Research article

Trichome-like emergences in Croton of Brazilian highland rock outcrops: Evidences for atmospheric water uptake

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Background and aims: Although trichomes are typical features of the species-rich genus Croton, little is known about their development, anatomical structure and function. This study aims to characterize the anatomy of leaf trichomes of three Croton species restricted to Brazilian rock outcrops, and investigate their functional role in an environment with limited soil water availability.

Methods: Samples of leaves at different stages of maturity were submitted to standard anatomical light and scanning electron microscopy techniques to perform a structural and developmental study. Atmospheric water uptake was investigated through the absorption of aqueous solutions of berberine hemisulfate and Indian ink.

Key results: The so-called trichomes actually correspond to emergences because they originate from protodermal and ground meristem activity rather than from epidermal tissue. These emergences show a complex anatomy, are closely associated with the mesophyll sclereids and might be involved in leaf atmospheric water uptake. The outermost cells of the emergences are arranged radially and present non-lignified thick walls. We demonstrate that an aqueous solution can effectively penetrate through the leaf emergences, continuing through sclereids and reaching the vascular bundle or flowing across the mesophyll maximizing water distribution in the leaf tissue.

Conclusions: The complex structure and hypothesised function of Croton trichome-like emergences represent a novel finding in Euphorbiaceae. In the three species studied, these leaf emergences might play a role in atmospheric water absorption and they may be one of the clues for the occurrence of Croton in habitats with limited soil water supply where this genus is usually species-rich and abundant.

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1. Introduction

Trichomes of diverse types characterize Croton L. (Euphorbiaceae), one of the largest genera of flowering plants (Berry et al., 2005) with over 1200 species (Covaerts et al., 2000). Because of their great diversity in morphology, density, and distribution patterns on the plant, trichomes have been used by Croton systematists as a rich source of characters at different taxonomic levels (Webster, 1993; Lima and Pirani, 2003, 2008; Riina et al., 2010; Caruzo et al., 2011; van Ee and Berry, 2011; Vitarelli et al., 2015).

Plant trichomes are specialized epidermal structures originating from protodermal cells. The existence of a connection between trichomes and structures in the mesophyll has been reported in several species of Croton (Solereder, 1908; Metcalfe and Chalk, 1950; Sá-Haiad, 1987; Starling, 1993; Louro et al., 2003; Appezzato-da-Glória and Carmello-Guerreiro, 2006; Lucena and Sales, 2006; Silva, 2006; Sá-Haiad et al., 2009; Barros and Soares, 2013), but without any experimental demonstration about the trichomes functional role.

In Croton, seven types of trichomes (stellate, fasciculate, multiadiate-rosulate, dendritic, lepidote, papillose, and glandular) have been described (Webster et al., 1996). Trichomes are important features in systematics, physiology and ecology, however studies about their anatomy and development are scarce. We
set to conduct a detailed study of leaf trichomes of three closely related species of *Croton* section Lamprocroton, namely *Croton erythroxyloides* Baill., *C. pygmaeus* L.R. Lima, and *C. splendidus* Mart. ex Colla. In a recent taxonomic study, lepidote trichomes were reported for the abaxial side of the leaves of *C. pygmaeus* and *C. splendidus* and on both leaf surfaces in *C. erythroxyloides*, and stelate trichomes on the adaxial side of the leaves of *C. pygmaeus* and *C. splendidus* (Lima and Pirani, 2008).

*Croton erythroxyloides*, *C. pygmaeus* and *C. splendidus* are common in high elevation rocky habitats in southeastern Brazil; the so-called *campos rupestres* and *campos de altitude* (Lima and Pirani, 2008). Plants living in these ecosystems are exposed to similar extreme environmental conditions, such as high daily variation of temperature, risk of frost, intense sun irradiation, strong winds, and the nutrient-impoverished shallow soils with a low water-holding capacity that leads to long periods of severe water limitation for plant productivity (e.g., Safford, 1999; Benites et al., 2003; Lüttege et al., 1998; Morales et al., 2015; Silveira et al., 2015). These conditions exert continuous selective pressure on plants colonizing these environments, which could result in the evolution of morphoanatomical and/or physiological adaptations (Eames and McDaniels, 1947; Metcalfe and Chalk, 1983; Hamba et al., 2002; Alcantara et al., 2015), especially those involved in water economy. Many species have superficial root systems that would allow for rapid use of rain pulses (Nobel et al., 1990). Whilst leaves of resurrection plants are physiologically inactive during the dry season, differing from deciduous species in that the former retain leaf nutrients during the dry season (Griffiths et al., 2014). Aerial roots with velamen or pseudostems are common in Velloziaceae (Porembiski and Barthlott, 1995), Bromeliaceae (Pita and Menezes, 2002) and Orchidaceae (Silva et al., 2010). Velamen may play a role in accessing moisture from dew and rain in *campos rupestres* and *campos de altitude* (Moreira et al., 2009; Silva et al., 2010) or could be more important in preventing water loss than in moisture or nutrient uptake (Dycus and Knudson, 1957).

