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Original Paper

Adaptive morphoanatomy and ecophysiology of *Billbergia euphemiae*, a hemiepiphyte Bromeliaceae

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Abstract

Habitats under distinct selective pressures exert adaptative pressures that can lead individuals of the same species to present different life strategies for their survival. The aim of this study was to analyse morphoanatomical and physiological traits for identification of adaptive ecological strategies related to both terrestrial and epiphytic life phases of *Billbergia euphemiae.* It was verified that *B. euphemiae* showed lower height, as well smaller length, width and foliar area in epiphytic phase than in terrestrial phase. Concerning to foliar anatomy, the thicknesses of leaf and water-storage parenchyma were higher in terrestrial phase, as densities of stomata and scales on the abaxial surface were higher in epiphytic phase. About the contents of photosynthetic pigments, only chlorophyll *a*/*b* ratio showed differences between life phases. In both habits, plants exhibited roots with absorption hair. In epiphytic phase, roots exhibited higher velamen thickness, smaller outer cortex, higher number of inner cortex cell layers and higher number of protoxylem poles. Thus, *B. euphemiae* individuals in epiphytic exhibited lots of traits related to water retention, once these plants are not into the ground. Besides, the plasticity observed may contribute for survival of this group in habitats submitted to modifications (*e.g.*, climate change and other variations caused by human interference).

Key words: anatomy, epiphyte, leaf, photosynthetic pigments, root.

Resumo

Habitats com pressões seletivas diferentes exercem pressões adaptativas que podem levar indivíduos de uma mesma espécie a apresentar diferentes estratégias de vida para sua sobrevivência. O objetivo desse estudo foi analisar características funcionais para identificação de estratégias ecológicas adaptativas relacionadas às fases de vida terrestre e epifítica de *Billbergia euphemiae*. Foi verificado que *B. euphemiae* na fase epifítica possui menor altura, bem como folhas de menor comprimento, largura e área em relação às plantas na fase terrestre. Em relação à anatomia foliar, a espessura do limbo e do parênquima aquífero foram maiores em plantas na fase terrestre, enquanto a densidade de estômatos e de escamas na face abaxial foram maiores na fase epifítica. Em relação ao teor de pigmentos fotossintéticos, apenas clorofila *a/b* mostrou diferença entre as fases de vida. Em ambos os habitats, as plantas exibiram raízes com pelos absorventes. Na fase epifítica, as raízes exibiram maior espessura do velame, menor córtex externo, maior número de camadas celulares no córtex interno e maior número de polos de protoxilema. Dessa forma, indivíduos de *B. euphemiae* na fase epifítica exibiram muitas características relacionadas à retenção de água, uma vez que essas plantas não estão inseridas no solo. Além disso, a plasticidade observada pode contribuir para sobrevivência desse grupo em habitats sujeitos a mudanças (*e.g.*, mudanças climáticas e outras variações causadas por interferência humana).

Palavras-chave: anatomia, epífita, folha, pigmentos fotossintéticos, raiz.

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Introduction

Habitats under distinct selective pressures exert adaptative pressures that can lead individuals of the same species to present different life strategies for their survival (Bradshaw 1965; Ackerly 2003; Nicotra *et al.* 2010). Hemiepiphytes are known for being epiphytes that show a phase of life associated to the ground (Kress 1986). They can be classified as primary hemiepiphytes when individuals start their lives as epiphytes and extend the roots to the ground, or as secondary hemiepiphytes, when individuals germinate in ground and adhere themselves on phorophytes through propagation stolons (Granados-Sánchez *et al.* 2003). These plants exhibit contrasting ecological strategies according to different phases of life (Holbrook & Putz 1996a; Granados-Sánchez *et al.* 2003), which can be described by variations of anatomical, morphological and physiological traits (Violle *et al.* 2007).

In the past decades, many studies shown morphology and ecophysiology differences in epiphytic and terrestrial species in relation to environment (edaphic, luminosity, moisture) (Parrilla-Diaz & Ackerman 1990; Rada & Jaimez 1992; Scarano *et al.* 2002), biotic factors (Lawton & Williams-Linera 1996) and evolution (Benzing 2000) in different vegetative organs (Moreira *et al.* 2009). Although the epiphytism has received general attention in literature, still few studies have explored adaptive strategies in hemiepiphytes (Ting *et al.* 1987; Holbrook & Putz 1996b). In addition, among such studies much are restricts to genus *Ficus* sp. (Holbrook & Putz 1996b), still being unclear functional and ecological consequences of hemiepiphytism when take into account species belonging to other botanic family, as Bromeliaceae.

