for this

bromeliad species, might be due to either the adjustments in the extraction buffer and/or the modified method used to obtain the membranes. Despite these results, both studies have proved that most of the vesicles extracted are indeed from the tonoplast.

Similar to the rates observed for *Kalanchoë daigremontiana* (White and Smith 1989), the rates of ATP-dependent proton transport in the presence of the three anions may indicate a greater permeability of the tonoplast by fumarate, rather than by malate and citrate for all of the CAM bromeliad species. On the other hand, different from *K. daigremontiana*, which seems to present similar ATP- and PPi-dependent H^+ transport in tonoplast vesicles, all of the bromeliad species that we used in this study showed a higher tonoplast ATPase activity when compared with PPiase activity. In this way, the proton transport in the six CAM species seems to exhibit a preference for ATPase instead of PPiase. A possible explanation for this ATPase preference rather than PPiase, might be connected with the high activity of pyrophosphate dependent 6-phosphofructokinase (PPi-PFK) in bromeliads. The phosphofructokinase (PFK) enzyme is responsible for catalyzing the conversion of fructose-6-phosphate to fructose 1,6- biphosphate (Pollack and Williams 1986). In plants two types of PFK have been described, ATP- and PPi-dependent (Carnal and Black 1983; Mertens 1991; Alves *et al.* 2001). Carnal and Black (1983) reported that angiosperms with PPi-PFK activities 4 to 70 times higher than ATP-PFK tend to be succulent and exhibit CAM photosynthesis. These same authors showed in *A. comosus* that PPi-PFK activity, but not ATP-PFK activity, would be sufficient to support the rate of glycolytic carbohydrate processing required for acid accumulation (Carnal and Black 1989). Therefore, PPi-PFK might be competing for PPi with PPiase in all six CAM bromeliad species and it may be why the tonoplast ATPase has been selected as the primary H^+ pump in bromeliads.

Previous studies conducted with *Kalanchoë daigremontiana* did not consider the ATP- or PPi-dependent H^+ transport in the absence of anions (control) (White and Smith

1989; White *et al.* 1990; Bettey and Smith 1993; Lüttge *et al.* 2000; Lüttge 2000). In addition the protons' transport rates were not checked without anions in *Ananas comosus* (Mc. Rae *et al.* 2002) and *Mesembryanthemum crystallinum* (Barkla *et al.* 1995). In contrast, this study tested this transport in the absence of fumarate, malate and citrate anions; the observations were that a significant ATP- and PPi-dependent proton transport occurred in all of the bromeliad species. Also, in the majority of the species, the ATP- or PPi-dependent H^+ transport in the control (no anion) was higher than in the presence of citrate (this anion was also not tested in most of the studies previously described). To our knowledge, this is the first time that this result has been observed. Further experiments were carried out in order to verify the validity of these results. We made different buffers to use in the pH measurements, we tested different pH levels for the buffers, and for the different anions we prepared solutions using different ATP concentrations; despite the changes, in all of the tests the control still maintained a significant proton transport rate (data are not shown). Thereby, proton transport in the absence of anions seems to be an integral characteristic of the family Bromeliaceae due to the fact that the H^+ transport without anion occurred in all of the species used in this study. On the other hand, because we performed all of the assays considering the presence or absence of the anions, it is possible to discuss the real physiological significance of our results once the background rates are subtracted in all of the bromeliad species.

Besides the proton transport into the vacuole, many studies have shown the accumulation of organic acids, mainly malate and citrate, during the dark period in CAM plants and used it as a parameter to describe the degree of CAM intensity (Maxwell *et al.* 1994; Pereira *et al.* 2013). Based on the ATP- and PPi-dependent proton transport and the nocturnal malate and citrate accumulation, we verified the highest degree of CAM intensity in *B. pyramidalis*, while *T. usneoides* showed the lowest level compared with the other CAM bromeliad species. On the other hand, as we expected, *V. sucrei*, the control used for all of the

experiments, revealed a diurnal malate accumulation as well as a low proton and anions transport into the vacuole, a characteristic response of C_3 species. For all six CAM species, Δmalate values were considerably higher than Δcitrate values. The ratio averaged between Δmalate and Δcitrate was 5 times higher across the six CAM bromeliad species. In addition, these data reveal a correlation between the accumulation of malate and citrate during the night and the ATP- or PPi-dependent proton transport rates in the presence of malate or citrate anions. The quinacrine fluorescence-quenching experiments done for all CAM species used in this study showed that the permeability of the tonoplast membrane for malate appears to be higher than its permeability for citrate. This result is consistent with a higher nocturnal malate accumulation in the vacuole compared with a citrate accumulation.

In summary, this study has compared the ATP- and PPi-dependent proton transport in tonoplast vesicles of one C_3 and six CAM bromeliad species from two subfamilies, Bromelioideae and Tillandsioideae. All of the bromeliad species showed a preference for the tonoplast ATPase activity, which can be seen in the higher H^+ transport rates when the species were in the presence of this proton pump compared with the tonoplast PPiase activity. All of the species from the subfamily Bromelioideae exhibited a higher accumulation of organic acids during the night and also a higher ATP-dependent proton transport rate compared with the members of the subfamily Tillandsioideae. In addition, this study demonstrated the order of effectiveness of ATP- and PPi-dependent H^+ transport, and the relative permeability by fumarate, malate and citrate in six CAM bromeliad species. In regards to PPi-dependent proton transport no pattern in order of effectiveness was observed for the CAM species. However, a pattern is presented for all of the CAM bromeliad species tested, for ATP-dependent H^+ transport: fumarate > malate > citrate. Finally, the average rate of nocturnal malate accumulation across the six CAM bromeliad species was 5 times higher than the citrate accumulation. This organic acids storage into the vacuole during the night in

all six CAM bromeliad species was found to be related to the quinacrine fluorescence-quenching experiments which showed that the permeability of the tonoplast membrane for malate appears to be much higher than its permeability for citrate.

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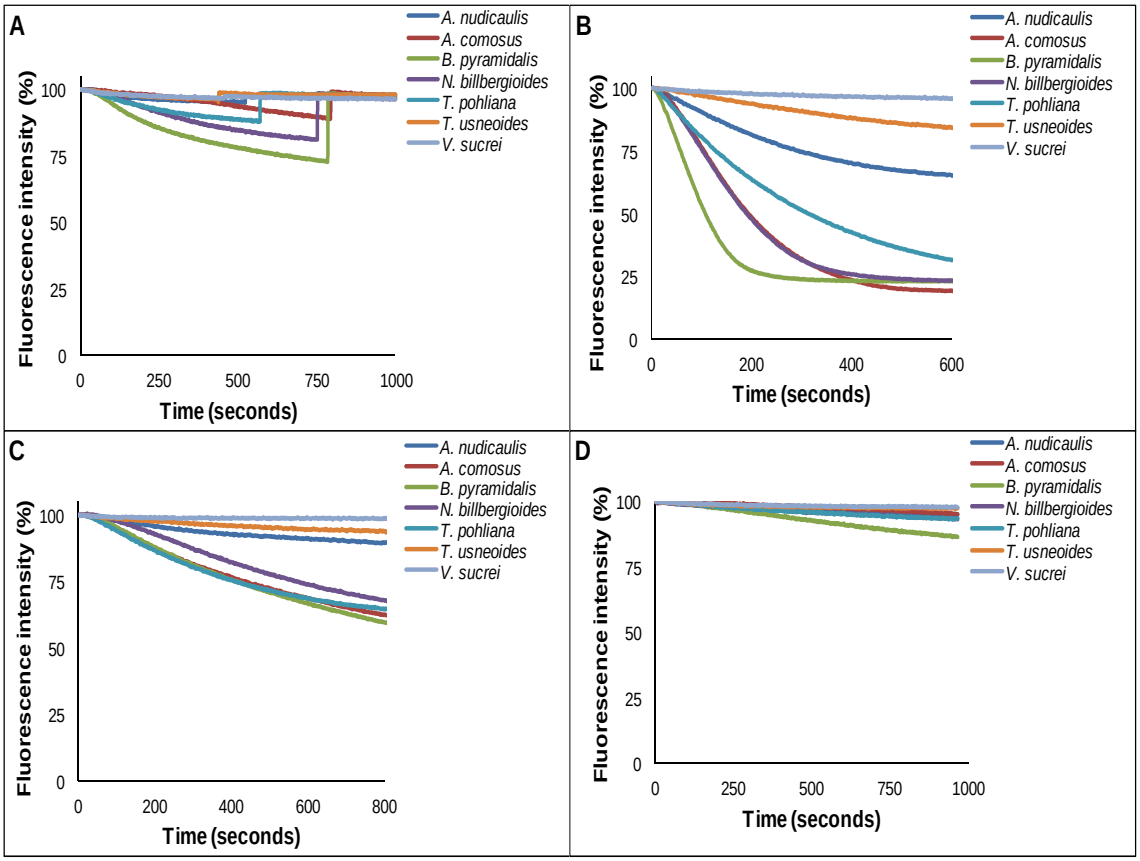
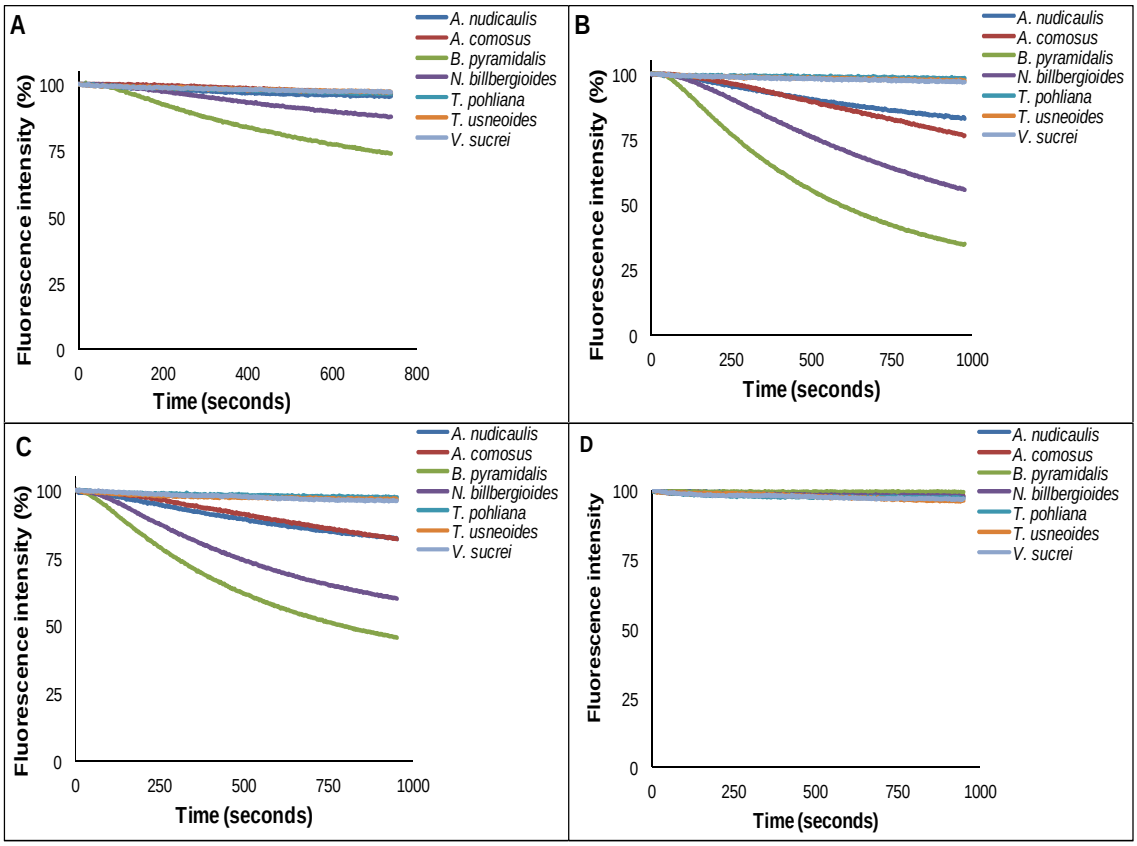
Supplementary Material



Supplementary Figure 1 ATP-dependent proton transport monitored by the quenching of quinacrine fluorescence in the control (no anion) (A), presence of fumarate (B), malate (C) and citrate (D) in tonoplast vesicles extracted from *Aechmea nudicaulis*, *Ananas comosus*, *Billbergia pyramidalis*, *Nidularium billbergioides*, *Tillandsia pohliana*, *Tillandsia usneoides* and *Vriesea sucrei*. Proton transport is expressed relative to the rate observed after adding ATP (3.0 mM) into the cuvette. In A, between 400-800 seconds (depending on the species), 2.0 mM NH_4^+ was added. Data are from one experiment, representative of a total of three preparations.



Supplementary Figure 2 PPi-dependent proton transport monitored by the quenching of quinacrine fluorescence in the control (no anion) (A), presence of fumarate (B), malate (C) and citrate (D) in tonoplast vesicles extracted from *Aechmea nudicaulis*, *Ananas comosus*, *Billbergia pyramidalis*, *Nidularium billbergioides*, *Tillandsia pohliana*, *Tillandsia usneoides* and *Vriesea sucrei*. Proton transport is expressed relative to the rate observed after adding PPi (3.0 µM) into the cuvette. Data are from one experiment, representative of a total of three preparations.

CHAPTER 2

Nitrate increases CAM intensity in two *Kalanchoë* species providing a higher proton transport across the tonoplast vesicles

Abstract

Among the species that perform CAM photosynthesis, the *Kalanchoë* species have been one of the largest groups studied. The influence of different nitrogen sources and their relative concentrations have on PEPC activity, organic acids accumulation into the vacuole during the night, and the rate of nocturnal CO_2 fixation have been tested in select representatives from this genus. However, there has been little discussion regarding the interrelationship among nitrogen sources (nitrate and ammonium), their concentrations, the rate of proton transport into the vacuole and the levels of nocturnal organic acids accumulation in *Kalanchoë* species. The present study verified ATP- and PPi-dependent proton transport rates in the presence of three anions: fumarate, malate and citrate. In addition, we also showed the accumulation of nocturnal malic, fumaric and citric acids in *Kalanchoë laxiflora* and *Kalanchoë tubiflora* cultivated with Hoagland's solution modified with different nitrogen sources and their relative concentrations. Both species that were kept in 2.5 mM of nitrate presented the highest ATP- and PPi-dependent proton transport rates and the highest levels of nocturnal organic acids accumulation, while the plants cultivated with 5.0 mM of ammonium showed the lowest values. The order of effectiveness for ATP-dependent proton transport for these two *Kalanchoë* species was: fumarate > malate > citrate. A preference for the tonoplast ATPase activity rather than PPiase activity was also observed in both species. Higher ATP- or PPi-dependent proton transport rates and higher levels of nocturnal organic acids accumulation were verified in *K. tubiflora* compared with in *K. laxiflora*. In addition, these results showed

a higher degree of CAM expression in *K. tubiflora* rather than in *K. laxiflora*. Finally, inorganic nitrogen sources and their relative concentrations influenced the intensity of the CAM in these two *Kalanchoë* species.

Keywords: crassulacean acid metabolism, nitrogen, organic acids, vacuole.

Abbreviations: ATP, adenosine triphosphate; CAM, crassulacean acid metabolism; DW, dry weight; PEPC, phospho*enol*pyruvate carboxylase; PPi, inorganic pyrophosphate.

Introduction

Crassulacean acid metabolism (CAM) is a photosynthetic pathway characterized by nocturnal CO_2 fixation performed by the enzyme phospho*enol*pyruvate carboxylase (PEPC), the accumulation of organic acids during the night, mainly malic acid, and the efficiency of water-use (Ting 1985; Cushman *et al.* 2008; Borland *et al.* 2011; Borland *et al.* 2014). At least 343 genera from 35 plant families perform CAM photosynthesis (Smith & Winter 1996; Silvera *et al.* 2010). Among these genera, *Kalanchoë* species have been one of the largest groups studied (Kluge and Ting 1978; Osmond 1978; Kluge *et al.* 1991). It has been suggested that most species from the genus *Kalanchoë* are able to perform CAM, and also a correlation has been established between the capacity to perform CAM and the climate of the habitats where the samples were collected (Kluge *et al.* 1991).

Numerous studies have been conducted upon *Kalanchoë blossfeldiana* to determine how it performs CAM photosynthesis, in the presence of varying environmental factors such as the availability of nutrients (Ota 1988a, 1988b; Ota and Yamamoto 1991). In one of the works analyzing this species, researchers observed an interrelationship between CAM and the availability of nutrients; a higher expression of CAM photosynthesis was observed when the plants were in the presence of 1.0 mM of nitrate compared with in the presence of 1.0 mM of ammonium. However, this species exhibited the highest CAM expression when both nitrogen sources were removed from the nutritive solution (nitrogen starvation) and a water deficit was imposed for the first time upon these plants (Ota 1988a). This suggests that for *K. blossfeldiana* N-withdrawal status is an important factor in the plants ability to reach their highest CAM performance. In another study, plants from this same species received 5.0 mM of nitrate or 5.0 mM of ammonium without being subjected to a water deficit. After 2 months a higher CAM intensification was demonstrated in the plants cultivated with 5.0 mM of nitrate compared with those cultivated with ammonium (Ota 1988b). In addition to the

nitrogen source, the concentration of nitrogen seems to be an important factor in the intensification of CAM in *Kalanchoë* species. In *Kalanchoë pinnata* (Crassulaceae), another typical CAM plant, researchers investigated the influence of different nitrate concentrations (0.6 mM or 24.0 mM) on the CAM photosynthetic pathway. Plants cultivated with a higher nitrate concentration showed higher PEPC activity when compared with plants cultivated with a lower nitrate concentration (Winter *et al.* 1982). All of these studies support the idea that both inorganic nitrogen sources and their concentrations influence the magnitude of CAM expression.

Additional studies used another model CAM plant, *Kalanchoë daigremontiana,* to develop a method to check ATP- and PPi-dependent proton transport in tonoplast vesicles (White & Smith 1989; White *et al.* 1990; Bettey & Smith 1993; Bartholomew *et al.* 1995; Pantoja & Smith 2002). White and Smith (1989) observed that malate was more effective than chloride in stimulating ATP- and PPi-dependent vesicle acidification in *K. daigremontiana*. This is evidence which shows that malate is more readily transported across the tonoplast compared with chloride. Also, the rates of vesicle acidification were checked in these tonoplast vesicles and it was reported that fumarate, followed by malate, supported higher rates than chloride (White & Smith 1989). Another work done with *Nicotiana tabacum* (Solanaceae), a C_3 species, showed the influence of both nitrogen source and concentration on proton transport across the vacuole and on malate accumulation (Lüttge *et al.* 2000). The highest malate accumulation and the strongest ATP-dependent malate transport into the vacuole was observed when the species was in the presence of either 10.0 or 20.0 mM of nitrate.

These studies presented before raise some important questions. How much higher/lower would the H^+ transport across the tonoplast be in other *Kalanchoë* species that perform a stronger/weaker CAM than *K. daigremontiana* performs? How permeable would

the tonoplast vesicles be for citrate, fumarate and malate anions in *Kalanchoë laxiflora* and *Kalanchoë tubiflora*? Would distinct nitrogen sources and their different concentrations alter proton transport across the vacuole, and/or affect organic acids accumulation during the night in this organelle, in *Kalanchoë* species that perform different degrees of CAM photosynthesis? Based on these questions, the present study is primarily focused on the influence of the presence/absence of different nitrogen sources (NH_4^+ and/or NO_3^-) and final concentrations of these nitrogen sources (2.5 mM or 5.0 mM) on CAM intensity through a combination of physiological and biochemical approaches. Also, we show for the first time, to our knowledge, how nitrogen sources and their concentrations influence ATP- and PPi-dependent proton transport in the presence of fumarate, malate or citrate anions in two *Kalanchoë* species that perform different degrees of CAM, *K. laxiflora* and *K. tubiflora* (Crassulaceae). This work also examines the interrelationship among citrate, fumarate and malate nocturnal accumulation and how the sources of nitrogen and their concentrations affect proton transport in tonoplast vesicles. Finally, this study discusses what influence the presence/absence of NH_4^+ and/or NO_3^- has on the preference of H^+ transport in tonoplast vesicles for ATPase or PPiase, as well as evaluates the order of effectiveness of fumarate, malate and citrate in these two species from the family Crassulaceae.

Material and Methods

Plant material and growth conditions

Adult *Kalanchoë laxiflora* (Baker) and *Kalanchoë tubiflora* (Harvey) R. Hamet plants were taken from the garden in the Department of Botany at the University of São Paulo, São Paulo, Brazil, and were transferred to controlled environment chambers, under a photosynthetic flux density (PFD) of about 200 µmol m^{-2} s^{-1} of photosynthetic active

radiation to the top leaf surfaces of the *Kalanchoë* species, 12 h photoperiod, a day/night air temperature of 25/22ºC, and a day/night relative humidity of 60/70%.

The plants were cultivated in pots containing fine sand, with one plant per pot, and were acclimated for two weeks. After this acclimation period, experiments were performed for 60 days and each species was divided into six treatments based on nitrogen sources and their concentrations: nitrogen-deficient, used as a control, 2.5 mM of ammonium + 2.5 mM of nitrate; 2.5 mM of ammonium; 2.5 mM of nitrate; 5.0 mM of ammonium; and 5.0 mM of nitrate. The plants were watered every two days for a total of 60 days with Hoagland's solution (Hoagland & Arnon 1950) modified with the concentrations of KNO_3^-, $Ca(NO_3)_2$ and/or $(NH_4)_2SO_4$, described before. After 60 d, leaf samples were taken from six plants kept in each treatment, and were then analyzed to determine the proton transport rates in the tonoplast vesicles, and the organic acids accumulation during the night.

Tonoplast Isolation

Fully expanded leaves from *K. laxiflora* and *K. tubiflora* were harvested 1-1.5 hours after the commencement of the light period. Tonoplast fractions from the mesophyll tissue were isolated in accordance with White & Smith (1989) with minor modifications. The leaf margins were removed and the mesophyll tissue (approximately 80 g fresh mass) was suspended in 250mL of ice-cold extraction buffer containing the following: 450 mM mannitol, 3.0 mM $MgSO_4$, 10.0 mM ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA), 10 mM dithiothreitol (DTT), 0.5% (w/v) polyvinylpyrrolidone (PVP-40) and 100 mM tris (hydroxymethyl)aminomethane (Trizma® base) buffered to pH 8.0 with HCl. The homogenate was filtered through two layers of cheesecloth and the filtrate was centrifuged at 18000 × g for 20 min. The resulting supernatant was centrifuged at 80000 × g for 60 min. The resulting pellet was layered over a 23% (w/v) sucrose cushion containing 1.1 M glycerol,

1.0 mM EDTA, 10 mM tricine and 2.0 mM DTT buffered to pH 8.0 with bis- TRIS- propane (BTP). The gradients were centrifuged at 100.000 × g for 70 min. Tonoplast vesicles were removed from the interface using a Pasteur pipette. Vesicles were then pelleted at 100.000 × g for 50 min. The resulting pellet was finally re-suspended in the same buffer as the first pellet. All steps were performed at 4^{o}C. Preparations were stored at -80^{o}C and were used within one month.

Measurement of pH.

Measurement of pH was determined according to the method described by White & Smith (1989) and the **Chapter 1** with minor modifications. Initial rates of H^+ transport at 25^{o}C were determined from the initial rates of fluorescence quenching at the time of the addition of 3.0 mM Tris-ATP or 500 µM Na_4PPi to the reaction media. For assays of ATP-dependent H^+ transport, the reaction medium contained 3.0 µM quinacrine, 6.0 mM $MgSO_4$,0.3 mM disodium ethylenediaminetetraacetate (EDTA), 150 mM Mannitol and 25 mM BTP buffered to pH 8.0 with Mes. For measurements of PPi-dependent H^+ transport, the reaction medium was identical except that the $MgSO_4$ concentration was increased to 7.5 mM and the medium also contained 100 mM K-Mes. 50 mM of the anions (fumarate, malate and citrate), 50 mM potassium nitrate (inhibitor of vacuolar membrane) or 100 µM Sodium orthovanadate plus 100 µM Sodium azide (inhibitor of non-vacuolar membrane) buffered to pH 8.0 with BTP were used in the ATP- and PPi-dependent proton transport assays. Approximately 3 µg of protein was used in all of the ATP- and PPi-dependent proton transport assays. Fluorescence quenching was measured using a model LS-55 luminescence spectrometer (Llantrisant, UK) with excitation at 422 nm and emission at 495 nm, both with a slit width of 5 nm. Quinacrine fluorescence intensity (%F) was calculated based on the slopes

after adding ATP or PPi for each organic acids or control tested for all of the vesicles extracted from these two *Kalanchoë* species used in this study.

