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Intermediate C_3-CAM metabolism in *Bulbophyllum involutum*: A species with limited leaf morphological variation in relation to light

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ABSTRACT

Bulbophyllum involutum (Ochidaceae) grow in areas with outcrops and high light heterogeneity. We considered that this intermediate C_3-CAM species should have more expressive CAM metabolism, and leaf succulence (mainly in tissues/structures related to water storage or economy) in individuals that have grown exposed to high light intensities. Here, we investigated (1) if the leaves of *B. involutum* undergo significant morphological and anatomical changes when growing under different light regimes; (2) which leaf structural features were most influenced by different light levels; and (3) what is the interplay between such variation and the photosynthetic metabolism, and its relation to the ecophysiology of *B. involutum*. For that, the plants were selected along a transect (across the outcrop) into three classes according to the light condition under which they grown: sun (receiving above 35 mol m^{-2} day^{-1} of photosynthetically active radiation [PAR]); partially shaded (between 14 and 23 mol m^{-2} day^{-1} of PAR); and shaded (less than 8 mol m^{-2} day^{-1} of PAR). The leaves of plants growing in direct sunlight were smaller, thicker and showed high leaf specific mass. The dry mass of leaf per unit area increased during the dry season, indicating that some carbohydrates play important role in the acid metabolism (PEP synthesis). They could help to reduce cell water potential in leaves, facilitating water flux and the maintenance of leaf succulence throughout the year. The leaf succulence increases the possibility of organic acid storage during the night by the C_3-CAM photosynthetic pathway. The low flexibility of C_3-CAM metabolism in *B. involutum* leaves didn't show high expression of CAM in dry season, but seems to be helpful to these plants' establishment at variable light environments.

1. Introduction

Crassulacean acid metabolism (CAM) is a highly variable process, and can show different physiotypes depending on environmental factors such as CO_2 concentrations, water and light availability, as well as temperature (Dodd et al., 2002; Lüttge, 2004). Some organisms keep their stomata closed during most of the day and night, while fixing CO_2 produced from the metabolism of organic acids. Some CAM species open their stomata during the day to assimilate direct atmospheric CO_2 *via* rubisco, and this CO_2 is fixed as organic acids *via* PEP-carboxylase at night (Lüttge, 2004); other species though exhibit C_3 photosynthesis under favorable environmental conditions, while expressing CAM metabolism under stress circumstances (such as those produced through water deficits) (Lüttge, 2004). Switching from C_3 to CAM expression occurs not only in response to changes in environmental conditions, but can also occur during plant development (Herppich et al., 1998). The flexibility of the C_3-CAM plants, allows grow under larger ecological amplitudes, with better performance under variable stress in time and space (Lüttge, 1999). However, the environmental factors that affect CAM expression, may also affect leaf structure.

CAM leaves are usually described as succulent and are characterized by the presence of large vacuolated cells in the mesophyll, small intercellular spaces, a thick cuticle, and low stomatal density (Cushman, 2001; Nelson et al., 2005; Moreira et al., 2013a). Variation in plant morphological traits has been widely discussed in the literature, especially in terms of tissue responses to different light gradients (*e.g.*,Gratani et al., 2006; Valladares and Niinemets, 2008). The leaves that were fully exposed to the sun usually have smaller total area, higher specific leaf mass, are thicker (due to the greater number of palisade and spongy parenchyma layers), have thicker cuticles and epidermises, and high stomatal density (Gratani et al., 2006; Valladares and Niinemets, 2008). Nevertheless, the effects of light on succulent leaves are not extensively studied.

The relationships between leaf structures and photosynthetic pathways were discussed by Winter et al. (1983) for many plant species, including *Bulbophyllum* orchids, with C_3 and CAM species. The five *taxa* reported to be CAM species (*B. aurantiacum* F. Muell., *B. baileyi* F. Muell., *B. crassulifolium* [A. Cunn.] Rupp, *B. macphersonii* Rupp., and *B. minutissimum* [F. Muell.] F. Muell.) have epiphytic or

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rupiculous habits and succulent leaves. These characteristics were also observed in *B. involutum* Borba, Semir & F. Barros, suggesting that it is a CAM species. This species preferentially grows in high light intensities, although it can be found in a number of different light environments in the *Campos Rupestres*. The *Campos Rupestres* constitute a typical phytophysiognomy from Brazilian Subtropical savanna that lies at altitudes above 1000 m (Giulietti and Pirani, 1988). They are characterized by open, herbaceous vegetation on sandy, stony soils mixed with herbs and shrubs growing on outcropping islands of quartzite, sandstone, gneiss or iron ore rocks. A rich mosaic of floristically related if formed, but physiognomically distinct communities reflect local variations in topography, substrate, soil depth, and microclimate (Giulietti and Pirani, 1988). In the *Campos Rupestres* of the Serra do Caraça, southeastern Brazil, at least 50% of the individuals of *B. involutum* are directly exposed to the sun and receive more than 35 mol m^{-2} day^{-1} of photosynthetically active radiation (PAR), 35% receive between 14 and 23 mol m^{-2} day^{-1} of PAR, while only 15% of individuals are found growing under shaded conditions, receiving less than 8 mol m^{-2} day^{-1} of PAR (Moreira et al., 2013b). Considering that *B. involutum* occupies niches with different levels of light availability, we hypothesized that individuals that grow at higher intensities of light are going to have more expressive CAM metabolism, and leaf succulence, mainly in tissues related to water (and organic acids) storage. In the present study we addressed the following questions: does the occupation of sites characterized by different levels of PAR imply significant leaf morphological and anatomical differences? If this variation exists, which leaf structural features are most influenced by the different light levels? What is the interplay between such variation and the photosynthetic metabolism, and its relation to the ecophysiology of *B. involutum*?