Among the anatomical traits, trichomes stand out by their widespread presence in phylogenetically unrelated species (Araújo et al., 2010; Lusta et al., 2014; Francino et al., 2015). They might have different functions, such as protection against herbivores either by physical (Levin, 1973; Wagner, 1991) or chemical defense (Levin, 1973), reduction of the fraction of radiation that is absorbed by leaves (Fahn, 1986; Karabourniotis and Esseas, 1996), protection of stomata and reduction of water loss by evapotranspiration (Fahn, 1986). Trichomes may play a role in atmospheric water uptake, performing as specialized epidermal structures that could speed the atmospheric water absorption process (Stuart, 1968; Benzing et al., 1976; Martin and von Willert, 2000; Fernández et al., 2014). Besides trichomes, other epidermal structures, such as hydathodes and cuticle structure, could be related to atmospheric water absorption (Grundell, 1933; Meidner, 1954; Leyton and Armitage, 1968; Riederer 2006; Eller et al., 2013). All of these epidermal structures seem to provide an important alternative water supply, especially in environments where soil water availability is deficient. Several experiments could be conducted to evaluate the water pathway through epidermal structures, for example, using berberine sulfate fluochrome (Heide-Jorgensen, 1990), aqueous safranin solution (Milanez and Machado, 2008), and measuring the hydric potential in wettable leaves (Fernández et al., 2014).

This study aims to perform anatomical, histochemical and ontogenetic characterization of leaf trichomes of three closely related *Croton* species occurring in similar habitats, i.e., *campos rupestres* and *campos de altitude*, as well as to evaluate their functional role in plants growing in such extreme and harsh environments characterized by limited soil water availability.

### 2. Materials and methods

#### 2.1. Tissue sampling and study site

*Croton erythroxyloides*, *C. pygmaeus* and *C. splendidus* were chosen because they present different types of trichomes (lepidote, stellate, and fasciculate) and occur in extreme habitats, allowing us to compare trichome structure, development, and function under similar environmental conditions. Leaf samples were obtained from natural populations or from herbarium material (Table 1). Voucher specimens were deposited in the herbarium of the Universidade Federal de Viçosa (VIC).

*Croton erythroxyloides* and *C. splendidus* were collected from natural populations (Fig. 1A–E) in *campos de altitude* above 1700 m elevation on ‘Serra das cabeças’, ‘Parque Estadual da Serra do Brigadeiro’ (PESB), Minas Gerais State, Brazil (Fig. 1A–C). *Campos de altitude* (altitudinal grasslands) are rupestrian ecosystems of igneous formation that occur mostly on the ‘Serra da Mantiqueira’ and ‘Serra do Mar’ ranges in southeastern Brazil. *Campos rupestres* and *campos de altitude* share several plant genera and are known to harbor considerable numbers of endemic species (Safford, 2007; Alves and Kolbek, 2010). In spite of their floristic and phytosociological similarities, they are geologically distinct (Benites et al., 2007; Alves and Kolbek, 2010). For example, *campos de altitude* have more floristic affinities with the páramo vegetation of the Andes than *campos rupestres* (Alves and Kolbek, 2010). On the other hand, both ecosystems have soils developed on ironstones that are shallow and acidic with a low water-holding capacity.

The PESB is located at the summit of ‘Serra da Mantiqueira’ between 42°20’ and 42°40’S and 20°20’ and 21°00’W (Engenix, 1995). The regional climate is classified as mesothermal medium – CWb (Koeppen, 1948), the average annual precipitation is 1300 mm. Although, the annual precipitation does not imply a xeric environment, the high sun light exposition, shallow depth and high permeability of soils and intense wind, produce an environment with extreme low water supply (Benites, 1998). The rainy season is from November to March and the dry season from May to September. Leaf samples were collected in both the dry and rainy season. The average annual temperature is 18 °C (64.4 °F) (Engenix, 1995), with June, July and August as the coldest months. The recorded minimum temperature was below 0 °C. The presence of clouds covering the peaks of the PESB is observed daily, especially in early morning hours (Fig. 1B and C). The individuals sampled grow in dense grass tussocks directly on bare rock outcrops with low water-holding capacity (Fig. 1C).

Samples of *Croton pygmaeus* were obtained from herbarium material (Table 1). We were unable to obtain fresh material from this species because of the difficult and expensive logistics due to its disjunct location from the other two species. According to the herbarium collection labels the individuals sampled are shrubs 1–2 m tall, growing directly on rocky substrates in the state of Rio Grande do Sul, southern Brazil (approximate location at 30°32′6.92″S, 53°33′2.14″W).

Leaves at different developmental stages (leaf primordia, young and mature leaves) from each specimen were used in this study. Four specimens of *Croton splendidus*, four of *C. erythroxyloides* and two of *C. pygmaeus* were used as replicates (Table 1), using a minimum of three leaves per specimen. Sections of leaf samples were taken from the apical, median and basal portions of the blade (including midrib, margin, and region between them).

