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Utilization of urea by leaves of bromeliad *Vriesea gigantea* under water deficit: much more than a nitrogen source

A. MATIZ[1], P.T. MIOTO[1], M.P.M. AIDAR[2], and H. MERCIER[1]*

Department of Botany, Institute of Biosciences, University of São Paulo, CEP 05422-970, São Paulo, SP, Brazil[1]
Institute of Botany, IBT, CEP 04045-972, São Paulo, SP, Brazil[2]

Abstract

Vriesea gigantea Gaudichaud is an epiphytic bromeliad with a high capacity to take up urea. In plants, urea is hydrolyzed by urease into ammonium and CO_2, providing nitrogen to the plant. Most studies of urea nutrition have focused only on nitrogen metabolism, whereas scarce attention has been given to CO_2 assimilation. Therefore, this study attempted to investigate whether urea could play an important role as a carbon source, which could be of a significant importance under water deficit conditions because of the limitation in atmospheric CO_2 influx into the leaves due to stomatal closure. In this study, detached leaves of *V. gigantea* were exposed to water deficit and supplied with urea. The most photosynthetic parts of the leaf (mainly the apical leaf portion) showed higher urease activities and CO_2 buildup near chloroplasts, particularly during the nighttime under water deficit conditions when compared to urea application without the water deficit. Moreover, part of the CO_2 generated from urea hydrolysis was fixed into malate, probably *via* phospho*enol*pyruvate carboxylase. Therefore, urea may contribute to the carbon balance of plants under water deficit conditions. Our data suggest that, besides being a source of nitrogen, urea might also be an important carbon source during CO_2-limited conditions in leaves of epiphytic bromeliads.

Additional key words: carbon dioxide assimilation, malate, phospho*enol*pyruvate carboxylase, urease.

Introduction

The epiphytic habitat is highly limited in water and nutrients, which are available only occasionally or seasonally through rainfall (Benzing 2000). For this reason, plants that live in this environment must cope with these conditions to survive. As a result, some of the main, widespread adaptive traits developed by several epiphytes to endure this xeric habitat include an efficient use of nutrients, particular morphological and anatomical characteristics, and a highly efficient use of water (Benzing 1976). An interesting morphological characteristic of some epiphytic bromeliads is the presence of a tank. This is a structure formed by the imbrications of the leaves in a rosette conformation, which allows accumulation of water and organic matter that fall into it. Water and nutrient absorption is possible because of a high density of trichomes in the basal portion of the leaves, which come into contact with the accumulated solution in the tank (Benzing *et al*. 1976, Benzing 1990, 2000, Takahashi *et al.* 2007, Matiz *et al*. 2013).

Therefore, the leaves of epiphytic-tank bromeliads are the most important vegetative organs (Benzing 2000), in which the apical portion is mainly responsible for photosynthesis, whereas the leaf bases mostly perform functions related to absorption (Benzing *et al*. 1976, Benzing 2000, Zotz *et al*. 2002, Takahashi *et al*. 2007). In fact, several physiological and anatomical differences were observed along the length of the leaf, such as an increasing gradient of organic acids from the base to the apical portion (Popp *et al*. 2003, Freschi *et al*. 2010, Mioto and Mercier 2013), nitrogen (Medina *et al*. 1994), chlorophyll content, as well as nitrogen assimilation enzyme (Takahashi and Mercier 2011) and photosynthetic enzyme activities (Freschi *et al*. 2010, Mioto and Mercier 2013).

Among mineral nutrients, nitrogen is the one required

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Abbreviations: CAM - crassulacean acid metabolism; PEG 6000 - polyethylene glycol 6000; PEPC - phospho*enol*pyruvate carboxylase; PPFD - photosynthetic photon flux density; RWC - relative water content.
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* Corresponding author; fax: (+55) 1130917547; e-mail: hmercier@usp.br

in higher quantities by the plants (Amtmann and Blatt 2008). For a very long time, it was believed that inorganic nitrogen sources, such as ammonium and nitrate, are not only more abundant in soils but also the sole forms absorbed by plants. Consequently, most studies of nitrogen nutrition have focused on inorganic nitrogen sources (Paungfoo-Lonhienne *et al*. 2012). Nevertheless, several studies have shown that plants are able to uptake organic nitrogen as well such as amino acids (Persson and Näsholm 2001, Inselsbacher *et al*. 2007, Jämtgård *et al*. 2008, Näsholm *et al*. 2009, Svennerstam *et al*. 2011, Gruffman *et al*. 2014), peptides (Paungfoo-Lonhienne *et al*. 2008, Näsholm *et al*. 2009, Cambui *et al*. 2011), and urea (Witte *et al*. 2002, Inselsbacher *et al*. 2007, Mérigout *et al*. 2008).

In addition, organic sources are very important for plant nutrition mainly in environments with a low availability of inorganic nitrogen and, thus, are likely to be very important in epiphytic habitats (Chapin *et al*. 1993, Kielland 1994, Schimel and Chapin 1996, Benzing 2000). In these habitats, part of the nutrients can be transported by air, and its soluble fraction could be available to the plants (Lipson and Näshlom 2001, Cape *et al*. 2011). Furthermore, epiphytic-tank bromeliads can act as support cores for organisms such as amphibians, small invertebrates, other plants, and microorganisms. Thus, excretes released into the tank from animals that live in or use the tank as a shelter constitute an important organic nitrogen resource for the bromeliads (Benzing 1990, Lopez *et al*. 1999, Romero *et al*. 2006, 2008, 2010, Leroy *et al*. 2013).