To address these questions, we evaluated individuals of *B. involutum* growing in *Campos Rupestres* under different light conditions in terms of the morphological and anatomical structures of their leaves, and the results were compared to their photosynthetic pathways.

2. Materials and methods

2.1. Plant material

Samples of *B. involutum* (Orchidaceae) leaves were collected in the Serra do Caraça, southeastern Brazil (~43°30′11″W × 20°05′ 03″ S), in outcrops with many fissures, and high light heterogeneity. This light heterogeneity is attributable to the wide diversity of plant species growing there, with their distinct architectures and phenologies. Additionally, the positions of individuals on the rocky outcrops and the presence of fissures modify the shade conditions where the plants grow, because of changes in the incident angles of the sun throughout the day and the year (Moreira et al., 2013b). Temperature and relative humidity were monitored from 7:00 a.m. to 6:00 p.m. in the field for four distinct months during the course of the year (July, October, January and May). July represents the dry season and January represents the rainy season, while October and May are the transitional months between these two remarkable seasons. The minimum temperature recorded was 13 °C in July, and the maximum was 37 °C in May. The relative humidity varied from 13% to 78% in July (dry season), 40% to 80% in October, and from 15% to 68% in May. It rained throughout the day in January (rainy season).

Bulbophyllum involutum is a rupicolous and endemic species of Brazil. Thirty individuals were selected according to their degrees of exposure to sunlight: 11 within the "sun" class (exposed to direct sunlight); 11 within the "partially shaded" class (interspersed with shrubs and subshrubs, or growing between shallow cracks); and eight within the "shaded" class (under shrubs or tree formations), as described by Moreira et al. (2013b). The "sun" class comprises individuals that received more than 35 mol m^{-2} day^{-1} of photosynthetic active radiation (PAR), the "partially shaded" individuals received between 14 and 23 mol m^{-2} day^{-1}, and the "shaded" class had less than 8 mol m^{-2} day^{-1}. The amount of PAR was directly measured individual by individual using a quantum sensor (Li-cor USA). The measurements were done between 8 am and 6 pm every hour, of each season (July, October and May), except in January due to the heavy season rains.

2.2. Diurnal variations of organic acid concentrations and carbon isotope discrimination

Photosynthetic metabolism was determined by diurnal titratable acid fluctuations and carbon isotope discrimination. For evaluations of acidity (ΔH^+), 200 mg of fresh leaves (FM) were collected from each individual at 08:00 and at 18:00. The material was kept at low temperatures until analyzed. After weighing, the samples were boiled in distilled water for 5 min, cooled to room temperature, and the extracts titrated with 0.01 N NaOH (pH 7.0), as described by Hartsock and Nobel (1976).

The $^{13}C/^{12}C$ ($\delta^{13}C$) ratios were evaluated according to Ehleringer and Osmond (1989). Fragments of leaves were collected and dried at 50 °C for subsequent maceration and cryogenic grinding. Approximately 50 μg of dry material was placed in tin capsules and analyzed in a mass spectrometer (Delta-S, Finnigan MAT, Bremen, Germany). Photosynthetic metabolism classes were determined by comparing these results with the values proposed by Ehleringer and Osmond (1989). The CAM photosynthetic pathway was attributed to plants that presented δ^3C values between −10 and −22 ‰, while plants with C_3 metabolism expressed δ^3C values between −20 and −35 ‰.

2.3. Structural analysis

Leaf areas were calculated using fully expanded leaves collected in full sunlight, partially shaded, or shaded sites (n = 30). The leaves were scanned and analyzed using ImageJ 1.45s software (National Institutes of Health, USA).

For scanning electron microscopy, leaf samples were fixed in 4% Karnovsky in 0.1 M phosphate buffer (pH 7.2) (Karnovsky, 1965), then fixed in 1% osmium tetroxide for 2 h, dehydrated in an ethanol series (Johansen, 1940), and dried in a critical point apparatus (Bal-Tec). The fragments were metallized with 30 nm of gold in a sputter coater (Sputtering Bal-Tec), and analyzed in a scanning electron microscope (LEO EVO 40 PVX).

Histological slides were prepared from material fixed in FAA_{50} (37% formaldehyde, acetic acid, and ethyl alcohol, 1:1:18, *v*/v). Part of this material was dehydrated in an ethanol series and embedded in Historesin Leica® according to the manufacturer's instructions. The slides were stained with 0.05% toluidine blue (pH 6.8) (O'Brien and McCully, 1981), and examined using a light microscope (Olympus BH2-BHS). The thickness of the mesophyll, epidermis, and cuticle on the abaxial and adaxial sides of the leaves were measured (n = 30) from digital images of leaf cross sections captured with a camera coupled to the microscope.