#### 2.2. Light microscopy

Leaf tissue sampled from herbarium specimens was boiled in distilled water for 10 min, treated with 2% potassium hydroxide for 2 h at room temperature, rinsed in tap water five times, dehy-
Table 1
List of species, emergence type, and voucher information.

<table>
<thead>
<tr>
<th>Species</th>
<th>Emergence type</th>
<th>Voucher (herbarium)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Croton erythroxyloides Baill.</td>
<td>Lepidote (adaxial and abaxial)</td>
<td>Vitarelli 002 (VIC), Vitarelli 003 (VIC), Vitarelli 005 (VIC), Vitarelli s.n. (VIC)</td>
</tr>
<tr>
<td>Croton pygmaeus L.R. Lima</td>
<td>Stellate (adaxial) Lepidote (abaxial)</td>
<td>Falkenberg 6483 (BHC), Marchett 549 (BHC)</td>
</tr>
<tr>
<td>Croton splendidos Mart. ex. Colla</td>
<td>Fasciculate (adaxial) Lepidote (abaxial)</td>
<td>Alvim s.n. (HUEMG), Alvim 40 (HUEMG), Vitarelli s.n. (VIC), Vitarelli s.n. (VIC)</td>
</tr>
</tbody>
</table>

Fig. 1. *Campos de altitude of 'Serra das Cabeças' ('Parque Estadual da Serra do Brigadeiro', Minas Gerais, Brazil). (A) View from the summit with a clear sky at 12:00 noon. (B) View from the summit with sky covered by fog, early morning. (C) View of vegetation on the summit. *Croton* populations grow, along with dense grass tussocks, directly on bare rock. (D) *Croton splendidus*, detail of leaf surfaces showing a discolor aspect due to the high density of lepidote emergences. (E) *C. erythroxyloides*, showing high density of lepidote emergences, visible to the naked eye, as brown pits.

Fresh leaf material was fixed in the field either in FAA (glacial acetic acid, formalin, 50% ethanol, 1:1:18 by volume) (Johansen, 1940) or NBF (neutral buffered formalin) for general characterization and histochemical testing (Clark, 1973). After fixation, the samples were also stored in 70% ethanol. Part of the samples was dehydrated in an ethanol series (30–70%) and stored in 70% ethanol (Smith and Smith, 1942). Fresh leaf material was fixed in the field either in FAA (glacial acetic acid, formalin, 50% ethanol, 1:1:18 by volume) (Johansen, 1940) or NBF (neutral buffered formalin) for general characterization and histochemical testing (Clark, 1973). After fixation, the samples were also stored in 70% ethanol. Part of the samples was dehydrated in an ethanol series and embedded in methacrylate (Historesin, Leica Microsystems Nussloch GmbH, Heidelberg, Germany, prepared according to the manufacturer instructions). The embedded samples were cross and longitudinally sectioned (5 μm thick) in an automatic rotary microtome (RM2155, Leica Microsystems Inc., Deerfield, USA) using glass knives, and the sections were stained with toluidine blue pH 4.0 (O’Brien and McCully, 1981).

Hand-cut sections were obtained from both fresh and fixed samples for performing the following histochemical tests: Sudan IV (Pearse, 1980) and neutral red under fluorescence (Kirk, 1970) for detection of total lipids; phloroglucinol for lignin (Johansen, 1940); Rutenium red (Johansen, 1940) for pectins. Some sections of methacrylate embedded samples were tested for total polysaccharides using periodic acid Schiff (PAS) (Mcmanus, 1948).

For the description of leaf surface characters, estimation of density of trichome-like emergences and measurements of stalk length of the lepidote emergences, whole leaves were cleared using a solution of 10% sodium hydroxide and 20% sodium hypochlorite, interspersed with successive washes in distilled water (Johansen, 1940, modified). The leaf material was stained with safranin (1% alcoholic solution) and mounted in glycerinated gelatin.
Density of trichome-like emergences and stalk length were measured on the following specimens: *Croton pygmaeus* (Falkenberg 6483; Marchett 549), *Croton splendidus* (Alvim s.n.; Vitarelli s.n.) and *Croton erythroxyloides* (Vitarelli 002; Vitarelli 003) (Table 1). Trichome-like emergences were counted at the middle region of both adaxial and abaxial leaf surfaces, excluding the mid-vein area. The stalk length of lepidote emergences was measured on leaf cross sections from embedded samples.

Observations and photographs were obtained using a light microscope (AX70TRF, Olympus Optical, Tokyo, Japan) equipped with a U-photo system and digital camera (AxioCam HRC: Zeiss, Göttingen, Germany), and an epifluorescence HBO 50W mercury vapor lamp and filter block A (exciter filter BP 340–380, dichroic mirror 450, barrier filter LP-430).

The denomination of trichome-like emergence types follows the classification by Webster et al. (1996).

