SECRETORY STRUCTURES IN LEAVES AND FLOWERS OF TWO DRAGON'S BLOOD *CROTON* (EUPHORBIACEAE): NEW EVIDENCE AND INTERPRETATIONS

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Editor: Patrick S. Herendeen

Premise of research.   Previous studies of secretory structures in species of the Neotropical dragon's blood *Croton* (section *Cyclostigma*) show inconsistencies in their classification. An accurate assessment of the identity and homology of such structures is essential for taxonomic and evolutionary studies.

Methodology.   Field-collected leaves, stipules, and flowers at different developmental stages were sampled. The material was subjected to standard anatomical study by light microscopy and SEM, and secretions were evaluated by histochemical analyses.

Pivotal results.   Leaves and flowers of *Croton echinocarpus* and *Croton urucurana* present five secretory structures (idioblasts, laticifers, colleters, extrafloral nectaries, and floral nectaries) with high similarity between the two species. Idioblasts secrete compounds of a mixed nature; laticifers are branched and nonarticulated; and colleters and nectaries present hydrophilic secretion. The leaf marginal glands previously described as extrafloral nectaries are actually colleters of the standard type. We found colleters in staminate and pistillate flowers. The histochemical tests detected proteins in the secretions of all structures.

Conclusions.   The classes of secondary metabolites detected support the biological activities of secretion described in the literature. The correct identification of colleters in flowers establishes a new register of these structures in flowers of this genus. We show that an approach integrating anatomic structure, histochemistry, and period of secretion activity allows for a more accurate classification and homology assessment of secretory elements in this genus, which is exceptionally rich in this type of structures.

Keywords: anatomy, colleters, Crotoneae, histochemistry, nectaries, stipules.

Introduction

Croton echinocarpus Müll. Arg. and *Croton urucurana* Baill. are members of section *Cyclostigma*, a group of tree species popularly known as dragon's blood because of the exudation of abundant red latex (Riina et al. 2009; van Ee et al. 2011) that is used for medicinal purposes across the Neotropics. Besides laticifers, four other secretory structures (colleters, idioblasts, extrafloral nectaries, and secretory trichomes) have been identified in the leaves of several *Croton* lineages from sections other than *Cyclostigma* (Vitarelli et al. 2015). Previous anatomical surveys on members of *Cyclostigma* included the two species studied here and described secretory structures from both leaves and flowers (Sá-Haiad et al. 2009; De-Paula et al. 2011); however, classification and homology assessment of such structures remain unclear and require further investigation.

Extrafloral nectaries have been recorded to occur on the margin, base, and blade of leaves of *C. echinocarpus* and *C. urucurana* (Sá-Haiad et al. 2009). However, similar mar-

ginal glands have been described as colleters of the standard type in different sections of *Croton* and other Crotoneae genera (*Astraea* Klotzsch and *Brasiliocroton* P.E. Berry & Cordeiro; Riina et al. 2014, 2015; Vitarelli et al. 2015). De-Paula et al. (2011), in a study of the floral morphology and anatomy of several Crotoneae species, including *C. urucurana*, found glands alternate to floral nectaries that they described as filamentous structures. These authors also reported glands along the surface of sepals as secretory trichomes, although they noted their similarity to colleters (De-Paula et al. 2011).

For an accurate classification of a secretory structure, its anatomical characterization must be accompanied by an analysis of the chemical nature of its exudate and an evaluation of the period of secretory activity. Descriptions based solely on morphology are not sufficient to assign a role to a structure (Lersten and Curtis 1996) or to determine the importance of the exudate for the plant. In addition, morphologically similar structures may play different functional roles. Extrafloral nectaries can be confused with other secretory structures, such as colleters, because the latter are also external structures that can have similar anatomy and topology. However, the period of activity and the composition of the secretion show that the activity of colleters is earlier than that of nectaries, and the colleters' secre-

tion is characterized by the presence of mucilage, lipids, and proteins and the absence of sugar (Fahn 1979; Thomas 1991; Cruz et al. 2002; Klein et al. 2004; Barreiro and Machado 2007). Nectaries, on the other hand, secrete nectar, a sugary substance consisting mainly of glucose, fructose, and sucrose (Fahn 1979; Bentley and Elias 1983).