Thirty additional epidermal fragments were immersed in 50% sodium hypochlorite, washed in water, stained with 1% safranin in 50°GL ethanol (Berlyn and Miksche, 1976), and mounted in Kaiser gelatin (Kraus and Arduin, 1997) to calculate stomatal densities (n_{st}), and stomatal pore areas (A_{st}). The numbers of stomata per area were counted, and stomata pore areas were measured using ImageJ® software. The area occupied by the stomatal pores in 1 mm^2 of leaf surface (n_{ast}) was expressed as percentages, being calculated by multiplying the average values of the stomatal pore areas (A_{st}) by the stomatal density.

Specific leaf mass (SLM) and saturated water content (SWC) were evaluated in 1 cm^2 fragments excised from three leaves for each individual (n = 30). The LSM was obtained by calculating the ratio of dry weight (DW) to the tissue area (A), as in Witkowski and Lamont (1991), where $LSM = DW/A$. The SWC was determined as the difference between the fully hydrated leaf weight (HW) and the dried leaf weight

(DW) after incubation for 48 h at 80 °C, normalized by the tissue dry mass, where $SWC = (HW - DW) / DW$ (Ogburn and Edwards, 2012).

2.4. Relative distance plasticity index — RDPI and data analysis

We evaluated the variation of the histometric parameters of the leaves of *B. involutum* growing under different light conditions using the Relative Distance Plasticity Index - RDPI, as described by Valladares et al. (2006). The relative distances (RD) between the values of the characteristics were calculated for all pairs of individuals from the same population grown under different light conditions. The RD was calculated using the formula $RD_{ij \to i'j'} = d_{ij} \to i'j' / (x_{i'j'} + x_{ij})$, where j and j' are individuals belonging to the distinct light conditions i and i'. The RDPI can range from 0 (no plasticity) to 1 (maximum plasticity), and is obtained as $RDPI = \Sigma (d_{ij} \to i'j' / (x_{i'j'} + x_{ij})) / n$, where n is the total RD. The RDPIs were compared using ANOVA, followed by Tukey test. All statistical analyses were performed using JMP software (SAS Institute, U.S., 1989–2002).

To compare the light treatment parameters, the data were evaluated for normality and homogeneity. The normal features were compared using ANOVA, considering light source variations as treatments. The Tukey test was used to compare the means. Nonparametric data were compared separately by the Kruskal-Wallis test.

3. Results

3.1. Photosynthetic pathways

Bulbophyllum involutum showed mean values of ΔH^+ between 5.3 ± 7.0 and 19.9 ± 12.7 g μeq H^+ g^{-1} FM (Table 1) for sun, partially shaded or shaded individuals. The mean values (with their respective standard deviation) are represented in Table 1, and no statistical differences were observed among individuals growing under the three different light conditions or during the year. The absolute values clearly showed wide amplitude of variation in organic acid content, including some negative values, and 0 to 37 μeq H^+ g^{-1}MF (ΔH^+) (the absolute values of the leaf acidity changes between phases II and IV of the CAM metabolism for each individual of *B. involutum* studied here are represented in Appendix 1). The values for carbon discrimination (δ‰) were also similar for individuals exposed to the three different light conditions (Table 1), both in the dry (July) and rainy (January) seasons. These values ranged from −20 to −22‰.

3.2. Morphological traits

Individuals of *Bulbophyllum involutum* exposed to high visible light radiation conditions showed significantly lower leaf areas. Leaf areas increased approximately 33% in plants belonging to the "partially shaded" class, and around 110% in plants of the "shaded" class (Table 2). The leaves of the individuals belonging to the "sun" and "partially shaded" classes showed LSM values up to 20% higher than the individuals in the "shaded" class. LSM values were higher in July for the individuals that have grown exposed to sun or under partially shaded conditions (Table 3). The lowest SWC values were found for the sun class leaves (~20% lower than shaded class leaves), followed by partially shaded class leaves (~10% lower than shaded class leaves), with no statistically significant differences during the year (Table 3).

The leaves had simple epidermises, homogeneous mesophylls, and collateral vascular bundles. A thick and rough cuticle protruded above the stomata, forming supra-stomatic chambers (Fig. 1a and b). No significant differences occurred in cuticle and epidermis thicknesses among the different light classes (Table 4). Glandular trichomes were composed of three cells – two basal cells with prominent, thickened walls, and one superior secretory cell, consistently covered by fungal hyphae (Fig. 1b and c). The mesophyll was composed of a homogeneous chlorenchyma with vacuolated cells, reduced intercellular spaces, idioblasts with raphides, and tracheoidal elements of different shapes and sizes (Fig. 1d and e). Although structurally similar, the mesophyll was significantly thicker in plants belonging to the "sun" class, with an approximately 10% reduction among individuals of the "partially shaded" class, and a 16% reduction for individuals in the "shaded class" (Table 4). Collateral vascular bundles showed two groups of pericyclic fibers (Fig. 1f), and cells with reticulated thickenings in the layers nearest the phloem.