Protein Determination

Protein concentration was measured using the method described by Bradford (1976), with bovine serum albumin as the standard.

Organic Acid Quantifications

To determine the nocturnal accumulation of organic acids (Δ citrate, Δ fumarate and Δ malate), fully developed leaf samples (100 mg) from six individual plants were collected 1 hour after the start of the light period (dawn), and 1 hour before the end of the light period (dusk). Afterwards, the samples were ground in liquid nitrogen and subsequently homogenized with 500 µL of MCW solution (methanol, chloroform and water, 12:5:1) containing benzoic acid which was used as the standard (1 mg mL^{-1} of methanol), the samples were then incubated at 60ºC for 30 min. All samples were centrifuged at 16.000 × g at 4ºC for 10 min. The supernatant was collected (50 µL) and dried for 1 hour at 60ºC in a SPEED-VAC. Then, the dried sample was re-suspended in 25 µL of pyridine and 25 µL of N- tert- butyldimethylsilyl- N- methyltrifluoroacetamide (MTBSTFA) and derivatized for 1 h at 92ºC. An aliquot of 1µL of the derivatized sample was used to quantify the organic acids by gas chromatography - mass spectrometry (GC-MS), a chromatographic system (Shimadzu- QP2010SE), column (Agilent- DB5MS) and an auto sampler (Shimadzu- AOC-20i). Standard curves of citric, fumaric and malic acids were used to determine the concentrations of individual organic acids in the samples. Results are expressed as millimole per gram of dry weight (mmol g^{-1} DW).

Statistical Analysis

All data were presented as mean values with ± standard deviation (SD). The significant differences among the distinct anions or treatments in the same species were contrasted by the Tukey–Kramer test as $P < 0.05$, using JMP 5.01 software (SAS Institute 2002). The difference between the inhibitors in the same treatment and species was contrasted by Student's *t*-test at 5% significance ($P < 0.05$).

Results

The tonoplast vesicles used in all of the proton transport assays were extracted in three independent experiments, and three anions: fumarate^{-2}, malate^{-2} and citrate^{-3} were used to check their % quinacrine fluorescence-quenching (F) min^{-1} mg protein^{-1}.

To determine the relative contribution of vacuolar and non-vacuolar membranes in these two species kept in different nitrogen treatments, we used a specific inhibitor of vacuolar membranes (V-ATPases) (KNO_3) or non-vacuolar membranes (non V-ATPases) (NaN_3 plus Na_3VO_4) (Table 1). In all of the nitrogen treatments that *K. laxiflora* and *K. tubiflora* plants were given, a lower ATP-dependent proton transport was verified while the membranes were in the presence of KNO_3 compared with the control (absence of inhibitors). When the membranes extracted were in the presence of NaN_3 plus Na_3VO_4 there was a slight decrease in proton transport, however, this transport was significantly higher than when the membranes were in the presence of an inhibitor of vacuolar membranes (Table 1). These results confirm that most of the extracted vesicles in this study are indeed from the tonoplast. All of these treatments, for both *Kalanchoë* species, showed an inhibition percentage of ATP-dependent proton transport, in the presence of KNO_3, higher than 54% (Table 1). On the other hand, in the presence of NaN_3 plus Na_3VO_4, all of the other treatments showed an inhibition percentage between 2 and 32% when a specific inhibitors of non V-ATPases was added (Table 1).

Table 1 ATP-dependent proton transport in the tonoplast vesicles of *Kalanchoë laxiflora* and *Kalanchoë tubiflora*, in the presence of fumarate and of a specific inhibitors of the non-vacuolar membrane (NaN_3 plus Na_3VO_4) or a specific inhibitor of the vacuolar membrane (KNO_3) or no-inhibitor (control), measured as initial rates of quinacrine fluorescence-quenching. The treatments were: nitrogen-deficient (used as a control), 2.5 mM of ammonium + 2.5 mM of nitrate, 2.5 mM of ammonium, 2.5 mM of nitrate, 5.0 mM of ammonium and 5.0 mM of nitrate. Results are expressed as the mean (±SD) for three preparations. The rates of proton transport were measured in % H^+ min^{-1} mg $protein^{-1}$ and % of inhibition. Different capital letters indicate an average that is significantly different among the treatments in the same species (Tukey–Kramer test; $P < 0.05$). Asterisks indicate significant differences between inhibitors in the same treatment in each species by Student's T-test at 5% significance.

	Specific activity (% min^{-1} mg $protein^{-1}$) (inhibition relative to control)		
Kalanchoë laxiflora	**Control**	**+ KNO_3**	**NaN_3 + Na_4VO_3**
Nitrogen-deficient	3279 ± 30.8 A	161 ± 3.5 C (95%) *	2857 ± 21.7 B (13%)
2.5 mM Ammonium + 2.5 mM Nitrate	4501 ± 76.2 A	171 ± 4.8 C (96%) *	3487 ± 43.3 B (23%)
2.5 mM Ammonium	2781 ± 19.1 A	92.0 ± 18.9 C (97%) *	2254 ± 78.9 B (19%)
2.5 mM Nitrate	4498 ± 33.4 A	58.6 ± 6.5 C (99%) *	4396 ± 31.9 B (2.3%)
5.0 mM Ammonium	697 ± 27.5 A	71.8 ± 3.2 C (90%) *	612 ± 26.4 B (13%)
5.0 mM Nitrate	1119 ± 47.2 A	89.8 ± 6.2 C (92%) *	838 ± 94.4 B (25%) *
Kalanchoë tubiflora	**Control**	**+ KNO_3**	**NaN_3 + Na_4VO_3**
Nitrogen-deficient	3735 ± 54.6 A	459 ± 50.9 C (88%) *	3010 ± 30.8 B (19%)
2.5 mM Ammonium + 2.5 mM Nitrate	3936 ± 136.1 A	203 ± 47.5 C (95%) *	2659 ± 86.2 B (32%)
2.5 mM Ammonium	3673 ± 97.01 A	232 ± 31.2 B (94%) *	3580 ± 108.7 A (2.4%)
2.5 mM Nitrate	4757 ± 146.7 A	269 ± 23.5 B (94%) *	4622 ± 168.7 A (2.8%)
5.0 mM Ammonium	311 ± 28.2 A	141 ± 13.4 C (54%) *	237.8 ± 13.1 B (23%)
5.0 mM Nitrate	1964 ± 132.4 A	264 ± 57.0 C (86%) *	1628 ± 55.6 B (17%)

ATP-dependent proton transport rates (%F) in *K. laxiflora*'s and *K. tubiflora*'s tonopast vesicles in the presence of fumarate, malate and citrate anions were calculated using the slope in Supplementary Figures 1 and 2.

Fumarate supported the highest rates of vesicle acidification in all of the treatments that *K. laxiflora* and *K. tubiflora* were submitted to, while malate was the anion responsible for the second highest rate of acidification in the tonoplast vesicles (not considering the control). In the presence of fumarate, *K. laxiflora* plants cultivated in 2.5 mM of nitrate presented the highest ATP-dependent proton transport rates, about 4061% min^{-1} mg $protein^{-1}$, (Table 2), followed by plants cultivated in the presence of 2.5 mM of ammonium+2.5 mM of nitrate, nitrogen-deficient, 2.5 mM of ammonium, 5.0 mM of nitrate and 5.0 mM of ammonium (Table 2). For comparison, ATP-dependent proton transport in the presence of 2.5 mM of nitrate was about 9 times higher than in the presence of 5.0 mM of ammonium. For *K. tubiflora*, in the presence of fumarate, the highest proton transport rates were also observed in plants cultivated in 2.5 mM of nitrate, 4172% min^{-1} mg $protein^{-1}$, followed by 2.5 mM of ammonium+2.5 mM of nitrate, 2.5 mM of ammonium, nitrogen-deficient, 5.0 mM of nitrate and 5.0 mM of ammonium. When this *Kalanchoë* species was cultivated in 2.5 mM of nitrate, ATP-dependent H^+ transport was about 3.5 times higher than when the species was in the presence of 5.0 mM of ammonium.

In the presence of malate, the H^+ transport rates showed a similar pattern for both *Kalanchoë* species. *Kalanchoë* plants cultivated with 2.5 mM of nitrate presented the highest proton transport rates, followed by the plants cultivated in the presence of 2.5 mM of ammonium+2.5 mM of nitrate, nitrogen-deficient, 2.5 mM of ammonium, 5.0 mM of nitrate and 5.0 mM of ammonium (Table 2). For *K. laxiflora*, the highest proton transport rate in the presence of malate was about 11 times higher than the lowest H^+ transport rate, while for *K.*

tubiflora this proton transport rate was about 4 times higher in the plants cultivated with 2.5 mM of nitrate compared with the plants kept in 5.0 mM of ammonium (Table 2).

In the presence of citrate, the proton transport rates in *K. laxiflora* and *K. tubiflora* did not show any discernible pattern. The highest H^+ transport rates for *K. laxiflora* were described in the presence of 2.5 mM of nitrate and 2.5 mM of ammonium, while the lowest rates were observed in the presence of 2.5 mM of ammonium+2.5 mM of nitrate and nitrogen-deficient (Table 2). For *K. tubiflora*, the highest H^+ transport rates were observed in the presence of 2.5 mM of ammonium, while the lowest were observed in the presence of 5.0 mM of ammonium (table 2). Thereby, the order of effectiveness (not considering the control) was fumarate > malate > citrate for *K. laxiflora* and *K. tubiflora* plants kept in all of the treatments. The increase or decrease of ATP-dependent proton transport rates observed in both *Kalanchoë* species were dependent upon nitrogen sources and their relative concentrations.

Generally, ATP-dependent proton transport in all of the treatments in *K. tubiflora* showed significantly higher rates while in the presence of fumarate or malate compared with when in the presence of all of the treatments for *K. laxiflora*. On the other hand, the pattern of H^+ transport in the presence of fumarate and malate was very similar in both species (Table 2).

Table 2 ATP-dependent proton transport measured as initial rates of quinacrine fluorescence-quenching in the control (no anion), presence of fumarate, malate and citrate (present as their BTP-salts at 50 mM) in the tonoplast vesicles of *Kalanchoë laxiflora* and *Kalanchoë tubiflora*, in nitrogen-deficient (used as a control), 2.5 mM of ammonium + 2.5 mM of nitrate, 2.5 mM of ammonium, 2.5 mM of nitrate, 5.0 mM of ammonium and 5.0 mM of nitrate. Results are expressed as the mean (± SD) and are relative to rates of proton transport measured in % H^+. min^{-1}. mg $protein^{-1}$. Different capital letters indicate averages that are

significantly different among treatments that use the same anion (Tukey–Kramer test; $P < 0.05$). Different lower case letters indicate averages that are significantly different among anions/control in the same treatment (Tukey–Kramer test; $P < 0.05$).

	Specific activity (% min^{-1} mg protein^{-1})			
Kalanchoë laxiflora	**Fumarate**	**Malate**	**Citrate**	**Control**
Nitrogen-deficient	3040 ± 9.6 C a	1053 ± 15.3 C b	38.8 ± 7.6 D d	274 ± 6.8 A c
2.5 mM Ammonium + 2.5 mM Nitrate	3113 ± 14.9 B a	1516 ± 15.3 B b	53.08 ± 9.0 CD b	208 ± 7.0 C c
2.5 mM Ammonium	2131 ± 12.6 D a	522 ± 6.9 D b	126 ± 7.7 AB c	21.7 ±2.3 F d
2.5 mM Nitrate	4061 ± 24.2 A a	1623 ± 12.0 A b	142 ± 10.7 A d	239 ± 5.1 B c
5.0 mM Ammonium	445 ± 7.7 F a	149 ± 8.4 F c	73.0 ± 6.2 C d	190 ± 10.5 D b
5.0 mM Nitrate	878 ± 16.4 E a	210 ± 6.0 E b	111 ± 8.1 B c	104 ± 3.5 E c
Kalanchoë tubiflora	**Fumarate**	**Malate**	**Citrate**	**Control**
Nitrogen-deficient	2809 ± 8.2 D a	1424 ± 6.8 C b	73.3 ± 5.8 C d	145 ± 4.02 C c
2.5 mM Ammonium + 2.5 mM Nitrate	3776 ± 5.3 B a	1810 ± 6.7 B b	107 ± 9.1 B d	243 ± 3.5 A c
2.5 mM Ammonium	3715 ± 5.01 C a	1310 ±10.01 D b	142 ± 7.4 A d	220 ± 12.2 B c
2.5 mM Nitrate	4172 ± 15.5 A a	2088 ±6.9 A b	108 ± 10.4 B c	108 ± 7.0 D c
5.0 mM Ammonium	1207 ± 8.9 F a	531 ± 7.6 F b	21.4 ± 1.0 E d	67.2 ± 5.4 E c
5.0 mM Nitrate	2009 ± 5.2 E a	774 ± 9.9 E b	45.2 ± 8.3 D d	26.7 ± 1.2 F c

The PPi-dependent proton transport rates (%F) in *K. laxiflora*'s and *K. tubiflora*'s tonoplast vesicles in the presence of different anions were also calculated using the slopes in Supplementary Figures 3 and 4.

The pattern of PPi-dependent H^+ transport rates in the presence of all of the anions was different when compared with ATP-dependent proton transport in *K. laxiflora* and *K. tubiflora*. For *K. laxiflora*, in the presence of fumarate, the highest H^+ transport rates were observed in 2.5 mM of nitrate, 2124% min^{-1} mg protein^{-1}, followed by 2.5 mM of ammonium+2.5 mM of nitrate, 2.5 mM of ammonium, 5.0 mM of nitrate, and 5.0 mM of ammonium, while the lowest rate was observed in the nitrogen-deficient treatment (Table 3). For *K. tubiflora*, in the presence of the same anion, the highest proton transport rates were observed in 2.5 mM of nitrate followed by 2.5 mM of ammonium+2.5 mM of nitrate, 2.5 mM

of ammonium, nitrogen-deficient, and 5.0 mM of nitrate, and the lowest rate in 5.0 mM of ammonium (Table 3).

In the presence of malate, the highest rates were observed in *K. laxiflora* plants cultivated with 2.5 mM of nitrate followed by 2.5 mM of ammonium+2.5 mM of nitrate, nitrogen-deficient and 2.5 mM of ammonium and the lowest rates in 5.0 mM of ammonium and 5.0 mM of nitrate (Table 3). On the other hand, *K. tubiflora* presented the highest H^+ transport rates in 2.5 mM of nitrate followed by 2.5 mM of ammonium+2.5 mM of nitrate, nitrogen-deficient and 2.5 mM of ammonium, 5.0 mM of nitrate and the lowest rate in 5.0 mM of ammonium (Table 3).

Finally, in the presence of citrate, *K. laxiflora* plants cultivated in 2.5 mM of ammonium+2.5 mM of nitrate showed the highest proton transport rates, while the lowest rates were observed in 2.5 mM of ammonium, 5.0 mM of ammonium and nitrogen-deficient (Table 3). For *K. tubiflora*, the PPi-dependent proton transport rates in the presence of citrate were the highest in 5.0 mM of ammonium and 2.5 mM of nitrate, while the lowest rate was observed in 2.5 mM of ammonium+2.5 mM of nitrate (Table 3). Thereby, the order of effectiveness (not considering the control) was fumarate > malate > citrate for *K. laxiflora* plants kept in all of the treatments. On the other hand, for *K. tubiflora*, the order of effectiveness was very different from that observed previously in *K. laxiflora*. In 2.5 mM of ammonium+2.5 mM of nitrate, nitrogen-deficient and 2.5 mM of nitrate, the order of effectiveness (not considering the control) was fumarate = malate > citrate; while in 2.5 mM of ammonium, 5.0 mM of ammonium and 5.0 mM of nitrate it was fumarate > malate > citrate (Table 3). The variations in the PPi-dependent proton transport rates in this species were also dependent upon the different nitrogen sources and their relative concentrations.

Table 3 PPi-dependent proton transport measured as initial rates of quinacrine fluorescence-quenching in the control (no anion), presence of fumarate, malate and citrate (present as their BTP-salts at 50 mM) in the tonoplast vesicles of *Kalanchoë laxiflora* and *Kalanchoë tubiflora*, in nitrogen-deficient conditions (used as a control), 2.5 mM of ammonium + 2.5 mM of nitrate, 2.5 mM of ammonium, 2.5 mM of nitrate, 5.0 mM of ammonium and 5.0 mM of nitrate. Results are expressed as the mean (± SD) and are relative to rates of proton transport measured in % H^+ min^{-1} mg $protein^{-1}$. Different capital letters indicate averages that are significantly different among treatments that use the same anion (Tukey–Kramer test; $P < 0.05$). Different lower case letters indicate averages that are significantly different among anions/control in the same treatment (Tukey–Kramer test; $P < 0.05$).

	Specific activity (% min^{-1} mg $protein^{-1}$)			
Kalanchoë laxiflora	**Fumarate**	**Malate**	**Citrate**	**Control**
Nitrogen-deficient	315 ± 7.1 E a	203 ± 8.5 C b	33.2 1.7 C d	51.8 ± 4.5 D c
2.5 mM Ammonium + 2.5 mM Nitrate	688 ± 3.7 B a	536 ± 6.8 B b	219 ± 8.4 A c	87.3 ± 5.3 C d
2.5 mM Ammonium	511 ± 10.2 C a	220 ± 12.1 C b	25.3 ± 4.6 C d	51.0 ± 3.8 D c
2.5 mM Nitrate	2125 ± 25.01 A a	753 ±15.3 A b	75.4 ± 3.9 B d	126 ± 5.2 A c
5.0 mM Ammonium	404 ± 13.9 D a	119 ± 5.1 D b	20.8 ± 0.7 C c	106 ± 5.2 B b
5.0 mM Nitrate	523 ± 8.9 C a	142 ± 8.1 D b	87.7 ± 7.8 B c	73.3 ± 6.7 C c
Kalanchoë tubiflora	**Fumarate**	**Malate**	**Citrate**	**Control**
Nitrogen-deficient	829 ± 16.5 D a	906 ± 54.9 C a	34.4 ± 0.7 CD b	70.2 ± 3.4 DE b
2.5 mM Ammonium + 2.5 mM Nitrate	1125 ± 21.8 B a	1057 ± 10.04 B a	23.4 ± 2.43 D b	49.8 ± 13.3 E b
2.5 mM Ammonium	1025 ± 5.01 C a	854 ± 10.02 C b	42.0 ± 7.1 CD d	90.6 ± 9.2 CD c
2.5 mM Nitrate	1280 ± 6.7 A a	1303 ± 11.9 A a	66.3 ± 1.53 AB c	108 ± 13.1 BC b
5.0 mM Ammonium	468 ± 10.1 E a	405 ± 8.3 E b	82.9 ± 11.9 A d	141 ± 7.9 A c
5.0 mM Nitrate	846 ± 10.8 D a	674 ± 1.5 D b	49.7 ± 10.02 BC d	120 ± 8.5 AB c

In both species, *K. laxiflora* and *K. tubiflora*, ATP- or PPi-dependent proton transport rates presented a control (no anion used) higher than zero, and in most of the treatments the rates in the control were higher than the rates measured in the presence of citrate; only *K.*

laxiflora plants kept in 5.0 mM of ammonium, exhibited higher ATP-dependent proton transport rates in the control compared with in the presence of malate and citrate (Table 2).

Generally, ATP- or PPi-dependent proton transport rates were higher in the presence of fumarate than when in the presence of the other anions in both species, and in all of the treatments. Also, this transport seems to exhibit a preference for ATPase rather than PPiase, because the H^+ transport rates in the presence of ATP were always higher than in the presence of PPi.

The nocturnal malate, fumarate and citrate accumulation was measured in the leaves of *K. laxiflora* and *K. tubiflora* (Fig. 1). In all of the treatments for both *Kalanchoë* species, the highest nocturnal organic acid accumulation was malate. For example, in *K. laxiflora* plants cultivated with 2.5 mM of nitrate, the amount of malate accumulated during the dark period was about 8 times higher than fumarate and about 72 times higher than citrate in this same treatment. The second highest level of nocturnal malate accumulation in this species was observed in 2.5 mM of ammonium+2.5 mM of nitrate followed by nitrogen-deficient and 2.5 mM of ammonium. The treatments that resulted in the lowest amount of malate accumulated during the night were 5.0 mM of nitrate followed by 5.0 mM of ammonium (Fig. 1A). *K tubiflora* plants cultivated in 2.5 mM of nitrate, the treatment that showed the highest amount of organic acids accumulated during the night, exhibited nocturnal malate accumulation of about 20 times higher than fumarate, and about 4 times higher than citrate. 2.5 mM of ammonium+2.5 mM of nitrate and 2.5 mM of ammonium were the treatments that showed the second highest levels of nocturnal accumulation of this organic acid, followed by nitrogen-deficient, 5.0 mM of nitrate and the lowest accumulation was observed in 5.0 mM of ammonium (Fig. 1B).

For fumarate, *K. laxiflora* presented the highest nocturnal accumulation in the presence of 2.5 mM of nitrate followed by nitrogen-deficient and 2.5 mM of ammonium+2.5

mM of nitrate (Fig. 1A). While *K. tubiflora* demonstrated the highest nocturnal accumulation of this organic acid in the presence of 2.5 mM of nitrate, 2.5 mM of ammonium + 2.5 mM of nitrate, nitrogen-deficient and 2.5 mM of ammonium (Fig. 1B).

For citrate, the highest night accumulation was revealed in *K. laxiflora* plants cultivated with 2.5 mM of nitrate and 2.5 mM of ammonium (Fig. 1A). While for *K. tubiflora*, the highest nocturnal accumulation of this organic acid was observed in 2.5 mM of nitrate followed by 2.5 mM of ammonium + 2.5 mM of nitrate and 2.5 mM of ammonium (Fig 1B).