Because of inconsistencies in distinguishing between nectaries and colleters in previous studies of *Croton* and the importance of an accurate classification of such structures for taxonomy and evolutionary studies requiring accurate homology assessment, we analyze the structure and histochemistry of leaf and floral secretory structures of *C. echinocarpus* and *C. urucurana*. We also discuss the relationship between structure, secretion composition, and function as well as the chemical nature of the secretions and its connections to the phytochemistry findings and medicinal uses reported for both species in the literature.

Material and Methods

Croton echinocarpus is endemic to the Atlantic Forest of southeastern Brazil (Caruzo and dos Santos 2015), while *Croton urucurana* is more widely distributed in southern South America (Brazil, Argentina, Paraguay, and Bolivia), occurring as a pioneer species in riparian forest (Caruzo and Cordeiro 2007; Cordeiro et al. 2015). Both species occur spontaneously in Minas Gerais state, as well as around the Universidade Federal de Viçosa campus (20°45′10.109″S, 42°52′16.167″W) and along the BR-356 road (20°54′3.939″S, 42°39′12.857″W), where fieldwork for this study was conducted. Samples were collected from vegetative and reproductive branches of both species. Herbarium vouchers were deposited at the VIC herbarium (Coutinho et al. 337, 338, 25/I/2014 [VIC]). Leaves at different developmental stages (shoot apex, leaf primordium, and mature leaves), stipules, and staminate and pistillate flowers in different stages of development were sampled (from bud to early fruit).

Three fixative treatments were used for three different sets of samples. The first set was fixed in FAA (formalin : acetic acid : ethanol 50%, 1 : 1 : 18 by volume; Johansen 1940) for 24 h to carry out a structural characterization with light microscopy and to test for hydrophilic compounds. The second was fixed in neutral buffered formalin for 48 h (Lillie 1965), to detect lipophilic compounds, and in ferrous sulfate in formalin (Johansen 1940), a fixative utilized for detection of total phenolic compounds. Finally, the third group of samples was fixed in formaldehyde-glutaraldehyde 2.5% (phosphate buffer 0.1 M, pH 7.3; Karnovsky 1965) for complementary structural and histochemical analysis.

For the description of surface characters, entire leaves were cleared with a solution of 10% sodium hydroxide and 20% sodium hypochlorite, interspersed with successive washes in distilled water (Shobe and Lersten 1967). The material was stained with basic fuchsin (0.5% alcoholic solution), and slides were mounted with glycerinated gelatin (Kaiser 1880).

Part of each sample was dehydrated in an ethanol series and embedded in methacrylate (Historesin, Leica Instruments, prepared according to the manufacturer's instructions). The samples were cross- and longitudinally sectioned at 4–6 μm in an automatic rotary microtome (model RM2265, Leica Biosystems, Nussloch, Germany) with glass knives (Leica Biosystems). Sections were stained with toluidine blue, pH 4.6 (O'Brien

et al. 1965), and slides were mounted with synthetic resin (Permount, Fisher Scientific, Fair Lawn, NJ).

Part of each sample was also dehydrated through a tert-butanol series (Johansen 1940), embedded in histological paraffin with DMSO (Histosec, Merck, Darmstadt, Germany), and serially sectioned at 10–12-μm thickness on a rotary microtome (Spencer 820, American Optical, Buffalo, NY). For structural characterization, longitudinal and cross sections were stained with Astra blue 1% (Kropp 1972) and Safranin O 1% (Bukatsch 1972, adapted) and mounted with synthetic resin. For structural characterization and histochemical tests, floral samples were dehydrated through tert-butanol series and embedded in histological paraffin with DMSO.