Moreover, it has been reported that epiphytic bromeliads have a high capacity to effectively use organic sources (Inselsbacher *et al*. 2007, Takahashi and Mercier 2011). Several studies with tank bromeliads have shown their high capacity to use urea as an organic nitrogen source (Mercier *et al*. 1997, Endres and Mercier 2001, Inselsbacher *et al*. 2007, Cambui *et al*. 2009, Takahashi and Mercier 2011), as it has been observed in *in vitro* grown *Vriesea gigantea*, which shows a good growth when kept in a medium with urea (Endres and Mercier 2001). In addition, the importance of urea in *V. gigantea* metabolism was also evidenced by the high capacity of their leaves to absorb this organic compound. *V. gigantea* plants show an unusual kinetics for urea absorption, in which no saturation is observed, even at a 10 mM concentration (Inselsbacher *et al*. 2007).

Once urea is absorbed by plant cells, it must be assimilated and/or stored. Most studies suggest that nitrogen present in urea is only available for plant metabolism if urea is previously hydrolyzed by urease (EC 3.5.1.5, a nickel metalloenzyme; Gerendás and Sattelmacher 1999, Cao *et al*. 2010) giving rise to ammonium and CO_2 (Follmer 2008, Witte 2011, Zambelli *et al*. 2011).

Previous studies focused on the importance of urea as a nitrogen source for plants, but since this molecule releases CO_2 when hydrolyzed, we believe it could function as a source of carbon, as well. Conditions that reduce the uptake of CO_2, such as water limitation, could represent a scenario in which urease activity may partially compensate for lack of CO_2 in plants by providing it from urea hydrolysis. To investigate this hypothesis, we chose the tank-bromeliad *V. gigantea* due to its high capacity to uptake urea and to the functional specialization along its leaves.

Materials and methods

Plant growth, treatments, and sampling: For the experiments, plants of *Vriesea gigantea* Gaudichaud approximately 10-year-old were used. Their seeds were germinated *in vitro* and then the plantlets were cultivated in a medium with macronutrients according to Knudson (1946) and micronutrients according to Murashige and Skoog (1962) until reaching an average height of 3 cm. After that, the plants were transferred to a shaded greenhouse in the Department of Botany of the University of São Paulo (São Paulo, Brazil) and maintained in a mix of commercial organic substrate composed of pine bark and *Tropstrato*®. These plants were subsequently acclimated in a growth chamber at a temperature of 25 ± 2 °C, a relative humidity of 60 - 70 %, a photosynthetic photon flux density (PPFD) of 200 μmol(photon) m^{-2} $s^{-,1}$ and a 12-h photoperiod for two weeks. Then, leaves from the 8^{th} to 15^{th} nodes, representing the youngest fully expanded leaves, were detached under water and maintained in water (controls) or in a 30 % (m/v) polyethylene glycol (PEG 6000) solution (simulating water deficit) for eight days. On the eighth day, 5 mM urea and 0.5 μM $NiCl_2$ (an urease cofactor) were added to the water or PEG solutions. The pH of the solutions was set to 5.8.

For biochemical analyses, detached leaves (n = 5) belonging to at least four individuals were divided into three portions: a base, corresponding to the purplish part of the leaf, with the remaining part of the leaf blade equally divided forming the corresponding middle part and apex.

Measurements of relative water content (RWC): The RWC was determined according to Barrs and Weatherley (1962). Leaf discs with an area of 1 cm^2 were collected one hour after dawn and immediately weighed to determine fresh mass (FM). Then, the samples were maintained in distilled water for 72 h to obtain water saturated mass (WSM), and after that, the samples were dried at 65 °C until a constant dry mass (DM) was obtained. The RWC was calculated using a formula [(FM - DM)/WSM - DM)] × 100. The measurements were made in triplicate.

Leaf gas exchange: Gas-exchange measurements were made continuously on the middle and apical portions of the detached leaves from the control and water deficit treatments over 24 h by using an infrared gas exchange system (*LI-6400*, *Li-Cor*, USA). Every leaf portion was enclosed in a leaf chamber with a temperature and a relative humidity the same as in the growth chamber, PPFD on the adaxial surface of the leaf was set to 200 μmol m^{-2} s^{-1} during the daytime, and a CO_2 concentration at 390 μmol mol^{-1}.

Phospho*enol*pyruvate carboxylase (PEPC) activity: Quantification of PEPC (EC. 4.1.1.31) activity was performed according to the method described by Freschi *et al*. (2010). For this, 0.5 g of different fresh leaf portions were collected at the transition of dark to light and ground to a fine powder. Then, the powder was mixed with five volumes of buffer containing 200 mM Tris-HCl (pH 8.0), 1 mM Na_2EDTA, 5 mM DTT, 10 mM $MgCl_2$, 10 % (v/v) glycerol and 0.5 % (m/v) bovine serum albumin. The homogenate was centrifuged for 5 min (4 °C, 15 000 *g*), and the supernatant was immediately used for enzyme assay. Activity of PEPC was performed at 30 °C in 2 cm^3 of a standard reaction medium containing 50 mM Tris-HCl (pH 8.0), 1 mM DTT, 10 mM $MgCl_2$, 10 mM $NaHCO_3$, 200 mM NADH, and 3 mM phospho*enol*pyruvate. The reaction was started by adding 0.4 cm^3 of the enzyme extract, and consumption of NADH was measured at 340 nm for 5 min. The measurements were made in triplicate.

Urease activity and endogenous urea content: Urease activity was determined by quantifying NH_4^+ released from urea hydrolysis by the method of Hogan *et al*. (1983) and Takahashi and Mercier (2011). A sample of 0.5 g of fresh leaf material was incubated in 2 cm^3 of a 100 mM phosphate buffer (KH_2PO_4/K_2HPO_4; pH 7.5) containing 5 % (v/v) *n*-propanol and 200 mM urea. Tubes containing the samples were vacuum infiltrated for 1 min three times and incubated at 30 °C for 60 min. Aliquots of 0.1 cm^3 were taken from the incubating medium at the beginning and after 60 min of the assay. Ammonium produced was quantified by the phenol-hypochlorite reaction (McCullough 1967, Weatherburn 1967) and performed exactly as outlined in Takahashi and Mercier (2011). The measurements were made in triplicate at 0, 1, 4, 7, and 10 h after urea supply. In order to estimate the potential amount of ammonium produced through urease activity in the different portions of the leaf, the values of urease activity of both the daytime and the nighttime were integrated. Endogenous urea content was determined exactly as described in Takahashi and Mercier (2011).