The leaves of *B. involutum* had similar internal structures under the three different light conditions. Tetracytic stomata occurred (Fig. 1a)

Table 2
Means ± SD of leaf area (n = 30), stomata density (n = 30), stomata pore area (n = 40) and leaf area ratio occupied by stomata pores of *Bulbophyllum involutum* (Orchidaceae) individuals growing in the Serra do Caraça, southeastern Brazil, in light classes: sun, partially shaded and shaded. Values presenting different letters in a same column are significantly different at 0.05 level of Tukey's test.

Light classes of *Bulbophyllum involutum*	Leaf area (cm^2)	Stomata density (n_{st} cm^{-2})	Stomata pore area (μm^2)	A_{st}/A_{leaf} (%)
Sun	9.5 ± 4.7^{b}	31.3 ± 5.3^{a}	407.3 ± 64.2^{a}	1.3 ± 0.3^{a}
Partially shaded	13.2 ± 5.8^{ab}	30.3 ± 6.3^{a}	355.4 ± 93.2^{a}	1.1 ± 0.3^{ab}
Shaded	18.1 ± 6.9^{a}	22.1 ± 5.4^{b}	354.1 ± 60.9^{a}	0.7 ± 0.3^{b}

Table 3
Means ± SD of specific leaf mass and saturated water content of *Bulbophyllum involutum* (Orchidaceae) individuals growing in the Serra do Caraça, southeastern Brazil, in light classes: sun (n = 11), partially shaded (n = 11) and shaded (n = 8). Values presenting different capital letters in a same line or different lowercase letters in a same column are significantly different at 0.05 level of Tukey's test.

		Sun	Partially shaded	Shaded
Specific leaf mass (mg DM cm^{-2})	July/07	37.4 ± 3.0^{Aa}	28.4 ± 7.4^{Aa}	22.2 ± 3.3^{Ba}
	October/07	23.8 ± 2.6^{Ab}	24.7 ± 2.8^{Ab}	20.2 ± 2.7^{Ba}
	January/08	25.9 ± 3.0^{Aab}	24.2 ± 2.0^{Ab}	22.1 ± 3.2^{Ba}
	May/08	26.8 ± 2.8^{Aab}	23.6 ± 3.4^{Ab}	21.4 ± 2.7^{Ba}
	Annual mean	26.4 ± 3.3^{A}	24.8 ± 4.5^{A}	22.1 ± 4.4^{A}
Saturated water content (g H_2O g^{-2} MS)	July/07	5.70 ± 0.8^{Ca}	6.22 ± 1.2^{Ba}	7.02 ± 2.1^{Aa}
	October/07	6.50 ± 0.9^{Ca}	7.37 ± 0.9^{Ba}	7.73 ± 1.1^{Aa}
	January/08	6.29 ± 0.7^{Ca}	7.19 ± 5.9^{Ba}	7.93 ± 0.5^{Aa}
	May/08	5.56 ± 0.6^{Ca}	6.76 ± 0.7^{Ba}	7.01 ± 1.3^{Aa}
	Annual mean	6.01 ± 0.8^{C}	6.74 ± 0.9^{B}	7.64 ± 1.3^{A}

Table 1
Diurnal titratable acidity fluctuations (means ± SD) and relative carbon isotope composition ($\delta^{13}C$) (means ± SD) of leaves of *Bulbophyllum involutum* (Orchidaceae) individuals growing in the Serra do Caraça, southeastern Brazil, in the light classes sun, partially shaded and shaded. The samples were obtained at July and October 2007, and January and May 2008 for diurnal titratable acidity fluctuations, and at July 2007 and January 2008 for $\delta^{13}C$. No statistical differences among classes of light in a same date or different dates in same classes of light for ΔH^+ and $\delta^{13}C$ were detected according to Tukey and Kruskal-Wallis tests at 5% of probability.

Light classes of *B. involutum*		ΔH^+ (μeq g FM^{-1})					$\delta^{13}C$ (‰)	
	n	Jul/07	Oct/07	Jan/08	May/08	n	Jul/07	Jan/08
Sun	11	7.8 ± 6.6	8.9 ± 8.6	5.4 ± 7.0	19.9 ± 12.7	5	−20.8 ± 1.2	−21.5 ± 1.2
Partially shaded	11	12.3 ± 11.9	9.6 ± 7.5	13.4 ± 7.3	16.1 ± 7.0	6	−20.3 ± 1.7	−20.6 ± 1.0
Shaded	8	12.3 ± 8.8	17.6 ± 11.7	9.6 ± 8.5	10.7 ± 6.4	4	−21.2 ± 0.7	−20.5 ± 1.1