For detection of the main classes of secondary compounds, the following histochemical tests were carried out: periodic acid–Schiff reagent for total polysaccharides (McManus 1948); ruthenium red for acidic mucilage (Gregory and Baas 1989); tannic acid/ferric chloride for neutral mucilage (Pizzolato and Lillie 1973); Sudan black B (Pearse 1985) and neutral red (Kirk 1970) for total lipids in visible and UV light, respectively; NADI reagent for terpenoids (David and Carde 1964); copper acetate/rubeanic acid for fatty acids (Ganter and Jollés 1969–1970); Nile blue for neutral and acidic lipids (Cain 1947); vanillin/hydrochloric acid for tannins (Mace and Howell 1974); Wagner's reagent for alkaloids (Furr and Mahlberg 1981); and xilidine ponceau (O'Brien and McCully 1981) and ninhydrin/Schiff's reagent (Pearse 1985) for protein. Standard control procedures were carried out simultaneously as required for each test, and the slides were mounted in glycerinated gelatin (Kaiser 1880). To verify the occurrence of glucose in the secretion, Glicofita Plus (Accu-Chek Active, Hoffmann-La Roche) was used directly on the secretion.

Observations and photographic documentation were performed with a light microscope (Model AX70TRF, Olympus Optical, Tokyo) equipped with a U-Photo system and a digital camera (AxioCam HRc, Carl Zeiss, Göttingen, Germany), an epifluorescence HBO 50-W mercury vapor lamp, and a filter block A (exciter filter BP 340–380, dichroic mirror 450, barrier filter LP-430). Macro images were obtained with a stereomicroscope (Stemi 2000-C, Carl Zeiss Microscopy, Jena, Germany) coupled with a digital camera (AxioCam ERc5s, Carl Zeiss Microscopy).

For studies in scanning electron microscopy (SEM), the samples previously fixed in FAA or formaldehyde-glutaraldehyde 2.5% were dehydrated in ethanol and critical-point dried with CO_2 in an 020 CPD dryer (Bal-Tec; Balzers, Liechtenstein). The samples were mounted onto stubs and coated with gold in an FDU 010 sputter-coater (Bal-Tec). Examinations and image captures were conducted with a Leo 1430VP SEM (Zeiss, Cambridge, United Kingdom) at the Centro de Microscopia e Microanálises at the Universidade Federal de Viçosa.

For the classification of the laticifers we followed De Bary (1884). The colleters were classified according to Thomas (1991).