The order of total organic acids (malate + fumarate + citrate) accumulated during the night in *K. laxiflora*'s treatments was as follows: 2.5 mM of nitrate > 2.5 mM of ammonium+2.5 mM of nitrate = nitrogen-deficient > 2.5 mM of ammonium ≥ 5.0 mM of nitrate = 5.0 mM of ammonium (Fig. 1A). For *K. tubiflora*, this order showed a slight difference from that observed in *K. laxiflora*. It was: 2.5 mM of nitrate > 2.5 mM of ammonium + 2.5 mM of nitrate > 2.5 mM of ammonium > nitrogen-deficient > 5.0 mM of nitrate > 5.0 mM of ammonium (Fig. 1B). As shown in the previous results, the nocturnal organic acids accumulation varies according to the species, nitrogen sources and their concentrations. *K. tubiflora* exhibited higher nocturnal organic acids accumulation in all of the treatments compared with *K. laxiflora* (Fig. 1). The total amount of organic acid accumulated during the night in *K. tubiflora* was about 3.5 times higher than in *K. laxiflora* (Fig. 1)

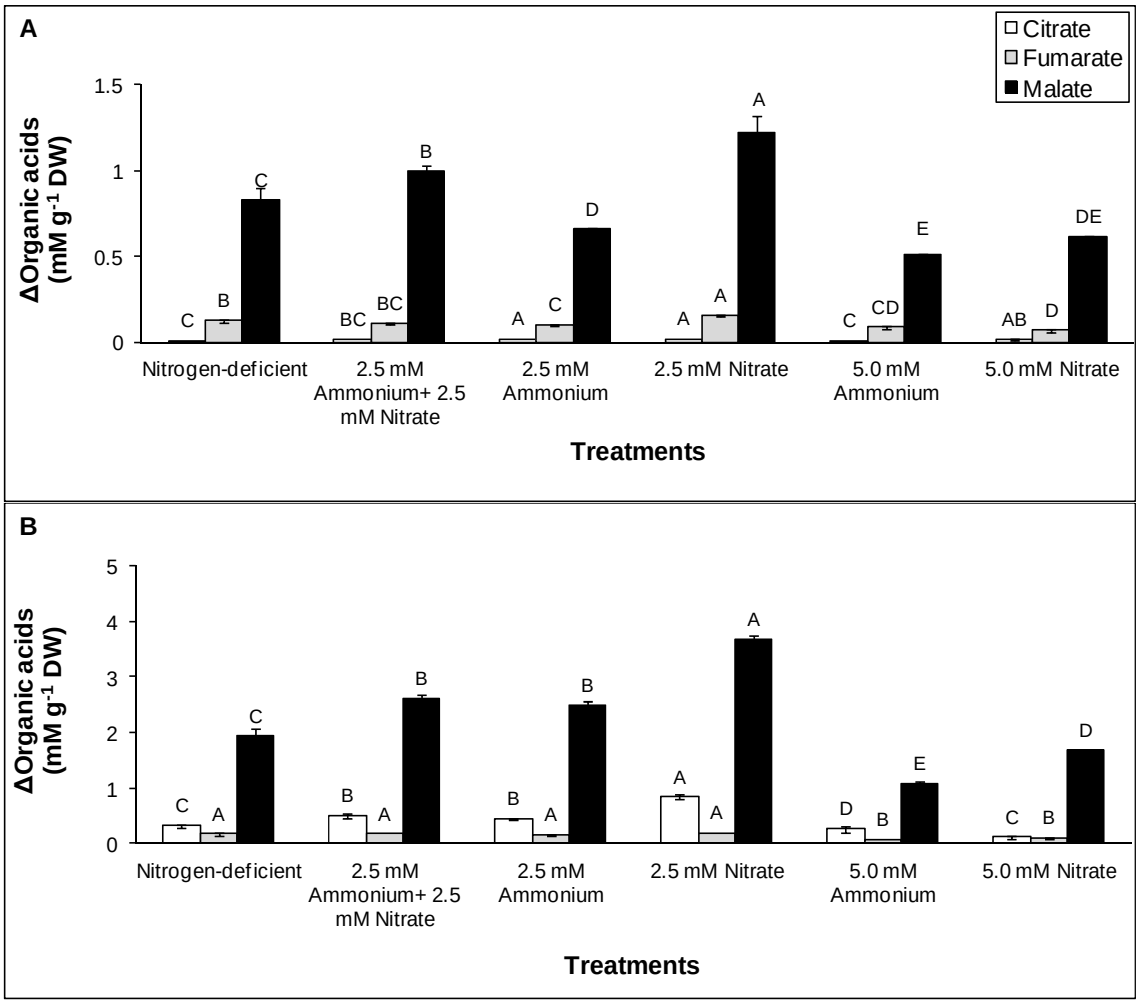


Fig.1 Nocturnal organic acids accumulation (citrate, fumarate and malate) in the leaves of *Kalanchoë laxiflora* (A) and *Kalanchoë tubiflora* (B) in nitrogen-deficient conditions (used as a control), 2.5 mM of ammonium + 2.5 mM of nitrate, 2.5 mM of ammonium, 2.5 mM of nitrate, 5.0 mM of ammonium and 5.0 mM of nitrate. Data are expressed as the mean (± SD) (n = 3; each biological replicate is a pool of three individual plants). Δ organic acids values were obtained from the dawn minus dusk values. Different letters indicate averages that are significantly different among treatments (Tukey Kramer test; $P < 0.05$).

Discussion

Many studies performed on *Kalanchoë* species have observed the influence that inorganic nitrogen sources and their concentrations have on CAM expression (Ota 1988a, 1988b; Ota and Yamammoto 1991; Santos and Salema 1991). However, this study is the first to show, to our knowledge, the influence of different sources and their concentrations on the transport of protons in tonoplast vesicles, and the relationship of these nitrogen sources and their concentrations with the nocturnal accumulation of malate, fumarate and citrate in two *Kalanchoë* species, *K. laxiflora* and *K. tubiflora*, which perform different degrees of CAM. Ota and Yamammoto (1991) noticed in *K. blossfeldiana* a higher CAM expression when these plants were kept in the presence of nitrate. Our results corroborate this study; we observed in *K. laxiflora* and *K. tubiflora* that the highest levels of organic acids, mainly malate, were accumulated during the night in the plants kept under 2.5 mM of nitrate (low concentration). Another study performed with *K. lateritia* found that both low and high concentrations of nitrogen (about 1.5 mM and 15.0 mM, respectively) decreased the PEPC activity and titratable acidity when compared with intermediate concentrations (3.0 mM) (Santos and Salema 1991). In *K blossfeldiana*, a higher ammonium concentration (10.0 mM) presented toxicity effects such as necrosis in the basal portion of the stem of this species (Ota 1988a). Additionally, in our investigation we verified in *K. laxiflora* and *K. tubiflora* that the highest ammonium and nitrate concentrations (5.0 mM) decreased the overall organic acids (malate, fumarate and citrate) accumulation during the night. It is probable that even 5.0 mM of ammonium would be toxic to *K. laxiflora* and *K. tubiflora*, and this toxicity might be responsible for the lowest rates of nocturnal organic acids accumulation in these species. Britto *et al.* (2001) explained NH_4^+ toxicity as a result of the high energetic cost associated with pumping this ion back out of the cells, after entering at high rates in ammonium sensitive species.

Generally, when we combine the results from this study and the observations made in most of the earlier publications with *Kalanchoë* species, the data support the theory that species from the genus *Kalanchoë* show a preference for NO_3^- instead of NH_4^+. However, the preference for the concentration of nitrate seems to be different among *Kalanchoë* species. For *K. pinnata*, a high nitrate concentration (24.0 mM) increased PEPC activity by about 3 times, when compared with a low concentration (0.6 mM) (Winter *et al.* 1982). For *K. blossfeldiana*, a slight increase in nocturnal malate accumulation was observed in the presence of a high nitrate concentration (10.0 mM), but, the CO_2 absorbed during the night tended to decrease in this high concentration (10.0 mM) (Ota and Yamamoto 1991). In this study, *K. laxiflora* and *K. tubiflora* seem to have a preference for lower nitrogen concentrations (2.5 mM) regardless of the source of nitrogen. Another revealing result is that these species prefer either the combination of nitrogen sources (2.5 mM of ammonium + 2.5 mM of nitrate) or the nitrogen-deficiency, rather than the high concentration of each nitrogen source individually. In addition to showing the influence of nitrogen sources and their concentrations on CAM photosynthetic expression, this study is also significant in that it differentiates between which types of organic acids the *Kalanchoë* species prefer to accumulate during the night in the vacuole. *K. laxiflora* exhibited the highest accumulation of malate followed by fumarate and citrate during the night, while *K. tubiflora* exhibited the preference for malate, followed by citrate and fumarate. Besides this, it is important to report that the levels of nocturnal organic acids which accumulated in *K. tubiflora* were significantly higher than in *K. laxiflora*. In this sense, *K. tubiflora* might perform a stronger CAM photosynthesis when compared with *K. laxiflora*.

As discussed before, high ammonium concentrations (5.0 or 10.0 mM) have been shown to have a toxic effect on most of the *Kalanchoë* species that have been previously studied, and it could be responsible for decreasing the CAM expression in these species (Ota

1988a). However, this raises the question why would a high nitrate concentration (5.0 mM) also decrease CAM expression in *K. laxiflora* and *K. tubiflora*? Studies have reported that NO_3^- could inhibit the tonoplast ATPase (Smith *et al.* 1984). Thereby, plants kept under a high nitrate concentration (5.0 mM), would not allow the movement of organic acids and protons into the vacuole, which would decrease CAM expression in these *Kalanchoë* species. However, low nitrate concentrations (2.5 mM) would not have an inhibitory effect on the tonoplast ATPase, as we saw in this study when this nitrogen treatment presented the highest ATP-dependent proton transport rates in *K. laxiflora* and *K. tubiflora*. In *N. tabacum* plants, the relative rates of ATP-dependent malate transport in the tonoplast vesicles were higher in the presence of 10.0 mM of nitrate rather than 20.0 mM of this inorganic nitrogen source (Lüttge *et al.* 2000). Thereby, the nitrate inhibitory effect seems to be directly related to nitrate concentration, as well as dependent upon the species. In *K. tubiflora* and *K. laxiflora*, 5.0 mM of nitrate was enough to decrease ATP-dependent proton transport.

In regard to proton and organic acids transport into the vacuole, White and Smith (1989) reported very similar ATP- and PPi-dependent proton transport rates in the presence of fumarate or D-malate for the plant *K. daigremontiana*, and this transport was more active in the presence of fumarate compared with malate. In *K. laxiflora* and *K. tubiflora* a significant preference for ATPase to the proton transport in the presence of fumarate or malate, rather than PPiase in all of the treatments was observed in this study. Moreover, fumarate was more effective than malate and citrate in all of the treatments, performed on both species, in stimulating ATP- or PPi-dependent vesicle acidification, indicating that the "preference" of the vacuolar channel for fumarate over malate seems to be an intrinsic property of this transport system in *K. laxiflora* and *K. tubiflora*. However, this does not imply that more fumarate will be accumulated than malate in the vacuole, as was shown in

our study, there is not more fumarate than malate available in the cytosol, therefore no more fumarate is able to be accumulated in the vacuole of both *Kalanchoë* species.

For PPi-dependent vesicle acidification, in *K. laxiflora*, fumarate was more effective than the other two organic acids tested in all of the treatments. In *K. tubiflora*, fumarate was more effective than citrate in all of the treatments, however it was only more effective than malate in 2.5 mM of ammonium and in higher nitrate and ammonium concentrations. ATP-dependent proton transport in *K. tubiflora*, in the presence of fumarate or malate, was significantly higher than in *K. laxiflora*. The contribution made by PPiase and V-ATPase in the proton transport reported to *M. crystallinum* showed a large difference, since the V-ATPase activity was about nine times higher than the PPiase when these plants were CAM induced by salinity stress. Bremberger *et al.* (1988) suggested that tonoplast ATPase is a more important enzyme than H^+-PPiase, in regards to driving the nocturnal accumulation of organic acids in this CAM plant. Our results corroborate this, as we noticed a higher proton transport in the vacuole in the presence of ATP rather than PPi for both *Kalanchoë* species used in this study. Besides a higher nocturnal organic acids accumulation in *K. tubiflora* compared with *K. laxiflora*, the quinacrine fluorescence-quenching experiments done for both species showed that the permeability of the tonoplast membrane for the anions used in this study appears to be higher in *K. tubiflora* rather than their permeability in *K. laxiflora*. This result is consistent with a higher nocturnal organic acids accumulation into the vacuole of *K. tubiflora* compared with the accumulation into the vacuole of *K. laxiflora*.

In conclusion, this study has provided evidence that both ATP- and PPi-dependent proton transport rates are influenced by inorganic nitrogen sources (simultaneous or individual occurrence) by their concentrations (2.5 or 5.0 mM) and by the *Kalanchoë* species itself. Moreover, we have shown to our knowledge for the first time in *Kalanchoë* species, that there is a slight ATP- and PPi-dependent proton transport in the absence of any anion,

that can be considered as a background, and also that there is a preference for ATPase rather than PPiase to the proton transport in both *Kalanchoë* species. Finally, our data indicate that a low nitrate concentration (2.5 mM) increases the nocturnal organic acids accumulation, increases ATP- and PPi-dependent proton transport into the vacuole in both *K. laxiflora* and *K. tubiflora*, and is responsible for the highest CAM expression in both *Kalanchoë* species; while a higher nitrate concentration (5.0 mM) decreases the amount of organic acids accumulated during the night, and also seems to have an inhibitory effect on ATP-dependent proton transport into the vacuole in both *K. laxiflora* and *K. tubifl*ora.

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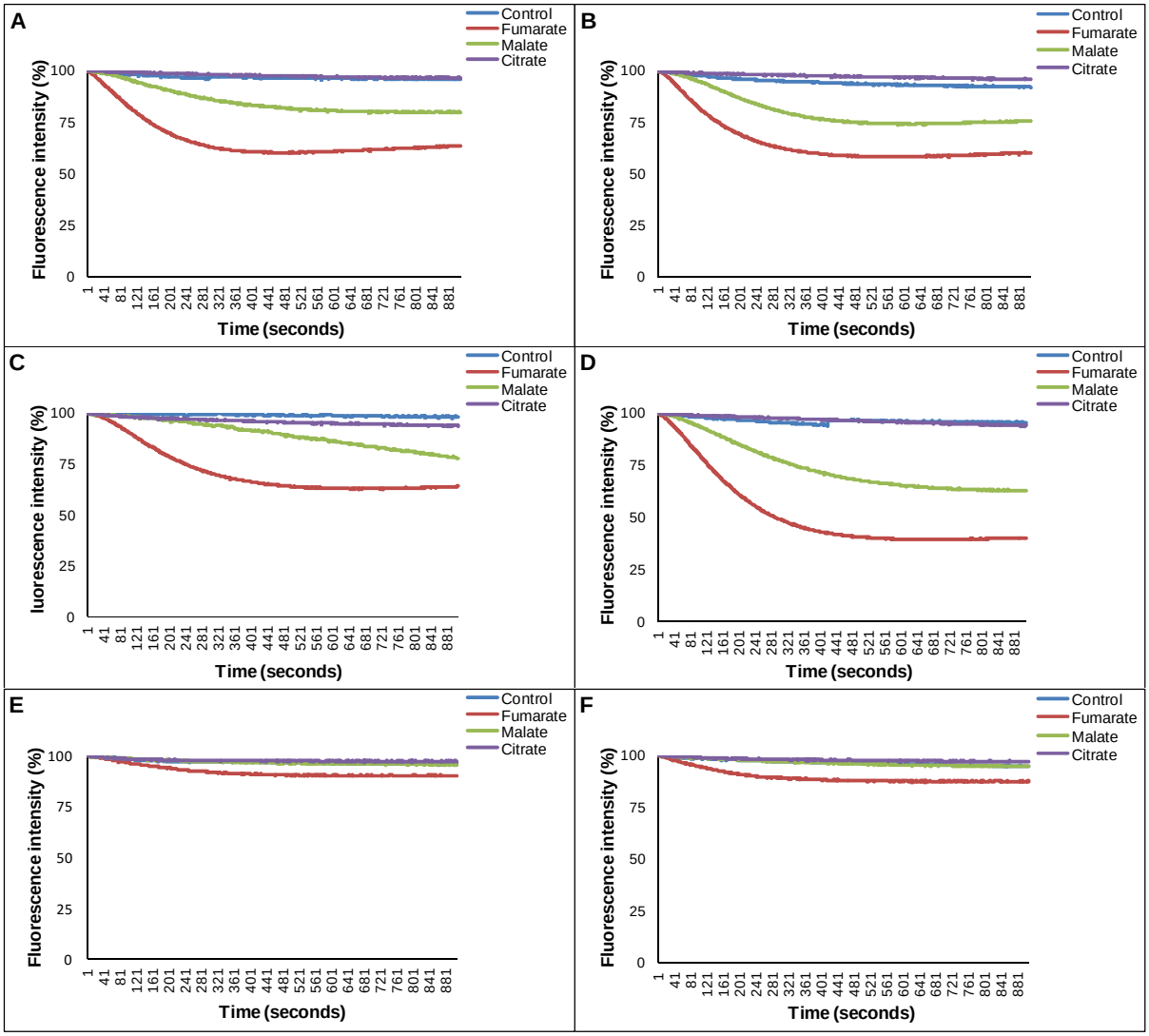
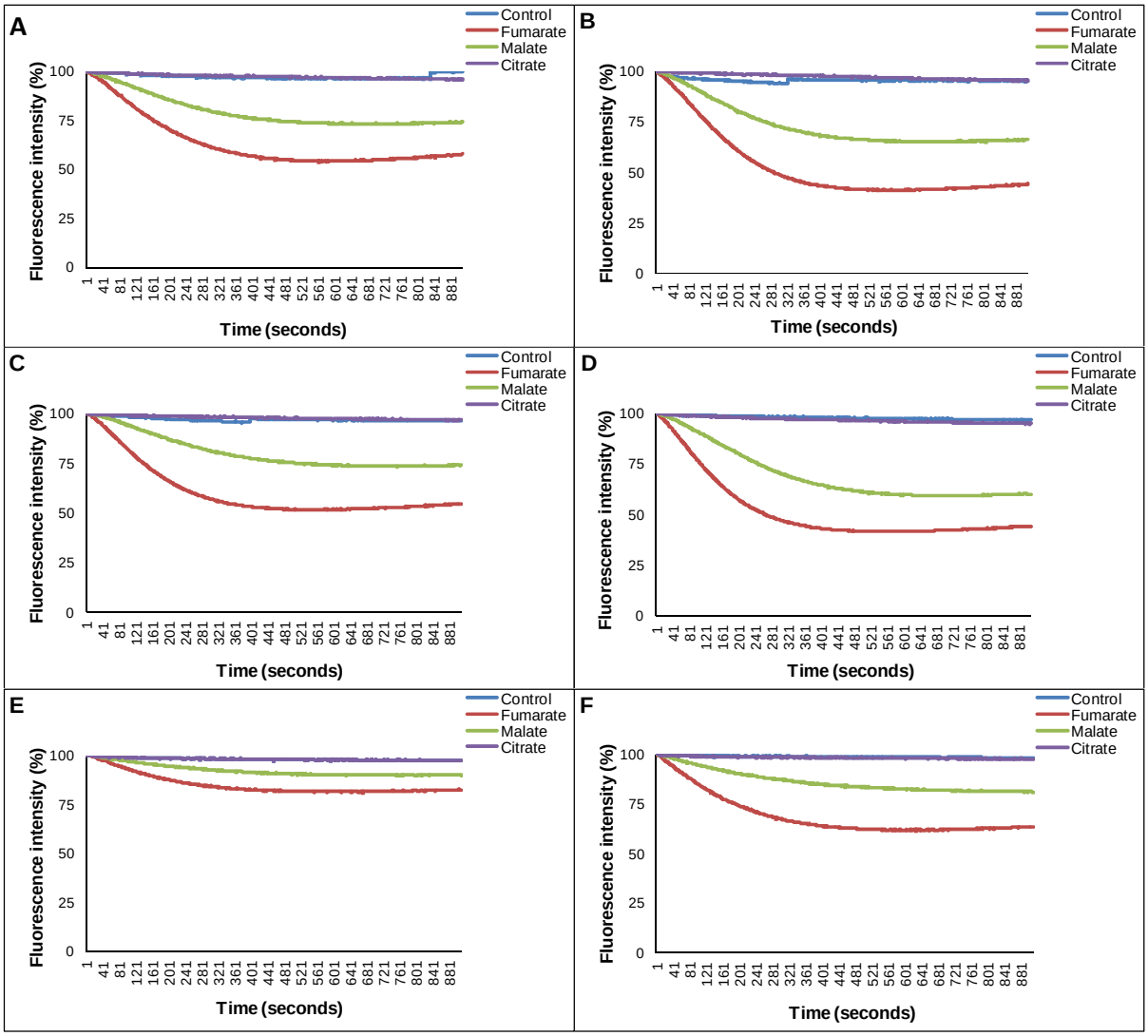
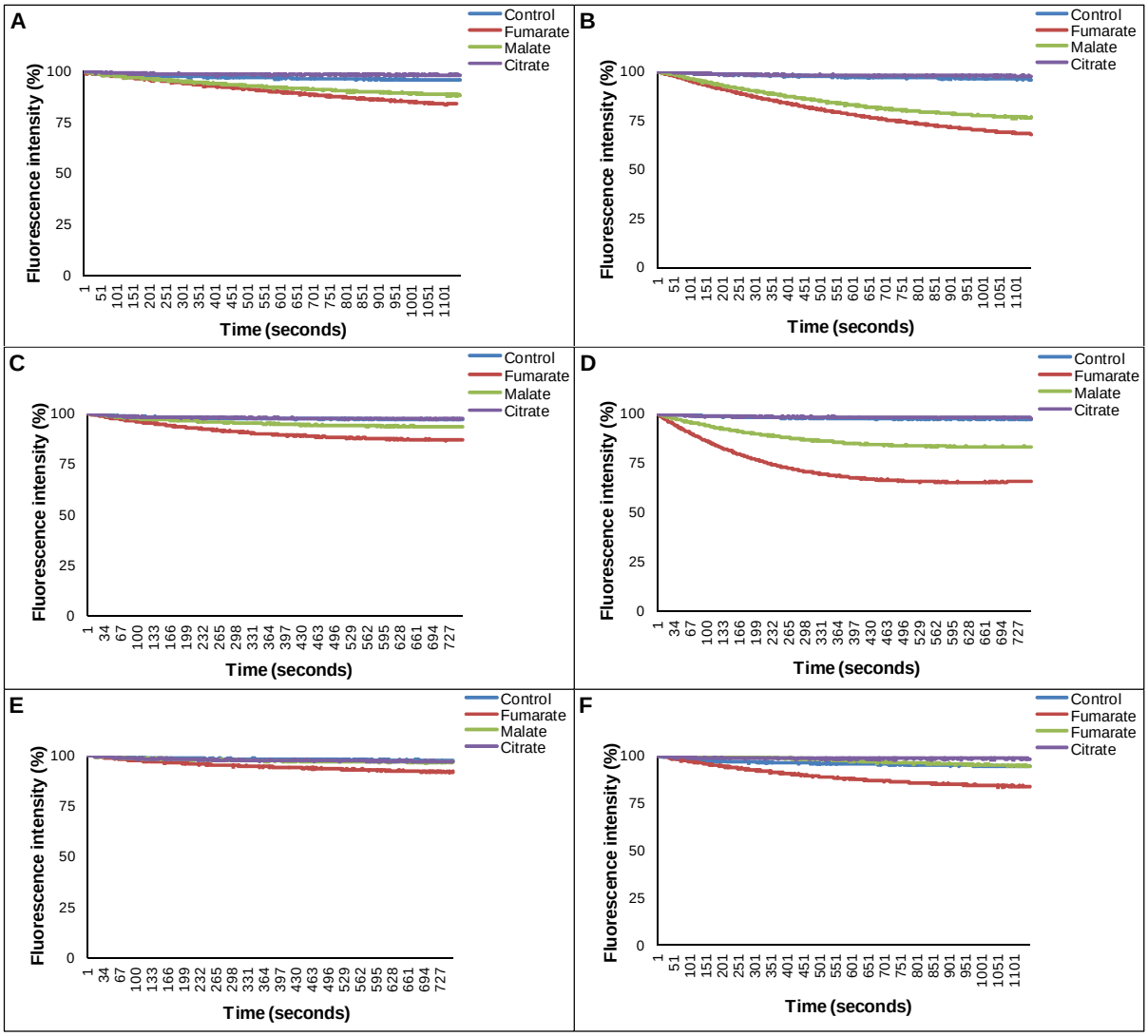
Supplementary Material



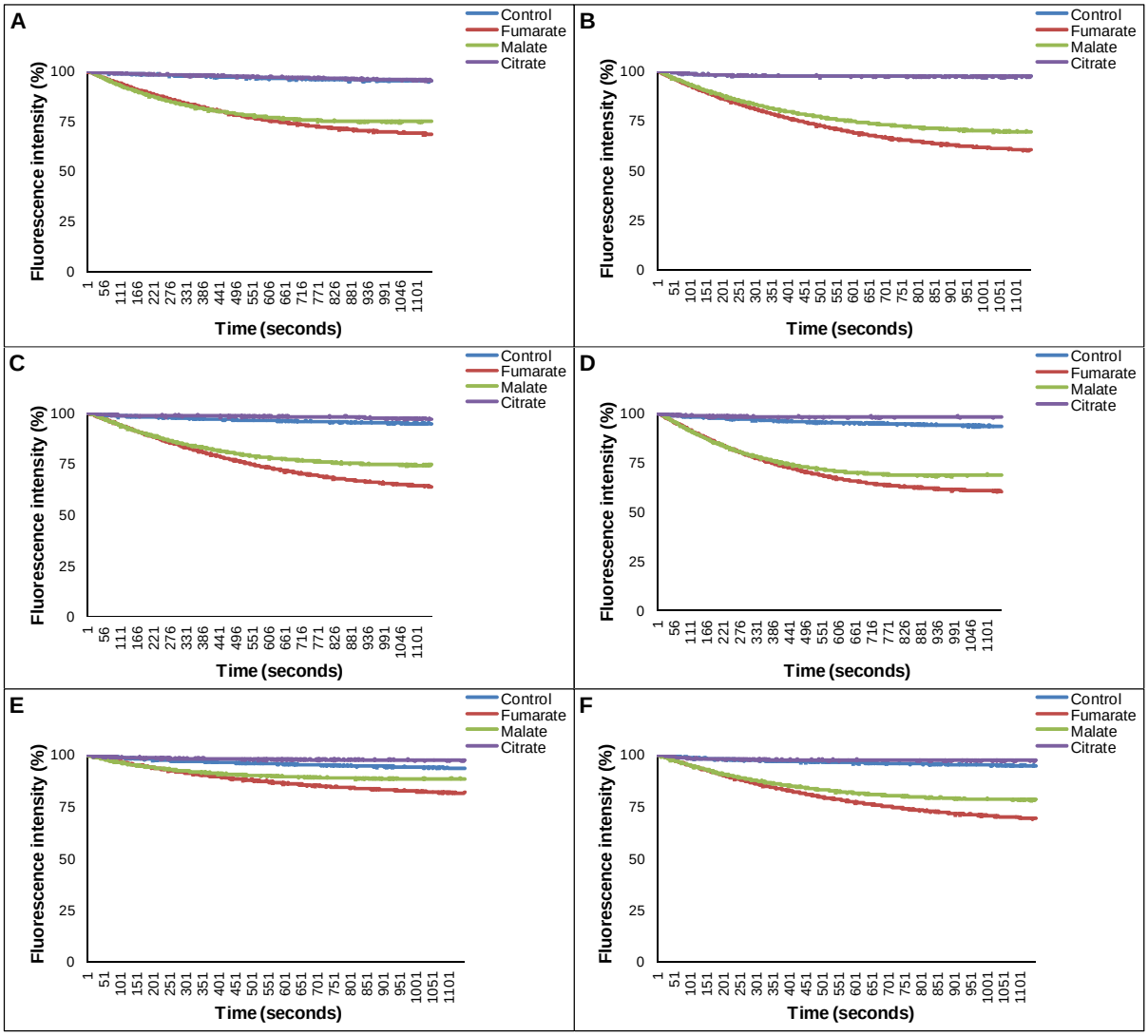
Supplementary Figure 1 ATP-dependent proton transport monitored by the quenching of quinacrine fluorescence in the control (no anion), presence of fumarate, malate and citrate in the tonoplast vesicles of *Kalanchoë laxiflora* in nitrogen-deficient (used as a control), 2.5 mM of ammonium + 2.5 mM of nitrate, 2.5 mM of ammonium, 2.5 mM of nitrate, 5.0 mM of ammonium and 5.0 mM of nitrate. Proton transport is expressed relative to the rate observed after adding ATP (3.0 mM) into the cuvette. Data are from one experiment, representative of a total of three preparations.