Cytochemical detection of CO_2: Cytochemical detection of CO_2 was performed in the apical portions of detached leaves in the dark period 7 h after a 5 mM urea solution was added. The assay was performed according the method used by Millanes *et al*. (2004). The apical parts of the leaves were sectioned into 1-mm pieces and incubated in a 100 mM phosphate buffer (pH 7.5) with 100 mM urea and 10 mM $CoCl_2$ at 30 °C for 60 min. The control of the reaction was prepared incubating tissues of the apexes of the leaves without the urea solution. After incubation, the leaf sections were washed thoroughly in distilled water and treated with $(NH_4)_2S$ for 30 s at room temperature. After that, the tissues were washed with distilled water and incubated with 1 % (m/v) osmium tetroxide in a phosphate buffer for 1.5 h and subsequently incubated with 0.5 % (m/v) uranylacetate overnight. The material was dehydrated in a graded series of ethanol and embedded in Spurr resin at 60 °C for one week. Ultrathin sections of leaves were obtained with a *Leica Ultracut UCT* ultramicrotome (Wetzlar, Germany) and analyzed using a *Philips CM100* transmission electron microscope (Eindhoven, The Netherlands).

The total percentage of isotopic enrichment of malate by CO_2 from urea hydrolysis: Leaf discs (5 mm in diameter) from the different leaf portions from either well-watered (control) or water deficit conditions were cut and incubated in solutions of either 5 mM [^{13}C]urea (99 atom % *Sigma-Aldrich*, St. Louis, USA) or urea at a natural isotopic abundance (as a GC-MS control). The discs were cut and exposed to urea at the beginning of the dark period and collected after 12 h (the end of the dark period) for GC-MS analyses.

The samples (0.1 g of fresh leaves) were ground in liquid nitrogen to a fine powder and mixed with 0.5 cm^3 of a 12:5:1 (m/m/m) mixture of methanol, chloroform, and water at 60 °C for 30 min. Salicylic acid was used as an internal standard (20 μg cm^{-3} of the methanol mixture) after confirming that no endogenous peaks of this compound are detected. Then, the samples were mixed with 0.5 cm^3 of water and centrifuged at 18 000 *g* for 10 min. Finally, 0.1 cm^3 of the supernatant was vacuum dried at 60 °C. The dried residue was dissolved with 0.03 cm^3 of pyridine (99.8 %, *Sigma-Aldrich*) and derivatized with 0.03 cm^3 of *MTBSTFA* (*Sigma-Aldrich*) at 92 °C for 1 h.

Aliquots of 1 mm^3 were injected (a splitless mode) into a GC-MS system (*QP2010SE*, *Shimadzu*, JP) attached to a quadrupole mass spectrometer, using helium as a carrier gas at a constant flow of 24 cm^3 min^{-1}. Gas chromatography was performed using a 30 m *Agilent DB-5ms* column (Santa Clara, USA) with an inner diameter of 0.25 mm and a stationary phase diameter of 0.25 μm. The initial running condition was set at 100 °C followed by a 6 °C min^{-1} temperature rise to 300 °C. This final heating was maintained for 10 min.

In order to differentiate unlabeled malate from [^{13}C]malate, the peak of an unlabeled malate commercial standard was compared to [^{13}C]malate produced by sequential PEPC and MDH reactions using [^{13}C]sodium bicarbonate (98-atom % ^{13}C, 99 %, *Sigma-Aldrich*).

The m/z ion masses to identify the compounds were 115, 419, and 287 for unlabeled malate and 115, 420, and 288 for [^{13}C]malate. The total percentage of isotopic

enrichment of [^{13}C]malate by CO_2 from urea hydrolysis was calculated using the 420 and 419 peaks from the labeled-urea and unlabeled-urea treated detached leaves. The formula is: $[A_{420}/(A_{420} + A_{419}) - B_{420}/(B_{420} + B_{419})] \times 100$, where the letter A indicates the peaks of the labeled-urea treated leaves, and the letter B indicates the peaks of detached leaves treated with urea at a natural isotopic abundance.

Data analysis: All data are shown as means with standard deviations. Student's *t*-test was used to confirm statistical significance ($\alpha = 0.05$) between the treatments.

Results

In order to induce stomata closure and consequently limit absorption of atmospheric CO_2 into the tissue, the leaves of *V. gigantea* were detached and maintained in flasks containing a 30 % (m/v) PEG 6000 solution (Mioto and Mercier 2013). The RWC measured on the 8th day of treatment decreased in all the leaf portions (apex, middle and base, Fig. 1) indicating that the PEG 6000 treatment effectively reduced the amount of water available for the leaves. The RWC of the basal portion decreased by about 60 %, whereas in the apical and middle portions by about 24 and 10 %, respectively.

In order to verify the limitation of atmospheric CO_2 available to the drought-stressed leaves, gas exchange was evaluated in the apical and middle portions only (Fig. 2) since the basal portion shows a reduced number of stomata and chlorophyll indicating little photosynthetic activity (Takahashi *et al*. 2007). Carbon dioxide uptake in the control leaves happened exclusively during the daytime for both apical and middle regions (Fig. 2*A*). However, in leaves exposed to the water deficit, the stomata remained mostly closed during the entire day, as evidenced by no positive net photosynthesis (Fig. 2*B*). These results confirm that the control leaves were photosynthetically active even after being detached for 8 d and that the water deficit treatment was effective in restricting CO_2 absorption into the leaves.