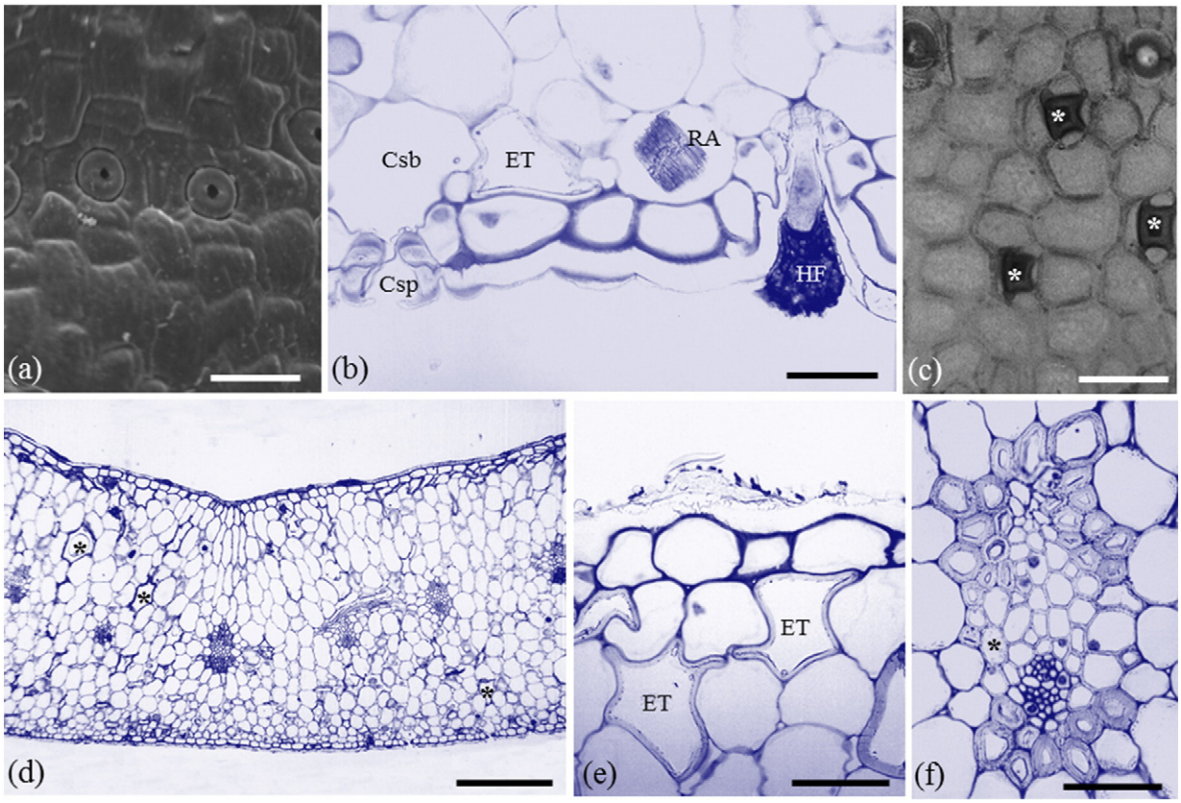

Fig. 1. Leaf anatomical features of *Bulbophyllum involutum* (Orchidaceae) growing on rocky outcrops in Serra do Caraça, southeastern Brazil. a) Electron Microscopic image showing the striated cuticle and tetracytic stomata. b) Abaxial epidermis with a thick cuticle, glandular trichomes with fungal hyphae (HF), raphides (RA), tracheoidal elements (ET), and stomata with substomatic (Csb) and suprastomatic (Csp) chambers. c) Parademic view of the abaxial epidermis with tetracytic stomata and glandular trichomes (*). d–f) Transverse sections. d) Mesophyll formed by voluminous and vacuolated cells, with sparse tracheoidal elements (*). e) Epidermis on the adaxial leaf surface, with tracheoidal elements (ET). f) Collateral vascular bundle, with pericycle fibers and cells with reticular thickened walls (*). Bars: a, b = 100 μm, c = 500 μm, d–f = 50 μm.

only on the abaxial surface, with significantly higher densities in individuals belonging to the "sun" and "partially shaded" classes (both with ~31 stomata cm^{-2}) as compared to the "shaded" class (~22 stomata cm^{-2}) (Table 2). However, the stomatal pore areas did not change, and so due to the higher stomatal densities observed on the leaves of individuals belonging to the "sun" class, higher proportions of their leaf areas were occupied by stomatal pores.

3.3. Relative distance plasticity index — RDPI

The RDPI values indicated that, except for cuticle thickness on the adaxial leaf surface and epidermal thickness, all other characters were significantly different among the different light treatments. Higher RDPI value variations occurred between individuals belonging to the "sun" and "shaded" classes (Table 5). The lowest variation was between individuals of the "sun" and "partially shaded" classes. The structural parameter showing the most variation was leaf area (with RDPI values between 0.22 and 0.36), which was substantially greater than the other structural parameters evaluated; significant variations were also seen among the characters related to gas exchange (with RDPI values between 0.07 and 0.29 for stomata density, stomata pore area, and A_{st}/A_{leaf}). The characters that varied least were those related to the leaf thickness, with RDPI values not exceeding 0.09.