Supplementary Figure 2 ATP-dependent proton transport monitored by the quenching of quinacrine fluorescence in the control (no anion), presence of fumarate, malate and citrate in the tonoplast vesicles of *Kalanchoë tubiflora* in nitrogen-deficient (used as a control), 2.5 mM of ammonium + 2.5 mM of nitrate, 2.5 mM of ammonium, 2.5 mM of nitrate, 5.0 mM of ammonium and 5.0 mM of nitrate. Proton transport is expressed relative to the rate observed after adding ATP (3.0 mM) into the cuvette. Data are from one experiment, representative of a total of three preparations.



Supplementary Figure 3 PPi-dependent proton transport monitored by the quenching of quinacrine fluorescence in the control (no anion), presence of fumarate, malate and citrate in the tonoplast vesicles of *Kalanchoë laxiflora* in nitrogen-deficient (used as a control), 2.5 mM of ammonium + 2.5 mM of nitrate, 2.5 mM of ammonium, 2.5 mM of nitrate, 5.0 mM of ammonium and 5.0 mM of nitrate. Proton transport is expressed relative to the rate observed after adding Na_4PPi (500µM) into the cuvette. Data are from one experiment, representative of a total of three preparations.



Supplementary Figure 4 PPi-dependent proton transport monitored by the quenching of quinacrine fluorescence in the control (no anion), presence of fumarate, malate and citrate in the tonoplast vesicles of *Kalanchoë tubiflora* in nitrogen-deficient (used as a control), 2.5 mM of ammonium + 2.5 mM of nitrate, 2.5 mM of ammonium, 2.5 mM of nitrate, 5.0 mM of ammonium and 5.0 mM of nitrate. Proton transport is expressed relative to the rate observed after adding Na_4PPi (500 µM) into the cuvette. Data are from one experiment, representative of a total of three preparations.

Ammonium intensifies CAM photosynthesis during water deficit increasing vacuolar malate channel expression and the tolerance to drought in leaves of *Guzmania monostachia* (Bromeliaceae)

Abstract

Guzmania monostachia is an epiphytic bromeliad capable of upregulating crassulacean acid metabolism (CAM) along the leaf in response to changing nutrients and water conditions. Previous studies have shown that under drought, the apical portion of the leaves increases CAM expression in comparison with the basal region. Additionally, absence of nitrogen combined with water deficit seems to increase CAM intensity in the apex of the leaves of this bromeliad. The present study has demonstrated the interrelationship between nitrate and/or ammonium and water deficit in the regulation of CAM intensity in *G. monostachia* leaves. The highest induction of the CAM photosynthesis was observed in the leaves kept in the presence of ammonium associated with a water deficit. For the first time, it was observed that NH_4^+ + water deficit is associated with the increase of ATP- and PPi-dependent proton transport into the vacuole and with higher expression levels of vacuolar malate channel, *ALMT9*. In addition, higher levels of total soluble sugars and an increase in antioxidant enzymatic activities (SOD, CAT, APX and GR) were observed in the presence of NH_4^+ + water deficit . In the presence of ammonium, the accumulation of soluble sugars and the activation of antioxidant defenses seem to be related to the increased of drought tolerance by osmotic adjustment and limitation of oxidative damage in the leaves of *G. monostachia*.

Keywords: ammonium, crassulacean acid metabolism, epiphytic bromeliad, malate transport, nitrate, tonoplast.

Abbreviations: ALMT9, aluminum-activated malate transporter 9; APX, ascorbate peroxidase; ATP, adenosine 5′-triphosphate; CAM, crassulacean acid metabolism; CAT, catalase; DW, dry weight; FB293, F-box protein 293; GR, glutathione reductase; MDH, malate dehydrogenase; PEG, polyethylene glycol; PEPC, phospho*enol*pyruvate carboxylase; PPi, inorganic pyrophosphate; RWC, relative water content; SOD, superoxide dismutase; TF2A, Transcription initiation factor IIA; TSS, total soluble sugar.

Introduction

In typical crassulacean acid metabolism (CAM), a water-saving photosynthetic pathway, the CO_2 is taken up at night and fixed by phospho*enol*pyruvate carboxylase (PEPC) as organic acids, mainly malic acid, into the vacuole (Crayn *et al.* 2004; Silvera and Lasso 2016). During the light period, organic acids are released from the vacuole and descarboxylated by malic enzyme (ME) or phosphoenopyruvate carboxykinase (PEPCK) and the CO_2 is reduced in the Calvin cycle (Ting 1985; Crayn *et al.* 2004; Silveira *et al.* 2009). CAM has been observed in 33 vascular plant families and in more than 6% of all vascular plants (Winter and Smith 1996; Crayn *et al.* 2015). Some plants are able to switch between C_3 and CAM photosynthesis in response to environmental factors, such as temperature, water, photon flux and nutrients (Winter *et al.* 2008; Freschi *et al.* 2010; Winter and Holtum 2011; Pereira *et al.* 2013). However, little has been discussed about the relationship between nutrients and CAM compared with the other environmental factors (Ota 1988a, 1988b; Santos and Salema 1991; Winter and Holtum 2011).

One of the most recent studies performed on *Calandrinia pohliandra* (Montiaceae), a facultative CAM species, showed a slight nocturnal CO_2 assimilation when KNO_3 fertilization was interrupted (Winter and Holtum 2011). Ota (1988a, 1988b) observed in *Kalanchoë blossfeldiana* (Crassuleaceae) the highest CAM expression in the presence of 1.0 or 5.0 mM of nitrate compared with 1.0 or 5.0 mM of ammonium. Rodrigues *et al.* (2014) demonstrated in the apical portion of the leaves of *Guzmania monostachia* (Bromeliaceae) a higher CAM expression in the absence of nitrogen compared with either the absence of potassium or phosphorus.

In relation to malate transport, the CAM orthologue of *Arabidopsis thaliana* protein, ALMT9 (aluminum activated malate transporter 9) seems to be the best candidate for this anion transport into the vacuole, however this vacuolar channel has not been

characterized in CAM species (Kovermann *et al.* 2007; Borland *et al.* 2009). On the other hand, the accumulation of malate as malic acid due to the H^+ transport in tonoplast vesicles conducted by H^+-ATPase and/or H^+-PPiase has been well described in *Kalanchoë daigremontiana* (Crassulaceae), an obligate CAM species (White and Smith 1989; Bartholomew *et al.* 1996; Lüttge *et al.* 2000). Lüttge *et al.* (2000) found in *Nicotiana tabacum* (Solanaceae), a C_3 species, higher malate accumulation and relative H^+ transport rates into the vacuole when the plants were cultivated in the presence of either 10.0 or 20.0 mM of nitrate compared with 3.0 and 6.0 mM of ammonium. In the same study, *K. daigremontiana* presented similar relative H^+ transport rates while in the presence of 10.0 or 20.0 mM of nitrate and 3.0 or 6.0 mM of ammonium (Lüttge *et al.* 2000). However, to our knowledge, no studies have shown the interrelationship among inorganic nitrogen sources, expression level of ALMT9 and ATP- and PPi-dependent proton transport into the vacuole in CAM plants.

Although past studies have shown that ammonium can be toxic and negatively affect the photosynthetic rates in some species, such as *Kalanchoë blossfeldiana* and *Moricandia arvensis* (Brassicaceae) (Winter *et al.* 1982; Ota 1988a), recent studies have verified higher photosynthetic activity in the presence of ammonium compared with nitrate (Guo *et al.* 2007; Hessini *et al.* 2013; Li *et al.* 2009; Zhonghua *et al.* 2011). *Spartina alterniflora* (Poaceae), a C_4 species, was able to maintain the highest photosynthetic rates and stomatal conductance when cultivated with ammonium, probably due to the increase of antioxidant enzymes, which limit oxidative damage and increase PEPC activity (Hessini *et al.* 2013). *Oryza sativa* (Poaceae) cultivated under water deficit (PEG 10%, MW: 6000) in medium containing ammonium presented higher CO_2 assimilation rates when compared with the plants cultivated in the presence of nitrate. In addition, Guo *et al.* (2007) inferred that

ammonium nutrition enhances the resistance to water stress in rice. However, little is known about the effects of combining ammonium with water deficit in CAM plants.

Here, we show that ammonium is more effective than nitrate in promoting an increase in CAM intensity in the leaves of *Guzmania monostachia* under drought conditions. This is probably because ammonium triggers the activation of ALMT9 and, consequently, the malate transport into the vacuole. Higher photosynthetic rates resulted in accumulation of soluble sugars, involved in the osmotic adjustment. Best performance of ammonium-treated leaves under water stress can also be related to an efficient activation of antioxidant enzymes.

Material and Methods

Plant material and Growth Conditions

Micropropagated plants of *Guzmania monostachia* (L.) Rusby ex Mez Var. *monostachia*, approximately 3 cm tall, were transferred to pots containing a commercial organic substrate (Tropstrato-"Vida Verde") and maintained in a greenhouse in the Department of Botany at the University of São Paulo, São Paulo, Brazil, until reaching the adult phase (approximately two and half years) (Pereira *et al.* 2013). After this period, plants were then transferred to controlled environmental chamber under 200 µmol m^{-2} s^{-1} of photosynthetic active radiation to the top leaf surfaces of the bromeliad, 12 h photoperiod, a day/night air temperature of 25/22ºC, and a day/night relative humidity of 60/70% for acclimation. During one month of acclimation all plants were watered with distilled water on a daily basis.

Water and Nutrient Deficit Treatment

Water deficit treatment was imposed based on Pereira *et al.* (2013) with modifications. After 30 days of acclimation in the chamber conditions described above, adult

plants that averaged 23.7 ± 0.9 cm tall with 32.7 ± 4.1 leaves and a tank volume of 40.5 ± 7.3 mL, had their 8th to 12th youngest fully developed leaves detached and individually transferred to glass flasks containing 10 mL of distilled water or 30% polyethylene glycol 6000 (PEG) (water deficit condition) diluted in: Knudson solution (Knudson, 1946) without nitrogen source (nitrogen-deficient) (hydric potential near ψ= -2.3 - MPa) or containing 2.5 mM of ammonium + 2.5 mM of nitrate ($+NH_4^+ + NO_3^-$) (hydric potential near ψ= -2.0 MPa), 5mM of ammonium ($+NH_4^+$) (hydric potential near ψ= -2.3 MPa) or 5mM of nitrate (NO_3^-) (hydric potential near ψ= -2.2 MPa) as the only source of nitrogen. The hydric potential was determined by a psychrometer (PSψPRO, Wescor, USA). Flasks with the detached leaves were kept in a controlled environment growth chamber as previously described. After 7 d, leaves from different nitrogen treatments were divided into three portions: a) basal, corresponding to the part of the leaf that forms the tank in the whole plant and contains lower levels of chlorophyll, b) apical, corresponding to the upper half of the green part of the leaf blade, and c) middle, corresponding to the lower half of the green part of the leaf blade. The apical portions were used for all biochemical and molecular analyses. The basal portion of the leaves was used only for the molecular assays. The middle region was used together with the apical portion to do the proton transport assays, because the fresh weight necessary to perform the method was 80 g.

Measurements of Relative Water Content (RWC)

Relative water content was determined according to the method described by Pereira *et al.* (2013). The fresh, dry and turgid weights of apical leaf discs (~ 1 cm^2) were weighed immediately after leaf harvest to determine the fresh weight (FW), and then these same discs were kept in contact with distilled water for 24 h. Afterwards, all leaf discs were weighed to determine the turgid weight (TW) and were subsequently maintained for 72 h at 60 ºC before

being weighed to determine the dry weight (DW). Tissue water content was calculated using the formula ((FW- DW)/ TW- DW)) x 100 (Martin and Schmitt, 1989). Measurements were made in triplicate.

Organic Acids Quantifications

The nocturnal accumulation of organic acids (Δ citrate and Δ malate) was determined according to the method described in **Chapter 2**. Leaf samples (100 mg) were collected 1 hour after the start of the light period (dawn), and 1 hour before the end of the light period (dusk). Afterwards, the samples were ground in liquid nitrogen and subsequently homogenized with 500 µL of MCW solution (methanol, chloroform and water, 12:5:1) containing benzoic acid which was used as the standard (1 mg mL^{-1} of methanol), the samples were then incubated at 60ºC for 30 min. All samples were centrifuged at 16.000 × g at 4ºC for 10 min. The supernatant was collected (50 µL) and dried for 1 hour at 60ºC in a SPEED-VAC (Labconco). Then, the dried sample was re-suspended in 25 µL of pyridine and 25 µL of N- tert- butyldimethylsilyl- N- methyltrifluoroacetamide (MTBSTFA) and derivatized for 1 h at 92ºC. An aliquot of 1µL of the derivatized sample was used to quantify the organic acids by gas chromatography - mass spectrometry (GC-MS), a chromatographic system (Shimadzu- QP2010SE), column (Agilent- DB5MS) and an auto sampler (Shimadzu- AOC-20i). Standard curves of citric and malic acids were used to determine the concentrations of individual organic acids in the samples. Results are expressed as micromole per gram of dry weight (µM g^{-1} DW).

PEPC and MDH Activities

Phospho*enol*pyruvate carboxylase (PEPC) and malate dehydrogenase (MDH) extractions and assays were performed as described by Pereira *et al.* (2013) with minor modification. Leaf samples (1 g) stored in liquid nitrogen were ground to a fine powder and

extracted in five volumes (v/w) of buffer containing 200 mM Tris–HCl (pH 8.0), 1 mM EDTA, 5 mM dithiothreitol (DTT), 10 mM $MgCl_2$, 10% (v/v) glycerol and 0.5% (w/v) bovine serum albumin (BSA) (5 mL of buffer per gram of tissue). The homogenate was centrifuged for 5 min at 15.000 × g, and the supernatant was used in the PEPC and MDH assays. The MDH activity (in the OAA-reducing direction) was assayed in a 2 mL reaction containing 50 mM Tris–HCl (pH 8.0), 2 mM OAA, 5 mM $MgCl_2$ and 200 µM NADH. For enzymatic determination, the reaction was initiated by adding an aliquot of 400 µL of the leaf extract. The PEPC activity was assayed in a 2 mL standard reaction medium containing 50 mM Tris–HCl (pH 8.0), 1 mM DTT, 10 mM $MgCl_2$, 10 mM $NaHCO_3$, 200 µM NADH and 3 mM phospho*enol*pyruvate. For enzymatic determination, the reaction was started by adding an aliquot of 200 µL of leaf extract. For both the PEPC and MDH activities determination, the change in absorbance was continuously measured at 340 nm after adding the leaf extracts, and all reported rates are from linear portions of absorbance versus time curves (usually between 0 and 10 min). The enzymes were assayed at 30°C. PEPC and MDH activities were expressed as µmol NADH consumed per minute per gram of dry weight (µmol NADH min^{-1} g^{-1} DW).

Soluble Sugars Quantifications

In order to quantify soluble sugars (glucose, fructose and sucrose), samples (100 mg) were ground in liquid nitrogen and subsequently homogenized with 500 µL of MCW solution (methanol, chloroform and water, 12:5:1) containing phenyl-b-glucopyranoside that was used as the standard (2 mg/mL of methanol), and the samples were then incubated at 60ºC for 30 min. All samples were centrifuged at 16.000 x g at 4ºC for 10 min. The supernatant was collected (50 µL) and dried for 1 hour at 60ºC in a SPEED-VAC (Labconco). Then, the dried sample was re-suspended in 25 µL of pyridine and 25 µL of Bis(trimethylsilyl)

trifluoroacetamide (MTBSTFA) and derivatized for 1 hour at 75ºC. An aliquot of 1µL of the derivatized sample was used to quantify soluble sugar by gas chromatography - mass spectrometry (GC-MS), a chromatographic system (Shimadzu- QP2010SE), column (Agilent- DB5MS) and an auto sampler (Shimadzu- AOC-20i). Standard curves of glucose, fructose and sucrose were used to determine the concentrations of individual soluble sugar in the samples. Results are expressed as micromole per gram of dry weight (µM g^{-1} DW).

Antioxidants Enzymes Activities

Fresh leaf samples of 200 mg harvested at 12h (light period) were ground with 2 mL of extraction solution described by Souza *et al.* (2013). Homogenates were centrifuged at 11000 *g*, 4°C for 30 min, and supernatants were kept at -80°C until analysis. All samples were analyzed in triplicates.

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

GR enzyme activity was determined following Shaedle and Bassham (1977). The reaction mixture contained 50 mM Tris-HCl (pH 7.5), 0.5 mM oxidized glutathione (GSSG), 0.15 mM NADPH and 3 mM $MgCl_2$. The reaction was started by adding 100 µL of leaf extract to the reaction mixture. NADPH consumption by GR was measured at 340 nm in a

spectrophotometer at 15 s intervals for 2 min. Activity was calculated with an extinction coefficient of 6.2 mM^{-1} cm^{-1} and expressed as µmol $NADP^+$ min^{-1} g^{-1} DW.

APX enzyme activity was assayed with the method described by Nakano and Asada (1981), modified by Weng *et al.* (2007). The reaction mixture contained 100 mM potassium phosphate (pH 7.0), EDTA 0.1 mM, 0.5 mM ascorbate and 0.2 mM H_2O_2. The reaction was started by adding 40 µL of leaf extract into the reaction mixture. The oxidation of ascorbate was measured at 290 nm in a spectrophotometer at 15 s intervals for 2 min. The activity was calculated with an extinction coefficient of 2.8 mM^{-1} cm^{-1} and expressed as µmol ascorbate min^{-1} g^{-1} DW.

CAT enzyme activity was quantified according to Luck (1974) with some modifications. The reaction solution contained 100 mM potassium phosphate (pH 7.5) and 15 mM H_2O_2. The reaction was started by adding 100 µL of leaf extract into the reaction solution. The consumption of H_2O_2 was measured at 240 nm in a spectrophotometer at 15 s intervals for 2 min. CAT activity was calculated with an extinction coefficient of 0.4 mM^{-1} cm^{-1} and expressed as µmol H_2O_2 min^{-1} g^{-1} DW.

Tonoplast Isolation

The method for tonoplast vesicle extraction was based on that used for Ananas comosus (McRae *et al.* 2002) and other bromeliad species (Chapter 1). Leaves were harvested in the flasks kept in controlled environment chambers 1.0 to 1.5 h after commencement of the light period. The sections of leaf lamina (apex and middle portions) totaling approximately 80 g fresh mass were suspended in 250 mL of ice-cold extraction buffer containing the following: 450 mM mannitol, 3.0 mM $MgSO_4$, 2.0 mM ethylenediaminetetraacetic acid disodium salt (EDTA), 10 mM DL-dithiothreitol (DTT), 1.0% (w/v) polyvinylpyrrolidone (PVP-40), 0.5% (w/v) bovine serum albumin, 100 mM tris(hydroxymethyl)aminomethane (Trizma® base,

adjusted to pH 8.0 with HCl), 1mM phenylmethysulphonyl fluoride, 1.1 M glycerol, 0.5 mM 3,5-di-tert-4-butylhydroxytoluene, 25.19 mM potassium disulfite and 1.0 mM benzamidine hydrochloride. After precooling, the tissue was homogenized in a commercial blender and the homogenate filtered through two layers of cheesecloth and then centrifuged at 18000 × g for 20 min. The resulting supernatant was centrifuged at 80000 × g for 60 min. The resulting pellet was layered over a 25% (w/v) sucrose cushion containing 1.1 M glycerol, 1.0 mM EDTA, 10 mM Tricine (N-[tris(hydroxymethyl)methyl]glycine), adjusted to pH 8.0 with BTP (1,3-bis[tris(hydroxymethyl)methylamino]propane), and 2.0 mM DTT. The gradients were centrifuged at 100 000 × g for 70 min, after which tonoplast vesicles were removed from the interface using a Pasteur pipette. Vesicles were then pelleted at 100 000 × g for 50 min and finally resuspended in the same buffer as the first pellet. All steps were performed at 4^{o}C. Preparations were stored at −80^{o}C until required.

Measurement of Vesicle Acidification.

Rates of intravesicular acidification on energization of the tonoplast H^+-ATPase or H^+-PPiase were determined according to the method described by White & Smith (1989) and in Chapter 1, with minor modification. Initial rates of H^+ transport at 25ºC were determined from the initial rates of fluorescence quenching upon the addition of 3.0 mM Tris-ATP or 500 mM Na_4PPi to the reaction medium. For assays of ATP-dependent H^+ transport, the reaction medium contained approx. 2–9 µg protein, 3.0 µM quinacrine (6-chloro-9-{[4-(diethylamino)-1-methylbutyl]amino}-2-methoxyacridine dihydrochloride), 6.0 mM $MgSO_4$, 0.3 mM EDTA, 150 mM mannitol and 25 mM BTP buffered to pH 8.0 with Mes (2-(N-morpholino) ethansulphonic acid). For measurements of PPi-dependent H^+ transport, the reaction medium was identical except that the $MgSO_4$ concentration was increased to 7.5 mM and the medium also contained 100 mM K-Mes, permeant anions to be tested were present at

50 mM (supplied as fumaric acid, malic acid, or citric acid, and buffered to pH 8.0 with BTP). Inhibitors tested were 50 mM potassium nitrate (inhibitor of vacuolar H^+-ATPase), 100 µM sodium orthovanadate (inhibitor of plasma membrane H^+-ATPase), 100 µM sodium azide (inhibitor of mitochondrial ATP synthase), and 2.0 mM ammonium sulphate (uncoupler of transmembrane pH gradients). Fluorescence quenching was measured using a model *LS-55 luminescence spectrometer* (*Llantrisant, UK)* with excitation at 422 nm and emission at 495 nm, both with a slit width of 5 nm.