Results

Field Observations of Morphological Features and Secretions

Flowers in the studied species are relatively small (<1 cm), unisexual, and arranged in thyrsoid inflorescences (fig. 1).
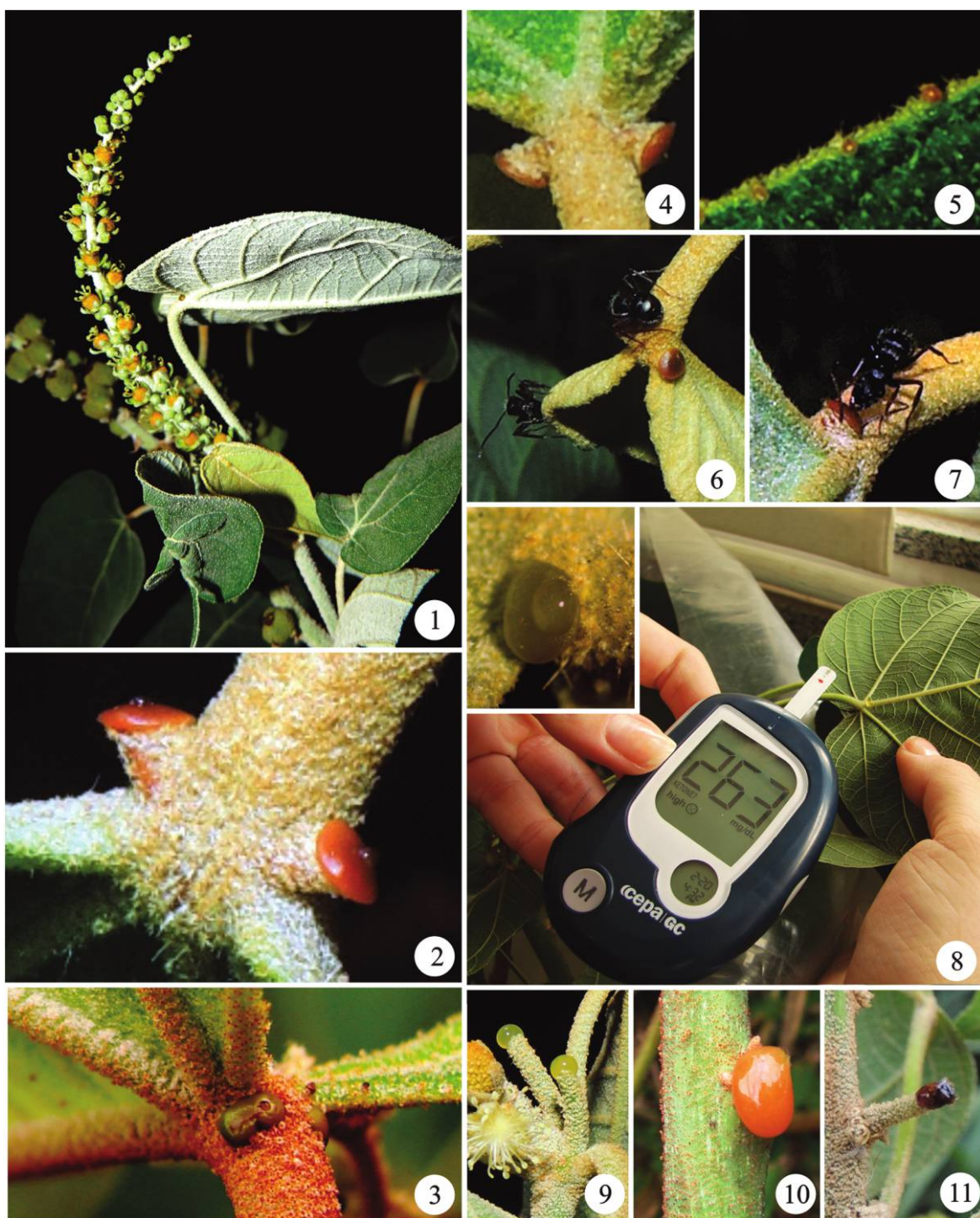
Figs. 1–11 *Croton urucurana* (*1*, *2*, *4*, *6–9*, *11*) and *Croton echinocarpus* (*3*, *5*, *10*) in the field. *1*, Unisexual flowers arranged in thyrsoid inflorescence. *2–4*, Acropetiolar/basilaminar extrafloral nectaries (EFNs): *2*, translucent secretion on the central portion of the EFN; *3*, globular and sessile EFN; *4*, Patelliform and stipitate EFN. *5*, Marginal colleters. *6*, *7*, Ants visiting the EFNs. *8*, Application of Glicofita, showing the glucose concentration. *9–11*, Secretions in different colors of latex exuding from wounded petiole: *9*, *10*, fresh secretions; *11*, dry and oxidized secretion (reddish). Photographs by I. A. C. Coutinho.

Floral nectaries (FNs) in pistillate and staminate flowers and glands along the leaf margin are minute, and at the time of fieldwork we did not observe any secretion with the naked eye. For this reason we were unable to perform the Glicofita Plus test on their secretions.

The leaves of both species present two types of glands. The acropetiolar/basilaminar glands, located at the junction of the petiole with the lamina, are easily noticed by their conspicuous size (figs. 2, 3). The translucent secretion of these glands is produced centrally on the surface of each gland (fig. 2). The glands are globular and sessile in *Croton echinocarpus* (fig. 3) and patelliform and stipitate in *Croton urucurana* (figs. 2, 4). Ovoid glandular structures, much smaller than the acropetiolar/basilaminar glands, are evident along the margin of young leaves and leaf primordia in both species (fig. 5). In mature leaves, these glands are persistent, with a brown coloration, in *C. echinocarpus* and deciduous, leaving a scar, in *C. urucurana*. Ants were observed only visiting (fig. 6) and colleting secretion (fig. 7) of the acropetiolar/basilaminar glands of both species.