4. Discussion

4.1. CAM metabolism in B. involutum

The variability of the acid content among the light classes or among the different months of the year (note the high standard deviation of the ΔH^+ values - Table 1) can mask acid fluctuations in the leaves of *B. involutum*. This high dispersion of ΔH^+ values was previously observed in leaves of *Welwitschia mirabilis* distributed along a gradient of water availability (a transect in direction to the coast of Namibia) (Willert et al., 2005). These authors pointed that even with more intense sampling efforts used, with a high number of replications; the high variability may still remain. Thus, the carbon discrimination ($\delta^{13}C$) helps us to clarify the photosynthetic metabolic processes — which showed values around −21‰ — suggesting intermediate C_3-CAM metabolism in *B. involutum* leaves. CAM metabolism is homoplastic, and occurs in a wide variety of species, especially in arid environments, suggesting high variation for this photosynthetic

Table 4
Means ± SD thickness of cuticle and epidermis in adaxial and abaxial faces of leaves, and mesophyll thickness (n = 30, means ± SD) of *Bulbophyllum involutum* (Orchidaceae) individuals growing in the Serra do Caraça, southeastern Brazil, in light classes: sun, partially shaded, and shaded. Values presenting different letters in a same column are significantly different at 0.05 level of Tukey's test.

Light classes of *B. involutum*	n	Abaxial cuticle (μm)	Adaxial cuticle (μm)	Abaxial epidermis (μm)	Adaxial epidermis (μm)	Mesophyll (mm)
Sun	11	11.2 ± 0.4^a	11.6 ± 0.5^a	29.8 ± 0.9^a	47.8 ± 1.3^a	1.9 ± 0.1^a
Partially shaded	11	11.3 ± 0.3^a	11.6 ± 0.3^a	30.2 ± 0.8^a	45.9 ± 1.2^a	1.7 ± 0.1^{ab}
Shaded	8	9.3 ± 0.4^a	10.8 ± 0.4^a	30.3 ± 1.0^a	47.7 ± 1.2^a	1.6 ± 0.2^b

Table 5
Variation of histometry data in leaves of *Bulbophyllum involutum* (Orchidaceae) individuals growing in the Serra do Caraça, southeastern Brazil, in the light classes sun, partially shaded and shaded. The variation was obtained using Relative Distance Plasticity Index (RDPI, Valladares et al., 2006). Values presenting different letters in a same line are significantly different at 0.05 level of Tukey's test.

	Sun × partially shaded	Partially shaded × shaded	Sun × shaded
Leaf area	0.23 ± 0.1^{b}	0.22 ± 0.1^{b}	0.36 ± 0.2^{a}
Cuticle thickness in abaxial face of leaves	0.06 ± 0.03^{b}	0.12 ± 0.07^{a}	0.13 ± 0.08^{a}
Cuticle thickness in adaxial face of leaves	0.06 ± 0.04^{a}	0.07 ± 0.04^{a}	0.07 ± 0.04^{a}
Epidermis thickness in abaxial face of leaves	0.06 ± 0.003^{a}	0.06 ± 0.003^{a}	0.06 ± 0.003^{a}
Epidermis thickness in adaxial face of leaves	0.03 ± 0.02^{a}	0.04 ± 0.03^{a}	0.04 ± 0.03^{a}
Mesophyll thickness	0.06 ± 0.05^{b}	0.07 ± 0.04^{b}	0.09 ± 0.05^{a}
Stomata density	0.07 ± 0.05^{b}	0.17 ± 0.09^{ab}	0.18 ± 0.1^{a}
Stomata pore area	0.10 ± 0.06^{b}	0.13 ± 0.09^{ab}	0.15 ± 0.1^{a}
A_{st}/A_{leaf}	0.13 ± 0.09^{b}	0.25 ± 0.2^{a}	0.29 ± 0.2^{a}

process (Dodd et al., 2002; Silvera et al., 2010). Comparatively, intermediate C_3-CAM species exhibit greater plasticity than exclusive CAM species (Ting, 1985; Dodd et al., 2002) and have different pathways of carbon assimilation. CO_2 can be obtained exclusively through respiratory activity, or from stomata that open only at night, from the continuous assimilation of atmospheric CO_2 during 24 h, or from variations of all three of these conditions (Dodd et al., 2002; Lüttge, 2004).

During dry periods, intermediate C_3-CAM species generally reduce their relative water content, followed by increases in CO_2 fixation *via* PEP-carboxylase — which indicates a tendency towards CAM metabolism (Dodd et al., 2002). The *B. involutum* leaves didn't show reduction in the water content during the dry season, and the ΔH^+ values were variable throughout the year (with low variation in $\delta^{13}C$, and no relation with water availability). For many authors, there is strong evidence that intermediate C_3-CAM species are a transitional group between obligatory C_3 and obligatory CAM species, representing an advantage for colonizing new habitats that are often quite arid (Kluge et al., 2001; Dodd et al., 2002; Lüttge, 2004). However, Lüttge (1999) argued the case of *Clusia* species, a genus with high variation in CAM metabolism expression. Besides the comment that reduction in water availability amplifies CAM expression in C_3-CAM intermediate *Clusia* species (statement based in studies from Borland et al., 1993; Zotz and Winter, 1994; Borland et al., 1996; Zotz and Winter, 1996; Borland et al., 1998), the obligate C_3 species *C. multiflora* and the C_3-CAM intermediate species *C. minor* are sympatric, and the C_3 species is dominant in fully exposed sites. The C_3-CAM intermediate species seemed to perform better in environments with low radiation, and the flexibility of the C_3-CAM metabolism seemed to encourage a better performance under variable stress in time and space (Lüttge, 1999). Moreira et al. (2013b) described the same population of *B. involutum* occurring preferentially in rock surfaces exposed to high solar radiation and temperatures, but also occupying niches with different light availabilities. The light heterogeneity in this rocky outcrop was a product from shrubs and subshrubs with different phenology, architectures, shape and size of leaves, and the variations in micro-topography that determine different daily and seasonal changes in photosynthetic active radiation.