Protein Determination

Protein concentration was measured according to Bradford (1976), using bovine serum albumin as the standard.

RNA Extraction and RT-PCR

Total RNA was extracted from 100 mg (FW) of powdered leaf material (apex and basal portions collected separately at dusk) using PureLink RNA Mini Kit (Ambion) following the manufacturer's recommendations. Genomic DNA contamination was removed by treatment with DNAse I (Invitrogen). The cDNA was synthesized using the SuperScript III One-Step RT-PCR System (Invitrogen) as recommended by the manufacturer. Amplifications were carried out in total volume of 10 uL with SYBR Select Master Mix (Applied Biosystems) on StepOnePlus Real-Time PCR (Applied Biosystems). PCR conditions consisted of an initial heating step at 95°C for 10 min, followed by 95°C for 15 s, 40 cycles of 54°C for ALMT9 or 52°C for the reference genes for 30 s, 72°C for 30 s. After cycling, melting curves were run from 60°C to 95°C for 20 min, to confirm that a single PCR product was amplified. The analyses of expression stability of the reference genes were performed with BestKeeper (Pfaffl 2004). The relative expression level of target genes was calculated as described by Pfaffl (2001), with the expression values normalized against the

geometric mean of two reference genes (Pfall 2001). All reactions were performed using three biological replicates and two technical replicates. Primers used in this study were designed on partial *G. monostachia* sequences obtained in the transcriptomic analysis of this species performed by our group (unpublished data). The primers pairs used are shown in Supplementary Table S1.

Statistical Analysis

All data were presented as mean values with ± standard deviation (SD). The differences between averages in relation to watered and drought conditions were assessed by t-Student's average comparison test at $P < 0.05$. The significant differences among the different treatments were contrasted by Tukey-Kramer's test at $P < 0.05$.

Results

PEG 6000 (30%) induces a water deficit in detached leaves

The leaves of *G. monostachia* kept under water deficit for 7 days by the addition of PEG 6000 showed a decrease in the relative water content (RWC) independent from the nitrogen treatment when compared with the leaves kept in water. Besides, NH_4^+ + water deficit provided a lower decrease in the relative water content compared with the other nitrogen treatments under water deficit (Fig.1). These results indicated that PEG 6000 (30%) can induce a deficiency of water in the leaves of *G. monostachia*.

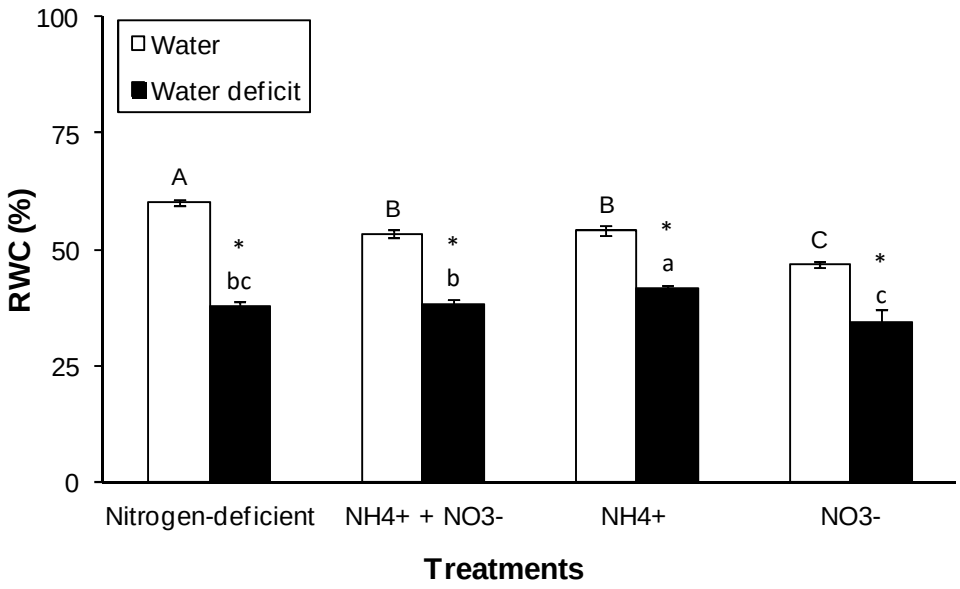


Fig.1. Relative water content in the leaves of *G. monostachia* kept for 7 days in water or water deficit (PEG 6000) associated with the presence or absence of ammonium and/or nitrate. Data are expressed as the mean (±SD) of three replicate samples. Asterisks indicate a significant difference between control (water) and water deficit in the same nitrogen treatment ($P < 0.05$%; Student's t-test). Different capital letters indicate averages that are significantly different among the nitrogen treatments + water ($P < 0.05$; Tukey-Kramer's test). Different lower case letters indicate averages that are significantly different among nitrogen treatments + water deficit ($P < 0.05$; Tukey-Kramer's test).

CAM induction in the leaves of G. monostachia under different nitrogen sources

One of the most important parameters used to check the occurrence of CAM photosynthesis is the nocturnal accumulation of organic acids. As shown in Fig. 2A and 2B, the leaves kept under NH_4^+ + water deficit presented the highest levels of malate and citrate accumulation during the night. The nocturnal malate accumulation levels in the apical portion of the leaves kept under water stress condition were1.3, 2.3 and 1.9 times higher when the leaves were in the presence of ammonium rather than in the nitrogen- deficient, $NH_4^+ + NO_3^-$ or NO_3^-, respectively (Fig. 2A). In Figure 2B, the leaves kept under NH_4^+ followed by the

presence of NO_3^- presented the highest citrate nocturnal levels. Malate accumulation during the night was always higher than citrate accumulation independent of the nitrogen treatment (Fig.2).

In addition to the accumulation of nocturnal organic acids, PEPC and MDH activities were analyzed to check the CAM expression in the leaves of *G. monostachia* (Fig. 2). In accordance with the higher accumulation of organic acids, the highest PEPC activity was observed in the leaves kept under a water deficit associated with NH_4^+ (Fig. 2C), followed by leaves maintained in nitrogen-deficiency + water deficit. In the presence of NH_4^+ + water deficit, PEPC activity was about 1.3 to 2.2 times higher than in the other nitrogen treatments that were associated with water deficit (Fig. 2C). Except for NH_4^+ + NO_3^-, all nitrogen treatments that were associated with a water deficit presented higher PEPC activity than when in the presence of water (Fig. 2C). In relation to MDH, the highest values of activity were observed in the leaves kept in the presence of NH_4^+ + water deficit and nitrogen deficient + water deficit conditions (Fig. 2D). The influence of ammonium or nitrate concentrations on the PEPC activity was also checked in the leaves of *G. monostachia* (Fig S1). The PEPC activity was always higher in the leaves kept in the presence of NH_4^+ rather than NO_3^- (Fig S1). Higher ammonium concentrations increased the PEPC activities, while no significant changes were detected in PEPC activities under different nitrate concentrations (Fig S1).

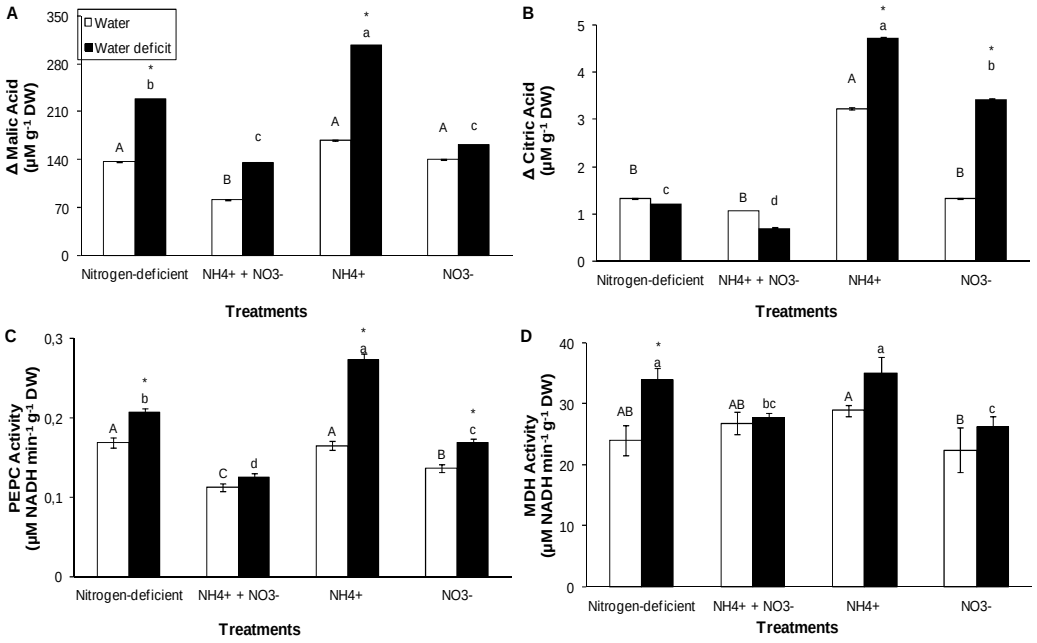


Fig.2. Nocturnal malic (A) and citric (B) acids accumulation, PEPC (C) and MDH (D) activities in the leaves of *G. monostachia* kept for 7 days in water or water deficit associated with the presence or absence of ammonium and/or nitrate. Data are expressed as the mean (±SD) of three replicate samples. Asterisks indicate a significant difference between water and water deficit in the same nitrogen treatment ($P < 0.05\%$; Student's t-test). Different capital letters indicate averages that are significantly different among the nitrogen treatments + water ($P < 0.05$; Tukey-Kramer's test). Different lower case letters indicate averages that are significantly different among nitrogen treatments + water deficit ($P < 0.05$; Tukey-Kramer's test).

Effect of water deficit and N sources on soluble sugars content

Fructose, glucose and sucrose were quantified in *G. monostachia* leaves kept under different nitrogen treatments associated with water deficit and their respective controls (Fig. 3). The main soluble carbohydrate accumulated was glucose followed by fructose and sucrose depending on the nitrogen treatment and water condition (Fig 3A, 3B and 3C). The leaves kept in NH_4^+ + water deficit presented the highest total soluble sugars (TSS) levels

(Fig. 3D). In general, the leaves kept in water deficit presented an increase in the TSS, mainly glucose and sucrose contents, compared with the leaves kept in water in all nitrogen treatments (Fig.3).

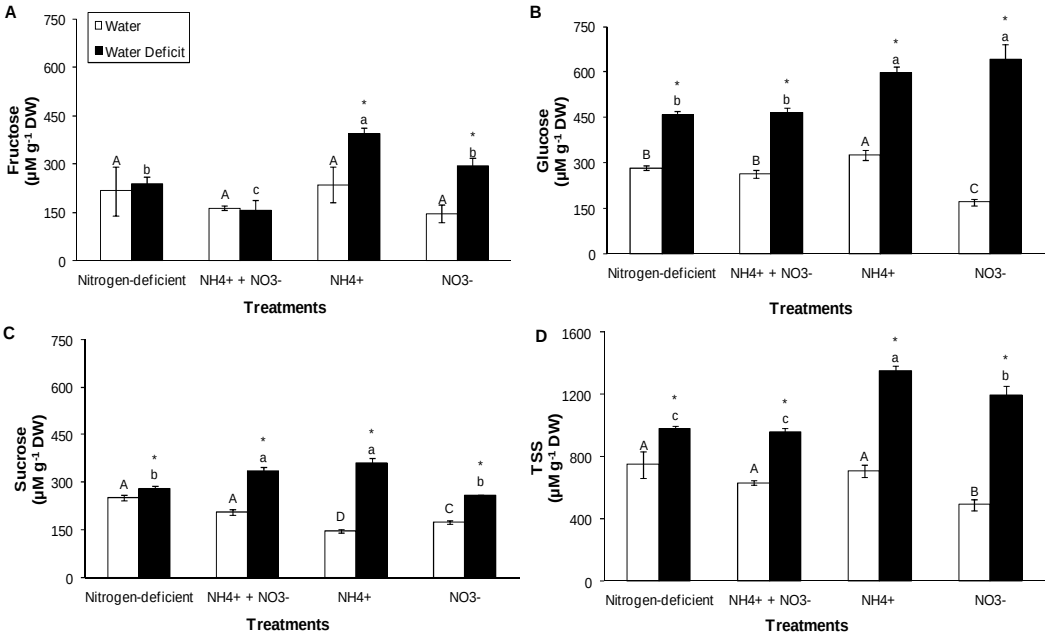


Fig.3. Fructose (A), Glucose (B), Sucrose (C) and total soluble sugars (TSS) (D) content in *G. monostachia* leaves kept for 7 days in water or water deficit associated with the presence or absence of ammonium and/or nitrate. Data are expressed as the mean (±SD) of three replicate samples. Asterisks indicate a significant difference between water and water deficit in the same nitrogen treatment ($P < 0.05$%; Student's t-test). Different capital letters indicate averages that are significantly different among the nitrogen treatments + water ($P < 0.05$; Tukey-Kramer's test). Different lower case letters indicate averages that are significantly different among nitrogen treatments + water deficit ($P < 0.05$; Tukey-Kramer's test).

Effect of water deficit and N sources on antioxidant enzymes activities

Under water deficit conditions the activities of the antioxidant enzymes (SOD, CAT, APX and GR) were higher in the leaves kept in $NH4^+$ + water deficit, compared with the leaves kept in water, as well as the leaves kept under the other nitrogen treatments + water

deficit (Fig. 4). For SOD, the enzyme that exhibited the highest activity levels compared with the other antioxidants enzymes, all of the nitrogen treatments associated with water deficit exhibited higher levels of activity rather than in the presence of water (Fig. 4A). Water deficit decreased the APX activity for all nitrogen treatments, excepting for $NH4^+$ that showed a significant increase in the activity of this enzyme.

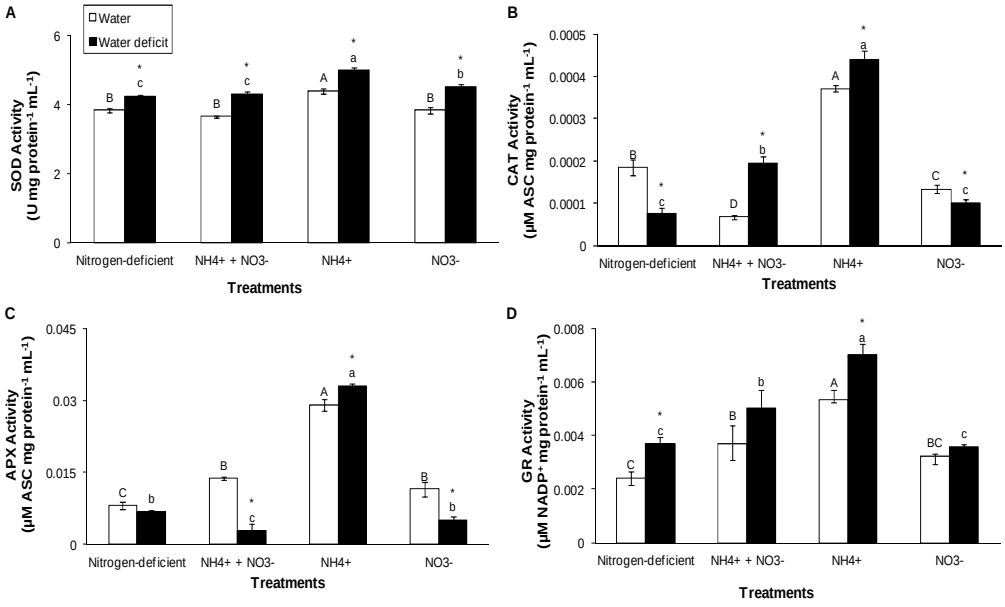


Fig.4. Antioxidant enzymatic activities of SOD (A), CAT (B), APX (C) and GR(D) in *G. monostachia* leaves kept for 7 days in water or water deficit associated with the presence or absence of ammonium and/or nitrate. Data are expressed as the mean (±SD) of three replicate samples. Asterisks indicate a significant difference between water and water deficit in the same nitrogen treatment ($P < 0.05$%; Student's t-test). Different capital letters indicate averages that are significantly different among the nitrogen treatments + water ($P < 0.05$; Tukey-Kramer's test). Different lower case letters indicate averages that are significantly different among nitrogen treatments + water deficit ($P < 0.05$; Tukey-Kramer's test).

Effect of water deficit and N sources on ATP and PPi-dependent proton transport rates

To determine the relative contribution of vacuolar and non-vacuolar membranes in the green portion of the leaf blade of *G. monostachia* leaves kept in different nitrogen treatments, we used a specific inhibitor of vacuolar membranes (V-ATPases) (KNO_3) or non-vacuolar membranes (non V-ATPases) (NaN_3 plus Na_3VO_4) to show that most of the membranes used were vacuolar (Table S1).

The leaves of *G. monostachia* exhibited higher ATP-dependent proton transport rates in the presence of fumarate compared with malate and citrate independent from the nitrogen treatments and water conditions (Table 1). In the presence of fumarate, the leaves kept in NH_4^+ + water deficit presented the highest ATP-dependent proton transport, while the leaves kept in the presence of NO_3^- showed the lowest proton transport rates in the presence of the same anion (Table 1). ATP-dependent proton transport rates were about two times higher in the leaves kept in the presence of NH_4^+ + water deficit than in the leaves maintained in NO_3^- + water deficit (Table 1). For all of the nitrogen treatments, the order of effectiveness of ATP-dependent proton transport was always fumarate > malate > citrate (Table 1).

Table 1 ATP-dependent proton transport was measured as initial rates of quinacrine-fluorescence quenching in the presence of fumarate, malate and citrate (present as their BTP-salts at 50 mM) in the tonoplast vesicles of *G. monostachia* leaves kept in water or PEG 30% associated with the presence or absence of ammonium and/or nitrate. Results are expressed as the mean (±SD) and are relative to rates of proton transport measured in % H^+ min^{-1} mg $protein^{-1}$. Different capital letters indicate averages that are significantly different among treatments that use the same anion in the same portion of the leaves ($P < 0.05$; Tukey-Kramer's test). Different lower case letters indicate averages that are significantly different

among anions in the same treatment in each portion of the leaves ($P < 0.05$; Tukey-Kramer's test).

	Specific activity (% min^{-1} mg $protein^{-1}$)		
Treatments	**Fumarate**	**Malate**	**Citrate**
Nitrogen-deficient + water	162 ±1.5 D a	72.4 ± 0.3 C b	35.6 ± 1.3 C c
Nitrogen-deficient + water deficit	197 ± 0.3 B a	80.3 ± 1.4 B b	52.7 ± 0.7 A c
NH_4^+ + NO_3^- + water	164 ± 1.2 D a	59.4 ± 1.0 D b	42.03 ± 0.4 BC c
NH_4^+ + NO_3^- + water deficit	189 ± 6.2 BC a	78.1 ± 1.3 BC b	53.2 ± 1.2 A c
NH_4^+ + water	124 ± 1.5 E a	72.8 ± 3.3 C b	47.1 ± 1.1 AB c
NH_4^+ + water deficit	317 ± 1.6 A a	89.1 ± 0.7 A b	34.8 ± 1.1 C c
NO_3^- + water	117 ± 3.3 E a	62.4 ± 1.2 D b	53.4 ±1.3 A c
NO_3^- + water deficit	182 ± 3.7 C a	76.4 ±1.6 BC b	51.2 ±0.8 A c

PPi-dependent proton transport in the presence of fumarate, malate and citrate in the leaves kept under a water deficit showed higher proton transport rates compared with the leaves kept in water. (Table S3). The order of effectiveness of proton transport for the leaves maintained in water deficit was always fumarate > malate > citrate. In general, the PPi-dependent proton transport was lower than the ATP proton transport in all of the nitrogen treatments.

Effect of water deficit and N sources on ALMT9 relative expression

The *ALMT9* gene, which encodes a vacuolar malate channel, was up-regulated under water deficit, showing a higher induction (about 2 fold) when water deficit was associated to NH_4^+ nutrition in comparison to NO_3^-, $NH4^+$ + NO_3^- or nitrogen deficiency in the apical portion of the leaves of *G. monostachia* (Fig. 5). All nitrogen treatments led to the repression of this gene in the absence of water deficit. The *ALMT9* expression in the basal portion of the leaves was always lower than in the apical one (Fig. S2).

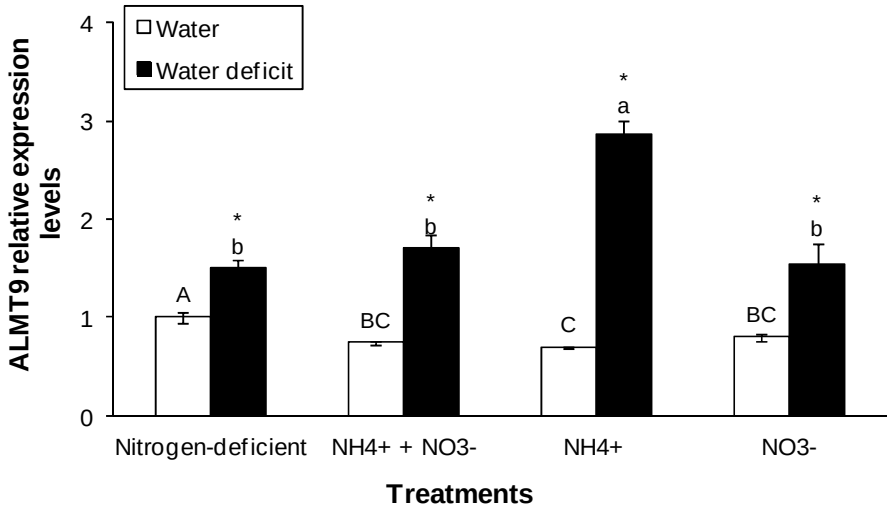


Fig. 5. qRT-PCR analysis of the expression of *ALMT9* gene in the apical portion of the leaves of *G. monostachia* kept in water or water deficit associated with ammonium and/or nitrate deficiencies for 7 days. Relative expression in each treatment was compared with a normalized control (nitrogen-deficient + water). Data are expressed as the mean (± SD). The mean relative expression was calculated from the means of two technical replicates from three biological replicates and normalized against the reference genes (FB293 and TF2A). Asterisks indicate significant differences between water and water deficit in the same nitrogen treatment ($P < 0.05\%$; Student's t-test). Different capital letters indicate averages that are significantly different among treatments + water in the same portion of the leaves ($P < 0.05$; Tukey-Kramer's test). Different lower case letters indicate averages that are significantly different among treatments + water deficit in the same portion of the leaves ($P < 0.05$; Tukey-Kramer's test).

Discussion

The data presented in this study highlight the important role that inorganic sources of nitrogen play in controlling the intensity of CAM photosynthesis. Our assays showed that presence of NH_4^+ + water deficit increases this photosynthetic pathway in the leaves of the bromeliad *Guzmania monostachia*, when compared with the other nitrogen treatments. Some

studies have shown the opposite response in *Kalanchoë blossfeldiana* (Ota 1988b; Ota and Yamammoto 1991). The highest nocturnal CO_2 fixation and malate accumulation during the night were observed in the presence of 1.0 mM of nitrate. On the other hand, lower concentrations (0.2 mM) of ammonium increased CO_2 uptake at night when compared with the same concentration of nitrate (Ota and Yamamoto 1991). When this species was cultivated in higher concentrations of nitrate or ammonium (10.0 mM), a higher CAM expression was observed in the presence of nitrate, while the plants kept under ammonium presented toxicity effects and died (Ota 1988b). For *G. monostachia* leaves, higher ammonium concentrations (5.0 mM) induced a higher CAM expression than lower concentrations (1.25 or 2.5 mM). On the other hand, when lower concentrations of ammonium were associated with nitrate the PEPC activity decreased more than when the nitrogen sources were applied separately. The decrease in PEPC activity in the presence of both nitrogen sources was likely a result of the lower ammonium concentrations, which decrease this enzymes activity and the presence of nitrate. Nitrate is known to increase the cytokinins levels, which act as negative regulator of PEPC activity in *G. monostachia* and *Mesembryanthemum crystallinum* (Schmitt and Piepenbrock 1992; Pereira *et al.* 2013).

