



Original Papers

Tissue investments related to water absorption and retention in Bromeliaceae: exploring variations in CAM metabolism expressing between dry and rainy seasons

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Abstract

Structure and function are strongly related in bromeliad leaves. Some species do not develop tank rosettes and must rely on other structural and physiological attributes to deal with environmental stressors. Three species of Bromeliaceae without tanks were compared according to their leaf structure (anatomy and pectin composition), water retention abilities, and photosynthetic pathway expressions. *Tillandsia stricta* is an epiphyte, shaded by the canopy in a riparian forest, *T. usneoides* occurs on palm trees in an adjacent opened area, while *Dyckia minarum* is terrestrial and exposed to high light intensities in rupestrian fields. Despite limiting light and/or water conditions in each habitat, these species occur in adjacent areas. While they express CAM metabolism, *D. minarum* demonstrated more expressive acidity. Its greater investments in tissues related to water absorption and retention may reflect its high exposure to sunlight, poor nutrient availability for growth, and CAM expression. *Tillandsia usneoides* modifies its water-storing capacity between seasons, a property possibly associated with the presence of high methyl-esterified HGs in its cell walls. The higher succulence and relative water content during the rainy season may stimulate photosynthetic activity and maximize CAM expression.

Key words: bromeliads, CAM metabolism, cell wall, immunocytochemistry, leaf succulence.

Resumo

Estrutura e função estão fortemente associadas nas folhas de bromélias. Algumas espécies não desenvolvem rosetas em tanque e devem apresentar outros atributos estruturais e fisiológicos para lidar com fatores estressores do ambiente. Três espécies de Bromeliaceae sem tanque foram comparadas de acordo com sua estrutura foliar (anatomia e composição pética), capacidade de retenção de água e expressão da via fotossintética. Delas, *Tillandsia stricta* é epífita sombreada pela copa de uma mata ciliar, *T. usneoides* ocorre em palmeiras em uma área adjacente aberta e *Dyckia minarum* é terrestre e exposta a altas intensidades luminosas em campo rupestre. Apesar das condições limitantes de luz e/ou água em cada habitat, estas espécies ocorrem em áreas adjacentes. As três espécies expressam metabolismo CAM, mas *D. minarum* apresentou acidez mais expressiva. Seu investimento em tecidos relacionados à absorção e retenção de água pode ser relacionado à alta luminosidade, à baixa disponibilidade de nutrientes em que crescem e à expressão do CAM. *Tillandsia usneoides* alterou sua capacidade de armazenamento de água entre as estações, o que pode estar associada à presença de HGs com alta metil-esterificação nas paredes celulares. A maior suculência e teor relativo de água na estação chuvosa podem estimular a fotossíntese e a expressão do CAM.

Palavras-chave: bromélias, metabolismo CAM, parede celular, imunocitoquímica, suculência foliar.

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Introduction

The Bromeliaceae constitutes a group of plants whose presence contributes to biological diversity, as many species are used as a food source by many animals, and their tanks provide shelter for highly specialized organisms (Rocha *et al.* 2004). Bromeliad leaves exhibit structural and physiological differences according to their positions within the rosette and the apex-base axis that maximize water absorption and photosynthetic activity (Hermes *et al.* 2018). Bromeliaceae leaves generally have an epidermis with solitary silica bodies and peltate scales that protect the stomata and enhance water and nutrient absorption (Tomlinson 1969; Braga 1977). Water-storage parenchyma, longitudinal air channels, and collateral vascular bundles surrounded by a double sheath are present (Scatena & Segecin 2005). The presence of leaf traits such as water-storage parenchyma can lead to expressive succulence and may be associated with Crassulacean Acid Metabolism (CAM) (Moreira *et al.* 2009, 2013). CAM metabolism seems to be predominant within the *Tillandsia* and *Dyckia* genera of Bromeliaceae (Martin 1994; Reinert *et al.* 2003). This type of metabolism involves the nocturnal fixation of atmospheric CO₂ by phosphoenolpyruvate carboxylase (PEP-carboxylase), with organic acids being produced and stored in vacuole (Cushman 2005; Freschi *et al.* 2010). The stomata open during the night, thus reducing both transpiration and water to the environment. Each morning, malate (the main organic acid produced) is decarboxylated, and CO₂ is made available to be assimilated by the Calvin Cycle and used for carbohydrate synthesis (Cushman 2005; Freschi *et al.* 2010).

Thus, the main leaf trait associated with succulence and CAM metabolism in bromeliads is the presence of a water-storage parenchyma whose capacity for cell wall elongation and flexibility can determine the cell water storage (Hermes *et al.* 2018). In fact, the patterns of cellulose microfibril deposition and pectins with different degrees of methyl esterification apparently play important roles in cell elongation and shape acquisition (Albersheim *et al.* 2011; Bou Daher *et al.* 2018; Tucker *et al.* 2018). Pectins are complex polysaccharides with multiple functions in plants. They are represented by homogalacturonan (HG), rhamnogalacturonan-I, and rhamnogalacturonan-II (Willats *et al.* 2001a; Albersheim *et al.* 2011; Wolf & Greiner 2012; Bou Daher *et al.* 2018). Some studies have shown that the degrees of HG methyl-

esterification domains influence cell compressive strength, elasticity, water-holding capacity, and porosity (Willats *et al.* 2001b; Cosgrove 2018). HGs are synthesized in the Golgi apparatus in a high methyl-esterified form, increasing the water-holding capacities of the cells and reducing cell wall porosity, while the un-esterified HG residues can form Ca²⁺ linkages and promote the formation of an 'egg box' structure and cell wall stiffening, thus reducing the ability of the cell wall to elongate (Knox 1992; Hongo *et al.* 2012). Other polysaccharides in the cell walls include rhamnogalacturonan -I (RGI), which are composed of (1→2)-α-L rhamnosyl-(1→2)-α-D-galacturonyl subunits with galactan chains (Wydra & Beri 2006; Atmodjo *et al.* 2013) and, in association with HGs and cellulose, appears to be important to defining cell wall functionality (Chebli & Geitmann 2017).