The application of Glicofita Plus on the secretion of acropetiolar/basilaminar glands detected significant glucose concentration (263 mg/dL; fig. 8), confirming that these glandular structures are best interpreted as extrafloral nectaries (EFNs). Unfortunately, the secretion of the glands along the leaf margin could not be tested in the field because of the minute size of such glands.

In relation to the latex, we observed a sticky secretion when young branches were cut or damaged. When fresh, the latex was green in *C. urucurana* (fig. 9) and light brown *C. echinocarpus* (fig. 10), and it quickly turned reddish by oxidation of its compounds in both species (fig. 11). The same sticky secretion was observed when we made cuts directly on the trunk bark.

Internal Secretory Structures

Secretory idioblasts are dispersed in ground tissues and epidermis. Leaf epidermal idioblasts, in all developmental stages, are larger than ordinary epidermal cells and are projected outside the surface (fig. 12). In few cases, idioblasts are present at the base of nonglandular trichomes (fig. 13). In pistillate and staminate flowers, secretory idioblasts in different development stages are dispersed in the ground tissues of all floral whorls. Regardless of the region and development phase, idioblasts are fully differentiated, with heavily stained content in flowers and leaves.

Nonarticulated, branched laticifers (fig. 14) are dispersed in the ground tissues of both leaves and flowers. Our observations of shoot meristems show these laticifers to have a Y-shaped branching pattern (inset fig. 14) and evident secretory activity.

External Secretory Structures

Colleters occur on the margin of leaves (fig. 15), on the base, margins, and apex of stipules (fig. 16 and its inset), and in pistillate (figs. 17, 19) and staminate (fig. 18) flowers, where they alternate with FNs. Colleters are fully developed and active in young organs, and they are present in shoot meristems, developing leaves (fig. 15), flower buds (figs. 17–19), and flowers in preanthesis (fig. 20). The structure of these colleters is of the standard type (fig. 16 inset, fig. 21), since they are composed of secretory palisade epidermis covered with a thick cuticle, arranged radially to a nonsecretory and nonvascularized parenchymal central axis. Secretory idioblasts, laticifers, and cells with druse crystals are common among the cells of the central axis of colleters (fig. 21).