Once it was confirmed that the CO_2 absorption in

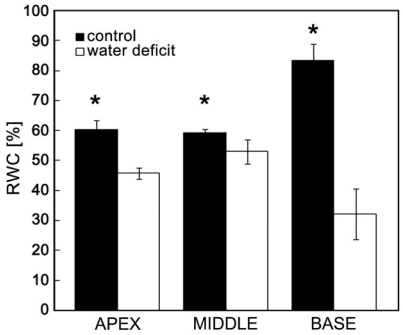

Fig. 1. Relative water content (RWC) of different portions of *Vriesea gigantea* detached leaves after 8 d under well-watered (*black bars*) and water deficit (*white bars*) treatments. Means ± SDs of three replicate samples. *Asterisks* indicate significant differences between the treatments (*t*-test; $P < 0.05$).

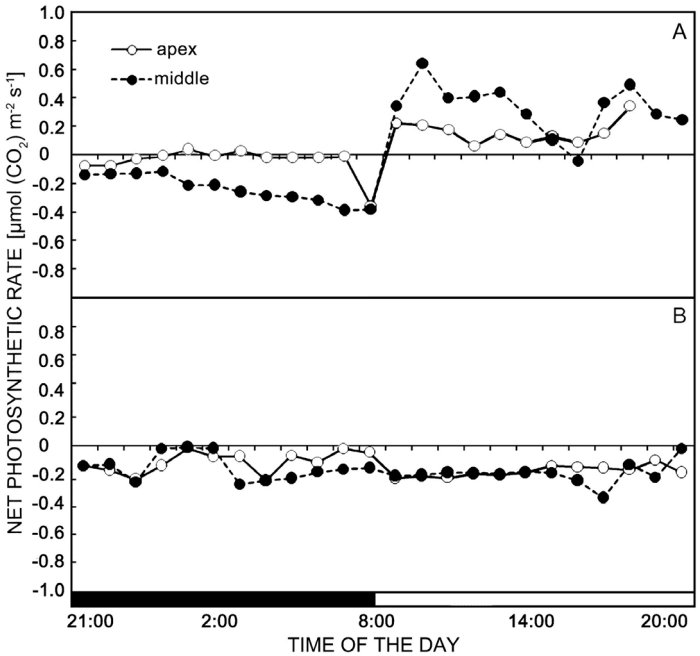

Fig. 2. The diurnal pattern of net photosynthetic rate in apical (*open circles*) and middle (*closed circles*) leaf portions of detached leaves of *Vriesea gigantea* after 8 d under well-watered (*A*) and water deficit (*B*) treatments. The *dark bar* in the x-axis indicates the period of darkness.

leaves under water deficit was restricted, a potential urease activity was analyzed after supplying the leaves with urea during either the daytime or nighttime (Fig. 3). During the light period, there were slight differences in urease activities between the treatments in the apical and middle portions (Fig. 3*A*, *B*), in which the total urease activities were slightly higher under water deficit than in control leaves (Table 1). Urease activities (Fig. 3*C*,*F*) and endogenous urea content (Fig. 4*C*,*F*) in the basal portion of leaves under water deficit were significantly lower when compared with well-watered leaves during both the daytime and nighttime. However, leaves subjected to water deficit showed a significantly higher urease activity in the apical portion during the night period when compared to the control (Fig. 3*D*).

The apical part of leaves under water deficit was responsible for 27.2 % of the total leaf urease activity, being almost five-fold higher than in control leaves (Table 1). On the other hand, during the light period, the apical portions of the control and water deficit treatments accounted for 6.3 and 12.1 % of the total urease-generated ammonium, respectively. In both the periods of the day when comparing leaves under water deficit with the control, the middle portions showed little difference in urease activity (Fig. 3*B,E*) and urea content (Fig. 4*B*,*E*). Nevertheless, in terms of the potential

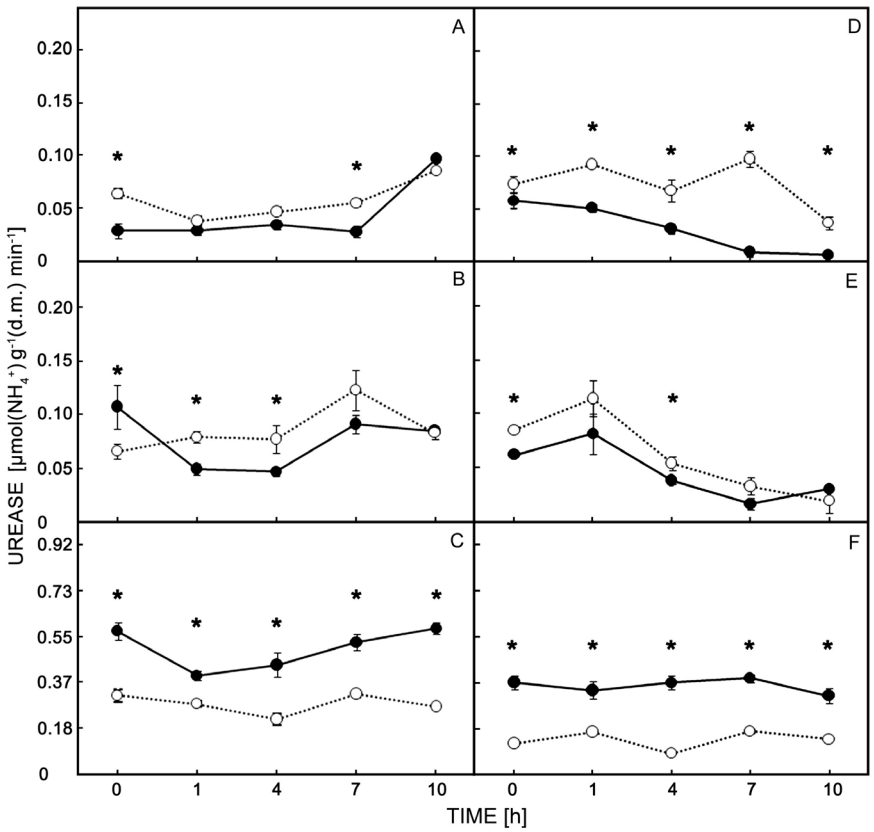

Fig. 3. Daytime (*A,B,C*) and nighttime (*D,E,F*) potential activities of urease in apical (*A*,*D*), middle (*B*,*E*), and basal (*C*,*F*) regions of detached leaves of *Vriesea gigantea* at 0, 1, 4, 7, and 10 h after supplying 5 mM urea to plants grown under well-watered (*closed circles*) and water deficit (*open circles*) conditions for 8 d. Means ± SDs of three replicate samples. *Asterisks* indicate significant differences between treatments (*t*-test; $P < 0.05$).