Epiphytes consist in a representative group in Bromeliaceae family, an ecologically diverse family, exhibiting high species richness among vascular plants in the neotropics (Kress 1986; Zotz 2013), having estimated 3,352 species belonging to 58 genus (Luther 2012). Furthermore, Bromeliaceae is a group known for having several adaptations to limiting conditions (Benzing 2000). Some examples of those adaptations are presence of epidermal scales (Segecin & Scatena 2004; Proença & Sajo 2007; Pereira *et al.* 2013), velamen (Benzing 2000; Proença & Sajo 2008) and CAM metabolism (Crassulacean Acid Metabolism) (Crayn *et al.* 2004). Such adaptations probably enabled these individuals to occupy a wide range of habitats, like those with low water and nutrients availability (Martin 1994), specially in Bromelioideae subfamily. This is one of eight subfamily of Bromeliaceae (Givnish *et al.* 2011; Goetze *et al.* 2016), with more of 500 species occurring in phytophysiognomies of Atlantic Forest (Martinelli *et al.* 2008).

Billbergia euphemiae E. Morren, is a secondary hemiepiphyte (Kress 1986; Granados-Sánchez *et al.* 2003), native of Brazil, which belongs to Bromelioideae subfamily (Bromelieae tribe) (BFG 2018). Such species occur normally in Atlantic Forest, including montane and submontane phytophysiognomies (Fontoura 1991), and is typical of restinga areas (Magnago *et al.* 2007). Its distribution is submitted to the influence of environmental factors variations, such as luminosity, humidity, availability of nutrients in ground and salinity, as well as biological factors, like reproduction and seed dispersion (Lüttge 2008). Overall, leaf anatomy of *Billbergia euphemiae* include an uniseriate and lignified epidermis with reduced lumen, cutinized. The stomata are in the same level of epidermis on abaxial surface of leaves and arranged in longitudinal rows. Peltate scales cover all surface in both surface of the epidermis, and disc cells do not differ from wing cells and have round shaped. This specie has water storage parenchyma, and subjacent, chlorenchyma with aeration channels and collateral vascular bundles in single series in the lower half of the leaf blade (Pereira *et al.* 2011).

In this context, understanding anatomy, morphology and physiology of hemiepiphytes is important for knowing how these plants behave according to environmental conditions in different phases of life. Moreover, this study allow to complement a big range of studies that hemiepiphytes anatomy focused on taxonomy (Aoyama & Sajo 2003; Ferreira *et al.* 2007; Monteiro *et al.* 2011; Zotz 2013) and ecology of Bromeliaceae (Dickison 2000; Bonnet & Queiroz 2006; Voltolini & Santos 2011; Males & Griffiths 2017). In this context, the aim of this study was to investigate adaptive strategies of *B. euphemiae*, in both terrestrial and epiphytic phases. Our hypothesis is that in epiphytic phase there are more traits related to high incidence of light and to water retention, once the roots are not into the ground.

Material and Methods

Study area

This study was carried out within the dry forest formation of restinga located at Paulo César Vinha State Park, a coastal plain of nearly 1,500 ha in Espírito Santo state, Brazil (20º33'–20º38'S, 40º23'–40º26'W). Weather of region is Aw type according to Köppen classification (1948), with annual temperature averaging 23.3º, annual rainfall of 1,307 mm and relative humidity of 80%. Such climate is characterized by existence of rainy summers and dry winters (Fabris 1995).

Botanical material

There were sampled mature leaves, collected on median areas of both rosette and roots that presented larger diameters, from five adult individuals in terrestrial phase and five individuals in epiphytic phase of *Billbergia euphemiae* (Fig. 1). We deposited the voucher of the species samples in VIES herbarium at Federal University of Espírito Santo, identified with the register number VIES 37156 in 12/09/2012 by collector B.B. Zorger s.n.

