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Nitrogen metabolism in leaves of a tank epiphytic bromeliad: Characterization of a spatial and functional division

Cassia Ayumi Takahashi, Helenice Mercier*

Department of Botany, Institution of Bioscience, University of São Paulo, Rua do Matão 277, 05508-090 São Paulo, SP, Brazil

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ABSTRACT

The leaf is considered the most important vegetative organ of tank epiphytic bromeliads due to its ability to absorb and assimilate nutrients. However, little is known about the physiological characteristics of nutrient uptake and assimilation. In order to better understand the mechanisms utilized by some tank epiphytic bromeliads to optimize the nitrogen acquisition and assimilation, a study was proposed to verify the existence of a differential capacity to assimilate nitrogen in different leaf portions. The experiments were conducted using young plants of *Vriesea gigantea*. A nutrient solution containing NO_3^-/NH_4^+ or urea as the sole nitrogen source was supplied to the tank of these plants and the activities of urease, nitrate reductase (NR), glutamine synthetase (GS) and glutamate dehydrogenase (NADH-GDH) were quantified in apical and basal leaf portions after 1, 3, 6, 9, 12, 24 and 48 h. The endogenous ammonium and urea contents were also analyzed. Independent of the nitrogen sources utilized, NR and urease activities were higher in the basal portions of leaves in all the period analyzed. On the contrary, GS and GDH activities were higher in apical part. It was also observed that the endogenous ammonium and urea had the highest contents detected in the basal region. These results suggest that the basal portion was preferentially involved in nitrate reduction and urea hydrolysis, while the apical region could be the main area responsible for ammonium assimilation through the action of GS and GDH activities. Moreover, it was possible to infer that ammonium may be transported from the base, to the apex of the leaves. In conclusion, it was suggested that a spatial and functional division in nitrogen absorption and NH_4^+ assimilation between basal and apical leaf areas exists, ensuring that the majority of nitrogen available inside the tank is quickly used by bromeliad's leaves.

Introduction

The Bromeliaceae family has members with different growth habits, such as terrestrial and epiphytic (Benzing, 1990). The tank epiphytic bromeliads have a very special structure formed by closely imbricated leaves, which creates a reservoir of water and organic debris. This debris can be used as nutrients by epiphytic bromeliads (Benzing and Renfrow, 1974). The leaf is considered the most important vegetative organ of tank epiphytic bromeliads due to its ability to absorb and assimilate nutrients through the trichomes, which are specialized structures present on the leaf surface (Benzing, 1990). The epiphytic bromeliad *Aechmea fasciata* took up ^{32}P through leaf trichomes when this nutrient was added in the tank. About 88% of this ion removed from the tank reappeared in the plant tissues (Winkler and Zotz, 2009). The leaves of tank epiphytic bromeliads can have the main function to absorb nutrients from tank but the roots of epiphytic bromeliads are not completely incapable of taking up nutrients. The *A. fasciata* roots were able to remove about 7% of the ^{32}P from the incubation medium when only roots were supplied with this source (Winkler and Zotz, 2009).

The leaves can be divided into at least three main parts: the basal leaf portion, forming the tank structure and the middle and the apical ones, which both receive more light during the lifespan of the leaf. Research showing the existence of physiological differences between leaf parts is scarce. Popp et al. (2003) detected an increase of carbohydrate and organic solute concentrations from the basal region to the apex of the leaves of *Ananas comosus*. Medina et al. (1994) quantified leaf nitrogen and lipid contents of the *A. comosus* and verified higher values of both metabolic compounds in the apex and middle parts of leaves compared to the basal parts. Freschi et al. (2010) showed that different leaf sections of the facultative C3-CAM bromeliad *Guzmania monostachia* can perform distinct functional roles in response to water shortage since the leaf base seemed to represent the main water reservoir for maintaining the high-

Abbreviations: αKG, alpha ketoglutaric acid; Glu, glutamate; GDH, glutamate dehydrogenase; GOGAT, glutamate synthase; Gln, glutamine; GS, glutamine synthetase; NR, nitrate reductase; NiR, nitrite reductase; St, starch; S, stomata density; Chls, total chlorophyll; T, trichome density.