Table 1. Potential ammonium amount generated from urea hydrolysis [µmol(NH_4^+) g^{-1}(d.m.)] obtained by integration of the urease activity curves during the entire daytime and nighttime at the apical, middle, and basal regions of detached leaves of *Vriesea gigantea.* Detached leaves were supplied with 5 mM urea after 8 d at well-watered (control) and PEG (water deficit) treatments. Values in brackets indicate the percentage of urease contribution from each leaf portion related to the whole leaf.

Leaf portion	Light period control	water deficit	Dark period control	water deficit
Apex	22.4 (6.3)	30.0 (12.1)	14.8 (5.8)	43.0 (27.7)
Middle	38.5 (10.9)	50.8 (20.5)	22.5 (8.9)	31.3 (19.8)
Base	292.9 (82.8)	166.8 (67.4)	216.5 (85.3)	83.6 (53.0)

ammonium production and, consequently, CO_2 yield through urease activity, the middle and apical portions of leaves under water deficit showed a higher contribution than those of control leaves (Table 1). Moreover, during the light and dark phases, the apical and middle portions of both the treatments showed a slight increase of urea content 1 h after urea application suggesting that part of the urea was transported from the basal portion to the upper portion of leaves (Fig. 4*A*-*D*).

To gain more insight into the elevated urease activities noted in the apical portions of leaves under water deficit particularly during the night, additional experiments were conducted in order to analyze CO_2 availability from urea hydrolysis. Cytochemical detection of CO_2 was performed in the apical leaf region after 7 h of urea application during the dark phase (the moment in which the highest urease activity was observed (Fig. 3*D*). In leaves under water deficit, a more prominent CO_2 deposition was noted in the cytoplasm near chloroplasts (Fig. 5*D*,*E*) and intercellular space (Fig. 5*F*) than in well-watered leaves (Fig. 5*A*,*B*,*C*). In order to ensure that CO_2 detection was provided by urea hydrolysis, a control of the cytochemical reaction was performed, in which the leaf tissues were not incubated in urea, resulting in no CO_2 deposits into the cells (Fig. 5*G*,*H*).

Considering the increase of CO_2 availability through urea hydrolysis in the upper parts of leaves under water deficit during the night, we wondered whether in this period of the day, the leaves were capable of fixing CO_2 produced by urea hydrolysis. Therefore, due to lack of Rubisco activity at night, PEPC activity was measured (Fig. 6). The results suggest an enhanced capacity to fix CO_2 in the apical part of drought-exposed leaves through PEPC due to a greater activity found in these leaves than in well-hydrated ones. This was further corroborated by applying labeled urea during the night and determining the relative percentage of [^{13}C]malate at the end of the night (Fig. 7). The data show greater labeling malate in the apical and middle portions of leaves from the water deficit treatment than of control leaves. Moreover, the highest percentage of labeled malate was detected in the basal portion of leaves, but it decreased under water shortage probably due to a decrease in PEPC and urease activities when compared to the base of well-hydrated leaves.

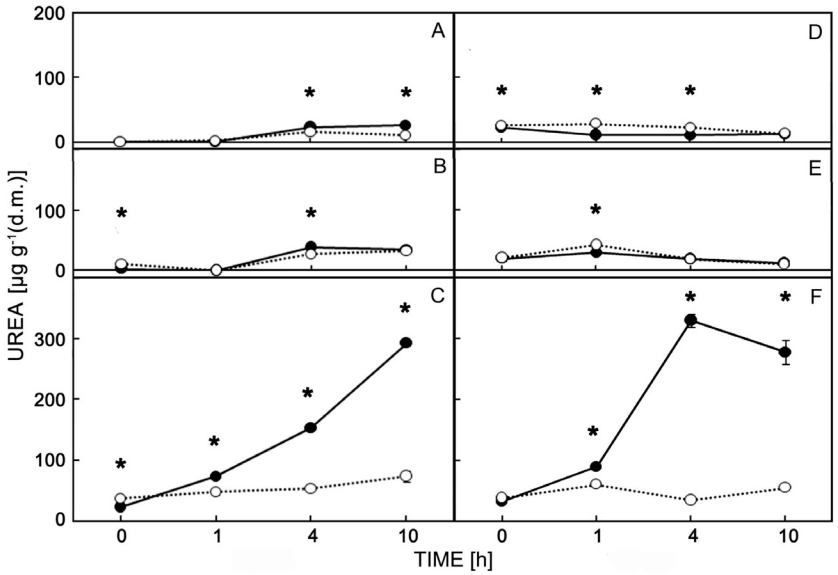

Fig. 4. Daytime (*A,B,C*) and nighttime (*D,E,F*) content of urea in apical (*A*,*D*), middle (*B*,*E*), and basal (*C*,*F*) regions of detached leaves of *Vriesea gigantea* at 0, 1, 4, and 10 h after supplying with 5 mM urea to plants grown under well-watered (*closed circles*) and water deficit (*open circles*) conditions for 8 d. Means ± SDs of three replicate samples. *Asterisks* indicate significant differences between treatments (*t*-test; $P < 0.05$).