Morphological measures analysis

We measured length (cm), area (cm^2), dry mass per area - LMA ($g\ cm^{-2}$) and foliar succulence ($g\ cm^{-2}$) - obtained by fresh mass minus foliar dry mass of foliar area divided by foliar area - one leaf per plant, totalling five leaves. We also measured the height from the ground (cm) of each sampled individual the upper boundary of the photosynthetic tissue (i.e. except inflorescence, seeds or fruits) (Pérez-Harguindeguy *et al*. 2013). Length, width and height were measured by a measuring tape. The leaf area was measured using an Area Meter LI-COR 3100 (Lincoln, USA). To obtain dry mass, leaves were weight after collecting them. As for dry mass, samples were weight after drying in the oven at 60 ºC until obtain constant mass (Garnier *et al.* 2001).

Determination of leaf angle

We measured leaf angle (º) in relation to the ground in one leaf from each individual, totalling five measurements, using a clinometer (James & Bell 2000).

Anatomical and histochemical analysis

We fixed leaves and roots from five individuals in FAA 50 (a mixture of formaldehyde, ethanol and acetic acid) (Johansen 1940) and stored in 70% ethanol. We made transversal sections by freehand, with razor blades, in median foliar third and 1 cm from the top of the root. We did the colouring process with safrablau solution, set them between the blade and the coverslip with glycerine (Kraus & Arduin 1997). On the leaves, we quantified leaf thickness (µm), chlorenchyma, and water-storage parenchyma thickness. We also analysed stomata and scales densities ($nº\ mm^{-2}$) in both surfaces of epidermis through epidermal impressions, using a drop of cyanoacrylate ester adhesive (Super Bonder®) on a histological blade (Wilson *et al.* 1981). In roots, we measured the diameter (µm) of metaxylem conducting elements and thickness

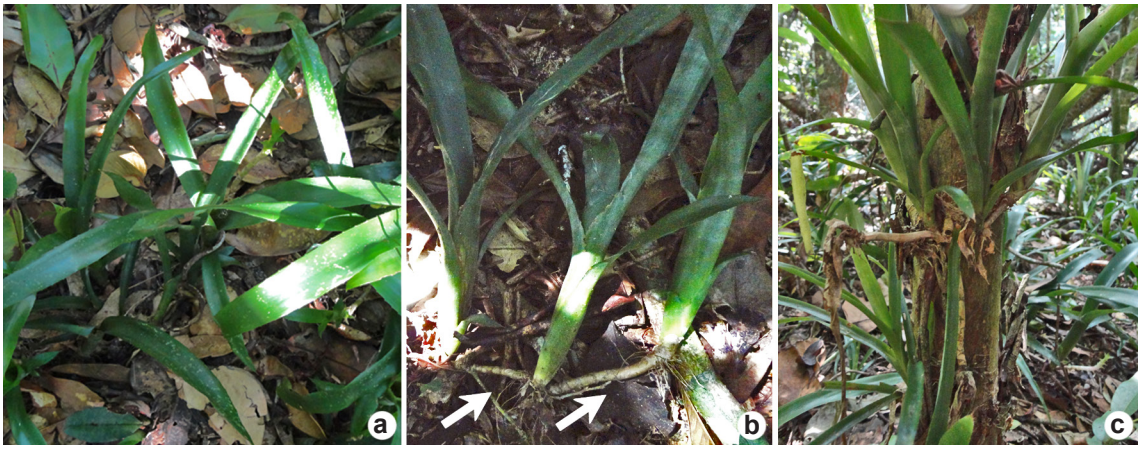

Figure 1 – a-c. Individuals of *Billbergia euphemiae* in dry forest formation of restinga in Paulo César Vinha State Park, ES, Brazil – a,b. terrestrial phase; c. epiphytic phase. Arrow indicates stolon of propagation.

of root, velamen, outer cortex or exoderm, inner cortex, poles numbers of protoxylem, and then identified presence/absence of epidermal hair and intercellular spaces on inner cortex. For each analysed parameter, we made eight measures in every sample (leaf and root) of five terrestrial individuals and from five epiphytes individuals. We measured all of them through a capture image system linked to Nikon E200 (Tokyo, Japan) microscope using Tsview v.6.1.3.2 software (Tucsen Imaging Technology Co. Limited). Results were documented using photomicrographs.

For histochemical analysis, freehand sections of roots were submitted to the reagent phloroglucinol in acid medium (Sass 1951) to verify possible lignified walls in cells of velamen.