We analyzed three different species of bromeliads: *Tillandsia stricta* Sol. ex Ker Gawl (an epiphyte shaded by the canopy of a riparian forest), *T. usneoides* (L.) L. (which occurs on trunks of palm trees in an adjacent opened area), and *Dyckia minarum* Mez (a terrestrial species that grows directly on rocky outcrops in rupestrian fields and is exposed to high light intensities). Despite the limiting light and/or water conditions in each habitat, these three species occur in adjacent sites. According to the literature, they can exhibit CAM metabolism (Griffiths & Smith 1983; Martin 1994; Reinert *et al.* 2003), but we hypothesize that *D. minarum* should evidence more expressive acidity and greater investments in tissues related to water absorption and retention that would provide higher rates of light exposure in their growth area. Additionally, this expressive acidity should be related to the detection of pectins with high methyl-esterified form in the cells of the water-storage parenchyma, increasing their capacity for elongation as well as water and organic acid storage.

Material and Methods

The Serra da Canastra region has two well-defined seasons; a dry season that extends from April to September, and a rainy season usually initiates in October, with periods of higher rainfall between November and March (INMET 2010). *Dyckia minarum* is rupicolous, and the individuals (n = 10) examined in this study were distributed on a rocky outcrop adjacent to a riparian forest (20°25.183'S, 46°40.281'W, in Delfinópolis, Minas Gerais state in southeastern Brazil), where individuals of both *T. stricta* (n = 10), and *T.*

usneoides (n = 10) were also studied (Fig. 1). These species are epiphytic, with individuals found distributed on different phorophytes along the watercourse. Two leaves were collected from each plant during the dry (August) and rainy (January) seasons according to their availability on each individual and their positioning within the rosette (3rd leaf from fully expanded leaves). For the following analysis, only the mid-sections of the leaves were used.

Diurnal variation of organic acids and carbon isotope discrimination

Photosynthetic metabolism was determined based on the diurnal variation of organic acids as well as carbon isotope discrimination. For the assessment of season effects on CAM expression,

acidity was determined using 200 mg of fresh leaf mass from each plant (n = 10) collected at 08:00 and at 18:00 hr. The collected material was kept at low temperatures until analyzed in the laboratory (approximately 1 hour after collection). The samples were boiled in distilled water for 5 min and then cooled to room temperature. The extracts were titrated with 0.01N NaOH, pH 7.0, according to the procedure described by Hartsock and Nobel (1976).

Carbon isotope ratios ($^{13}\text{C}/^{12}\text{C} - \delta^{13}\text{C}$) were evaluated according to the techniques described by Ehleringer & Osmond (1989). Leaf samples were collected and dried at 50 °C for maceration and subsequent cryogenic milling. Approximately 50 µg of dry leaf mass was held in tin capsules and subjected to mass spectrometry (Delta-S, Finnigan MAT, Bremen, Germany). The type of