Discussion

Urea plays a relevant role in agriculture since it is a frequently used nitrogen fertilizer worldwide (Cao *et al*. 2010). Nevertheless, in habitats limited in inorganic nitrogen, such as epiphytic, urea could be of a greater ecological importance. The importance of urea in nitrogen nutrition in epiphytic tank-bromeliads has been evidenced by a better growth (Endres and Mercier 2001) and high capacity of *V. gigantea* to absorb this organic nitrogen source (Inselsbacher *et al*. 2007). Although urea must apparently be hydrolyzed by urease to allow ammonia nitrogen assimilation (Gerendás and Sattelmacher 1999, Wang *et al*. 2008, Cao *et al*. 2010), there is little information about fixed CO_2 released from urea hydrolysis and the importance of urease under

CO_2-limited conditions such as water stress. Thus, in this study, we investigated whether urea could be an important carbon source for *V. gigantea* leaves under water deficit conditions.

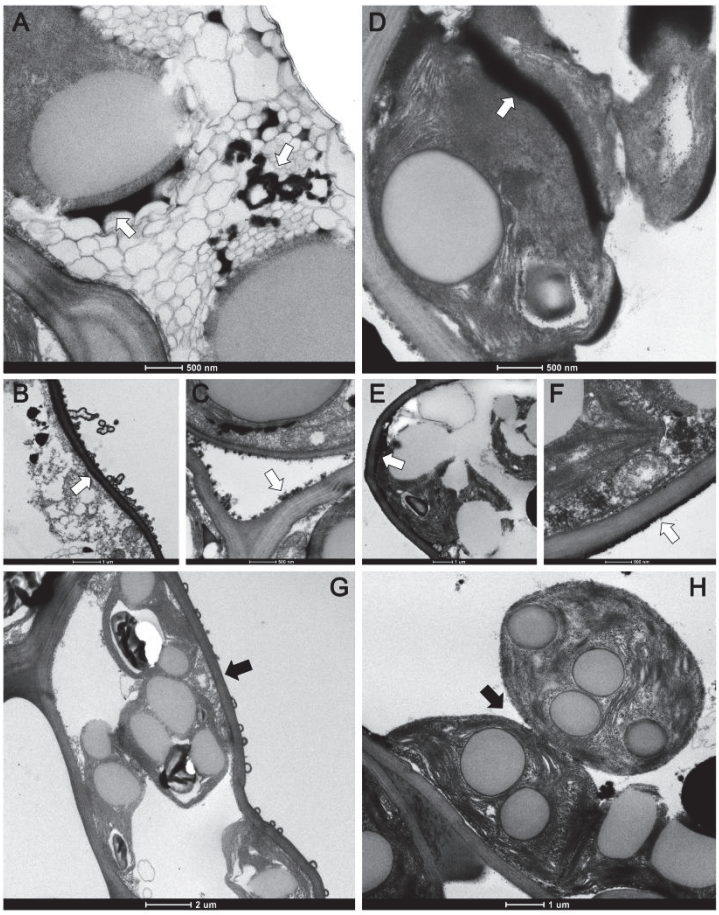

Fig. 5. Cytolocalization of CO_2 originated from urea hydrolysis at the apical region of detached leaves of *Vriesea gigantea* by transmission electron microscopy. The assay was performed during the dark phase 7 h after the detached leaves were supplied with 5 mM urea (*A*-*F*). Prior to the application of urea, the detached leaves were kept for 8 d under well-watered (control) (*A*,*B*,*C*) or water deficit (*D*,*E*,*F*) conditions. Control cells of the apical leaf portion of *V. gigantea* were kept under well-watered (*G*) or water deficit (*H*) conditions and incubated in the absence of urea. *White arrows* indicate the cytochemical localization of CO_2. *Black arrows* show no electron-dense deposits of CO_2.

Considering the fact that *V. gigantea* plants in the epiphytic habitat are exposed to a sporadic or seasonal availability of water through rainfall, these plants must frequently cope with water deficit events. During these events, atmospheric CO_2 absorption into the leaves could be impaired due to stomata closure (Wilkinson and Davies 2002). Therefore, in order to restrict the net CO_2 uptake and test our hypothesis, we submitted detached *V. gigantea* leaves to a moderate water deficit (-1.85 MPa) over 8 d. Under mild water deficit conditions, it has been suggested that net CO_2 absorption into leaves is reduced mainly due to stomata closure, which causes a decrease in CO_2 availability to chloroplasts (Cornic 2000, Cornic and Fresnau 2002).

After eight days of water deficit, the apex and middle portions of leaves lost 24 and 10 % of their RWC, respectively (Fig. 1). Several studies have suggested that an RWC decrease of 0 to 25 % decreases net photosynthetic rate mostly as a result of stomatal limitation (Cornic and Fresnau 2002, Lawlor and Cornic 2002). Indeed, our results show stomata closure in leaves under the water deficit as evidenced by no net photosynthesis (Fig. 2*B*). A higher water loss was observed in the basal portion in drought-stressed leaves (Fig. 1), suggesting water remobilization to the photosynthetic parts of leaves (the middle and apical portions). Likewise, other studies conducted in epiphytic bromeliads have shown a similar water distribution along the leaf after exposure to water shortage (Freschi *et al*. 2010, Mioto and Mercier 2013, Rodrigues *et al*. 2016), indicating a prioritization of the apical portion in terms of water distribution. Since the upper parts of bromeliad leaves are the main tissues responsible for photosynthetic activity when compared to the basal leaf portions (Zotz *et al*. 2002, Takahashi *et al*. 2007, Freschi *et al*. 2010, Mioto and Mercier 2013), it is conceivable that a region

with a large hydrenchyma (the basal portion) provides water to the upper parts of the leaf in order to avoid damage to the photosynthetic apparatus under water deficit conditions. It has been suggested that some aquaporins may be involved during water redistribution since these membrane channels can facilitate water transport among tissues (Kjellbom *et al*. 1999) and can be up-regulated under water deficit conditions (Lian *et al*. 2004, Alexandersson *et al*. 2005, Vera-Estrella *et al*. 2012) or nitrogen supply (Gaspar *et al*. 2003, Liu *et al*. 2003). Therefore, aquaporins may facilitate water and urea transport to other parts of the leaf, even under a low xylem flow resulting from a low transpiration rate caused by stomata closure.