4.2. Leaf structure

Bulbophyllum involutum leaves are structurally similar another CAM plants (Cushman, 2001; Nelson et al., 2005; Moreira et al., 2013a), in which the anatomical and morphological features reduce water loss. The mesophyll is homogeneous, with few or reduced intercellular spaces, and constituted by large and vacuolated cells which enhance water storage capacity. The retention of water in the leaves depends both on its storage capacity and on the control of the conductance (or greater resistance) for water vapor diffusing out of leaves. The *B. involutum* leaves showed epidermis covered by a thick cuticle which form suprastomatic chamber. The conductance for cuticular transpiration can be lower for xerophytes with thick cuticles, while stomata features such as density, pore depth and aperture radius (Nobel, 1999). In this way, the conspicuous suprastomatic chamber could offer a new resistance for water vapor diffusing out of leaves.

CAM metabolism is associated with distinct leaf structures generally associated with succulence (Cushman, 2001; Nelson et al., 2005). Regardless of the degree of succulence, leaf size is usually affected by exposure to sunlight (Gratani et al., 2006; Valladares and Niinemets, 2008). Thus, as expected, individuals of *B. involutum* belonging to the "sun" class had smaller and thicker leaves. The large areas and diminished thicknesses of shaded leaves offset lower light levels per unit area by increasing absorption surfaces (Gratani et al., 2006).

The lower specific leaf mass observed in individuals of *B. involutum* in the "shaded" class (LSM 31% lower than leaves exposed to direct sunlight) was also noted by Gratani et al. (2006) among woody species. Higher SLM in plants under high radiation is consistent with the higher density and greater thickness of their mesophyll, as well as greater sclerophylly (Sack et al., 2003; Gratani et al., 2006). Pyankov et al. (1999) observed that leaf thickness may determine the efficiency with which photons are captured and used by plants. Leaves with voluminous cells containing high numbers of chloroplasts tend to absorb more photons, leaving less residual energy available to penetrate into the deeper cells of the chlorenchyma (thus restricting its numbers of functional layers).

The build-up of sugars may suppress the photosynthetic activities in C_3 species (Paul and Pellny, 2003). However, the carbohydrate contents in CAM species may increase, not resulting in down-regulation of the photosynthesis (Drennan and Nobel, 2000). During the CAM metabolism, the carbohydrates are essentials; water-insoluble polysaccharides (as starch and glucans) or water-soluble sugars are required to furnish the carbon to form PEP for malate synthesis (Black et al., 1996), and indirectly, the free sugars combined with the organic acids would increase the cell osmolality, and reduce the water potential (Lüttge, 2007). The dynamic rule of the carbohydrates in CAM plants results in almost 8 to 20% of variation of the total dry matter in leaves during a single day (Black et al., 1996). In this way, the higher dry mass per unit area observed in *B. involutum* leaves during the dry season could be a product from the accumulation of starch and non-structural carbohydrates, increasing the osmolality in leaves (reducing the water potential), the water flux in direction to leaves, and consequently the water absorption (in the periods with low water availability). In fact, the saturated water content in *B. involutum* leaves during the year remained stable, even in the dry season.

Water storage tissues can support physiological metabolism in the absence of external water availability, and the maintenance of leaf saturated water content (SWC) could be a strategy of water stress avoidance at the cellular and tissue levels (Ogburn and Edwards, 2012). In this case, the CAM metabolism in leaves of *B. involutum* was not affected (positively or negatively) by seasonal water fluctuations. However, the lowest SWC values found for the "sun" class individuals probably occurred due the higher dry mass per unit area in some cases, and due the high water loss product from high irradiance and temperature that they are exposed to.

Stomatal density values for *B. involutum* were lower than those previously described for the Orchidaceae (Yukawa et al., 1992), although differences were observed among individuals exposed to different light conditions. The lower densities and stomatal pore areas of the individuals of *B. involutum* in the "shaded" class were similar to those reported for other understory/shaded species (Gratani et al., 2006; Valladares and Niinemets, 2008). The high stomata densities observed in full sunlight conditions are often associated with efficient controls of water losses. Additionally, the presence of a thick cuticle forming

supra-stomatal chambers may aid water retention, as these chambers are not always open (intact cuticle), and even when open, the sizes of their openings are often reduced as compared to stomatal pores themselves. As these chambers may confer additional resistance to gas exchange, their positive influence on water use efficiency in *B. involutum* is assumed.