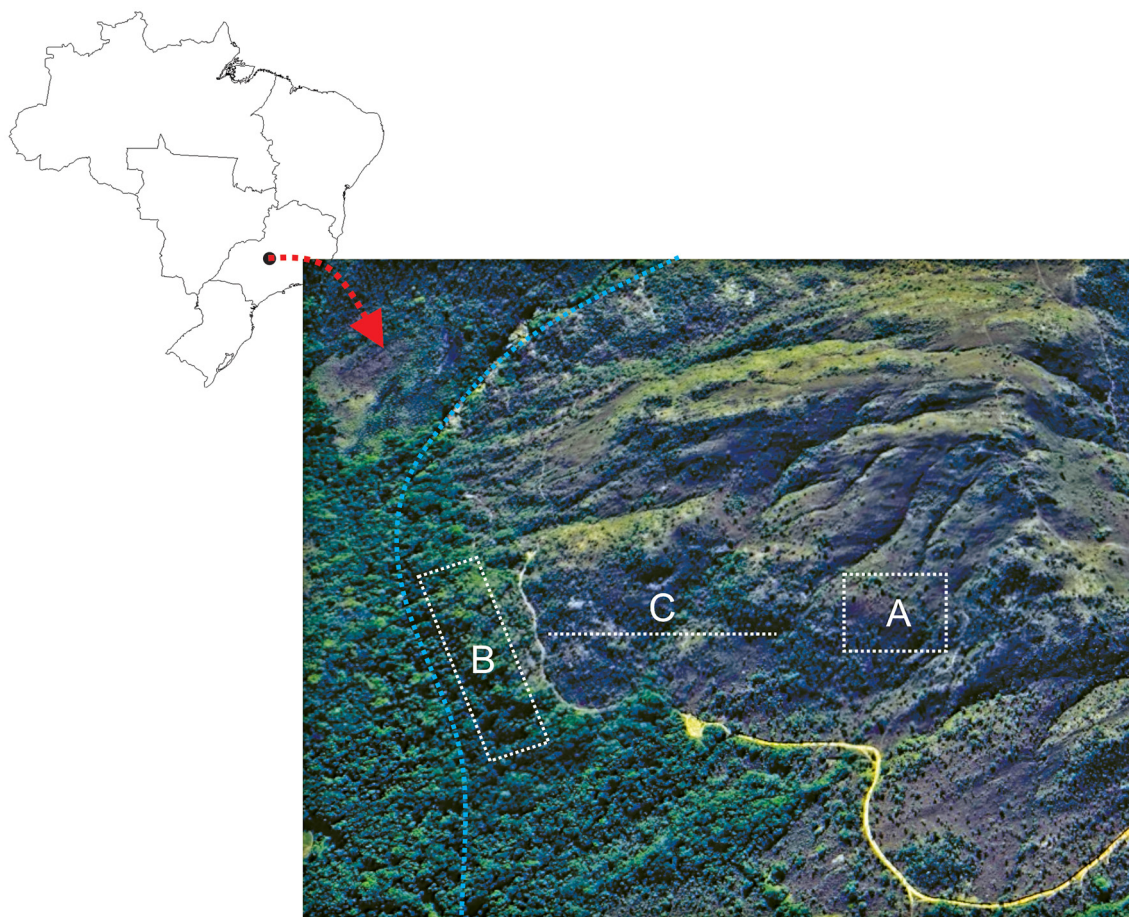


Figure 1 – The study was conducted in Serra da Canastra, in the municipality of Delfinópolis, Minas Gerais state, Brazil. *Dyckia minarum* is rupicolous and was found on a rocky outcrop (A). *Tillandsia stricta* (epiphyte) was encountered in a riparian forest (B) and *T. usneoides* (epiphyte) (C) was encountered on palm trees scattered between the *D. minarum* and *T. stricta* populations. The dashed blue line corresponds to the watercourse.

photosynthetic metabolism (C3 or CAM) was determined by comparing the $\delta^{13}\text{C}$ leaf values with those described by Ehleringer & Osmond (1989); with $\delta^{13}\text{C}$ values between -20‰ and -35‰ indicating C3 metabolism and $\delta^{13}\text{C}$ values between -10‰ and -22‰ indicating CAM metabolism.

Succulence, relative water content, and specific leaf mass

Analyses of succulence (SU), relative water content (RWC), and specific leaf mass (SLM) were performed between 08:00 and 09:00 h using 1 cm² leaf fragments from ten individuals of each species. SU was determined according to Ogburn & Edwards (2012), *i.e.*, $\text{SU} = (\text{TM} - \text{DM})/\text{DM}$, with TM being the turgid mass (after 24 h immersion in distilled water) and DM the dry mass (dried for 48 h at 80 °C). RWC was determined using the formula proposed by Turner (1981), where $\text{RWC} = [(\text{TM} - \text{FM})/(\text{TM} - \text{DM})] \times 100$; with FM representing the fresh mass. The specific leaf mass ($\text{SLM} = \text{DM}/\text{A}$) was calculated by the ratio between dry mass DM and leaf area (A) (Witkowski & Lamont 1991).

Structural and immunocytochemical analyses

Leaf fragments were fixed in FAA (37% formaldehyde, acetic acid, and 50% ethanol) for at least 48 hours and then stored in 70% ethanol (Johansen 1940). To prepare semi-permanent slides, free-hand transverse sections were clarified in 50% sodium hypochlorite, rinsed in water, and then stained with safranin and astra blue (Kraus & Arduin 1997). To prepare permanent slides, longitudinal and transverse sections were obtained with a microtome (7–10 µm) after fragment dehydration and embedding in Leica® historesin according to the manufacturer's instructions. The material was stained with 0.01% toluidine blue (O'Brien *et al.* 1964) and transverse sections were photographed using a light microscope (Olympus BX51, USA). Mesophyll and parenchyma thickness (water-storage, chlorophyllous, and spongy parenchymas) were measured using ImageJ 1.45s (National Institutes of Health, USA), considering 30 fields from ten leaves of each species. For epidermis analysis, 1 cm² fragments were immersed in 50% sodium hypochlorite at 50 °C, until epidermis detachment, and then washed and stained with 1% safranin in 50% ethanol (Berlyn & Miksche 1976). The paradermic slides were mounted in glycerin jelly (Kraus & Arduin 1997). The thickness of the

mesophyll, the water-storage, chlorophyllous, and spongy parenchyma were measured using ImageJ 1.45s software (National Institutes of Health, USA).

For scanning electron microscopy (SEM) analyses, the samples were fixed in Karnovsky's solution in 0.1 M phosphate buffer, pH 7.2, gradually dehydrated in an ethanol series, dried in a CO₂ critical-point-drier, sputter-coated with gold to 35 nm (O'Brien & McCully 1981), and then observed using a SEM (Leo Evo 40 XVP).

For the immunocytochemical analyses, leaf fragments were fixed in 2.5% glutaraldehyde and 2% formaldehyde in 0.1 M phosphate buffer, pH 7.2 (Roland & Vian 1991), dehydrated in an ethanol series, and processed according to the usual methodology for Historesin® embedding. Transverse sections were initially immersed in a blocking solution of Molico® milk protein/phosphate-buffered saline (PBS) for 30 minutes and then incubated with the following monoclonal primary antibodies (MAbs) diluted 1:10 in milk/PBS for 2 hours: JIM5 (Homogalacturonans (HGA) methyl-esterified), JIM7 (methyl-esterified HGA), JIM13 (arabinogalactan proteins), LM5 [(1 → 4) β-D-galactan], and LM6 [(1 → 5) α-L-arabinan] (Centre for Plant Science, University of Leeds, UK). The sections were washed in PBS and then incubated with FITC secondary antibody (1:100 in 3% milk/PBS) for 2 hours in the dark. Leaf samples without primary antibodies were used as the control for immunocytochemistry analyses. The sections were washed again in PBS, mounted in 50% glycerin, and evaluated under a fluorescence microscope (Olympus BX51, USA). Another set of samples was stained with Calcofluor White Stain® for cellulose detection.

Statistical analysis

The results were tested for normality. Data for the dry and rainy seasons were compared using the paired t-test (parametric data) or Wilcoxon test (non-parametric data). The three species were compared among themselves using ANOVA (parametric data) or the Kruskal-Wallis test (nonparametric data).