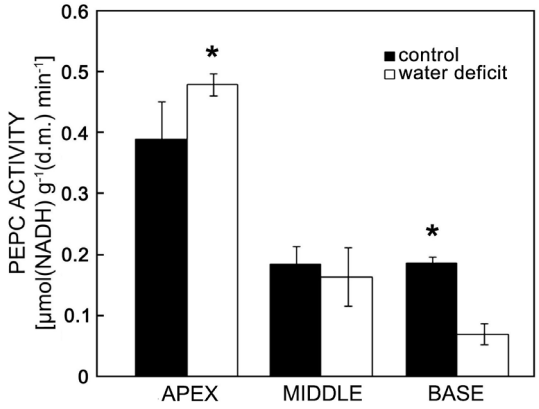

Fig. 6. Activity of phospho*enol*pyruvate carboxylase (PEPC) of different portions of *Vriesea gigantea* detached leaves after 8 d under well-watered (*black bars*) and water deficit (*white bars*) conditions. Means ± SDs of three replicate samples. *Asterisks* indicate significant differences between treatments (*t*-test; $P < 0.05$).

As expected, the drought induced changes in gas exchange rates (Fig. 2). In general, the apical and middle leaf portions showed a maximal photosynthetic rate restricted to the light period in well-watered conditions (Fig. 2*A*). Despite the fact that the leaves were detached, they were photosynthetically active even after 8 d. In addition, the values of diurnal CO_2 uptake were similar to those obtained in previous field and laboratory studies of epiphytic-tank bromeliad plants under well-watered conditions (Maxwell *et al*. 1995, Maxwell 2002, Freschi *et al*. 2010). Furthermore, the water deficit treatment through PEG 6000 exposure limited CO_2 entrance into the leaves during the entire daytime and nighttime period (Fig 2*B*).

Once CO_2 absorption into the leaves under water deficit was restricted, a potential urease activity was analyzed after the application of urea (Fig. 3) in order to determine whether urease could increase its activity under this condition and, subsequently, provide CO_2 to the cells. Endogenous urease activity was observed in all the leaf portions during both the daytime and nighttime (Fig. 3). These results agree with previous findings that showed urease to be a ubiquitous enzyme in plant tissues (Gerendás and Sattelmacher 1999, Witte *et al*. 2002, Cao *et al*. 2010). In addition, urease activities in the base of leaves from the control and water deficit treatments did not show significant changes during the daytime and nighttime (Fig. 3*C*,*F*). Despite the elevated urease activities in this leaf region, it was possible to observe a significant urea content (Fig. 4*C*,*F*), suggesting compartmentalization in the vacuole. Witte *et al*. (2002) showed a considerable urea accumulation in the first 12 h after urea application in potato leaves even at a high urease activity, suggesting vacuolar storage. This event may occur after fertilization or animal urine excretion (Witte 2011) and is an important mechanism to cope with environments with a sporadic nutrient availability such as the epiphytic habitat.

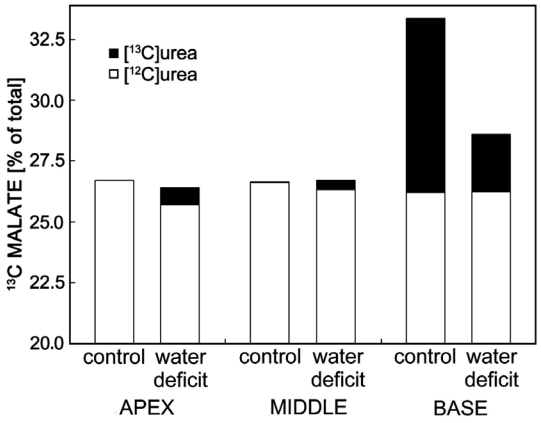

Fig. 7. Total labeled [^{13}C]malate during the nighttime in apical, middle and basal leaf portions of *Vriesea gigantea* after 8 d under well-watered or water deficit conditions. *White bars* indicate the natural abundance of [^{13}C]malate. *Black bars* indicate the isotopic enrichment of [^{13}C]malate by CO_2 originated from urea hydrolysis. Means of three replicate samples.

In addition, elevated urease activity in the basal portion of a leaf could help these plants benefit from the availability of organic nitrogen sources released during animal excretion, competing with other urea-consuming organisms present in the tank. Cambui *et al*. (2009) hypothesized that this bromeliad is also capable of secreting urease in the tank, which would allow a faster use of urea when this compound is available.

It has been suggested that plant urease activities are high enough to metabolize great amounts of urea, making the induction of this enzyme unnecessary (Cao *et al*. 2010). This seems plausible for the basal portions of the leaves, where the activity was considerable high and steady during the entire period. Nevertheless, urease was slightly induced after 10 h of urea exposition in the light period in both well-watered leaves and leaves under water deficit (Fig 3*A*) but, interestingly, only in the apical portion of drought-exposed leaves during the night (Fig. 3*D*). Despite the major contribution of the basal part of a leaf in terms of a potential ammonium and CO_2 generation by urease, apparently under water deficit, the

leaves increased the relative urease activity in the foliar portions, which are more related with photosynthetic functions (the apical and middle portions) (Table 1).