Before maturity, leaves exhibit conduplicate ptyxis and are sealed along the margins by both intertwining stellate trichomes and the sticky secretion produced by the marginal colleters (fig. 15). Likewise, the sticky secretion produced by colleters of floral buds overflows and helps to keep the buds closed. When the leaves fully expand and flowers become anthetic, the colleters senesce (figs. 22, 23) and turn brown (*C. echinocarpus* leaves) or are deciduous, each leaving a scar (fig. 25; *C. urucurana* leaves and flowers of both species).
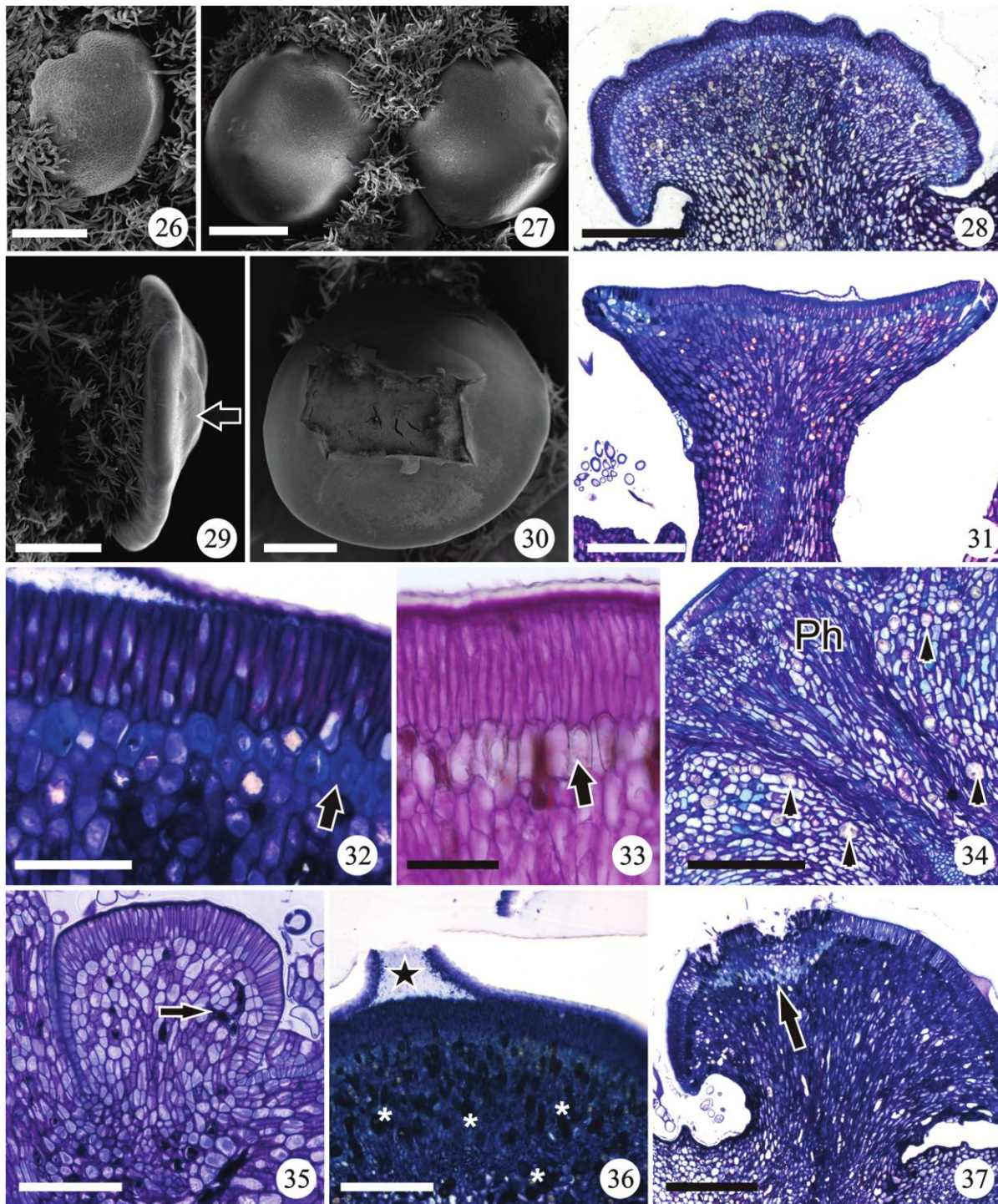
Although the two species present morphologically distinct EFNs—globular and sessile in *C. echinocarpus* (figs. 26–28) and patelliform and stipitate in *C. urucurana* (figs. 29–31)—they are anatomically similar. They have a convex surface consisting of a uniseriate palisade secretory epidermis (fig. 32), with dense protoplast, covered by a smooth and thick cuticle (figs. 28, 31, 32). Underlying the secretory epidermis, there are one or two layers of sclerenchyma (figs. 32, 33) and 10–12 layers of nectariferous parenchyma consisting of isodiametric cells with dense cytoplasmic contents (figs. 32–35). This parenchyma contains druse crystals, secretory idioblasts, and laticifers (figs. 32–36). The EFNs are vascularized by bundles originating from the lamina and/or the petiole. These bundles reach the entire nectariferous parenchyma and consist of both xylem and phloem, with the latter being more abundant (figs. 31, 34). At the base of the EFNs, nonglandular trichomes are also present (figs. 26, 27, 29).