2.3. Leaf absorption experiment

The capacity of the trichomes to absorb an aqueous solution was tested in fully expanded leaves from the thirteenth or fourth node of live plants (Fig. 1D). Fresh branch samples of *Croton splendidus* were sprayed with a 1% solution of fluorochrome berberine hemisulfate. These samples were kept in darkness overnight or longer. Hand-cut cross sections were made and the slides were immediately mounted in glycerol and visualized, to prevent secondary diffusion, under a fluorescent microscope. This methodology is based on experiments conducted by Heide-Jorgensen (1990).

Whole leaves were also dipped in an India ink aqueous solution, and kept in that solution during 4 h. Leaves were vertically positioned inside a recipient containing a solution of India ink in such a way that only the apical and median portions of the leaf blade were in contact with the solution. This was achieved by attaching the petiole to the upper part of the walls of the container. Leaves not subjected to the above treatments with berberine hemisulfate or India ink solutions were also analyzed as control samples.

The experiments using solution of fluorochrome berberine hemisulfate and India Ink solution were chosen to evaluate the leaf water absorption ability because they are simple and show direct results. This is important since it is very difficult to cultivate *Croton* plants out of their natural environment and there are many constraints to conduct experimental practices in the field due to the problematic access to the areas where the studied species occur.

2.4. Scanning electron microscopy

For analysis with a scanning electron microscope, samples fixed in Karnovsky solution (Karnovsky, 1965) were dehydrated in an ethanol series and submitted to critical-point drying (CPD 020; Bal-Tec, Balzers, Liechtenstein) using CO2 (Bozzola and Russel, 1992). The samples were then mounted onto stubs and coated with gold using a FDU 010 sputter coater (Bal-Tec, Balzers, Liechtenstein). Image captures were conducted using a Leo 1430VP SEM (Zeiss, Cambridge, United Kingdom).

3. Results

The epidermal structures observed in the three species studied are formed by the activity of protoderm and ground meristem. For this reason, from now on we refer to them as emergences instead of trichomes.

We observed three types of emergences: lepidote (Fig. 2A), fasciculate (Fig. 2B) and stellate (Fig. 2C, D). The lepidote type occurs on both leaf surfaces of *Croton erythroxyloides* and only on the abaxial side on the leaves of both *C. pygmaeus* and *C. splendidus*. The fasciculate emergences are present in the adaxial leaf side of *C. splendidus* and the stellate emergences are present on the adaxial side of the leaves of *C. pygmaeus*.

The lepidote emergences cover all the abaxial leaf surface and are visible to the naked eye (Fig. 1D, E) as brown dots. The density of lepidote emergences on the abaxial leaf surface was 3 emergences/μm² in *Croton erythroxyloides*, 5 emergences/μm² in *C. pygmaeus*, and 4 emergences/μm² in *C. splendidus*. On the adaxial leaf surface, the emergence density was 4 lepidote emergences/μm² in *C. erythroxyloides*, 1–2 stellate emergences/μm² in *C. pygmaeus*, and 4–5 fasciculate emergences/μm² in *C. splendidus*.

3.1. Ontogenetic development of emergences

Emergences in early and mature stages could be observed on the same leaf primordia due to their asynchrony and early development (Figs. 3 and 4). The beginning of the development of the three types of emergences is recognized by the presence of a protodermal cell that stands out because of its larger volume, evident nucleus and dense cytoplasm (Figs. 3A; 4A). The adjacent protodermal cells undergo a series of anticlinal divisions (Figs. 3B; 4B), give origin to a protuberance (Fig. 3C), in which the central cell shows an increasing in volume while the adjacent cells (lateral cells) display successive anticlinal divisions (Figs. 3C–F; 4B–D). The lateral cells are radially positioned to the central cell and will develop into lateral rays by stretching (Figs. 3G, H; 4D, E). The ray cells have peripheral nuclei (Fig. 3E, F), indicating their growing direction.

In the lepidote emergences, the lateral rays remain united by webbing and form a shield-like structure. Simultaneously with the ray cells stretching process protodermal cells, localized below the rays, become bulky and form a subradial disc (Fig. 3E–H). Under the subradial disc, protodermal and ground meristem cells form a multiserial stalk through periclinal divisions (Fig. 3C–G). At the central part of the stalk, cells arising from the ground meristem stretch out and become precursors of sclereids (Fig. 3H, F). The sclereids allow connection between the outer region of the emergences and the interior of the leaf (Fig. 5A, B, D–F) reaching the vascular bundles (Fig. 5A, E) and crossing the mesophyll (Fig. 5B). Ray cells are dead in mature emergences. The ray cell walls become thick, but non-lignified, as demonstrated by the negative reaction of phloroglucinol (Table 2; Fig. 5C). Additionally, the positive reaction to Rutenium red, along with the negative reaction to phloroglucinol, confirm that the lateral walls of the ray cells are rich in pectins and remain non-lignified even in mature structures (Table 2; Fig. 5C). In contrast, the central cell walls become thicker and undergo lignification (Table 2; Fig. 5C).

In completely mature lepidote emergences, the central cell is dead and shows a circular region at its centre (Fig. 5C, G, H), which probably is a more permeable zone since the anticlinal walls are thicker than the periclinal external wall. The anticlinal walls are lignified and pitted (Fig. 5C).