Results

Photosynthetic metabolism in Tillandsia stricta, T. usneoides, and D. minarum

The carbon isotope discrimination ($\delta^{13}\text{C}$) values were similar among all three species (Tab. 1), ranging from -14 to -17‰. Nocturnal acidification (ΔH^+) did not vary seasonally in *D. minarum* and

Table 1 – Diurnal titratable acidity fluctuation (ΔH^+ , $n = 10 \pm EP$) and carbon isotope discrimination ($\delta^{13}C$, $n = 4 \pm SE$) in leaves of three species of Bromeliaceae from Serra da Canastra, MG, Brazil.

	$\Delta H^+ (\mu eqH^+ g^{-1}FM)$			$\delta^{13}C (\text{‰})$
	Dry season	Rainy season		
<i>Dyckia minarum</i>	38.42 ± 7.15Aa	47.18 ± 7.68Aa	$P > 0.05$	-15.97 ± 0.1a
<i>Tillandsia stricta</i>	15.32 ± 3.26Ab	15.81 ± 5.52Ab	$P > 0.05$	-16.85 ± 0.04a
<i>Tillandsia usneoides</i>	9.02 ± 2.07Bb	17.87 ± 3.32Ab	$P < 0.05$	-14.96 ± 0.1a
	$p < 0.01$	$p < 0.01$		$p > 0.05$

* Data were compared between seasons (capital letters) or among distinct species (lower case). The means were contrasted by the Tukey test ($p \leq 0.05$).

T. stricta, while *T. usneoides* showed the greatest variation during the rainy season (17.9 $\pm$ 3.3 $\mu eqH^+ g^{-1}FM$ compared to 9.0 $\pm$ 2.1 $\mu eqH^+ g^{-1}FM$ during the dry season) (Tab. 1). *Dyckia minarum* had higher acidification than *T. stricta* and *T. usneoides*.

Succulence, specific leaf mass and relative water content

The leaves of the three Bromeliaceae species were analyzed during rainy and dry periods to compare their investments in dry mass with their ability for water uptake and retention (Fig. 2). *Dyckia minarum* had lower succulence than *T. stricta* and *T. usneoides*; and *T. usneoides* showed higher succulence values than *T. stricta* during the rainy season (~43% higher). Only *T. usneoides* showed changes in SU values between the dry and rainy months, with leaves being ~40% more succulent during the rainy season. All three species showed similar specific leaf mass values throughout the two seasons. However, *D. minarum* showed that values were 57% higher (~27.7 $\pm$ 0.7 mgDM mm⁻²) than those of *T. stricta* (~12.0 $\pm$ 0.3 mgDM mm⁻²) and 95% higher than those of *T. usneoides* (~1.05 $\pm$ 0.2 mgDM mm⁻²). RWC was

higher in *D. minarum* leaves than in the other two species when comparing only the values obtained during the dry season. Nevertheless, only *T. stricta* leaves showed variations in RWC variation between the two seasons, with 54% of the total leaf mass consisting of water during the dry season, as opposed to 73% during the rainy season.

Structural and immunocytochemical analysis

Dyckia minarum had scaly leaves and stomata distributed in furrows (Fig. 3a). Simple epidermal cells with sinuous (Fig. 3b) and thickening walls were observed, especially in the outer periclinal walls, where silica bodies were present (Fig. 3c). The subepidermal layers were composed of fibers, while the other layers of the mesophyll were divided adaxially into water-storage parenchyma and, abaxially into chlorophyllous parenchyma (with spongy parenchyma bands towards the stomata) (Fig. 3c-d). The water-storage parenchyma represented about 40% of the total mesophyll thickness (Tab. 2). Vascular bundles were embedded in the mid-portion of the mesophyll (Fig. 3d). The epidermal cells and the

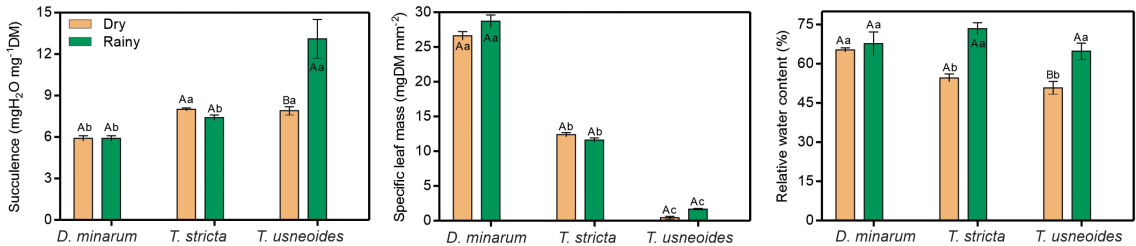


Figure 2 – Succulence (SU), specific leaf mass (SLM), and relative water content (RWC) in the leaves ($n = 30$, $\pm$ stand error) of three Bromeliaceae from Serra da Canastra, MG, Brazil. Data were collected during the dry (August) and rainy seasons (January). Capital letters compare different seasons; lower-case letters compare the different species ($p < 0.05$).

subepidermal fibers evidenced intense staining with Calcofluor White (Fig. 3e), demonstrating the presence of cellulose in their cell walls. Also, the outer periclinal walls of the epidermal cells showed intense labeling for epitopes of low methyl-esterified HGs, recognized by JIM5 antibodies

(Fig. 3f). Epitopes of high methyl-esterified HGs, recognized by JIM7, were detected mainly in the cell junctions of the water-storage parenchyma cells (Fig. 3g). Epitopes of arabinan recognized by LM6 were found in the inner anticlinal walls of the endodermis (Fig. 3h).