Determination of photosynthetic pigments contents

We made the photosynthetic pigments extraction according to Arnon's method (1949) that uses acetone 80% as extractor. Therefore, we macerated 0.200 g of fresh plant material in 25 mL of acetone. We made the whole process with samples maintained under low temperatures in a dark chamber using green light 40 W. We made extracts measurements using a Genesys 10 S UV-Vis Thermo Scientific spectrophotometer on 480, 645 and 663 nm absorbencies. To define photosynthetic pigments contents (μmol g^{-1} MF) we used the equations of Hendry & Grime (1993):

Chlorophyll *a* = $(12.7.A_{663} – 2.69.A_{645}) \times 1.119$

Chlorophyll *b* = $(22.9.A_{645} – 4.68.A_{663}) \times 1.102$

Carotenoids = $[A_{480} + (0.114.A_{663}) – (0.638.A_{645}) \times V] / 112.5.FM$

Where: A_{480} = a 480 nm; A_{645} = absorbance on 645 nm; A_{663} = absorbance on 663 nm; V = sample volume (mL) and FM = sample fresh mass (g).

Determination of photosynthetically active radiation (PAR)

We measured the Photosynthetically Active Radiation (μmol g^{-1} MF) at the occurrence area of terrestrial and epiphytic individuals using a FieldScout Quantum Light Meters reader (Plainfield, USA). We made five measurements at 9:00 AM, 11:00 AM and 13:00 PM, maintaining the equipment close to the individuals collected in each life phase and then we calculated the arithmetic mean of each one.

Data analysis

Data obtained through analysis of morphology, foliar angle, anatomy, photosynthetic pigments contents and photosynthetically active radiation were submitted to Shapiro-Wilk's test for normality. Once all data showed normal distribution, means were compared by t-test on STATISTICA 10 software (Statsoft 2006).

Results

Epiphytic phase of *Billbergia euphemiae* showed lower height; leaves with lowers length, width and area (Tab. 1). About photosynthetic pigments content, it was observed higher values of chlorophyll *a*/*b* ratio (Tab. 1). In relation to anatomic aspects, *B. euphemiae* in the epiphytic phase shown lower water-storage parenchyma thickness, lower leaf thickness and higher stomata and scales densities on abaxial epidermis, in relation to terrestrial phase (Fig. 2; Tab. 1). The following attributes showed no changes between terrestrial and epiphytic phases: foliar angle, succulence, LMA, chlorophyll *a*, chlorophyll *b*, total chlorophyll, PAR and carotenoids, as well as, chlorenchyma thickness and adaxial scales (Tab. 1).

Individual roots in the epiphytic phase showed higher total root thickness, thicker velamen with higher number of cell layers, lower thickness of outer cortex with lower number of cell layers, higher number of cellular layers of inner cortex and higher number of protoxylem poles, in relation to terrestrial phase (Fig. 2; Tab. 1). Both terrestrial and epiphytic individuals have root hair on peripheral layer of velamen cell (epivelamen). On the other hand, only inner cortex in the terrestrial phase presented intercellular spaces. The metaxylem diameter was the same in both phases (Fig. 2; Tab. 1).

Results of histochemical analysis were negative for detection of lignified walls in velamen cells.

Discussion

Results indicate that *Billbergia euphemiae* individuals present part of attributes related to water conservation in epiphytic phase. It may be observed through the reduction of morphological measures, scales densities on abaxial epidermis, velamen thickness and number of poles on

Table 1 – Comparative between means ± standard deviation (mean ± SD) of *Billbergia euphemiae* individuals traits in terrestrial and epiphytic phase in dry forest of restinga in Paulo César Vinha State Park, ES, Brazil. Means with different letters indicates significant differences by t-test in level of 5% ($p < 0.05$) of probability.