Ammonium also showed a positive effect on the expression of *ALMT9* gene in the apical portion of the leaves of *G. monostachia*. This is the first study, to our knowledge, that has investigated the influence of inorganic nitrogen sources on the biochemical levels of CAM photosynthesis and molecular of *ALMT9* gene expression in the leaves of a C_3-CAM bromeliad species. The apical portion of *G. monostachia* leaves showed a higher *ALMT9* expression when in the presence of NH_4^+ + water deficit compared with the other nitrogen treatments and compared with the basal portion. Kovermann *et al.* (2007) reported that this gene is expressed in all organs and cytosolic malate and fumarate levels are responsible for activating *ALMT9* in *Arabidopsis*. *A. thaliana* wild-type plants exhibited a higher stomatal

conductance and aperture when compared with atalmt9 knockout mutants (De Angeli *et al.* 2013a). These results show the involvement of *ALMT9* in mediating chloride flux in the vacuole of guard cells to perform a stomatal aperture in *A. thaliana*. In *Nicotiana benthamiana* leaves, the heterologous expression of *AtALMT9* enhanced malate current densities across the mesophyll tonoplasts (Kovermann *et al.* 2007). In *Vitis vinifera* (Vitaceae) was observed that *VvALMT9* mediates malate and tartrate accumulation in the berries. This gene is expressed in mesocarp tissue and its transcriptional levels increases during the fruit maturation (De Angeli *et al.* 2013b). However, the major role of *ALMT9* described for *A. thaliana* may not be the same for other plant species, especially CAM plants. Borland *et al.* (2009) described *ALMT9* as the best candidate to be an inward-rectifying anion channel responsible for transporting malate into the vacuole through a voltage-gate. Our study corroborates the other publications since we verified that the highest *ALMT9* expression occurred in the apical portion of *G. monostachia* leaves kept under NH_4^+ + water deficit at dusk. Under this treatment, this region of the leaf presented a higher malate content, which was responsible for activating and increasing *ALMT9* expression levels. For *G. monostachia*, *ALMT9* seems to be related to the malate transport into the vacuole at night, when the organic acids accumulation into the vacuole is higher and CAM intensity is stronger in the apex of the leaves kept in NH_4^+ + water deficit. Although, more studies need to be performed in order to better understand the crucial role that *ALMT9* plays in *G. monostachia*.

In relation to proton and organic acids transport rates, another study performed with *Nicotiana tabacum* showed higher ATP-dependent proton transport and malate accumulation into the vacuole in the presence of nitrate rather than ammonium (Lüttge *et al.* 2000). However, no studies have shown what influence nitrogen sources have on proton and organic acids transport into the vacuole of CAM plants. Our results support the idea that NH_4^+

associated with water deficiency increases CAM intensity in the leaves of *G. monostachia* by increasing proton and organic acids transport into the vacuole.

Other species have shown a preference for ammonium rather than nitrate for photosynthesis and growth, since NH_4^+ seems to enhance the plant's tolerance to stress conditions (Polesskaya *et al.* 2003; Inselsbacher *et al.* 2007; Li *et al.* 2009; Fernández-Crespo *et al.* 2012; Hessini *et al.* 2013). NH_4^+ was the most important N source found in the tank of *Vriesea gigantea* (Bromeliaceae). The ammonium concentration in the tank and its foliar uptake for this bromeliad species was about 7 times higher than the nitrate concentration (Inselsbacher *et al.* 2007). In *Oryza sativa* a higher water uptake rate was observed in the presence of ammonium + PEG 10% (MW: 6000) (water deficit condition) compared with nitrate + PEG10% (MW: 6000) (Li *et al.* 2009). In another study performed with the same species a higher CO_2 assimilation rate was verified in the presence of NH_4^+ + PEG 10% (MW: 6000) rather than in the presence of nitrate or nitrate and ammonium + PEG 10% (MW: 6000) (Guo *et al.* 2007). These authors suggest that NH_4^+ nutrition enhanced *Oryza sativa* plants' tolerance for water stress (Guo *et al.* 2007; Li *et al.* 2009). In our study *G. monostachia* showed higher relative water content in the leaves kept under NH_4^+ + water deficit compared with the other nitrogen sources associated with water deficit. In this way, ammonium seems to enhance the tolerance of this bromeliad leaves to drought conditions.

Our results also show the highest PEPC and MDH activities in the leaves of *G. monostachia* kept under NH_4^+ + water deficit; this strengthens the idea that NH_4^+ increases CAM intensity in the leaves of this bromeliad. Similarly, *Spartina alterniflora* plants grown with NH_4^+ nutrition exhibited a higher photosynthetic activity due to an increase in PEPC activity. It was proposed for this species that ammonium could decrease the oxidative damage by the induction of antioxidant enzymes activities (Hessini *et al.* 2013). Additionally, for *G. monostachia*, the leaves that presented the highest CAM intensity in the presence of NH_4^+ +

water deficit also exhibited the highest SOD, CAT, APX and GR activities. This suggests that ammonium might decrease the oxidative damage in the leaves by increasing the antioxidant enzymes activities.

Under water stress conditions, plants accumulate sugars that can function as osmolytes which maintain the cell turgor, preventing and protecting membrane fusion and maintaining proteins functionality (Guo *et al.* 2007; Krasensky and Jonak 2012; Arabzadeh 2012; Sinay and Karuwal 2014; Ceusters *et al.* 2016). Lisar *et al.* (2012) reported that plants regulate the osmotic potential of the cell by increasing sugar contents in order to withstand drought conditions, mainly when drought stress increases from a weak stress to severe one. In *G. monostachia* the highest total soluble sugars levels (fructose, glucose and sucrose) were also observed in the leaves maintained under presence of NH_4^+ + water deficit. This result suggests that NH_4^+ enhances the tolerance of *G. monostachia* leaves to a severe water stress by increasing the soluble sugar content and promoting the reduction of water potential, which could increase the water-use efficiency and contribute to the better functioning of the CAM enzymes in the presence of this nitrogen source instead of nitrate or nitrogen-deficiency (Nimmo, HG 2000; Borland *et al.* 2011).

In conclusion, this study provides evidence of positive regulation of CAM intensity in the leaves of *G. monostachia*, when NH_4^+ is associated with water deficit, more specifically by increasing PEPC and MDH activities and organic acids vacuolar accumulation during the night. Proton transport into the vacuole and *GmALMT9* expression were increased in the presence of NH_4^+ + water deficit suggesting a regulation of CAM photosynthesis by this inorganic nitrogen source at the biochemical and molecular levels. Ammonium also promotes an increase in leaf tolerance to water deficit, probably by osmotic adjustment due to sugar accumulation and by improving leaf capacity to limit oxidative damage by stimulating

antioxidant enzymes activities, all of which seems to favor CAM photosynthesis in *G. monostachia*.

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Supplementary Material

Table S1. Primer pairs used for qRT-PCR analysis.

Annotation	Primer (forward/reverse)	Amplicon length (pb)	Efficiency (%)	R^2
GmALMT9	5' GCAAAGGATTTAATCGGGGT 3' 5' ATAAGGCTTCATCGTCGGGTA 3'	171	90	0.9998
GmTF2A	5' GATGTCAATGTGGCTTATGAGG 3' 5' CTTTTGCGTTTTCCAGAGGAC 3'	110	89	0.9999
GmFB293	5' CTGAAGATGTGAACAAGCAAATCA 3' 5' CTGCCCAAACAGAAGAAGG 3'	137	83	0.9999

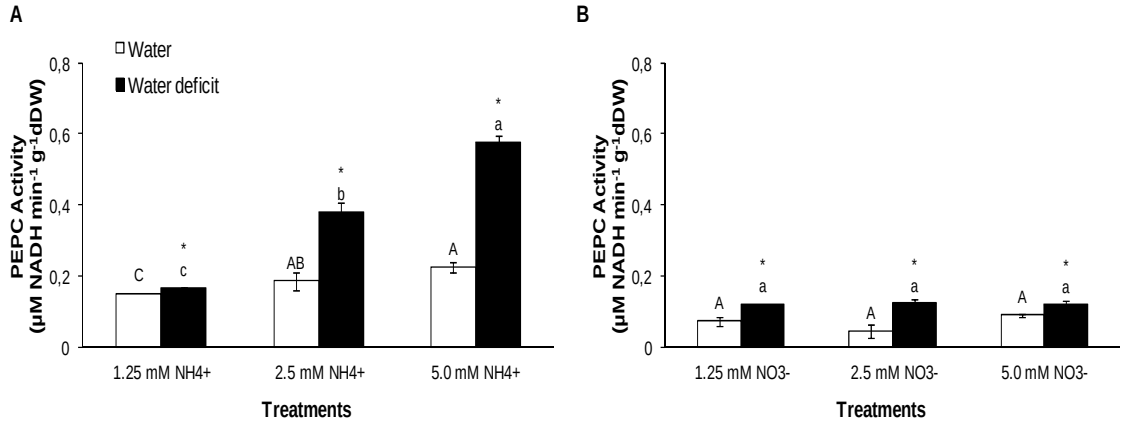

Figure S1. PEPC activity in *G. monostachia* leaves kept in water or water deficit associated with different ammonium (A) or nitrate (B) concentrations for 7 days. Data are expressed as the mean (±SD) of three replicate samples. Relative expression in each treatment was compared with a normalized control (nitrogen-deficient + water). Asterisks indicate a significant difference between water and water deficit in the same nitrogen treatment (P < 0.05%; Student's t-test). Different capital letters indicate averages that are significantly different among the nitrogen treatments + water (P < 0.05; Tukey-Kramer's test). Different lower case letters indicate averages that are significantly different among nitrogen treatments + water deficit (P < 0.05; Tukey-Kramer's test).

Table S2. ATP-dependent proton transport in the tonoplast vesicles of *G. monostachia* leaves in the presence of fumarate and of a specific inhibitors of the non-vacuolar membrane (NaN_3

plus Na_3VO_4) or a specific inhibitor of the vacuolar membrane (KNO_3) or no-inhibitor (control), measured as initial rates of quinacrine-fluorescence quenching. *G. monostachia* leaves were kept under water or PEG 30% associated with the presence or absence of ammonium and/or nitrate during 7 days. Results are expressed as the mean (±SD) for three preparations. The rates of proton transport were measured in % H^+ min^{-1} mg $protein^{-1}$ and % of inhibition. Different capital letters indicate averages that are significantly different among treatments that use the same anion in the same portion of the leaves ($P < 0.05$; Tukey-Kramer's test). Asterisks indicate averages that are significantly different Asterisks indicate significant differences between inhibitors in the same treatment by Student's T-test at 5% significance.

	Specific activity (% min^{-1} mg $protein^{-1}$) (inhibition relative to control)		
Treatments	**Control**	**+ KNO_3**	**NaN_3 + Na_4VO_3**
Nitrogen-deficient + water	322 ± 18.7 A	122 ± 9.2 C (62%)*	203 ± 17.6 B (37%)
Nitrogen-deficient + water deficit	365 ±10.2 A	95.8 ± 6.03 C (74%)*	301 ± 10.7 B (17%)
NH_4^+ + NO_3^- + water	300 ± 8.5 A	33.2 6.2 C (89%)*	188 ± 11.9 B (37%)
NH_4^+ + NO_3^- + water deficit	297 ± 17.5 A	63.7 ± 4.3 C (79%)*	255 ± 16.8 B (14%)
NH_4^+ + water	263 ± 13.9 A	43.9 ± 9.01 B (83%)*	258 ± 11.5 A (1.9%)
NH_4^+ + water deficit	407 ± 16.9 A	149 ± 14.9 C (63%)*	297 ± 8.9 B (27%)
NO_3^- + water	117 ± 8.9 A	22.6 ± 2.2 C (81%)*	80.4 ± 4.9 B (31%)
NO_3^- + water deficit	230 ± 4.9 A	68.4 ± 7.8 C (70%)*	169 ± 9.8 B (26%)

Table S3. PPi-dependent proton transport measured as initial rates of quinacrine-fluorescence quenching in the presence of fumarate, malate and citrate (present as their BTP-salts at 50 mM) in the tonoplast vesicles of *G. monostachia* leaves kept in water or PEG 30% associated with presence or absence of ammonium and/or nitrate. Results are expressed as the mean

(±SD) and are relative to rates of proton transport measured in % H^+ min^{-1} mg $protein^{-1}$. Different capital letters indicate averages that are significantly different among treatments that use the same anion in the same portion of the leaves ($P < 0.05$; Tukey-Kramer's test). Different lower case letters indicate averages that are significantly different among anions in the same treatment in each portion of the leaves ($P < 0.05$; Tukey-Kramer's test).

	Specific activity (% min^{-1} mg $protein^{-1}$)		
Treatments	**Fumarate**	**Malate**	**Citrate**
Nitrogen-deficient + water	168 ± 5.9 EF a	160 ± 1.2 B a	144 ± 6.9 A b
Nitrogen-deficient + water deficit	255 ± 7.2 B a	132 ± 7.7 C b	122 ± 6.4 B b
NH_4^+ + NO_3^- + water	107 ± 1.5 G a	100 ± 5.8 D a	88.9 ± 3.5 D b
NH_4^+ + NO_3^- + water deficit	155 ± 11.1 F a	117 ± 5.9 C b	97.9 ± 6.8 CD b
NH_4^+ + water	216 ± 3.1 C a	49.9 ± 1.1 E b	51.01 ± 3.2 F b
NH_4^+ + water deficit	293 ± 5.2 A a	186 ±5.7 A b	145 ±6.8 A c
NO_3^- + water	183 ± 5.4 DE a	185 ± 9.5 A a	113 ± 4.7 BC b
NO_3^- + water deficit	190 ± 5.5 D a	127 ± 1.2 C b	70.9 ± 6.8 E c

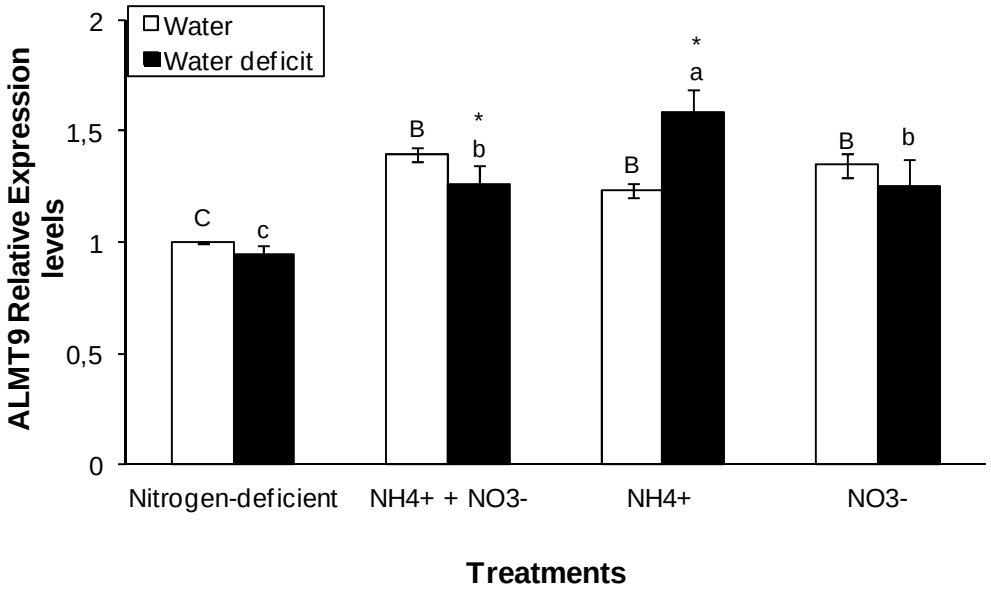

Figure S2. qRT-PCR analysis of the expression of *ALMT9* gene in the basal portion of the leaves of *G. monostachia* kept in water or water deficit associated with ammonium and/or nitrate deficiencies for 7 days. Relative expression in each treatment was compared with a normalized control (nitrogen-deficient + water) in each portion of the leaf. Data are expressed

as the mean (± SD). The mean relative expression was calculated from the means of two technical replicates from three biological replicates and normalized against the reference genes (FB293 and TF2A). Asterisks indicate significant differences between water and water deficit in the same nitrogen treatment ($P < 0.05$%; Student's t-test). Different capital letters indicate averages that are significantly different among treatments + water in the same portion of the leaves ($P < 0.05$; Tukey-Kramer's test). Different lower case letters indicate averages that are significantly different among treatments + PEG in the same portion of the leaves ($P < 0.05$; Tukey-Kramer's test).

Profiling of aquaporin gene expression in leaves of *Guzmania monostachia*: effects of day/night, nitrogen nutrition and water deficit regulation

Abstract

Leaves play an important role in the growth and development of plants, especially for tank epiphytic bromeliad species. In these plants, the basal portion of the leaves, where the tank is formed, is used to absorb and deliver water and nutrients to the leaf blade. Previous studies with the tank epiphytic bromeliad *Guzmania* monostachia have shown that the basal portion of the leaves is preferentially involved in the absorption of water and nutrients, while the apical region is mostly responsible for the assimilation of nutrients and photosynthesis, showing a functional compartmentalization in the leaves of this species. Aquaporins (AQP) have an important role in leaf growth, and movement, and in water and CO_2 transport. The present study attempted to investigate the expression of 11 aquaporin genes in the basal, middle and apical portions of the leaves of *G. monostachia* to discover if this functional leaf characteristic may be related to a differential regulation of distinct aquaporin genes by the time of day (dawn or dusk), water deficit and inorganic nitrogen sources. Most of the AQP genes were up-regulated at the beginning of light period (dawn) in all three portions of the leaves. Under water deficit induced by PEG coupled to nitrogen deficiency, most of the Plasma Membrane (PIP) aquaporin genes were up-regulated in the apex of the leaves. PIPs might increase membrane water permeability, water transport and CO_2 diffusion in the top of the leaves under water deficit. The apex is known to have the highest rates of CAM photosynthesis. The probable higher water and CO_2 transport in the apical region by PIPs could contribute to the higher CAM photosynthesis observed in the leaves of this species

under water deficiency **(Chapter 3)**. When NH_4^+ or NO_3^- were added to the medium containing PEG, the strongest up-regulation of AQP genes was observed in the basal and middle portions of the leaves in the presence of NH_4^+. Our data suggests that the increase of AQP expression by NH_4^+ enhances *G. monostachia*'s resistance to water deficit by increasing water absorption in the basal portion and facilitating the transport of water from both the base and middle regions to the apex of the leaves. This higher water content in the apical portion might help to increase the intensity of CAM photosynthesis previously observed in the apex of the leaves kept in the presence of NH_4^+ nutrition coupled to water deficit.

Keywords: Ammonium, Aquaporins, Epiphytic bromeliad, Gene expression, Nitrate, Water.

Abbreviations: AQPs, aquaporins; DW, dry weight; FB293, F-box protein 293; NIP, nodulin-26 like intrinsic protein; PEG, polyethylene glycol; PIP, plasma membrane intrinsic protein; RWC, relative water content; SIP, small basic intrinsic protein; TF2A, Transcription initiation factor IIA; TIP, tonoplast intrinsic protein.

Introduction

Bromeliad species occupy a wide range of habitats and they have developed strategies that help them survive in different environmental conditions (Smith & Till, 1998). One of the key adaptations in epiphytic bromeliad species is the presence of a "tank" structure, which is formed by the overlapping of leaf bases. This structure plays a central role in storing water and nutrients, which can be used in unfavorable environmental conditions, such as drought (Benzing 1980, 2000; Zotz and Thomas 1999; North *et al*. 2013). In these tank bromeliads, the base of the leaf is responsible for absorbing water and nutrients, and delivering them to the leaf blade (North *et al*. 2013). North *et al*. (2013) proposed that water taken up by leaf blades travels through the xylem and exits though the traqueids and the nearby cell walls in *Guzmania lingulata*. However, xylem elements are reduced in diameter in some bromeliad species and their reduction in the diameter limits their conducting capacity (Tomlinson 1969). Shatil-Cohen *et al*. (2011) suggest a possible involvement of aquaporins (AQPs) regulating water movement in and out of the veins in the leaves of *Arabidopsis thaliana* under drought conditions. However, little work has been done to understand the contribution of aquaporins to water relations in the leaves of tank epiphytic bromeliad species.

Aquaporins are water channels that belong to the major intrinsic protein family (MIP). These proteins are found in almost all living organisms and the number of AQPs vary among species (Agre *et al*. 1998). In *A. thaliana* 35 genes have been discovered (Qhigley *et al*. 2002), while for *Gossypium hirustum* over 71 genes have been documented (Park *et al*. 2010). Aquaporins are classified into five subfamilies according to sequence homology and subcellular location (Kruse *et al*., 2006; Maurel *et al*. 2008). These distinct subfamilies are: plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), small basic intrinsic proteins (SIPs), nodulin26-like intrinsic proteins (NIPs), and uncharacterized X intrinsic proteins (XIPs) (Johanson *et al*. 2001; Danielson and Johanson 2008). Although

AQPs have been described mainly as water channels, these proteins can also facilitate the passive transport of small solutes such as, glycerol, ammonia, urea, boric acid, silicon, arsenite, carbon dioxide (CO_2) and hydrogen peroxide (H_2O_2) (Maurel *et al*. 2008; Katsuhara *et al*. 2014).

Aquaporin expression can be regulated during plant development and by environmental factors (Heinen *et al*. 2009). In relation to developmental factors, AQPs seem to play a more important role in roots rather than leaves, under normal environmental conditions, since many PIP and TIP genes exhibited a higher expression in the roots rather than in the leaves (Johansson *et al*. 1996; Chaumont *et al*. 2000; Baiges *et al*. 2002; Smart *et al*. 2001). However, modulation of AQP expression has been shown to influence the leaf hydraulic conductivity and plant recovery from water stress, showing how important the AQPs are for leaf function (Parent *et al*. 2009, Postaire *et al*. 2010). The developmental stage also regulates AQPs expression. *Hordeum vulgare* showed a higher expression of *HvPIP1;3* in mature leaves, while *HvPIP1;5* and *HvPIP1;6* were up-regulated in the root elongation zone (Wei *et al*., 2007).

Among the environmental conditions that regulate AQP expression, drought stress has been the factor most studied in plants. Nevertheless, the overall pattern of AQP expression varies according to the genetic background, kind of stress imposition and developmental stage. Two-week old *A. thaliana* plants showed a 10-fold decrease in the transcript levels of *AtPIP1;5*, *AtPIP2;2*, *AtPIP2;3* and *AtPIP2;6* in the leaves when subjected to a mannitol-induced drought stress (Jang *et al*. 2004). In a more recent study, the pattern of drought regulation of PIPs was analyzed in five accessions of 4-week old *A. thaliana* after ceasing watering for 8-11 days of (Alexandersson *et al*. 2010). The authors showed that *AtPIP1;4* and *AtPIP2;5* were the only AQP transcripts up-regulated upon drought stress whereas most

AtPIPs were down-regulated, showing a pattern distinct from the observed by Jang *et al*. (2004).

The expression of few aquaporin genes was shown to be regulated by diurnal or circadian rhythm (Henzler *et al*. 1999; Lopez *et al*. 2003; Hachez *et al*. 2008; Sakurai-Ishikawa *et al*., 2011). Hachez *et al* (2008) showed that for maize leaves, most of the *ZmPIP* genes are up-regulated in the first hours of the light period and down-regulated at the end of the day. The oscillation in aquaporin expression during the day usually correlates well with the variation in plant hydraulic parameters, as root hydraulic conductivity (L_{pr}), transpiration and stomatal conductance (Henzler *et al*. 1999; Lopez *et al*. 2003; Beaudette *et al*., 2007; Sakurai-Ishikawa *et al*., 2011). Meanwhile, these studies were done on C3 or C4 species, which have a distinct pattern of hydraulic regulation when compared to CAM plants.