The central cell of the lepidote emergence is surrounded by a rounded shield formed by the union of the lateral ray cells (Fig. 5I), whose anticlinal walls bear pits (Fig. 5J). In the subradial region (or subshield region) there is a disc of cells with markedly sinuous outline and thick walls (Fig. 5J, K), that show bordered pits (Fig. 5K, detail). These sinuous cells remain metabolically active at maturity and contain numerous chloroplasts (Fig. 5H).

The above described structure (shield and subshield regions) is supported by a stalk of variable length: 80 μm on the abaxial leaf surface of *C. erythroxyloides*, 44.18–58.3 μm on the abaxial leaf surface of *C. pygmaeus*, and 80.72–101.09 μm on the abaxial leaf surface of *C. splendidus*. The stalk of lepidote emergences present on the adaxial leaf surface of *C. erythroxyloides* is almost sessile with approximately 40 μm of length (Figs. 3H; 5A, D), and stellate and fasciculate emergences are sessile (Fig. 5B). At the end
of the emergence development the stalk cells appear dead and a dense lipid layer is deposited over their anticlinal and periclinal walls constituting the external region of the stalk (Fig. 5D, G, H), as displayed by the positive reaction to Sudan IV (visible light) and neutral red (under fluorescent light, showing a bright white/blue color as shown in Fig. 5D, G, H).

Completely developed lepidote emergences are characterized by three different regions: the outermost part comprised by the central cell and the lateral rays or shield (Fig. 5C, I); the median region, formed by the subradial disc cells (Fig. 5J); and the basal region or stalk (Fig. 5G, H) internally constituted by sclereids (Fig. 5D).

The ontogenetic development of the stellate and fasciculate emergences is similar to the development of the lepidote ones. However, stellate and fasciculate emergences are always sessile or short-stalked, while in lepidote emergences the stalk can be long or short. In the sessile and short-stalked emergences the rays are directly connected with the sclereids of the mesophyll (Fig. 5D–F). Additionally, the central cell of the stellate and fasciculate emergences becomes more stretched, forming a central porrect ray (Fig. 4C–E). The lateral cells stretch and form the rays in a single whorl (Fig. 4E). The same process takes place in lepidote, stellate and fasciculate emergences. The difference between them is that in the lepidote ones the ray cells become totally or partially united by the lateral walls forming a rounded shield (Figs. 2A; 6 A). In the stellate emergences, the ray cells remain free in the same plane (Fig. 2C, D). The fasciculate emergences differ from the stellate ones in having ascending rays rather than positioned in a horizontal plane (Figs. 2B; 4E).

Table 2
Chemical nature of substances found in the cellular walls of different parts of leaf emergences.

<table>
<thead>
<tr>
<th>Region</th>
<th>Chemical nature of the substance</th>
<th>Reaction results/emergence type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ray cells</td>
<td>Lipids</td>
<td>(-) lepidote; (+) stellate; (+) fasciculate</td>
</tr>
<tr>
<td></td>
<td>Lignin</td>
<td>(-) lepidote; (+) stellate, in the rays basal region; (+) fasciculate, in the rays basal region</td>
</tr>
<tr>
<td></td>
<td>Pectin</td>
<td>(+) lepidote; (+) stellate; (+) fasciculate</td>
</tr>
<tr>
<td>Central cell</td>
<td>Lipids</td>
<td>(-) lepidote, stellate and fasciculate</td>
</tr>
<tr>
<td></td>
<td>Lignin</td>
<td>(+) lepidote, stellate and fasciculate</td>
</tr>
<tr>
<td></td>
<td>Pectin</td>
<td>(-) lepidote, stellate and fasciculate</td>
</tr>
<tr>
<td>Subradial disc cells</td>
<td>Lipids</td>
<td>(-) lepidote</td>
</tr>
<tr>
<td></td>
<td>Lignin</td>
<td>Inconclusive due the brown natural color of these cells</td>
</tr>
<tr>
<td></td>
<td>Pectin</td>
<td>(+) lepidote</td>
</tr>
<tr>
<td>Stalk cell walls</td>
<td>Lipids</td>
<td>(+) lepidote</td>
</tr>
<tr>
<td></td>
<td>Lignin</td>
<td>(-) lepidote</td>
</tr>
<tr>
<td></td>
<td>Pectin</td>
<td>(+) lepidote</td>
</tr>
<tr>
<td>Sclereids</td>
<td>Lipids</td>
<td>(-) lepidote, stellate and fasciculate</td>
</tr>
<tr>
<td></td>
<td>Lignin</td>
<td>(-) lepidote, stellate and fasciculate; or weak reaction in the three types</td>
</tr>
<tr>
<td></td>
<td>Pectin</td>
<td>(+) lepidote, stellate and fasciculate</td>
</tr>
</tbody>
</table>
3.2. Foliar absorption through emergences

The absorption capacity of the lepidote and fasciculate emergences was demonstrated by the experiments with berberine hemisulfate and India ink solutions. The water pathways were elucidated by these experiments and were observed by the yellow/blue fluorescence under UV light (Fig. 6B–E) after application of berberine hemisulfate spray. No fluorescence was produced by the control samples.