Traits	Life phases	
	Terrestrial	Epiphytic
Plant height (cm)	30.88 ± 2.56 a	19.36 ± 1.79 b
Foliar length (cm)	25.18 ± 1.8 a	16.12 ± 1.02 b
Foliar width (cm)	3.68 ± 0.31 a	1.84 ± 0.25 b
Foliar angle (º)	70 ± 9.61 a	50 ± 9.61 a
Leaf area (cm^2)	51.49 ± 2.01 a	19.97 ± 6.2 b
LMA ($g\ cm^{-2}$)	0.006 ± 0.001 a	0.007 ± 0.002 a
Foliar succulence ($g\ cm^{-2}$)	0.060 ± 0.008 a	0.052 ± 0.006 a
Chlorenchyma thickness (µm)	328.22 ± 8.89 a	339.04 ± 9.72 a
Water-storage parenchyma thickness (µm)	361.83 ± 8.81 a	248.06 ± 28.83 b
Leaf thickness (µm)	739.18 ± 10.06 a	603.12 ± 57.11b
Stomata density ($n^o\ mm^{-2}$)	10.41 ± 0.34 b	17.32 ± 0.88 a
Density of abaxial scales ($n^o\ mm^{-2}$)	11.01 ± 0.23 b	13.89 ± 0.49 a
Density of adaxial scales ($n^o\ mm^{-2}$)	8.27 ± 0.25 a	10. 64 ± 1.08 a
Chlorophyll *a* (µmol g^{-1} MF)	0.61 ± 0.07 a	0.52 ± 0.13 a
Chlorophyll *b* (µmol g^{-1} MF)	0.35 ± 0.15 a	0.25 ± 0.005 a
Chlorophyll *a/b* (µmol g^{-1} MF)	1.74 ± 0.36 b	2.08 ± 0.14 a
Total chlorophyll (µmol g^{-1} MF)	0. 96 ± 0.17 a	0.77 ± 0.21 a
RFA (µmol g^{-1} MF)	46.60 ± 0.15 a	61.80 ± 0.05 a
Carotenoids (µmol g^{-1} MF)	0.049 ± 0.007 a	0.044 ± 0.012 a
Number of velamen cell layers	3 a 4	5 a 7
Number of outer cortex cell layers	4 a 5	2 a 3
Number of inner cortex cell layers	1	3 a 4
Root hairs	Present	Present
Inner cortex with intercellular spaces	Present	Absence
Number of protoxylem poles	6	9
Total root thickness (µm)	513.45 ± 16.23 b	643.42 ± 15.61 a
Velamen thickness (µm)	106.81 ± 4.42 b	150.15 ± 5.04 a
Outer cortex thickness (µm)	49.61 ± 6.1 a	38.72 ± 1.69 b
Diameter of metaxylem poles (µm)	12.25 ± 2.05 a	14.94 ± 2.12 a

protoxylem. These attributes can be related to lower water and nutrients availability, since the roots of epiphytes plants are not into the ground (Benzing 2000).

The lower height found in *B. euphemiae* individuals of epiphytic phase, that propagated on phorophyte from stolon, may be explained by lower water and nutrients availability, which may contributes to lower cellular extension and photosynthetic efficiency (Lloyd & Farquhar 1996; Jaleel *et al.* 2009). In addition, the smaller height found may be a direct consequence of the smaller