As described above, AQP genes are regulated by distinct environmental and developmental factors. However, little has been discussed about how multiple associated environmental conditions, such as water deficit, diurnal oscillation and inorganic nitrogen sources, would affect the gene regulation of the four AQP subfamilies, PIP, TIP, NIP and SIP along the leaf of a CAM species.

In *Guzmania monostachia*, a tank epiphytic bromeliad species, plants kept under drought conditions showed a lower decrease of water content in the apex rather than in the basal portion of the leaves (Pereira *et al*. 2013). The authors proposed the possibility of water transport mediated by the aquaporins from the basal portion, which forms the tank where the water is stored, to the apical portion of the leaves, which presents higher photosynthetic rates (Pereira *et al*. 2013). In this study, we investigated RWC and aquaporin gene expression in the apical, middle and basal portions of the leaves of *G. monostachia* kept under different inorganic nitrogen sources, NO_3^- or NH_4^+ coupled to water deficit, for seven days. We observed lesser decrease of RWC in the apical portion of the leaves under NH_4^+ + water

deficit when compared with NO_3^- + water deficit. When in the presence of either NH_4^+ or NO_3^- + water deficit, most of the AQP genes were down-regulated at dawn and up-regulated at dusk, in the apical, middle and basal portions of the leaves. Under water deficit, *PIP1;2* genes were always up-regulated in the apical portion, while the NIP and SIP genes were always down- regulated in the same region of the leaves.

Material and Methods

Plant Material and Growth Conditions

Micropropagated plants of *Guzmania monostachia* (L.) Rusby ex Mez Var. *monostachia* were cultivated and obtained for the experiments using the method described by Pereira *et al*. (2013) and in **Chapter 3**. For the experiments, the adult plants were then transferred to controlled environmental chamber under 200 µmol m^{-2} s^{-1} of photosynthetic active radiation to the top leaf surfaces of the bromeliad, 12 h photoperiod, a day/night air temperature of 25/22ºC, and a day/night relative humidity of 60/70% for acclimation. During one month of acclimation, all plants were watered with distilled water on a daily basis.

Water and Nutrient Deficit Treatment

Water deficit treatment was imposed as described by Pereira *et al*. (2013) with modifications. After 30 days of acclimation in the chamber conditions, as described above, adult plants that averaged 23.7 ± 0.9 cm tall with 32.7 ± 4.1 leaves and a tank volume of 40.5 ± 7.3 mL, had their 8^{th} to 12^{th} youngest fully developed leaves detached. The leaves were individually transferred to glass flasks containing 10 mL of distilled water (control) or 30% polyethylene glycol 6000 (PEG) (water deficit) diluted in Knudson solution (Knudson, 1946) without nitrogen source (nitrogen-deficient) or containing 5.0 mM of ammonium (NH_4^+) or 5.0 mM of nitrate (NO_3^-) as the only source of nitrogen. Flasks with the detached leaves

were kept in a controlled environment growth chamber as previously described. After 7 d in the controlled environment, leaves from different nitrogen treatments were divided into three portions: a) basal, corresponding to the part of the leaf that forms the tank in the whole plant and contains lower levels of chlorophyll, b) middle, corresponding to the lower half of the green part of the leaf blade, and c) apical, corresponding to the upper half of the green part of the leaf blade. The portion of the base, which was in contact with the liquid in the flask glass was discarded, whereas the remainders of the basal, middle and apical portions were used for further analyses.

Measurements of Relative Water Content (RWC)

Relative water content was determined according to the method described by Pereira *et al*. (2013) and in **Chapter 3**. The fresh, dry and turgid weights of basal, middle and apical leaf discs (~ 1 cm^2) were weighed immediately after leaf harvest to determine the fresh weight (FW), and then these same discs were kept in contact with distilled water for 24 h. Afterwards, all leaf discs were weighed to determine the turgid weight (TW) and were subsequently maintained for 72 h at 60 ºC before being weighed to determine the dry weight (DW). Tissue water content was calculated using the formula ((FW- DW)/ TW- DW)) x 100 (Martin and Schmitt, 1989). Measurements were made in triplicates.

RNA Extraction and qRT-PCR

Total RNA was extracted from 100 mg (FW) of freeze-dried powdered leaf material (apex and basal portions collected separately) using PureLink RNA Mini Kit (Ambion) following the manufacturer's recommendations. Genomic DNA contamination was removed by DNAse I treatment (Invitrogen). The cDNA was synthesized using the SuperScript III One-Step RT-PCR System (Invitrogen) as recommended by the manufacturer. Amplifications were carried out in total volume of 10 uL with SYBR Select Master Mix (Applied

Biosystems) on StepOnePlus Real-Time PCR (Applied Biosystems). PCR conditions consisted of an initial heating step at 95°C for 10 min, followed by 95°C for 15 s 40 cycles of 52-60ºC for 30 s, depending on the primer (Table S1), and a final elongation step of 72°C for 30 s. After cycling, melting curves were run from 60°C to 95°C for 20 min, to confirm that a single PCR product was amplified. The analyses of expression stability of the reference genes were performed with BestKeeper (Pfaffl 2004). The relative expression level of target genes was calculated as described by Pfaffl (2001), with the expression values normalized against the geometric mean of two reference genes (Pfaffl 2001). All reactions were performed using three biological replicates and two technical replicates. Primers used in this study were designed on partial *G. monostachia* sequences obtained in the transcriptomic analysis of this species performed by our group (unpublished data). The primers are shown in Table S1.

Statistical Analysis

All data were presented as mean values with ± standard error (SE). The differences between averages in relation to water and water deficit were assessed by t-Student's average comparison test at $P < 0.05$. The significant differences among the different treatments were contrasted by Tukey-Kramer's test at $P < 0.05$.

Results

Relative water content in the leaves of Guzmania monostachia

The relative water content was determined in the basal, middle and apical portions of the leaves of *Guzmania monostachia* for 7 days (Fig. 1). Under water deficit, the base, middle and apex showed a marked reduction in RWC when compared with the same portions of the leaves kept in water over 7 days in almost all of the nitrogen treatments (Fig 1). In general, the decrease in the RWC in the basal portion of the leaves under water deficit was about 1.2

times higher than in the same portion of the control leaves for all of the nitrogen treatments on the 1st day and 1.3 higher on the 3rd day in the presence of NO_3^- (Figs. 1A, 1B). For the middle region, the decrease in the RWC was about 1.6 times in the presence of NO_3^- + water deficit on the 6th day and 1.2 to1.4 times on the 7th day in all nitrogen treatments + water deficit compared with the nitrogen treatments + water. The apical portion of the leaves kept in water showed the lower RWC when compared with the basal and middle portions of the leaves (Fig. 1). When comparing the apical region of the leaves under water deficit with those kept in water, the decrease in RWC was the highest on the 6th day, for the leaves kept in the presence of NO_3^- (Fig. 1E and 1F). On the other hand, for the apical portion of the leaves under water deficit the decrease in RWC was the lowest on the 5th day in the leaves kept in the presence of NH_4^+ (Fig. 1F). Overall, after 7 days under water stress, the apical region of the leaves exhibited the highest RWC in the presence of NH_4^+, whereas in the middle and basal portions, the highest RWC was observed in nitrogen-deficient and NH_4^+ treatments (Figs 1B, 1D and 1F). These results showed that PEG 6000 (30%) was effective in the induction of water deficit by decreasing the RWC over 7 days in all of the portions of detached leaves from *G. monostachia*. In general, when compared with the respective control (water), the highest decrease in the RWC was observed in the apical portion of the leaves kept in $+NO_3^-$ + water deficit, while the lowest decrease was verified in the basal portion of the leaves kept in nitrogen deficiency + water deficit.

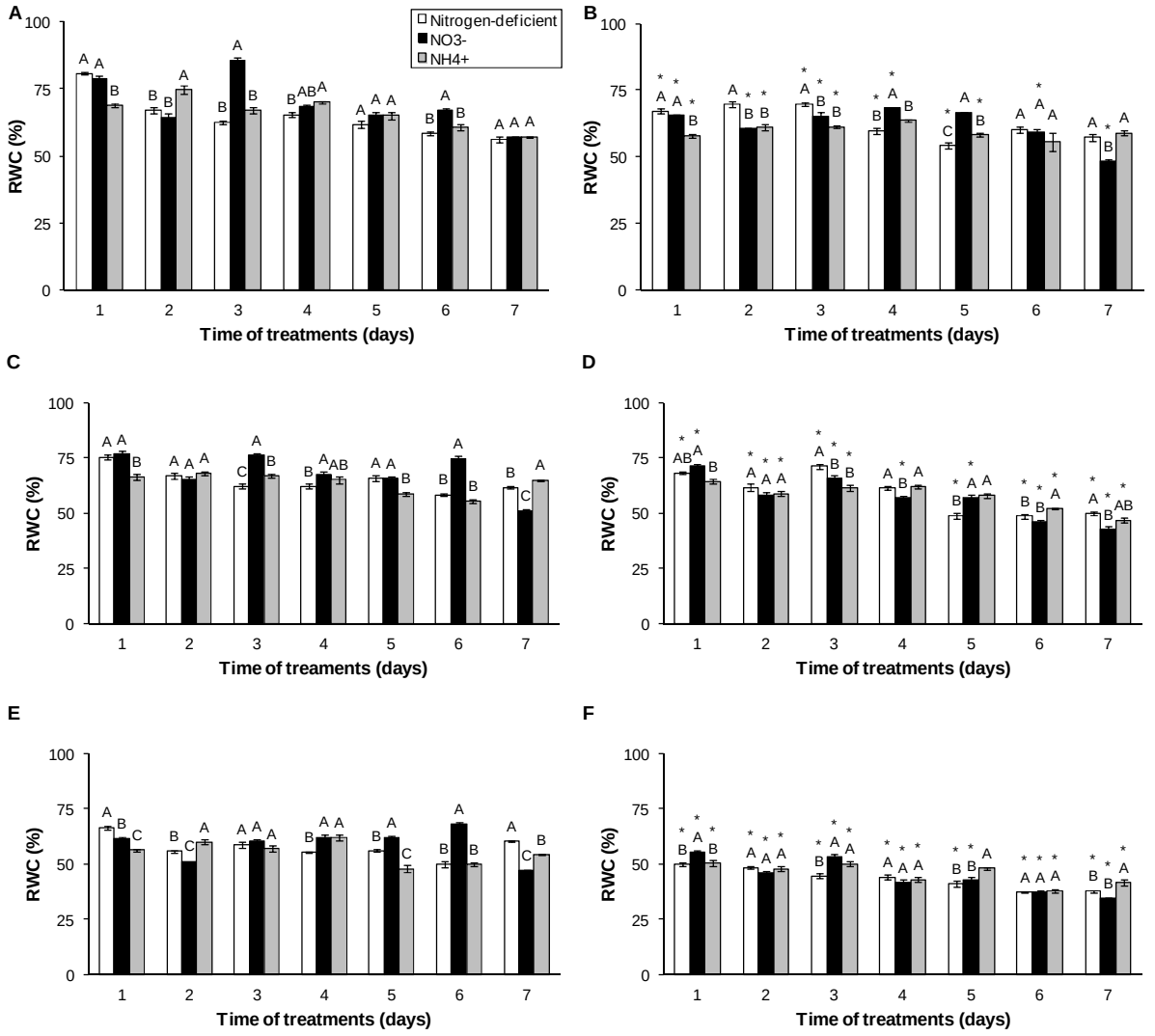

Fig.1. Relative water content in the basal portions of the leaves kept for 7 d in water (A) or water deficit (B), in the middle region of the leaves kept in water (C) or water deficit (D) or in the apical portion of the leaves of *G. monostachia* kept in water (E) or water deficit (F) associated with the absence of ammonium and/or nitrate. Data are expressed as the mean (±SE) of triplicate samples (each biological replicate is obtained from different leaves from different plants). Asterisks indicate a significant difference between water and water deficit in the same nitrogen treatment at the same time of the day ($P < 0.05$%; Student's t-test). Different capital letters indicate averages that are significantly different among the nitrogen treatments + water or water deficit at the same time of day ($P < 0.05$; Tukey-Kramer's test).

The effect of time of the day (dawn or dusk) on aquaporin gene expression

Most of the *G. monostachia* aquaporin genes were down-regulated at dusk in the apical, middle and basal portions of the leaves kept under nitrogen deficiency + water, excepting *TIP 4;3*, which presented a slight up-regulation in the middle portion of the leaves at dusk (0.6 fold-change) (Fig. 2). The highest up-regulation at dusk was observed for *TIP2;2b* (about 1.2 fold-change) in the basal portion of the leaves (Fig. 2). In the basal region, the only aquaporin gene that exhibited no transcriptional change was *PIP1;2a* (Fig. 2). In general, most aquaporin genes presented similar expression pattern in all of the portions of the leaves of *G. monostachia*.

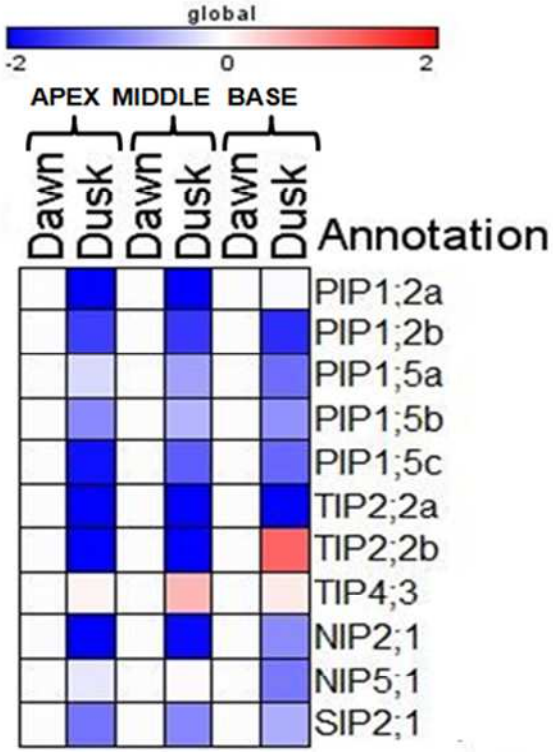

Fig.2. Heat map showing the expression profiles of aquaporin genes in the apical, middle and basal portions of the leaves of *G. monostachia.* Relative expression at dusk was compared with a normalized control (nitrogen deficient at dawn) in each portion of the leaf. The color scale represents FPKM normalized $\log_2$ transformed counts where blue indicates low expression and red indicates high expression.

The effect of water deficit on aquaporin gene expression

The *NIP2;1* gene exhibited the highest down-regulation in the middle (1.2 fold-change) portion of the leaves under nitrogen deficiency + water deficit at dawn, while *TIP2;2a* (1.8 fold-change in the apex and 1.9 fold-change in the middle) and *TIP2;2b* (1.8 fold-change in the apex and 2.1 fold-change in the middle) showed the highest down-regulation in these portions of the leaves at dusk (Fig. 3). On the other hand, in the basal portion of the leaves under water deficit at dawn, *PIP1;5a*, *PIP1;5c* and *TIP4;3* showed a slight down-regulation (0.6 fold-change), but still lower than the down-regulation observed for the same genes described before in the other regions of the leaf (Fig 3). The highest up-regulation was verified for *PIP1;2a* (1.2 fold-change at dawn and 2.2 fold-change at dusk) in the apical portion of the leaves under water deficit (Fig. 3). *NIP5;1* was the most up-regulated gene (1.7 fold-change) in the basal portion of the leaves at dusk (Fig 3). Most of the aquaporin genes exhibited a higher expression at dusk, when compared with the gene expressed at dawn under water deficit (Fig 3), showing that genes previously shown to be down-regulated at dusk compared to dawn (Fig. 2) were induced by water stress. *NIP5;1* (1.7 fold-change in the base) and *SIP2;1* (1.0 fold-change in the base) genes analyzed showed higher up-regulation in the basal portion of the leaves, kept in water deficit at dusk, rather than in the apical and middle regions. Conversely, all of the *PIP1;2* genes exhibited higher up-regulation in the apical portion of the leaves at both dawn and dusk, when compared with the up-regulation rates measured in the basal and middle portions (Fig. 3).

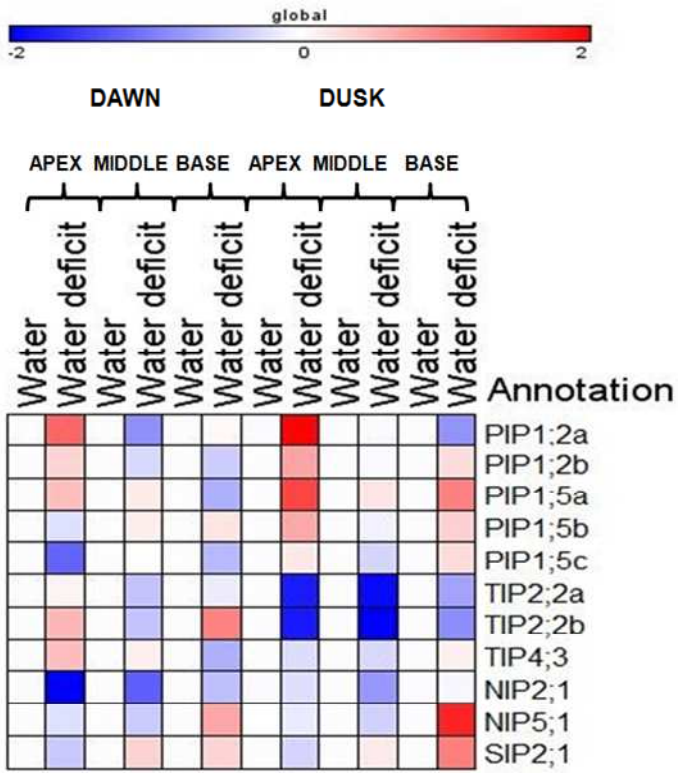

Fig.3. Heat map showing the expression profiles of the aquaporin genes in the apical, middle and basal portions of the leaves of *G. monostachia* at dawn and dusk. Relative expression in nitrogen treatments + water deficit was compared with a normalized control (nitrogen-deficient + water) in each portion of the leaf. The color scale represents FPKM normalized $\log_2$ transformed counts where blue indicates low expression and red indicates high expression.

The effect of inorganic nitrogen sources on aquaporin gene expression

The only genes up-regulated in the apex of the leaves under NO_3^- nutrition at dawn were *PIP1;2b*, *PIP1;5a* and *TIP4;3*. In this same nitrogen treatment, *PIP1;2b*, *PIP1;5b* and *TIPs* were up-regulated in the middle portion at dawn; while in the basal portion, the up-regulated genes were *PIP1;2a* (1.4 fold-change) and *SIP2;1* (1.0 fold-change) with a stronger up-regulation in *PIP1;2a* at dawn (Fig 4). For this same nitrogen treatment, *NIP2;1* was the most down-regulated genes in the apical (2.1 fold-change) and middle (0.8 fold-change) portions at dawn; while *PIP1;5c* (1.2 fold-change) and *NIP5;1* (1.6 fold-change) were the

most down-regulated genes in the basal portion at this same time of the day (Fig 4). The same genes that were up-regulated in the apex in the presence of NO_3^- were also up-regulated in the same portion of the leaves under NH_4^+; while in the middle portion, most of the *PIP1;5* and all *TIPs* were up-regulated in the presence of NH_4^+ (Fig. 4). For the basal portion, *PIP1;2a* (2.0 fold-change) and *TIP2;2b* (1.5 fold-change) showed the strongest up-regulation, however, most of the genes exhibited an up-regulation in the presence of NH_4^+ in the basal portion of the leaves of *G. monostachia* at dawn (Fig 4). The strongest down-regulated gene at dawn in the apical region under NH_4^+ was *NIP2;1* (1.2 fold-change); while in the middle region were *PIP1;2a* and *NIP2;1* (0.5 fold-change) and in the basal portion was *PIP1;5a* (1.4 fold-change) (Fig. 4). At dusk, in the apical portion of the leaves, most of the AQP genes were either up-regulated and few genes did not exhibited transcriptional changes in the presence of either NO_3^- or NH_4^+ (Fig. 4). In the middle region, most of the AQP genes were up-regulated and this regulation was stronger in the presence of NH_4^+ than NO_3^- (Fig. 4). In the basal portion, all genes were up-regulated and presented a very similar expression pattern under NO_3^- or NH_4^+ nutrition (Fig. 4).

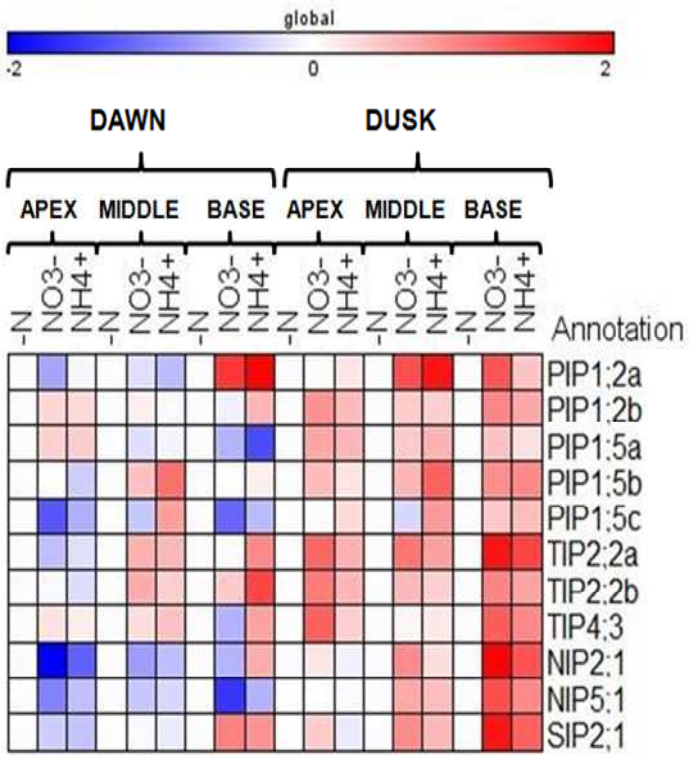

Fig.4. Heat map showing the expression profiles of the aquaporin genes in the apical, middle and basal portions of the leaves of *G. monostachia* at dawn and dusk. Relative expression in the presence of NH_4^+ or NO_3^- + water was compared with a normalized control (nitrogen-deficient + water) in each portion of the leaf. The color scale represents FPKM normalized $\log_2$ transformed counts where blue indicates low expression and red indicates high expression.

The coupled effect of inorganic nitrogen sources and water deficit on aquaporin gene expression

When water stress was associated to different nitrogen sources in the medium, changes in the expression profile of some genes were observed. For example, in the apical portion of the leaves submitted to water deficit under NO_3^- or NH4+ containing medium at dawn, the expression of *PIP1;5c* (0.5 fold-change), which was down-regulated by nitrogen sources under control water condition, was up-regulated by both nitrogen treatments under water stress. *NIP5;1* and *SIP2;1* were slightly up-regulated in the apex by NH4+ under water

stress at dawn (Fig 5). At dawn, most of the AQP genes exhibited a stronger up-regulation in the middle portion of the leaves kept in the presence of NH_4^+ when compared with NO_3^- (Fig. 5). In this leaf portion, *TIPs* and *NIP5;1* were up-regulated in the presence of NO_3^-, while *PIP1;2* genes and *NIPs* were up-regulated in the presence of NH_4^+ at dawn (Fig. 5). In the basal portion of the leaves, the strongest up-regulated gene at dawn was *PIP1;2a* either in the presence of NO_3^- (1.5 fold-change) or NH_4^+ (2.0 fold-change) (Fig. 5). The most down-regulated genes were *TIPs* in the apical portion of the leaves kept in NO_3^- at dawn (Fig 5). The transcript abundance of most of the aquaporin genes increased under both nitrogen treatments in the middle and base portion of the leaves at dusk. In the apex, distinct expression patterns were observed at dusk. *PIP1;5a* was the most down-regulated gene either in the presence of NO_3^- (0.8 fold-change) or NH_4^+ (1.1 fold-change) in the apical portion (Fig. 5). *TIP2;2b* did not exhibit transcriptional changes, *NIP2;1* was slightly down-regulated and all of the other AQP genes were up-regulated in the middle portion of the leaves kept in the presence of NO_3^- + water deficit. In contrast, under NH_4^+, all AQP genes were up-regulated in the middle region of the leaves at dusk (Fig. 5). In the basal region of the leaves kept in NO_3^- + water deficit, *PIP1;5a* and *TIP2;2a* showed no transcriptional changes, while *TIP2;2b* and *NIP5;1* were slightly down-regulated at dusk. The remaining AQP genes were up-regulated by NO_3^- associated to water deficit in this portion of the leaf (Fig 5). In contrast, at dusk, all AQP genes expressed in the basal portion of the leaves were up-regulated by NH_4^+ under water deficit. *PIP1;2a* (1.7 fold-change), *PIP1;2b* (1.4 fold-change) and *NIP2;1* (1.5 fold-change) were the most up-regulated genes in this portion of the leaf under the presence of NH_4^+ + water deficit (Fig. 5).