The thicker leaves of sun class plants are a consequence of increased tissue thicknesses, mainly of the chlorophyllous parenchyma and the epidermis, and are accompanied by increased cuticle thicknesses (Gratani et al., 2006). Increases in mesophyll thickness in *B. involutum* were mainly due to the increased thickness of the chlorenchyma, with intercellular spaces being reduced. It is noteworthy that the cuticle on its adaxial leaf surface is thicker. Characteristics such as large and vacuolated parenchymatous cells allow both the accumulation of organic acids and water storage in CAM plants, with the small intercellular spaces being responsible for low internal CO_2 conductance (Maxwell et al., 1997). The scattered tracheoidal elements with different shapes and sizes observed in the mesophyll cells of *B. involutum* were classified by Rao and Das (1979) as heteromorphic. These cells provide mechanical support, aid water retention by the leaves (Rao and Das, 1979; Stern and Judd, 2002), and could help the water flux in leaves. Their random and diffuse distributions, without any apparent connections to vascular bundles, are a strong indication of their functions in mesophyll regions devoid of venation.

Comparing Relative Distance Plant Index (RDPI) values, there are few visible structural differences between individuals belonging to "sun" and "partially shaded" classes, although more deeply shaded conditions produce significant changes in macro- and microscopic parameters, including greater leaf area changes, followed by characteristics related to the stomata and to tissue thicknesses. The wide variations observed in leaf areas favor the establishment of individuals of *B. involutum* in pronounced light gradients, as they allow maximum use of light resources in shaded sites (Niinemets and Valladares, 2004). The lower stomatal densities in individuals belonging to the "shaded" class may be due to their significantly greater leaf areas as compared to individuals in the "sun" class, as leaf area, together with changes in stomatal pore areas, and affect the proportions of the leaf area occupied by stomatal pores. This parameter is, in fact, a consequence of stomatal differentiation, even before leaf expansion (Glover, 2000). The high variation of parameters related to gas exchange was linked with the ability of plants to adjust to highly variable irradiance levels (Niinemets and Valladares, 2004). Despite the biochemical and ecophysiological flexibility of the C_3-CAM metabolic process (Lüttge, 1999; Cushman, 2001; Dodd et al., 2002), the leaf acidity and the carbon stable isotope content of *B. involutum* had non-significant differences among light intensities and seasonal water availability, with low morphological variation, and mesophyll tissue thickness largely unaffected.

5. Conclusions

The leaf area was the high leaf parameter affected by the different light intensities, and it benefits for "sun plants" are wide discussed in the literature; increasing stomata density and helping water economy. The other histometric leaf parameters and the leaf mass of the *B. involutum* individuals that grew under the three distinct light conditions (shade, partially shaded and sun) were similar. However, the dry mass of leaf per unit area increased during the dry season, an indicative of the high soluble sugars and starch content. These carbohydrates play an important role in the acid metabolism (PEP synthesis), and could reduce cell water potential in leaves, facilitating the maintenance of leaf succulence throughout the year, even among individuals growing under different light conditions. The degree of succulence is in agreement with C_3-CAM photosynthetic pathway, as expounded by Cushman (2001) arguing about CAM plants. The flexibility of C_3-CAM metabolism didn't show a tendency of high expression of CAM pathway in dry conditions (dry season), but seems to be helpful under variable stress in time and space. This condition was previous described by Moreira et al. (2013b) to the same population of *B. involutum*, occurring in heterogenic amplitude of light.

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Appendix 1. Absolute values of diurnal titratable acidity fluctuations of leaves of *Bulbophyllum involutum* (Orchidaceae) individuals growing in the Serra do Caraça, southeastern Brazil, in the light classes sun, partially shaded and shaded. These absolute values show that a single individual can vary their daily acidity from negative to positive values throughout the year, and that there is no pattern among individuals

Analyzed individuals in the three distinct light classes	ΔH+ (μeq g^{-1} FM)			
	July	October	January	May
Sun 1	18.26	10.83	0.81	23.61
Sun 2	3.65	7.30	13.80	32.60
Sun 3	18.56	−9.13	5.29	36.52
Sun 4	−1.32	14.61	16.32	13.74
Sun 5	10.85	9.13	13.68	23.49
Sun 6	10.92	11.03	2.28	34.26
Sun 7	3.65	14.75	−1.23	20.31
Sun 8	3.43	5.63	0.63	11.60
Sun 9	10.97	25.56	4.46	−1.96
Sun 10	5.48	1.82	8.75	24.02
Sun 11	1.18	7.32	−5.59	1.39
Partially shaded 1	0.06	5.52	14.98	12.72
Partially shaded 2	5.53	1.87	15.13	11.50
Partially shaded 3	−5.65	0.00	7.54	20.16
Partially shaded 4	3.75	3.62	0.47	14.41
Partially shaded 5	3.72	10.69	13.36	8.68
Partially shaded 6	27.39	7.59	14.75	31.56
Partially shaded 7	21.92	21.82	20.36	18.54
Partially shaded 8	18.36	20.09	26.43	5.55
Partially shaded 9	18.15	10.69	10.08	14.34
Partially shaded 10	10.72	5.63	5.16	20.19
Partially shaded 11	31.04	18.24	18.61	18.96
Shaded 1	0.00	14.61	8.77	10.48
Shaded 2	0.04	27.39	12.05	15.52
Shaded 3	18.36	14.95	−2.74	8.38
Shaded 4	18.02	14.45	11.03	6.15
Shaded 5	18.06	9.13	18.07	9.08
Shaded 6	23.74	−1.80	−2.71	19.98
Shaded 7	10.94	34.69	10.69	15.97
Shaded 8	9.13	27.41	20.92	−0.18

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