The EFNs present synchronism with leaf development. Before leaf lamina expansion, the EFNs are inactive, and the surface of each has an entire, thick cuticle (fig. 26). Upon leaf expansion, the nectaries become active and accumulate secretion beneath the cuticle of each EFN. This process promotes the distension of the cuticle, mainly toward the center of each EFN (figs. 27, 29), and also its subsequent rupture during the secretory phase (figs. 30, 36). When the EFNs become senescent, a necrotic parenchyma can sometimes be observed (fig. 37).

The FNs of pistillate and staminate flowers do not differ anatomically between the two species. Each flower has a five-lobed FN (fig. 38) located in the region between the outer wall of the ovary and the adaxial side of the sepals (figs. 39, 40). In longitudinal sections of flowers, the lobes are horn-like (figs. 39, 40), whereas in cross sections they present a convex surface with a slight concavity in the central part. The epidermis consists of cuboidal secretory cells, covered by a thin cuticle (figs. 41, 42) with few inactive stomata, through which the secretion of nectar occurs (inset, fig. 40). Longitudinal sections of flowers under fluorescent light show up to three layers of columnar sclerenchyma underlying the secretory epidermis of FN lobes (figs. 43, 44), with an arrangement similar to that in EFNs. The secretory parenchyma is well developed, consisting of vacuolated cells, secretory idioblasts with acidophile content heavily stained with safranin, druse crystals, and laticifers (figs. 41, 42). The vascularization is dense, derived mainly from the vascular system of the receptacle, with one or two branches of vascular bundles that reach the secretory parenchyma (fig. 45); some vascular bundles are surrounded by secretory idioblasts (fig. 41).
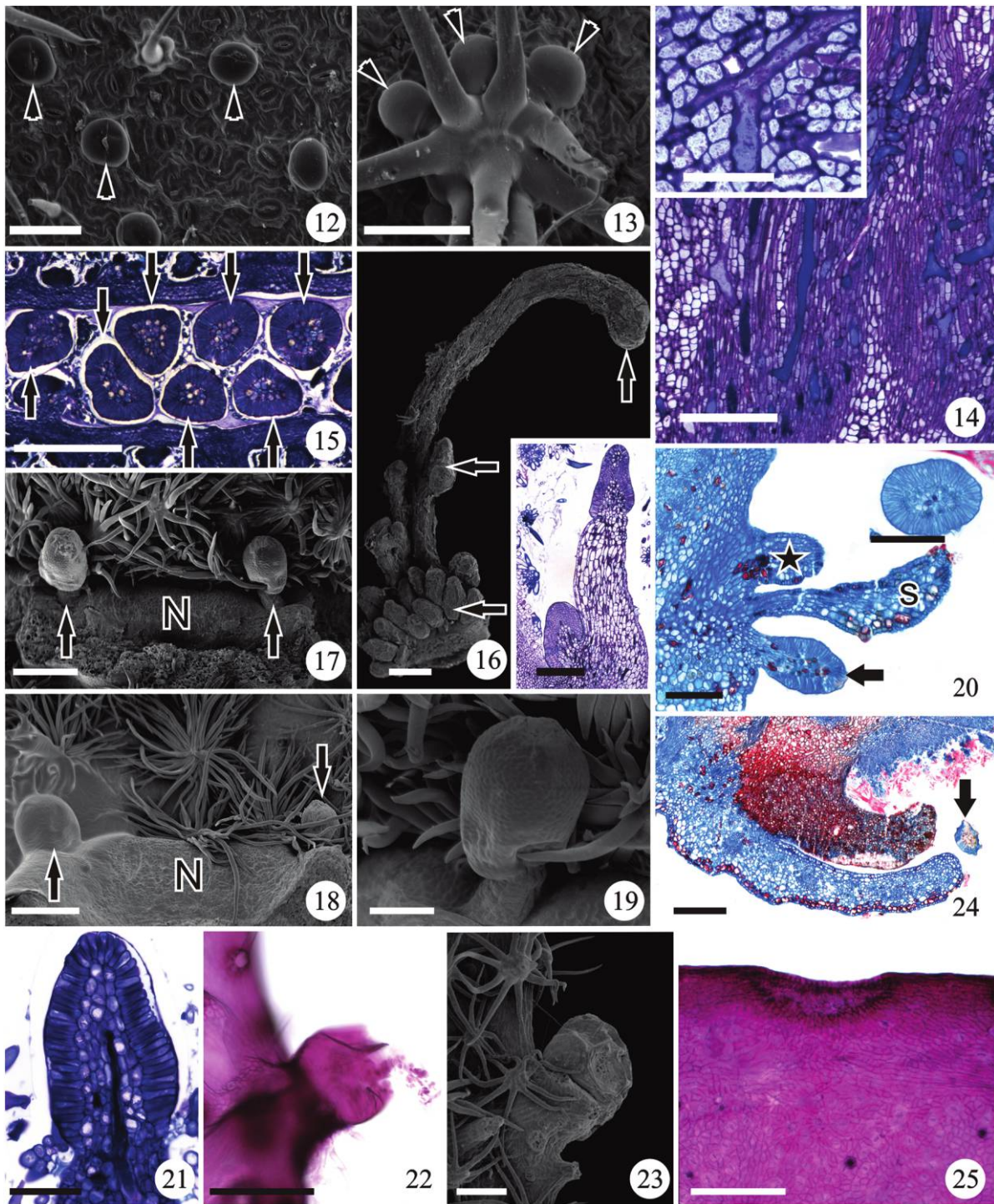