The lepidote, stellate and fasciculate emergences of the three Croton species studied are structurally complex. The rays of the emergences increase the contact area with the exterior (Fig. 5A–J), thus increasing the surface area for absorption of atmospheric moisture. The lepidote emergences are even more specialized in water capture than the other two (stellate and fasciculate) because they possess subradial disc cells (Fig. 5I) and many more rays forming their shield-like structure (Fig. 5I). These two features provide a larger surface of contact for the lepidote emergence.
Although the ray cell walls are thick, they are non-lignified (Fig. 5B, C), allowing water absorption from atmospheric moisture, which penetrates the structure via apoplastic pathway (Fig. 6C). In lepidote emergences, the lateral walls of the ray cells are connected and bear pits, allowing strong communication between such cells (Fig. 5). Due to this, we suggest that the absorbed water is radially distributed by the shield structure, and its flow is directed to the central area (central cell) (Fig. 6F). The pits of the central cell walls are responsible for the communication between the central cell and the rays (Fig. 5C). The subradial disc cells are a third potential way of water absorption (Fig. 6F). These cells remain alive at maturity (Fig. 5H), present thick walls with numerous pits, and have a markedly sinuous outline that increases their contact surface (Fig. 5J, K). We also observed bordered pits on the wall of the subradial disc cells (Fig. 5K). The aqueous solution absorbed by lepidote emergences flows initially through the apoplastic pathway until it reaches the stalk (Fig. 6C, F), which forces the symplastic pathway transport (Fig. 6D, F), thanks to the presence of lipidic compounds on the walls of the stalk cells.

We did not observe lipidic compounds in the basal area of walls of ray cells (Fig. 5E). For this reason, these two types of emergences might have a lower selectivity than the lepidote ones.

The solution absorbed penetrates toward the interior of the leaf through the sclereids, which maximizes the distribution of water in the mesophyll or into the vascular bundles (Fig. 6B).

### 4. Discussion

#### 4.1. Emergences instead of trichomes in Croton

The leaf structures we observed, which are called trichomes by Croton systematists, correspond to emergences as demonstrated by the protodermal and ground meristem activity detected in the present work. However, for practical reasons, we adopted Webster et al. (1996) trichome terminology (i.e., lepidote, stellate, etc.) to name these trichome-like emergences.

The types of emergences we found on the leaves of the studied species agree with trichome descriptions of Lima and Pirani (2008), except for *C. splendidus*, for which these authors reported the stellate type on the adaxial side of the leaf and we detected the fasciculate type instead. In the case of *C. splendidus*, we classified the emergences as fasciculate because of their ascending rays (Fig. 4D, E), which follows the definition of Webster et al. (1996). As we indicated above, the rays of a stellate emergence should be positioned in a horizontal plane (Fig. 2C, D).

#### 4.2. Functional and ecological role of Croton leaf emergences

The structure of the lepidote, stellate and fasciculate leaf emergences reported here suggests a function related to atmospheric water absorption. This finding is a new record in *Croton* and could
Fig. 5. (A) Cross sections of Croton erythoxyloides leaves embedded in methacrylate and submitted to PAS showing young sclereids connected to vascular bundle. (B) Hand cross section of C. splendidus leaf submitted to phloroglucinol, showing the central cell walls lignified and the lignified walls of basal region of the rays of fasciculate emergences (adaxial leaf side); note sclereids connecting the mesophyll reaching a lepidote emergence on the abaxial leaf side. (C) Hand cross section of C. erythoxyloides leaf submitted to phloroglucinol, showing negative reaction of ray cell walls and the presence of lignin only in the central cell wall (positive reaction). Pits can be observed on the anticlinal walls of central cell. (D–H) Hand cross section of fresh samples submitted to neutral red under UV light. (D) Lepidote emergence on adaxial leaf side of C. erythoxyloides; note the brightness from secondary fluorescence showing the presence of lipidic compounds in the short stalk cell walls. (E) Fasciculate emergences on the leaf adaxial side of C. splendidus; note sclereids connected with ray cells and reaching the vascular bundle. (F) Detail of pits on the scleroid cell wall. (G and H) Lepidote emergence of C. erythoxyloides leaf; note the bright yellow/white secondary fluorescence as a result of the presence of lipidic compounds in the stalk cell walls. The central cell with thick anticlinal walls and the subradial disc cells highlighted by red autofluorescence of plastids. (I–K) Lepidote emergence cells dissociated. (I) Frontal view of the radial cells almost totally connected to each other forming the shield. (J) Ray cells of the shield partially removed showing a frontal view of subradial disc cell stained with safranin; note lateral pits between ray cells. (K) Simous shape of the subradial disc cells; note the bordered pits (K detail). (Asterisk) Lipidic compounds in the stalk cell walls. (Aba) Abaxial leaf side. (Ada) Adaxial leaf side. (CC) Central cell. (Ch) Chloroplast. (PP) Palisade parenchyma. (Pt) Bordered pits. (Ra) Ray. (Sb) Subradial disc. (Sc) Sclereids. (SP) Spongy parenchyma. (VB) Vascular bundle. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
represent a survival strategy for populations of this genus living in environments with evident water restrictions such as the Brazilian rock outcrops (campos rupestres and campos de altitude). Water restriction in these habitats is caused by the low soil water retention due to the rocky surface and rough relief (Safford, 1999; Benites et al., 2003; Caiafa and Silva, 2007). Most of the literature on water absorption through leaves and stems in vascular plants has focused on the significance of this pathway for plants living in arid environments (Rundel, 1982). Even if the Brazilian rock outcrops are characterized by water soil restriction, there is a significant hydric and nutrition supplement available from the daily fogs (Gundel et al., 1994; Burgess and Dawson, 2004; Eller et al., 2013), which are present all year long. According to Eller et al. (2013), the occurrence of frequent fog events is a defining climatic attribute of tropical montane environments. Cavaleri et al. (1996) and Bruijnzeel et al. (2011) report that the direct contact of fog water droplets with the surface of stems or leaves causes water to drip to the soil, and this additional precipitation is considered to be a major hydrologi-
cal input in these tropical montane environments. Species that are able to use this atmospheric source of water and nutrients, as the three species studied here, might be more successful in colonizing these types of environment. It is important to highlight that fog is not pure water but a heterogeneous substance composed of water and dissolved ions (Houghton, 1955; Gundel et al., 1994), which could be also important for plant nutrition. Questions requiring further investigation are: how relevant is this atmospheric water supply to the hydric balance and nutrition of the studied species? and how significant is it for the species success in habitats with low availability of soil water?