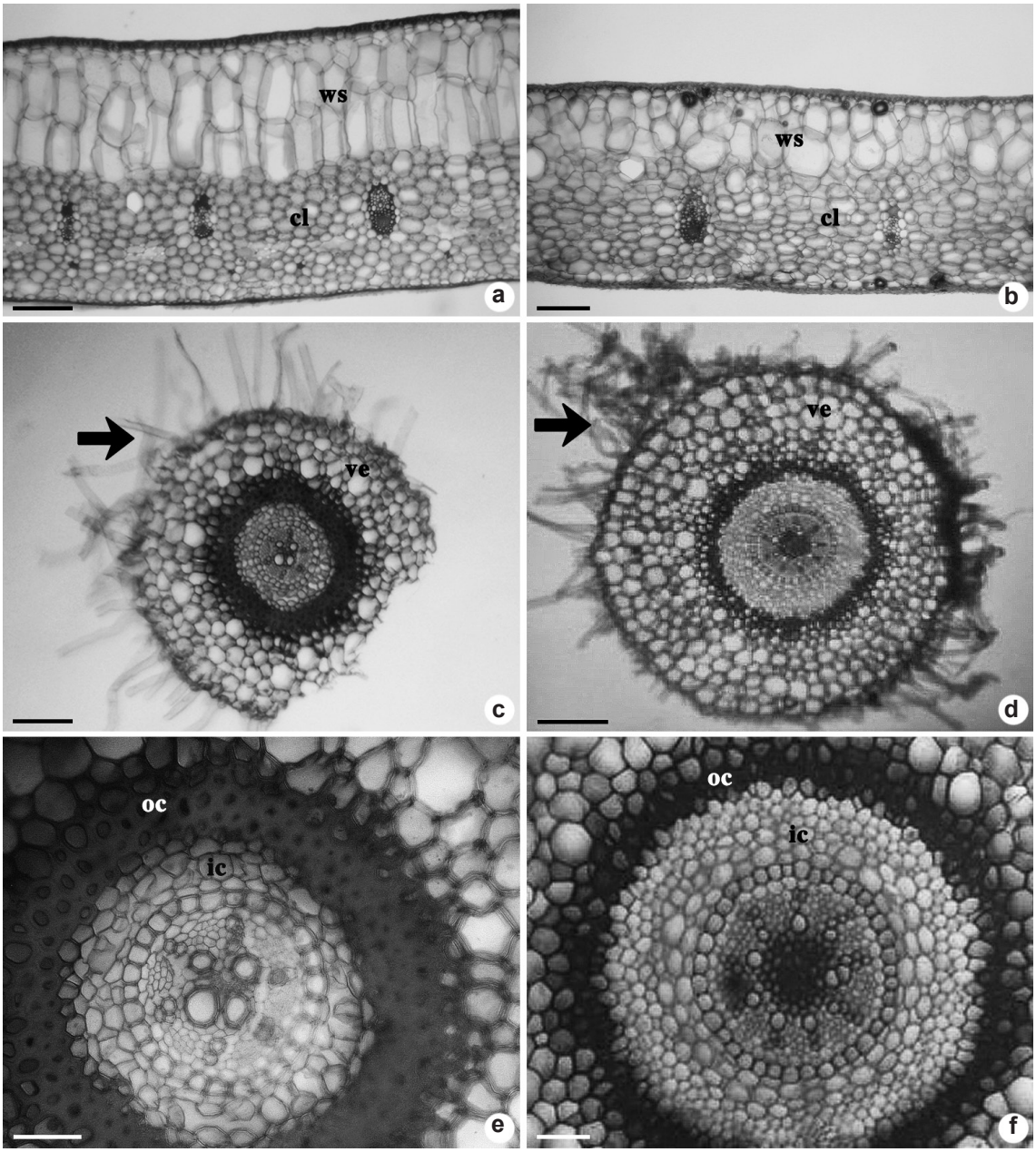

Figure 2 – a-f. Transverse sections of *Billbergia euphemiae* – a-b. leaves – a. terrestrial phase; b. epiphytic phase; c-f. roots – c,e. terrestrial phase; d,f. epiphytic phase. The arrow indicates epivelamen. (oc = outer cortex; ic = inner cortex; ws = water-storage parenchyma; ch = chlorenchyma; ve = velamen). Scale bars = 50 μm.

leaf size in the epiphytic phase. Lower length, width and leaf area in that phase can be seen as mechanisms for water retention (Reinert 1998). Smaller and straighter leaves are submitted to less resistance of air boundary layer which favours the faster cooling of the leaf and avoid water loss by transpiration (Parkhurst & Loucks 1972; Givnish & Vermeij 1976). This is a common trait in plants that are submitted to lower water availability, like individuals of *B. euphemiae* in epiphytic phase (Benzing 2000).

About the photosynthetic pigments contents only chlorophyll *a/b* changed between terrestrial and epiphytic phases. It means that in epiphytes, chlorophyll *b* is degraded faster in relation to chlorophyll *a* (Kramer & Kozlowski 1979; Ishida *et al.* 1999). It may happen due to a disorder of photosystem II caused by hydric stress (Souza *et al.* 2004). Other traits - foliar angle, LMA, succulence, chlorenchyma thickness, chlorophyll *a*, chlorophyll *b*, total chlorophyll and carotenoids - did not show changes in both terrestrial and epiphytic phase. As the light is similar in both habitats, even other factors like water and nutrients may not be strong enough to influence those foliar traits.