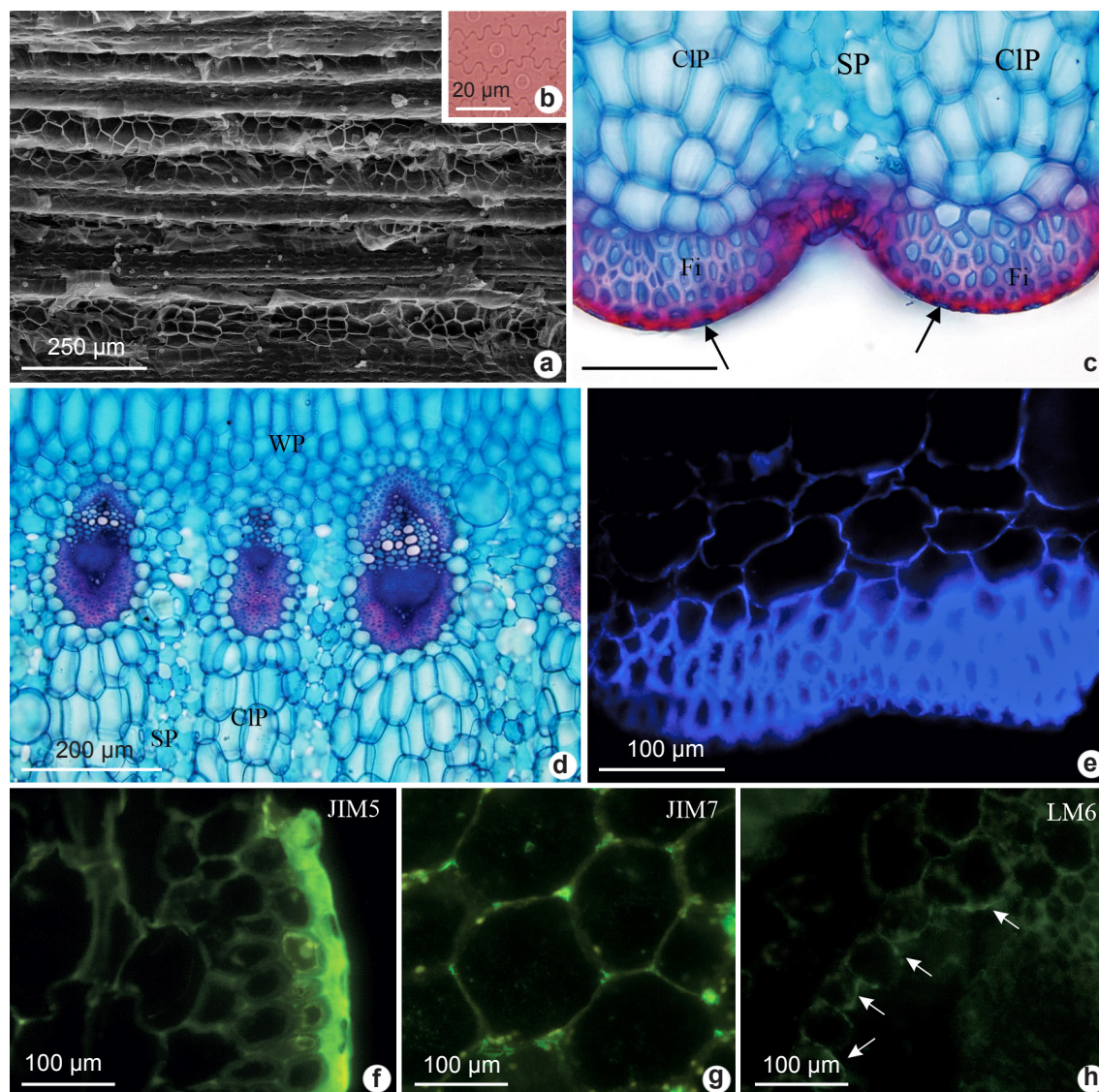


Figure 3 – a-h. *Dyckia minarum* – a. epidermis of the abaxial leaf surface as viewed using scanning electron microscopy (SEM). The small dots represent silica body encrustations; b. epidermis with sinuous cell walls; c. stomata inside furrows, silica bodies (arrows), and fibers in subepidermal layers; d. mesophyll with collateral vascular bundles; e. cellulose in the subepidermal layers labeled with calcofluor under fluorescence microscopy; f. epitopes of homogalacturonans with low methyl-esterification of the epidermis and fibers; g. epitopes of homogalacturonans with high methyl-esterification of the cell junctions of the water-storage parenchyma; h. epitopes of (1 → 5) α -L-arabinan in the inner periclinal cell walls of the endodermis (arrows). (Fi) fibers, (CIP) chlorophyllous parenchyma, (SP) spongy parenchyma, (WP) water-storage parenchyma. Scales are indicated in the photographs.

Table 2 – Thickness of mesophyll, water-storage parenchyma, chlorophyllian and spongy parenchyma (n = 30 ± SE) in leaves of three Bromeliaceae species from Serra da Canastra, MG, Brazil.

	Mesophyll thickness (µm)	Water-storage parenchyma (µm)	Chlorophyllian parenchyma (µm)	Spongy parenchyma (µm)
<i>Dyckia minarum</i>	1692.0 ± 20.5a	699.4 ± 18.1a	435.5 ± 4.4a	620.7 ± 8.7a
<i>Tillandsia stricta</i>	718.8 ± 10.7b	307.8 ± 7.1b	250.8 ± 5.7b	311.9 ± 5.5b
<i>Tillandsia usneoides</i>	949.3 ± 38.1b	-	-	-

* Data were among distinct species. The means were contrasted by the Tukey test (p ≤ 0.05).