* Corresponding author. Tel.: +55 11 30917592; fax: +55 11 30917547.
E-mail address: hmercier@usp.br (H. Mercier).

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est photosynthetic activity in apical leaf region. In addition, the CAM pathway was intensified specifically in the upper leaf section, which was the only region showing an almost constant water status during water deprivation (Freschi et al., 2010).

The tank epiphytic bromeliads are able to take up both inorganic and organic nitrogen. However, these bromeliads can have a possible preference for taking up and assimilating nitrogen from the urea and amino acids, as their presence during cultivation caused these plants to grow better (Endres and Mercier, 2001b). The nitrogen of nitrate or urea can be assimilated into amino acid when these compounds are reduced or hydrolyzed to ammonium by nitrate reductase (NR) (EC 1.6.6.1) or urease (EC 3.5.1.5), respectively (Marschner, 1995). NH_4^+ is incorporated into amino acids by the combined action of the enzymes glutamine synthetase (GS) (EC 6.3.1.2) and ferredoxin-dependent glutamate synthase (Fd-GOGAT) (EC 1.4.7.1). The NADH-dependent glutamate dehydrogenase (NADH-GDH) (EC 1.4.1.2) can also operate in the assimilation of ammonium, thereby complementing the enzymes of the GS/GOGAT cycle in the synthesis of glutamate (Miflin and Habash, 2002; Tercé-Laforgue et al., 2004).

Little is known about the importance of different foliar parts in the nutrition of the tank epiphytic bromeliads. Our previous experiments have shown that the apical foliar part of *Vriesea gigantea* may be involved with ammonium assimilation since the highest GS and GDH activities were found in this region (Takahashi et al., 2007). Hence, the present study examined the effects of inorganic (NO_3^-/NH_4^+; 3:2) or organic (urea) nitrogen sources on enzymes of the nitrogen metabolism in leaves of *V. gigantea*, a tank epiphytic bromeliad. We predicted that basal and apical leaf parts would play different roles in the NO_3^- reduction, urea hydrolysis and NH_4^+ assimilation. We used the activities of nitrate reductase (NR), urease, glutamine synthetase (GS) and glutamate dehydrogenase (NADH-GDH) to characterize nitrogen metabolism in leaf parts, as well as the changes in concentrations of endogenous ammonium and urea. Moreover, total chlorophyll, starch contents and stomata and trichome densities were also quantified in both regions of leaves.

Evidence is presented that nitrate reduction and urea hydrolysis occur predominantly in basal leaf part while ammonium is mainly assimilated in the apical portion of the leaves of *V. gigantea*.

Materials and methods

Plant species and culture conditions

Plants of the epiphytic tank bromeliad, *Vriesea gigantea* Gaudichaud, were obtained from the *in vitro* germination of seeds. After germination, the seedlings were cultivated in macronutrients from the Knudson medium (Knudson, 1946) and micronutrients from the Murashige and Skoog formulation (Murashige and Skoog, 1962) until reaching an average height of 3 cm. After that, the plants were transferred to pots with a commercial organic substrate (Rendmax®) and maintained in a greenhouse for approximately three years. Some growth stage features of the bromeliads used in the experiments are measured in five young *V. gigantea* plants and 12 leaves were used from each plant (from the 4th to 16th visible leaves in the rosette leaf arrangement). The average of total number of leaves per plant was 18.4 ± 2.07 (mean ± SD, $n = 5$ plants), leaf length per leaf was 18.27 ± 2.37 cm (mean ± SD, $n = 60$ leaves), length of apical leaf portion per leaf was 6.12 ± 0.79 cm (mean ± SD, $n = 60$ leaves), length of basal leaf portion per leaf was 6.19 ± 0.92 cm (mean ± SD, $n = 60$ leaves), fresh weight of apical leaf portion per leaf was 0.49 ± 0.11 g (mean ± SD, $n = 60$ leaves), fresh weight of basal leaf portion per leaf was 1.41 ± 0.45 g (mean ± SD, $n = 60$ leaves), dry weight of apical leaf portion per leaf was 0.08 ± 0.02 g (mean ± SD, $n = 60$ leaves) and dry weight of basal leaf portion per leaf was 0.20 ± 0.08 g (mean ± SD, $n = 60$ leaves).