The ability to capture water through leaves has been indirectly reported in species occurring in deserts (Martin and von Willert, 2000) but also in cloud-immersed mountain habitats (Berry and Smith, 2012, 2013; Berry et al., 2014; Eller et al., 2013) and coastal mountain regions where fog is a significant climatic contributor of moisture (Burgess and Dawson, 2004; Oliveira et al., 2005; Breshears et al., 2008; Limm et al., 2009; Simonin et al., 2009; Limm and Dawson, 2010; Berry et al., 2014; Gotsch et al., 2014). Croton species distributed in forest ecosystems also have sclereids associated with epidermal trichome-like emergences (Soares, 2013; Vitarelli, 2013), and these structures may play the same absorption role reported here, similarly to what has been described for species from tropical montane cloud forest such as Drimys brasiliensis Miers (Eller et al., 2013).

We believe that many other Croton species, if not all of them, have emergences that are similar to the ones described here, since several authors reported the presence of Croton trichomes associated with sclereids in the mesophyll (Metcalfe and Chalk, 1950; Starling, 1993; Louro et al., 2003; Appezzato-da-Glória and Carmello-Guerreiro, 2006; Sá-Haiad et al., 2009).

4.3. Atmospheric water uptake in Croton and other plant groups

The results of this study show the capacity of leaves from three species of Croton to absorb atmospheric water through the leaves. According to Rundel (1982), laboratory experiments demonstrated the ability of leaves to absorb water in different group of plants. However there are still controversies on the ecological and physiological significance of this way of water uptake. For water absorption and transfer to occur, a source of water and a favorable water potential gradient are necessary. In addition, morphological or anatomical structures must be present to speed the absorption process (Rundel, 1982). These structures may include specialized trichomes (Stuart, 1968; Benzing et al., 1976), specialized epidermal cells (Grundell, 1933; Meidner, 1954) and/or specialized cuticle features (Leyton and Armitage, 1968; Eller et al., 2013). There are only a few reports in the literature demonstrating leaf water absorption in flowering plants (see below), which might indicate that either leaf water absorption is a rare strategy or it has not been systematically studied across angiosperms.

The experiments with berberine hemisulfate and Indian ink solutions confirmed the absorption ability of the leaf emergences of the studied Croton species. Similar experiments have been performed in Hakea suaveolens R. Br. (Proteaceae) (Heide-Jorgensen, 1990) using berberine sulfate fluorochrome, and in Microlepis oleaeifolia (DC.) Triana (Melastomataceae) (Milanze and Machado, 2008) using aqueous safranin solution. In both cases, the permeability of the epidermis was demonstrated by the penetration of the solution through trichomes and sclereids to the mesophyll and vascular bundles. Fernández et al. (2014) studied the water absorption of holm oak leaves (Quercus ilex L.), an evergreen Mediterranean species, and found that the upper side of young and mature leaves was wettable and absorbed water, and they related this uptake mechanism to the presence of trichomes and trichome scars. Burrows et al. (2013) detected stellate trichomes with intrusive bases on the leaves of Solanum elaeagnifolium Cav. They reported that the trichome basal cells penetrate into the mesophyll and sometimes reach the bundle sheath of the vein, but without a direct contact with the xylem cells. The stellate trichomes of Solanum elaeagnifolium are very similar to the stellate emergences found in Croton. However, in S. elaeagnifolium the transport between trichomes and veins was not confirmed by sympatric (carboxylluorescin diacetate) or apoplastic (lucifer yellow) tracer dyes (Burrows et al., 2013). Trichomes associated with sclereids were also reported in Joveta humilis Guédès (Rubieceae), a Malagasy xerophytic shrub, and their structure suggests a water uptake function (Guédès, 1975).