The leaves of *T. stricta* had epidermal cells with rectangular shapes and sinuous anticlinal walls (Fig. 4a) that were thickened and lignified, with silica crystals inserted in the outer periclinal walls (Fig. 4b). High-density of peltate scales (Fig. 4c-d) was inserted in the epidermis through basal cells, and covered the stomata. The substomatal chambers communicated with the mesophyll air channels, with a mean thickness of 311.88 ± 30.04 µm (Tab. 2). The water-storage parenchyma was formed by anticlinally elongated cells with straight to slightly sinuous walls, representing about 40% of the total thickness of the mesophyll (Tab. 2). Collateral vascular bundles were present, ranging in size from larger to small, and they were fully or partially surrounded by fibers (Fig. 4e). Intense cellulose staining was observed in vascular bundle cells and epidermal walls (Fig. 4f-g). The phloem and the mesophyll cell junctions were labeled by JIM7, which recognizes the epitopes of high methyl-esterified pectins (Fig. 4h); epidermal cells, however, were not highlighted (Fig. 4i). In contrast, epitopes of β-D-galactans, recognized by LM5, were strongly detected in the water-storage parenchyma (Fig. 4j) and weakly detected in phloem and xylem (Fig. 4k).

Tillandsia usneoides had concave-convex leaves with terete morphology, a simple epidermis, homogeneous chlorophyllous parenchyma, and a vascular system composed of six collateral bundles (Fig. 5a). The abaxial and adaxial leaf surfaces had a high density of peltate scales (Fig. 5b-c) covering all of the stomata. The epidermal cell walls were not thickened and had no silica encrustations (Fig. 5d). The vascular bundles (Fig. 5e) were distributed along the axis of the adaxial surface. Calcofluor staining was observed in the epidermal cells and vascular bundle fibers. Epitopes of high methyl-esterified HGs, recognized by JIM7, were detected in the cell junctions of the

water-storage parenchyma (Fig. 5f), and epitopes of β-D-galactans, recognized by LM5, were mainly detected in the phloem and parenchyma cells (Fig. 5g).

Discussion

δ¹³C values are used to represent the carboxylation history of the plant, helping to clarify the expression of their photosynthetic pathways (Griffiths & Smith 1983). The δ¹³C values obtained here for *T. stricta*, *T. usneoides*, and *D. minarum* agree with the results of other studies on the same species (Griffiths & Smith 1983) and indicated CAM. The isotope results were corroborated by the measurements of day-night changes in titratable acidity. Most species of the genus *Tillandsia* show CAM (Medina & Troughton 1974; Griffiths & Smith 1983; Haslam *et al.* 2003; Crayn *et al.* 2004), and this metabolic pathway was previously detected in the genus *Dyckia* by other authors (Medina & Troughton 1974; Reinert *et al.* 2003; Crayn *et al.* 2004). However, constitutive CAM species do not seem to be common in the subfamily Pitcairnioideae (Griffiths & Smith 1983), despite the presence of many xeromorphic species.

Variations in diurnal titratable acidity represent instants in time for whole-plant physiology, and higher CAM expression was observed for *D. minarum*. However, only *T. usneoides* showed seasonal acidity variations, with higher values during the rainy season. CAM expression commonly varies with water availability, and increases in CAM expression due to drought have been observed in some studies (Martin 1994). Here, however, as well as in Martin & Schmitt (1989) and Haslam *et al.* (2003), nocturnal CO₂ uptake rates did not increase during drought conditions. Besides that, *T. usneoides* also showed low RWC values during the dry season and its leaves evidenced their its water retention capacity during the rainy season

(highest succulence values). Increased water content within plant tissues during the rainy season may be related to heightened photosynthetic activity and enhanced CAM expression, as evidenced by the elevated levels of tissue acidity observed during the night. Plants using CAM pathway usually have a mesophyll consisting of large vacuolated cells with high water storage capacities (Griffiths *et al.* 1986; Cushman 2001; Moreira *et al.* 2009, 2013). This feature enables the storage of organic acids produced from atmospheric CO₂ fixation overnight,

that will serve as a carbon reservoir for the Calvin cycle in the morning (Cushman 2005; Freschi *et al.* 2010).

Succulence may be defined as the plant's ability to store water in its tissues or organs, and represent a classic example of synergism between form and function for adaptation to stressful environments. High water contents lend a degree of physiological independence to the plants against a low water supply (Ogburn & Edwards 2012). Only *T. usneoides* showed changes in its water storage