The higher stomata density in epiphytic phase enable the increase of CO_2 absorption and is normally associated to a lower stomatal pore size that reduce water loss by transpiration through the rapid stomata closing (Abrams *et al*. 1992; Aasamaa *et al.* 2001; Hetherington & Woodward 2003). Furthermore, greater stomatal density may lead to greater transpiration if the stomatal opening and stomatal control in this species is less rigid, as found in plants under higher temperatures (Hetherington & Woodward 2003). This generally allows for greater leaf transpiration and, consequently, cooling of leaves (Hetherington & Woodward 2003; Cavallero *et al.* 2011; Voltolini & Santos 2011; Pereira *et al.* 2013). However, we did not observe in this study variation in solar radiation between habitats, which could cause variation in temperature to support this hypothesis. The density of epidermal scales in abaxial surface was also higher in epiphytic individuals and contributes to water and nutrients absorption in such phase (Dickison 2000). On the other hand, there was no difference of scales in adaxial surface, which may suggest an evolutionary conservation of this trait in *B. euphemiae* (de Bello *et al.* 2015).

The lower thickness of water-storage parenchyma in epiphytic phase differs from what has been found in literature (Benzing 2000) and does not show itself as a character related to the conservation of water. The data founded corroborates with Scarano *et al.* (2002) results, which in turn showed that leaves of *Aechmea bromellifolia* under high stress (individuals under high intensity of light and not submitted to flood events) have lower thickness of water-storage parenchyma in relation to individuals in more favourable habitats.

In epiphytes and terrestrial roots, epivelamen exhibit root hairs, which can be seen as an strategy for water and nutrients absorption (Segecin & Scatena 2004; Silva & Scatena 2011). Despite terrestrial bromeliads, roots have been described to have the exclusive function of fixation (Tomlinson 1969), roots of *B. euphemiae* in both phases are capable of practicing not only fixation, but also support and possibly absorption functions. However, even with the presence of root hairs, the absorptions efficiency, probably, is lower in relation to roots fixed into the ground. It happens with the epiphytes due to fast water drainage (Tomlinson 1969).

Individuals in epiphytic phase showed a thicker velamen. Such trait indicates that water loss is avoided via cortex and nutrients retention (Benzing 2000). Although, velamen confers mechanical protection and capacity of move water and nutrients in a fast and effective way for root system during the rain (Zotz & Winkler 2013). Water and nutrients absorption through the roots can be explained for lignin absence in velamen cellular walls, as shown by negative result with phloroglucinol, indicating be a characteristic that improve the water input in plants (Parrilla-Diaz & Ackerman 1990).

In both roots was possible to distinguish outer and inner cortex, as observed in *Billbergia zebrina* (Martins *et al.* 2016). Outer cortex is normally pluriestratified and lignified in Bromeliaceae group and avoids water loss (Tomlinson 1969). Although, according literature, the outer cortex is thicker and lignified in epiphytes plants (Dycus & Knudson 1957; Sanford & Adanlawo 1973), we observe that it is more developed in terrestrial phase than in epiphytic. One explication is that terrestrial phase individuals in restinga can be likely to soaked soil, during the rainy station (Scarano *et al.* 2002); and thicker outer cortex can work as an exaptation for prevention of suffocation, and input of microorganisms more prone in terrestrial phase (Hadley & Williamson 1972; Benzing *et al.* 1983; Parrilla-Diaz & Ackerman 1990). The number of cell layers in inner cortex was higher in epiphytic phase in relation to terrestrial phase, while in inner cortex of terrestrial phase were observed intercellular spaces. These spaces can transport water for capillarity

turning the process more efficient (Tomlinson 1969) or transport air (O_2 and CO_2), as speculated by Benzing *et al*. (1983), which can be important to respiration in water soaked soils in terrestrial phase. We observed protoxylem poles in higher number in epiphytic phase individuals, despite metaxylem elements are similar in diameter. This fact added to velamen-exoderm set, corroborates with Tomlinson (1969) who affirms that these elements groups are typical from individuals in epiphytic phase. Such traits can be an answer to the water and nutrients transportation need and substances to other plant organs (Tomlinson 1969), once plants in epiphytic phase face a problem of fast water flow from phorophyte. The similarity of metaxylem diameters measures can be related to evolutionary conservatism of this trait in *B. euphemiae*.

In conclusion, we hypothesis was partially accepted, once a substantial part of traits improved the water conservation in epiphytic phase. Furthemore, we shown that *B. euphemiae* worked as a good hemiepiphyte plant model showing that plants belonging to this group can show plasticity in most of functional traits in response to water availability, a strategy that can contribute to survivor of these plants in environment under changes (*e.g.*, climate changes and/or variations caused by human interference that lead to lower water availability). However, we indicate that part of traits in this study can reflect biotic interactions, defence to microorganisms, as well as, can be conserved within the phylogeny.

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