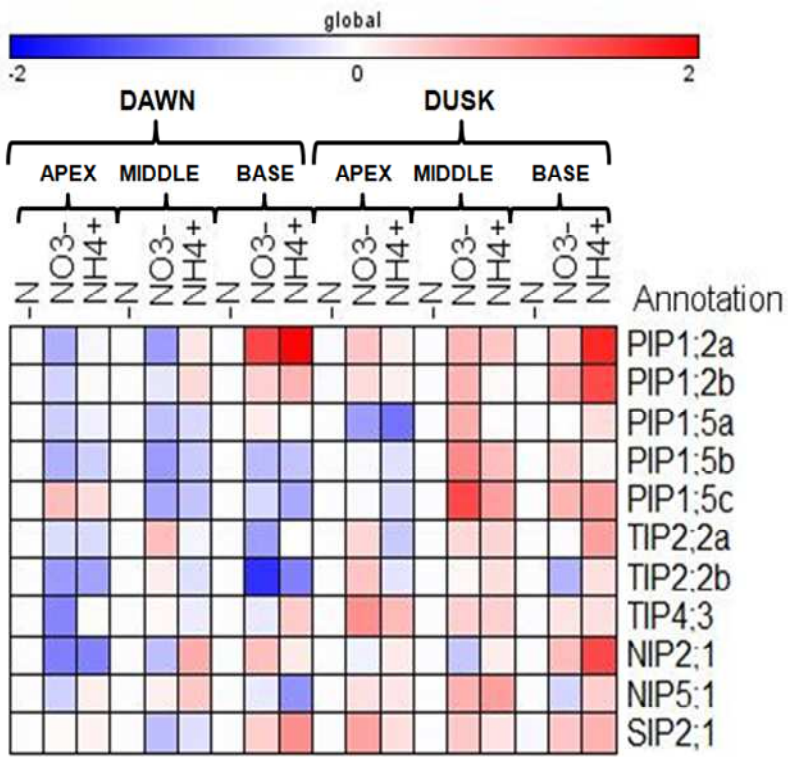

Fig.5. Heat map showing the expression profiles of the aquaporin genes in the apical, middle and basal portions of the leaves of *G. monostachia* at dawn and dusk. Relative expression in the presence of NH_4^+ or NO_3^- + water deficit was compared with a normalized control (nitrogen-deficient + water deficit) in each portion of the leaf. The color scale represents FPKM normalized $\log_2$ transformed counts where blue indicates low expression and red indicates high expression.

Discussion

Aquaporin expression is regulated by many environmental factors such as drought, flooding, salinity, cold and diurnal oscillations (Jang *et al*. 2004; Heinen *et al*. 2009; Vera-Estrella *et al*. 2012). In relation to diurnal cycle, Vera-Estrella *et al*. (2012) showed the transcriptional changes of McPIP1;4 in unstressed adult *Mesembryanthemum crystallinum* plants. The transcript levels of this aquaporin gene decreased at night and increased during the day. However, when *M. crystallinum* plants were under salt stress no changes were observed in the expression of PIP or TIP genes, during the day/night cycle (Vera-Estrella *et*

al. 2012). The diurnal expression of most of the ZmPIP genes in the mature zones of the leaves of *Zea mays* was higher in the first hours of the light period compared with at the end of the day (Hachez *et al*. 2008). In our study, most of the AQP genes (PIP, TIP, NIP and SIP) had their expression decreased at the end of the day (at dusk) in the apical, middle and basal portions of the leaves of *G. monostachia*. This result corroborates the highest AQP gene expression observed for *Z. mays* leaves in the first hours of the light period; the leaf of *G. monostachia* also showed a positive regulation in the first hours of the day for PIP, TIP, NIP and SIP genes and a higher expression at dawn compared with at dusk. Interestingly, all the genes analyzed in this study showed a day/night transcriptional regulation on, at least, one portion of *G. monostachia* leaf. Day/night regulation has been shown for few aquaporin genes until now, and mainly for root-specific genes, where the variation in expression levels was correlated to diurnal changes in root hydraulic conductivity. The leaves of *G. monostachia* are an interesting model to study the regulation of AQP genes, since it has been previously demonstrated (Pereira *et al*. 2013) that the basal portion of the leaf can function as a root, absorbing water and nutrients from the tank.

Drought is another environmental factor that regulates AQP expression, and its effect has been well-documented in the scientific literature (Smart *et al*. 2001; Jang *et al*. 2004; Da Silva *et al* 2013). *A. thaliana* seedlings presented a down-regulation of AtPIP1;5, AtPIP2;2; AtPIP2;3, AtPIP2;4 and AtPIP2;6 in the roots and in the aerial parts under drought stress., while only AtPIP2;1 and AtPIP2;5 genes were up-regulated over 2 weeks of drought (Jang *et al*. 2004). The transcript accumulation of *MIP2*, *MIP3*, *MIP4* decreased in *Nicotiana glauca* leaves of plants under drought-stress (Smart *et al*. 2001). In our study, we observed both upregulation and downregulation of different PIP genes of *G. monostachia* under water deficit, depending on the time of the day and leaf portion. The opposite profile observed for the same genes in the apex and basal portions of the leaves reinforce the different functions of

these tissues. The PIPs play an important role in water absorption and hydraulic maintenance in the leaves (Li *et al*. 2014; Xu *et al*. 2014). In addition, PIPs can facilitate CO_2 diffusion in the mesophyll and affect photosynthetic rates (Heckwolf *et al*. 2011). In general, most of the *PIP* genes showed a stronger up-regulation in the apical portion of the leaves of *G. monostachia* under water deficit compared with the other portions at dawn and at dusk. Under water deficit, the RWC was the lowest in the apex of the leaves of *G.monostachia*, and comparatively higher in the middle and basal portions. PIPs are known to function as water channels (Ariani and Gepts 2015); these genes were described to be down or up-regulated under drought conditions (Smart *et al*. 2001; Jang *et al*. 2004). Based on the observed for other plant species, the up-regulation of PIP genes can increase water and CO_2 permeability of the membrane facilitating their transport in the apical portion of the leaves. However, at dusk PIP genes are up-regulated in the base of the leaves when compared with the dawn; this up-regulation could help to increase water transport to the apical portion of the leaves under water deficit at dusk when photosynthesis rates are higher in this region of the leaf (Pereira *et al*. 2013).

TIPs are vacuolar membrane channels (tonoplast) involved in the regulation of osmotic potential and water flux through this compartment (Li *et al*. 2014). At dawn, the TIP genes were up-regulated in the apical portion of the leaves of *G. monostachia* under water deficit. On the contrary, at dusk all TIP genes were down-regulated. In this way, TIPs might be regulating and supporting water transport and osmotic potential in the apical portion of the leaves of this bromeliad at dawn, due to organic acids accumulation during the night and, consequently, higher turgor pressure, but not at dusk when turgor decreases after the remobilization of organic acids and water becomes available for plants (Lüttge 2004; Pereira *et al*. 2013).

The effect of inorganic nitrogen sources on water relations has been intensively studied in plants (Walch-Liu *et al*. 2000; Guo *et al*. 2007; Gao *et al*. 2010; Ding *et al*. 2015). In rice, a higher water absorption rate was observed in the presence of ammonium + water deficit rather than nitrate + water deficit (Gao *et al*. 2010). On the contrary, French bean plants showed a higher water uptake in the presence of nitrate rather than ammonium (Guo *et al*. 2007). In tobacco plants, no difference in water absorption rates was observed between the plants supplied with nitrate and those supplied with ammonium (Walch-Liu *et al*. 2000). Our results demonstrated a higher RWC in the apical, middle and basal portions of the leaves of *G. monostachia* kept under NH_4^+ + water deficit on the 7th d of drought stress, compared with NO_3^- + water deficit. For this epiphytic bromeliad species, NH_4^+ seems to improve water uptake ability and enhance leaf tolerance to water deficit.

For rice seedlings, an improvement in the water uptake by the roots was associated with the involvement of aquaporins (Gao *et al*. 2010; Ding *et al*. 2015). *Oryza sativa* seedlings showed higher aquaporin activity in the presence of ammonium than in the presence of nitrate (Gao *et al*. 2010). In this same study, the plants supplied with ammonium, rather than nitrate, exhibited both the highest levels of water uptake and the highest photosynthetic rates (Gao *et al*. 2010). Ding *et al*. (2015) showed an up-regulation of OsPIP1;2, OsPIP1;3, OsPIP2;2, OsPIP2;3 and OsPIP2;4 in the plants kept under NH_4^+ + PEG 10% (MW 6000). This increase in AQP gene expression in the presence of ammonium + water stress resulted in a higher permeability of the root hydraulic (Ding *et al*. 2015). In the leaves of *G. monostachia*, most of the AQP genes exhibited a stronger up-regulation under control water conditions and in the presence of both N sources, with higher AQP transcript accumulation under NH_4^+ nutrition in the basal and middle portions, rather than in the presence of NO_3^-. Additionally, under a PEG-induced water stress, a similar general profile of down-regulation at dawn and up-regulation at dusk was observed for both N sources, but

with some differences for specific genes. In general, AQP genes were induced by water deficit in the basal and middle portions in the presence of NH_4^+ and NO_3^- at dusk. As reported by North *et al*. (2013), the basal portion of the leaf in the epiphytic tank bromeliad species is the place where water and nutrients are absorbed, captured and delivered to the leaf blade. In *G. monostachia*, the up-regulation of most of the AQP genes in the basal and middle portions of the leaves kept under NH_4^+ + water or water deficit could be related to an improvement in water uptake and transport from the base and middle to the apex portion. The gradient of water content from the highest RWC observed in the base to the lowest in the apex is an evidence to support this hypothesis. In addition, higher photosynthetic rates were observed in the presence of NH_4^+ + water deficit compared with NO_3^- + water deficit (**Chapter 3**). Therefore, NH_4^+ may induce AQP gene expression in the basal and middle regions of the leaves under water deficit at dusk, mainly GmPIPs1;2 and GmPIPs1;5, which presented the strongest up-regulation. These PIPs were previously characterized as water and CO_2 channels and its increased expression might facilitate water transport and CO_2 diffusion from the base to the apical portion of the leaves. The induction of aquaporins in the base, pumping water to the apex, associated with the repression of some AQPs in the apex, avoiding water loss, could result in a higher water and CO_2 availability to the phosphoenolpyruvate carboxylase enzyme (PEPC), which exhibited higher activity in the apical portion of the leaves (Pereira *et al*. 2013; **Chapter 3**). This higher PEPC activity in the apex is responsible for a stronger CAM intensity in this portion of the leaves kept in NH_4^+ + water deficit rather than NO_3^- + water deficit (**Chapter 3**). In relation to the other aquaporin subfamilies analyzed in this study, NIP5;1 and NIP2;1 are described as urea, boron, arsenite and silicon transporters (Gu *et al*. 2012; Katsuhara *et al*. 2014; Ariani and Gepts 2015); while SIP2;1 seems to act as an endoplasmic reticulum (ER) channel for other small molecules, but not water (Ishikawa *et al*. 2015). In this way, the regulation of GmNIP and GmSIP genes by

nitrogen sources and water deficit in the leaves of *G. monostachia* is still unclear and more studies are necessary in order to better understand their regulation along the leaf of this epiphytic bromeliad.

Although there are probably many aquaporin genes in *G. monostachia*, if we consider the number of genes described for other species, we have examined only 11 isoforms that represent the main aquaporin subfamilies. Clearly, we must be cautious with regard to correlations between expression of AQPs and the water-stress response of *G.monostachia*, since the expression of the complete multigenic family and the corresponding functional analysis are still missing.

In conclusion, this study showed for the first time the regulation of AQP expression by different environmental factors along the leaf of *G. monostachia*. Taken together, our results suggest that aquaporin are differentially regulated by day/night cycle and inorganic nitrogen sources and are very responsive to water deficit, contributing to maintain the cellular water balance in the leaves of this bromeliad. Both nitrate and ammonium regulate aquaporin expression, with small differences among these two N sources, and a higher induction for the aquaporin genes expressed in the base of leaves, portion responsible for nitrogen and water uptake. Some of these AQPs may also be involved in the transport of small nitrogen molecules.

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Supplementary Material

Table S1. Primer pairs used for qRT-PCR analysis.

Annotation	Primer (forward/reverse)	Amplicon length (pb)	Tm (ºC)	Efficiency (%)	R^2
PIP1.2a	5' TGTTCTACATGGTGATGCAGTGCC 3' 5' TGCCCACAATCTCAGCACCCA 3'	156	60	87.5	0.9997
PIP1.2b	5' GTGAAGGGGTTCGAGAAGGG 3' 5' CTTGGCATCAGTGGCAGAGA 3'	150	54	86.5	0.9998
PIP1.5a	5' GTGTGGTGAAGGGGTTCCAA 3' 5' TGGCATCAGTGGCAGAGAAG 3'	153	55	89	0.9998
PIP1.5b	5' GTCGTCAAGGGCTTCCAGAA 3' 5' CGGAGAAGACGGTGTAGACG 3'	139	55	85	0.9998
PIP1.5c	5' AGGATGTACTGGTGGTACGC 3' 5' GTGGATCTTTTGGGTTGGGC 3'	66	54	91	0.9997
TIP2.2a	5' CCGCCATTGCCTACAACAAGTT 3' 5'CGACACCGCCACGCCCAG 3'	104	57	87	0.9997
TIP2.2b	5' CCGACCCAGTAGATCCAGTT 3' 5' CTCGTCTACACCGTCTACGC 3'	203	57	84	0.9998
TIP4.3	5' ATCATCACCGCCACCACCAG 3' 5' TCCTCACCTTCCTCTTCGTCTTCG 3'	133	57	93	0.9997
NIP2.1	5' GCCTCATCGTAACGGTGATGAT 3' 5' GGCACCTGAATCCAAGGGAA 3'	121	59	89	0.9998
NIP5.1	5' GGAGACGCTGATCGGCAATGC 3' 5' CCCAGGGGAAGTGGCGGAG 3'	137	60	90.5	0.9998
SIP2.1	5' GATTCCTCCAGTTAAATCAGAG 3' 5' TCATGAAAACATGGATATCAAGCA 3'	74	52	81	0.9960
TF2A	5' GATGTCAATGTGGCTTATGAGG 3' 5' CTTTTGCGTTTTCCAGAGGAC 3'	110	52	89	0.9999
FB293	5' CTGAAGATGTGAACAAGCAAATCA 3' 5' CTGCCCAAACAGAAGAAGG 3'	137	52	83	0.9999

Principais conclusões

As principais conclusões obtidas desse trabalho são descritas individualmente para cada capítulo apresentado.

No **capítulo 1**, entre as 6 espécies de bromélias CAM utilizadas nesse estudo, *Billbergia pyramidalis* apresentou as maiores taxas de transporte de prótons dependente de ATP ou PPi e maior acúmulo noturno de malato, enquanto *Tillandsia usneoides* exibiu as menores taxas de transporte de prótons e acúmulo de malato durante a noite. Todas bromélias mostraram uma preferência pela ATPase, em vez da PPiase, como força próton-motriz para o transporte de ácidos orgânicos no interior do vacúolo. A permeabilidade do tonoplasto na presença de ATP, para todas as bromélias CAM testadas foi fumarato > malato > citrato.

No **capítulo 2**, plantas de *Kalanchoë laxiflora* e *Kalanchoë tubiflora* cultivadas com 2.5 mM de nitrato apresentaram maiores taxas de transporte de prótons dependente de ATP ou PPi e maior acúmulo noturno de ácidos orgânicos no interior do vacúolo. Por outro lado, plantas de ambas as espécies mantidas em 5.0 mM de amônio exibiram os menores valores de transporte de prótons e acúmulo de ácidos orgânicos durante a noite. Ao comparar as duas espécies, os parâmetros descritos acima foram maiores nas plantas de *K. tubiflora* do que nas plantas de *K. laxiflora*. De modo geral, os resultados apresentados nesse capítulo sugerem que *K. tubiflora* apresenta um CAM mais forte comparado com *K. laxiflora* e as fontes inorgânicas de nitrogênio (NH_4^+ ou NO_3^-) , bem como suas concentrações, influenciam no grau de expressão do CAM nessas duas espécies de *Kalanchoë*.

No **capítulo 3**, os dados claramente mostram que a presença isolada de amônio (5.0 mM) associada ao déficit hídrico (PEG 6000 30%) favorece a maior expressão do CAM na porção apical das folhas de *Guzmania monostachia*, quando comparado com a presença isolada de nitrato (5.0 mM). Foi observado também o maior acúmulo de açucares solúveis (glicose, frutose e sacarose) e atividade das principais enzimas antioxidantes (SOD, CAT,

APX e GR) na porção apical das folhas dessa bromélia mantidas na presença de amônio. O transporte de prótons e ácidos orgânicos dependentes de ATP, bem como a expressão do gene ALMT9, que codifica o transportador de malato ativado por alumínio, foram aumentados na presença de amônio como única fonte isolada de nitrogênio. Esses resultados sugerem que o amônio melhorara a capacidade de limitar o dano oxidativo, bem como favorece o transporte de ácidos orgânicos no interior do vacúolo na porção apical das folhas de *G monostachia*.

No **capítulo 4**, nossos resultados sugerem que os 11 genes que codificam aquaporinas escolhidos nesse estudo são diferencialmente regulados pelo ciclo dia/noite e fontes de nitrogênio e são muito responsivos aos déficit hídrico, contribuindo para manter o balanço hídrico em folhas de *G. monostachia*. A presença de NH_4^+ parece favorecer a indução dos genes que codificam PIP1;2 e PIP1;5 nas porções basal e mediana no final do dia. O aumento na expressão desses genes na base, poderia auxiliar no transporte de água para o ápice, associado com a repressão desses genes na porção apical, evitando a perda de água, poderia resultar em uma maior disponibilidade de água e CO_2 para a enzima PEPC que exibiu maior atividade na porção apical das folhas, como destacado no **capítulo 3**.

Resumo

Historicamente, o metabolismo ácido das crassuláceas (CAM) tem sido bem estudado em espécies das famílias Bromeliaceae e, principalmente, Crassulaceae. Essa via fotossintética é caracterizada pelo acúmulo noturno de ácidos orgânicos dentro do vacúolo e pela fixação de CO_2 durante a noite pela enzima fosfoenolpiruvato carboxilase (PEPC). No entanto, pouco se sabe sobre a preferência pela atividade da enzima ATPase ou PPiase no transporte de prótons e ácidos orgânicos no interior das vesículas do tonoplasto em espécies CAM. A fotossíntese CAM pode ser induzida em plantas caracterizadas como CAM-facultativas por diversos fatores ambientais, por exemplo, déficit hídrico, termoperíodo, salinidade e deficiência de nutrientes. Contudo, pouco tem sido discutido sobre a influência dos nutrientes na indução do CAM. Esse estudo investigou o transporte de prótons através da membrana do tonoplasto em seis espécies de bromélias CAM e duas espécies de *Kalanchoë*. Todas as espécies usadas nesse estudo mostrou uma preferência pela ATPase do que pela PPiase para o transporte de prótons e ácidos orgânicos no interior das vesículas do tonoplasto. Nós também observamos uma maior expressão do CAM nas plantas de *Kalanchoë laxiflora* e *Kalanchoë tubiflora* mantidas na presença de 2.5 mM de NO_3^-. Por outro lado, *Guzmania monostachia*, uma espécie de bromélia epífita, exibiu a maior intensidade do CAM nas folhas mantidas na presença de NH_4^+ + déficit hídrico. Nessa espécie de bromélia, a maior expressão do gene *ALMT9* na porção apical das folhas, seguido pelas maiores taxas de transporte de prótons pela ATPase, acúmulo de açucares solúveis e a ativação das defesas antioxidantes parecem estar relacionados com o aumento da tolerância pelo ajuste osmótico e limitação do dano oxidativo nas folhas mantidas na presença de NH_4^+ + déficit hídrico. Uma outra explicação para a maior intensidade do CAM no ápice das folhas mantidas em NH_4^+ + déficit hídrico poderia ser a maior expressão dos genes que codificam aquaporinas nas regiões basal e mediana das folhas na presença dessa fonte de N, principalmente PIPs e TIPs, que talvez sejam

responsáveis pelo transporte de água das porções basal e mediana para a porção apical das folhas. O maior conteúdo de água conservado na porção apical poderia ajudar a aumentar a intensidade da fotossíntese CAM nessa porção das folhas de *G. monostachia* mantidas na presença de NH_4^+ + déficit hídrico.

Palavras-chave: Aquaporinas; Metabolismo ácido crassuláceo; Fontes de nitrogênio; Transporte de prótons.

Abstract

Historically, crassulacean acid metabolism (CAM) has been studied in many families, mainly Bromeliaceae and Crassulaceae. This photosynthetic pathway is characterized by the nocturnal organic acid accumulation in the vacuole as well as CO_2 fixation during the night by the phosphoenolpyruvate carboxylase enzyme (PEPC). However, little is known about the preference of ATPase or PPiase activities for the proton and organic acids transport in tonoplast vesicles in CAM species. CAM photosynthesis can be inducted in CAM-facultative species by environmental factors such as, water deficit, thermoperiod, salinity and nutrients deficiency. Although, little has been discussed about the influence of nutrients on CAM induction. This study investigated proton transport in CAM bromeliad species and two CAM *Kalanchoë* species. All of the species used in this study showed a preference for ATPase rather than PPiase for the proton and organic acids transport into the tonoplast vesicles. We also observed a higher CAM expression in *Kalanchoë laxiflora* and *Kalanchoë tubiflora* plants kept in the presence of 2.5 mM of NO_3^-. On the other hand, *Guzmania monostachia* plants, an epiphytic tank bromeliad species, exhibited the highest CAM intensity in the leaves kept in the presence of NH_4^+ + water deficit. In this same bromeliad, a malate transporter gene, *ALMT9*, showed its highest expression in the apical portion of the leaves and the highest proton transport rates into the vacuole by ATPase. Soluble sugars and antioxidant enzymes activities were also verified in this study in order to observe their influence on increasing the drought tolerance of *G.monostachia*. In the leaves kept in NH_4^+ + water deficit the highest antioxidant activities and accumulation of soluble sugars were observed, this suggests that this inorganic nitrogen source seems to increase the drought tolerance by osmotic adjustment and limitation of oxidative damage. These factors can favor the increase of CAM intensity in the leaves kept under NH_4^+ + water deficiency. Another explanation for why the highest CAM intensity was observed in the apical portion of the leaves kept in NH_4^+

+ water deficiency is because of the higher expression of aquaporin genes in the basal and middle regions of the leaves in the presence of this N source, mainly PIPs and TIPs, which might be responsible for transporting water from the basal and middle portions to the apical portion where these AQP genes are repressed. The fact that the highest water content is conserved in the apical portion might help to explain the increase in the intensity of CAM photosynthesis observed in the leaves of *G. monostachia* kept in the presence of NH_4^+ + water deficit.

Keywords: Aquaporins; Crassulacean acid Metabolism; Nitrogen sources; Proton transport.