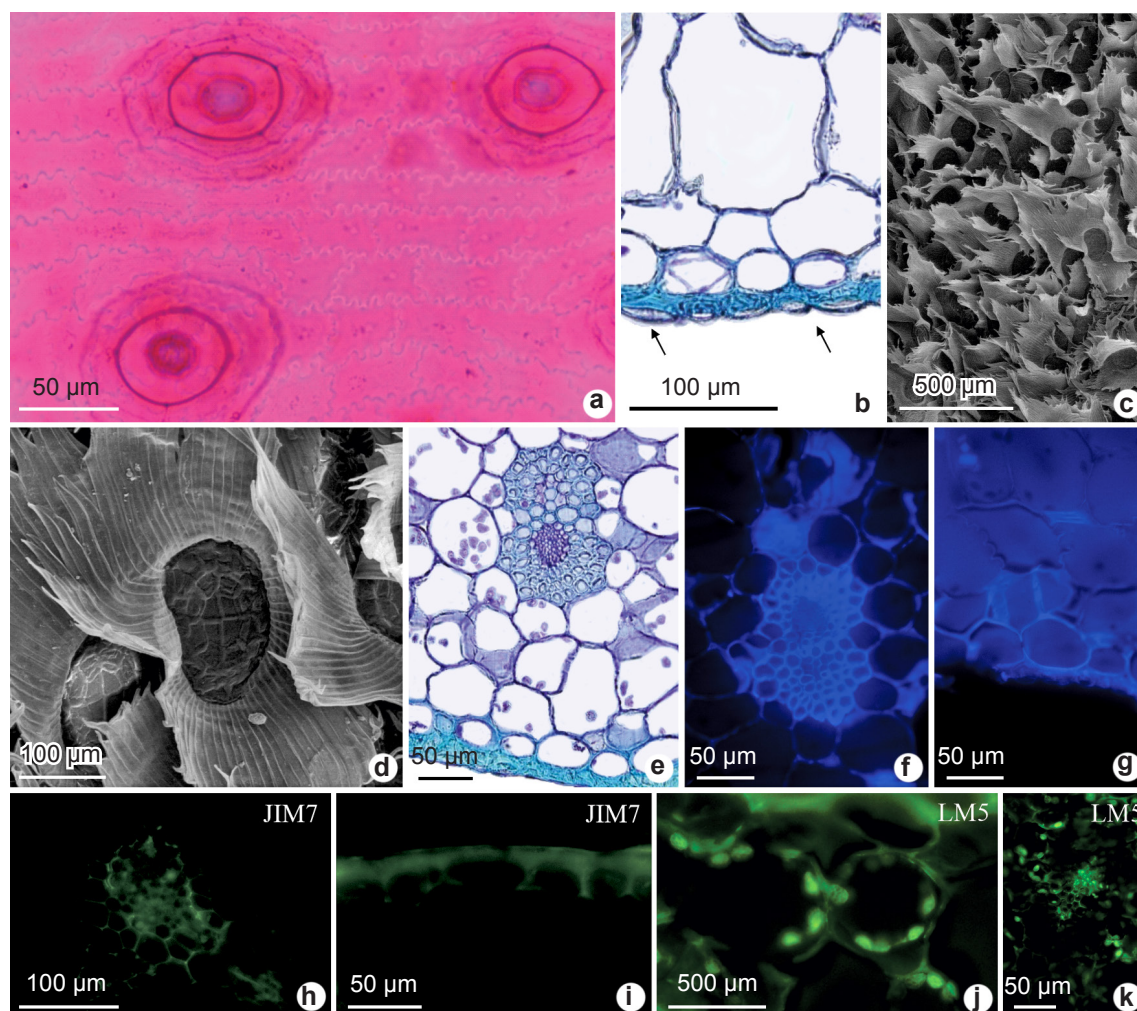


Figure 4 – a–k. *Tillandsia stricta* – a. epidermis with sinuous cell walls and scales; b. cross section showing silica body encrustations (arrows); c–d. scanning electron microscopy (SEM) images showing the high density of peltate scales (c) and a detail of it (d); e. collateral vascular bundle; f–g. cellulose in the vascular bundle labeled with calcofluor under fluorescence microscopy (f) and cellulose in water-storage parenchyma (g); h–i. epitopes of homogalacturonans with high methyl-esterification in the phloem (h) and cell junctions of the water-storage parenchyma (i); j–k. epitopes of homogalacturonans with low methyl-esterification in the water-storage parenchyma (j), xylem, and phloem (k). Scales are indicated in the photographs.