The overall structure of the emergences of Croton erythroxyloides, C. pygmaeus and C. splendidus is similar to the xerophytic trichomes described by Fahn (1986) in Correa backhouseana Hook. (Rutaceae) and Olea europaea L. (Oleaceae), which have lipidic compounds covering the walls of their basal cell and are also associated with atmospheric water absorption. However, C. backhouseana and O. europaea lack the complex structure of the stalk-sclereid and subradial disc cells described in the Croton emergences. According to Fahn (1986), the layer of lipidic compounds observed in basal or stalk cells of the trichomes of 12 xeromorphic species he studied plays a function similar to that of the endodermis, inducing a switch from the apoplastic to the symplastic pathway. A similar structure was observed in absorber trichomes in species of subtribe Pleurothallidinae in the family Orchidaceae (Pridgeon, 1981). The selectivity function of absorber trichomes (or other leaf epidermal structures) is relevant, since fog water is a solution of water and ions (Gundel et al., 1994; Houghton, 1955).

In the lepidote emergences of the Croton studied here, the stalk is the region where the selectivity of the absorbing solution occurs. This function is also assigned to the subradial disc cells, since those cells are apparently the only ones that remain alive and metabolically active in mature emergences, as attested by the presence of plastids. Nevertheless, there is no physical obstruction to prevent the apoplastic flow through the subradial disc cells. This region of the lepidote emergence could also be associated with movements that change the orientation of the lateral rays in response to changes in atmospheric humidity, resembling the movements of the Tillandsioideae scales in Bromeliaceae (Benzing et al., 1976). However, complementary ultrastructural and morphophysiological studies are required to confirm this hypothesis. Our results show that the subradial disc cells are highly specialized structures implied in absorption and transport of water due to their markedly sinuous outline and the presence of bordered pits on their walls. The subradial disc cells are similar to the modified tracheids present in the transfusion tissue of gymnosperms (von Mohl, 1871), which are responsible for short distance water transport.

Anatomically, the lepidote emergences on the leaves of Croton erythroxyloides, C. pygmaeus and C. splendidus are someway similar to the leaf scales of Tillandsioideae species (Tomlinson, 1969; Benzing et al., 1976). However in Tillandsioideae, the stalk cells (named dome cells) are alive and present a complex structure with invaginations of the plasmatic membrane and numerous mitochondria, being specialized in the absorption function (Benzing et al., 1976), whereas in Croton the stalk cells are specialized in the selectivity function. Besides this, the lepidote emergences in Croton show a greater structural complexity than the scales of Tillandsioideae, due to the presence of the subradial disc cells and the sclereids.

4.4. The emergence-sclereid complex in Croton

Sclereids are the most polymorphic cells in the plant body and can be found in clusters or as isolated cells (named idioblasts) (Evert, 2006). Several studies have focused on their morphologi-
The sclereids from lepidote, stellate and fasciculate emergences in *Croton* have thick and non-lignified (or weakly lignified) walls. This unusual pattern in sclereids was also observed in *Olea europaea* (Arzee, 1953) and in populations of *Androstachys johnsonii* Prain (Picrodendraceae) growing in environments with water restriction (Alvin, 1987). The connection between leaf trichomes and mesophyll sclereids has been directly related with leaf water uptake in *A. johnsonii* (Alvin, 1987). This author suggested the name "gelatinous sclereids" due to the structural similarity with gelatinous fibers, which possess a G-layer (so-called gelatinous layer) that gives them the hygroscopic capacity to catch and store water. In the leaves of *Hakea suaveolens*, the surface of the branches of mesophyll osteosclerides constitutes a pseudohydropemis and their internal arms are in contact with the modified tracheids in the vascular ends. The sclereids remain alive and take part in the water uptake function, allowing the water to flow from the leaf epidermis to the photosynthetic tissues (Heide-Jørgensen, 1990). In the studied *Croton* species, the sclereids carry water into the xylem and/or distribute it into the mesophyll, resembling the role of the bundle-sheath extension described by Plymale and Wylie (1944) and reported in xerophytic leaves (Fahn, 1986).

5. Conclusions

The so called trichomes present on the leaves of *Croton erythroxylonoides*, *C. pygaeus* and *C. splendidus* correspond to emergences because they originate from the protodermis and ground meristem. The anatomical origin, structure, as well as the functional role of these emergences are described in detail for the first time. Their structural complexity along with our experimental results provide strong evidence that they may play an important role in the absorption of atmospheric water by these *Croton* species and potentially by other species in the genus. This ability to capture water through the leaves may be one of the clues for the evolutionary success of this speciose group common in environments with limited soil water supply in tropics and subtropics worldwide.

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