Since the apical portions of water deficit leaves showed higher urease activities than the control group during the entire dark phase (Fig 3*D*) and, consequently, a high urease contribution with respect to the whole leaf (Table 1), we further investigated whether an elevated urease activity in the apical portion during the dark phase correlated with a higher CO_2 availability in the tissue. In fact, as shown in Fig. 5, cytolocalization of CO_2 during the night revealed more conspicuous electron-dense deposits in leaves under water deficit than in well-watered leaves, indicating a greater CO_2 availability in the area around chloroplasts, in the proximity of cell walls and in intercellular spaces. In a previous study, urease was localized in cytoplasmic, plasma-membrane, and cell-wall fractions of *V. gigantea* leaves by using both cytochemical detection and immuno-reaction assay (Cambui *et al*. 2009). Therefore, it is reasonable to localize the CO_2 from urea hydrolysis in intercellular spaces, cytosol near chloroplasts, and cell walls. Taking into account the fact that the samples were collected during the night, we believe that part of the CO_2 from urea hydrolysis remained accumulated around chloroplasts and that with the beginning of the light period, an elevated CO_2 concentration near chloroplasts may reduce the oxygenase action of Rubisco and, therefore, photorespiration. Thus, when urea is supplied, an extensive amount of CO_2 is likely deposited near chloroplasts. As suggested by Lawlor and Cornic (2002), the reduction of CO_2 assimilation due to a moderate water deficit can be alleviated by increasing the internal concentration of CO_2 in the plant. Thus, a high capacity for metabolizing urea may increase the internal concentrations of CO_2 to help mitigate the effects of drought. Therefore, when *V. gigantea* plants are subjected to intermittent water availability, as occurs in their natural environment, the affinity for urea could represent an adaptation to survive in these environments.

Interestingly, the apical portion of leaves under water deficit also showed a higher PEPC activity than control leaves (Fig. 6) indicating that at least part of the CO_2 produced by urea hydrolysis may be fixed during the night through PEPC, given that Rubisco is inactive during this period. Moreover, part of the $^{13}CO_2$ from ^{13}C-labeled urea clearly enriched the malate pool in photosynthetic tissues of drought-exposed leaves, confirming CO_2 fixation *via* PEPC at night (Fig. 7). Thus, the increase in urea-generated CO_2 fixation in the upper parts of the leaves may contribute to avoid further carbon losses during the night. On the other hand, in well-watered conditions, only the base of leaves showed relevant values of malate labeling. This indicates that CO_2 from urea may also play an important role under non-CO_2 limited conditions.

Phospho*enol*pyruvate carboxylase is an anaplerotic enzyme responsible for replenishing carbon skeleton intermediates of the tricarboxylic-acid cycle (O´Leary *et al*. 2011). In C_3 plants, PEPC is described as a non-photosynthetic enzyme, whereas in C_4 and CAM plants, this enzyme is involved in initial fixation of atmospheric CO_2 (Rademacher *et al*. 2002, O´Leary *et al*. 2011). Under conditions of limited CO_2 availability, such as those generated by stomata closure during drought, it seems that PEPC may play an important role in CO_2 fixation in order to avoid further carbon losses. Moreover, up-regulation of PEPC has been reported in several plants as a response to abiotic stresses, such as drought (Gonzalez *et al*. 2003, Aubry *et al*. 2011, Doubnerová and Ryslavá 2011, Cowling 2013, Hýsková *et al*. 2014, Mioto *et al*. 2015), suggesting that PEPC is a key enzyme involved in acclimation to abiotic constraints. Thus, *V. gigantea* might be able to use C_4 and CAM-like mechanisms to fix CO_2 under stress, as also reported for other C_3 plants (Moons *et al*. 1998, Hýsková *et al*. 2014, Mioto *et al*. 2015). It is also relevant to highlight the capacity of some species to switch from the C_3 photosynthetic pathway to CAM photosynthesis (C_3-CAM facultative species) in response to drought stress (Borland *et al*. 1998, Cushman and Borland 2002, Winter *et al*. 2008, Borland *et al*. 2011, Freschi and Mercier 2012). Therefore, it would be interesting to investigate whether *V. gigantea* could have some degree of flexibility to up-regulate a CAM-like photosynthesis under a limited water condition, as has been observed in other epiphyte tank bromeliads.

Interestingly, when the percentage of [^{13}C]malate at the end of the night is determined in leaves of *Guzmania monostachia*, an epiphytic bromeliad capable of performing CAM (Freschi *et al*. 2010, Mioto and Mercier 2013), a significant labeling of the [^{13}C]malate pool under both water deficit and well-watered conditions is observed (Fig. 1 Suppl.) indicating that carbon from urea could also be an important source for CAM plants, even in non-CO_2 limited conditions. Moreover, under water deficit, the leaves of *G. monostachia* showed a greater labeling of [^{13}C]malate than under well-watered conditions. The same pattern was seen in the apical and middle portions of *V. gigantea* leaves.

Nevertheless, both the high capacity of *V. gigantea* to metabolize urea in order to generate CO_2 and the increase of PEPC activity might be important mechanisms to endure drought stress since part of the CO_2 fixed into malate could be used as carbon skeletons for ammonium assimilation, for synthesis of important osmolytes to increase the capacity of osmotic adjustment, and/or for carbon storage. Furthermore, assimilation of nitrogen from sources other than urea, such as nitrate, could be more affected by lack of available carbon skeletons in a situation where the CO_2 uptake is a limitation for a plant. Thus, urea might offer a further advantage to plants in terms of nitrogen assimilation under water deficit.

Despite the scarce information about plant urease regulation, it is possible to notice differences in urease activities of leaves between the daytime and nighttime suggesting that this enzyme might be regulated differentially along the leaf by signals ruled by irradiance.

In addition, it seems that under CO_2-limiting conditions, such as drought, the contribution in terms of urease activity is increased significantly in the upper leaf portions, particularly during the dark phase. During this period, part of the CO_2 released from urea hydrolysis can be fixed *via* PEPC into malate. Therefore, urea may contribute to avoid further carbon losses during water deficit conditions. It is plausible that the high affinity of *V. gigantea* for urea is in part due to the capacity of this compound to provide CO_2 as well as ammonium. Further experimentation is required to determine the impact of CO_2 from urea in plants of *V. gigantea* under natural conditions.

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