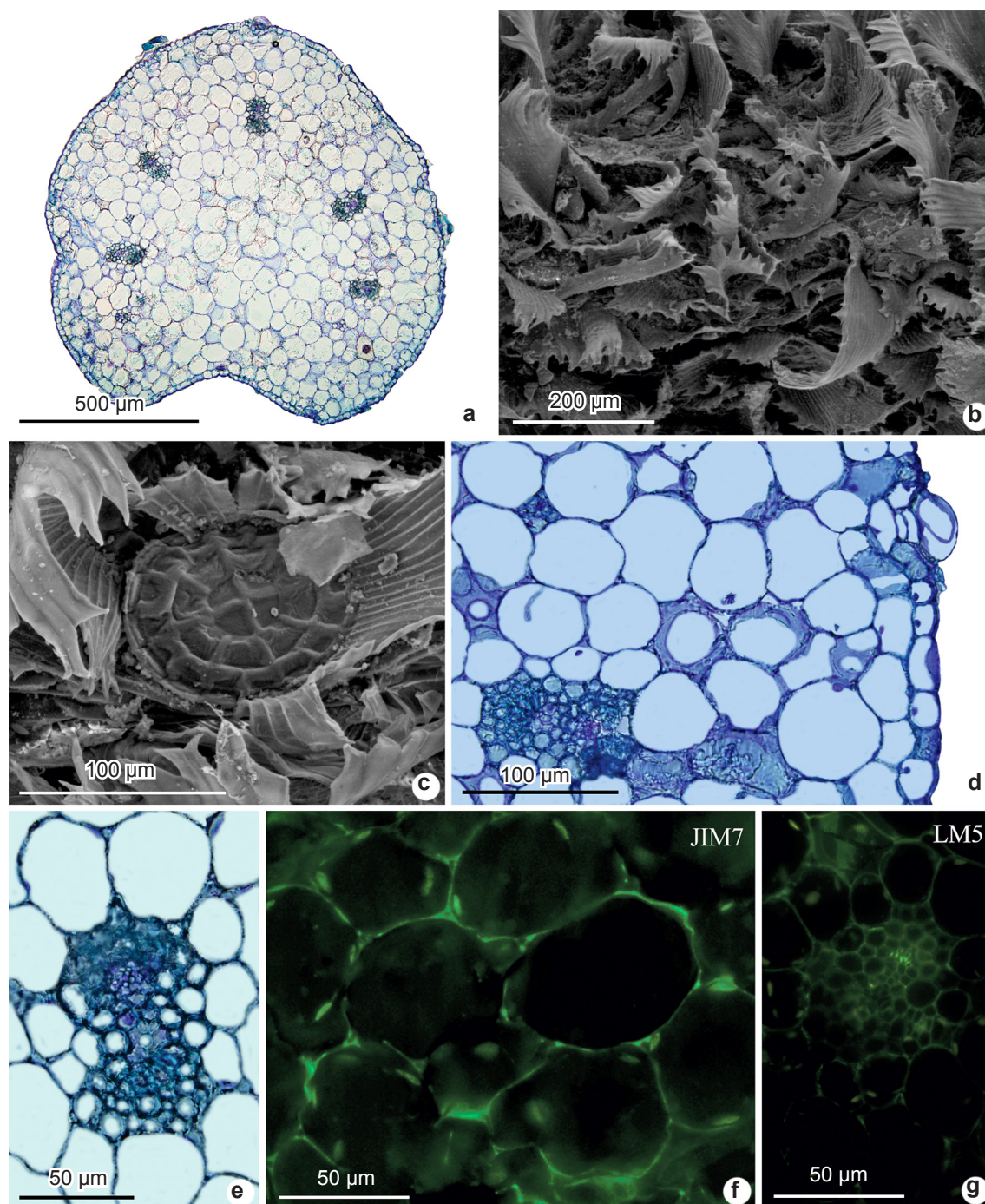


Figure 5 – a-g. *Tillandsia usneoides* – a. leaf cross section showing a concave-convex shape and a vascular system consisting of six collateral bundles. Mesophyll homogeneous; b-c. scanning electron microscopy (SEM) images showing the high density of peltate scales (c) and a detail of a scale; d. Thin-walled epidermal cells with no silica bodies. Homogeneous chlorophyllous parenchyma; e. collateral vascular bundle; f-g. fluorescence microscopy – f. epitopes of homogalacturonans with high methyl-esterification in parenchyma cells; g. (1 $\rightarrow$ 4) β -D-galactans in the phloem and parenchyma cells. Scales are indicated in the photographs.

capacity between the dry and rainy seasons, with an approximately 40% increase during the rainy season. The presence of HGs with high methyl-esterified groups in the cell walls of the water-storage parenchyma may confer high elasticity and elongation capacity to this specialized tissue. The presence of this HG epitope associated with galactans epitopes and cellulose microfibrils may confer elasticity to the cell walls (Knox *et al.* 1990; Albersheim *et al.* 2011) in agreement with the results showing the increased water storage capacity of *T. usneoides* during the rainy season. Although both *T. stricta* and *D. minarum* showed epitopes with high methyl-esterified HGs in the water-storage parenchyma, these species retained their water-storage capacities during both the rainy and dry seasons. In fact, the presence of high methyl-esterified HGs in the water-storage parenchyma of bromeliad species has been reported to be associated with tissue flexibility (Hermes *et al.* 2018).

A study using 12 species of *Tillandsia* by Loesch *et al.* (1993) showed that the percentages of leaf tissue representing water-storage parenchyma (also estimated from cross sections in the mid-region of the leaf) are highly variable, representing from 2.9 to 53% of the mesophyll. Although we did not identify a specific water-storage parenchyma in *T. usneoides* leaves (as Proença & Sajo 2007), other researchers have (Scatena & Segecin 2005). Tomlinson (1969) indicated that the development of water-storage parenchyma can vary among populations or even according to the environment. Functioning as a water storage organ, this tissue ensures leaf hydration even during the dry season (Brighigna *et al.* 1984).

Dyckia minarum showed higher SLM values than *T. stricta* and *T. usneoids*. These differences represented a greater tissue investment per unit of leaf area as a consequence of high mesophyll thickness and sclerification. This anatomic process is common in plants subject to water stress, mainly in situations of high irradiance and poor nutrient availability (Meziane & Shipley 2002), typical of rocky field conditions. The air channels connecting stomata and spongy parenchyma in leaves exhibiting high density or succulence can ensure the efficient airflow, even with high degrees of hydration. Although leaf tissue proportions varied according to their position in the rosette and between the apex and the base of the leaves (Hermes *et al.* 2018), the leaves of *D. minarum* and *T. stricta* showed higher proportions of water-storage and spongy parenchyma. *Tillandsia usneoides* showed

no tissue specialization, with low SLM values and high succulence, probably related to the terete morphology of their leaves.

The epidermis represents the main protective tissue of plants against mechanical injury, water loss, and invasion by pathogens. Wall thickenings of the epidermis among Bromeliaceae species can reduce water loss and prevent dehydration in xeric environments where these plants can commonly grow (Tomlinson 1969; Santos-Silva *et al.* 2013). The silica bodies in the epidermis confer resistance to the leaves and can act as a barrier against high light intensities by reflecting the excess of solar radiation - and avoiding overheating within the leaf (Krauss 1949; Yoshida *et al.* 1962). In some cases, silica bodies are also associated with the resistance of many bromeliads to insects and fungal infections (Krauss 1949; Yoshida *et al.* 1962).

Some Bromeliaceae species have no absorptive root system and lack tanks. In these species, the peltate scales that cover the entire leaf surface are responsible for nutrient capture and water uptake (Benzing *et al.* 1976; Griffiths & Smith 1983). Those scales also help prevent high transpiration rates (the stomata are covered by them), intercept excessive incident light, and reduce internal leaf tissue temperatures (Tomlinson 1969; Benzing 2000). Stomata are only present on the abaxial surface of *T. stricta* and *D. minarum* leaves, but *T. usneoides* had amphistomatic leaves with stomata on both sides of its leaves, but a reduced adaxial surface.

In summary, we confirm that the three species of bromeliads studied here express CAM metabolism. *Dyckia minarum* showed more expressive tissue acidity, greater leaf thickness, and higher SLM. The high investments in tissues involved in water absorption and retention of that species may be related to the high light incidence and poor nutrient availability in its natural habitat - typically rocky outcrops in rupestrian fields. *Tillandsia usneoides* showed no tissue specialization, but altered its water-storage capacity between seasons, a property that may be associated with the presence of high methyl-esterified HGs in the cell walls of its water-storage parenchyma. These results open new perspectives for the study of the water-storage parenchyma of CAM species, especially in terms of cell wall composition and dynamics. The higher succulence and relative water content during the rainy season may stimulate photosynthetic activity and maximize CAM expression, as evidenced by high tissue acidity values during the morning.

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Data availability statement

In accordance with Open Science communication practices, the authors inform that all data are available within the manuscript.

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