Inorganic or organic nitrogen source

Young bromeliads were cultivated in a greenhouse for three years, were transferred to a growth chamber and kept at a temperature of $25 \pm 2\,^\circ$C with a 12 h photoperiod (200 μmoles photons $m^{-2}\,s^{-1}$) and 70–80% relative humidity. After plants were acclimated in the growth chamber for one month, the organic debris that had been inside the tank was removed and leaves and roots were washed with the distilled water. Then, the bromeliads were transferred to a sand substrate, where they remained in a nutrient-free condition for ten days in order to deplete, at least in part, the endogenous nitrogen. Plants were watered with distilled water every two days. After this period, they were divided into two groups: tanks supplied with nutrient solutions containing an inorganic nitrogen source and those supplied with an organic nitrogen source, both at a concentration of 5 mM of total nitrogen. The solution with inorganic nitrogen (NO_3^-/NH_4^+, 3:2) was composed of half concentration of macronutrients from the Knudson formulation (Knudson, 1946) and of micronutrients from the Murashige and Skoog formulation (Murashige and Skoog, 1962). To the solution with organic nitrogen, or urea, 0.64 g L^{-1} of $CaSO_4$ was added, which was used to replace Ca^{+2} and SO_4^- ions since inorganic nitrogen salts had been removed. In addition, the urease cofactor was also added: 1 μmol L^{-1} of $NiCl_2 \cdot 6H_2O$. The nutrient solution, total volume about 30 mL, was applied strictly to the tank, always 1 h after the beginning of the light period in the growth chamber. The nutrient solution was applied carefully so that all leaf bases were in contact with the solution. After supplying plants with the nutrient sources, seven harvests were done during the light period: 1st, 3rd, 6th, 9th, 12th, 24th and 48th hour. At each harvest point, 33 leaves, removed from three bromeliads, were used. The leaves were divided in three portions: (a) base, corresponding to the region that has absence of chlorophyll and the greenish part of the leaf; (b) middle, corresponding to the lower half of the green portion of the leaf blade; and (c) apex, corresponding to upper half of green part of the leaf blade. The middle parts were discarded. Leaf apexes and bases, each portion with approximately 6 cm, were rinsed with distilled water, dried on filter paper, cut in small pieces and used in the biochemical analysis.

Analysis of NR activity (EC 1.6.6.1)

Nitrate reduction was measured using *in vivo* assay (Freschi et al., 2009). The fresh material (1 g) was weighed and incubated in 6 mL of 0.1 M phosphate buffer (pH 7.5) containing 3% propanol and 0.1 M KNO_3. Tubes containing the samples were vacuum infiltrated three times for 1 min and incubated in the dark at 30 °C for 60 min. Aliquots of 1 mL were removed from the incubating medium at the beginning and the end of essay. The nitrite produced was determined by adding 0.3 mL 1% sulphanilamide, which was prepared with HCl 3 M, and 0.3 mL 0.2% N-(1-naphthyl) ethylene-diamine. Absorbance was read at 540 nm after 30 min. Nitrate reductase activity was expressed as nmol nitrite produced per hour per gram of dry weight.

Extraction of the enzymes GS and NADH-GDH

The sample of fresh material (0.5 g) was macerated in a mortar with liquid nitrogen until a thin powder was obtained. This powder was transferred to centrifuge tubes previously cooled in liquid nitrogen. 4.5 mL of an extraction medium consisting of imidazole buffer 0.05 M, pH 7.9 and DTT 5 mM were pipetted into each tube. These samples were centrifuged at 10,000 × *g* at 4 °C, and the super-