Figs. 12–25 Idioblasts, laticifers, and colleters in *Croton echinocarpus* (*12*, *15*, *18*, *22*, *24*) and *Croton urucurana* (*13*, *14*, *16*, *17*, *19–21*, *23*, *25*). *12*, Idioblasts above the level of the other epidermal cells (arrowheads). *13*, Idioblasts at the base of nonglandular trichome (arrowheads). *14*, Laticifers dispersed in the ground tissues with (*inset*) a nonarticulated laticifer showing a Y-shaped branching pattern. *15*, Colleters (arrows) along the margin of a closed young leaf in a longitudinal section of the stem apex. *16*, Colleters at the base, margins, and apex of stipules (arrows); inset shows the longitudinal section of the stipule. *17*, Colleters (arrows) in pistillate flower, alternating with floral nectary (FN) lobes (N). *18*, Colleters (arrows) in staminate flower, alternating with FN lobes (N). *19*, Detail of colleter. *20*, Staminate flower in longitudinal section, showing the arrangement of floral nectary lobe (star), sepal (S), and colleter (arrow); inset shows the cross section of the colleter. *21*, Colleter of the standard type, showing the parenchymal central axis. *22*, Rupture of colleter. *23*, *24*, Colleters in senescence stage: *23*, colleter on leaf margin; *24*, colleter on pistillate flower in anthesis (arrow). *25*, Scar of leaf marginal colleter. Scale bars = 50 μm (*19*), 100 μm (*12*, *13*, *23*), 150 μm (*21*), 200 μm (*14* inset, *16–18*), 300 μm (*14*), 400 μm (*22*), 500 μm (*15*, *16* inset, *20*, *20* inset, *24*), 600 μm (*25*).

Figs. 26–37 Extrafloral nectaries (EFNs) of *Croton echinocarpus* (*26–28, 32, 34–37*) and *Croton urucurana* (*29–31, 33*). *26–28*, Globular and sessile. *29–31*, Patelliform and stipitate; note the distension of the cuticle in *29* (arrow). *32, 33*, Layers of sclerenchyma (arrow) between the secretory epidermis and nectariferous parenchyma. *34–36*, Nectariferous parenchyma vascularized mainly by phloem (Ph), with secretory idioblasts (asterisks), laticifers (arrow), and druse crystals (arrowheads) in different stages; note that in *36* the cuticle breaks during the secretory stage, yet with little secretion (star). *37*, EFN in senescence stage, with necrotic parenchyma (arrow). Scale bars = 100 μm (*36*), 200 μm (*26, 32, 33, 37*), 400 μm (*27, 29, 30*), 500 μm (*28, 31, 35*), 600 μm (*34*).
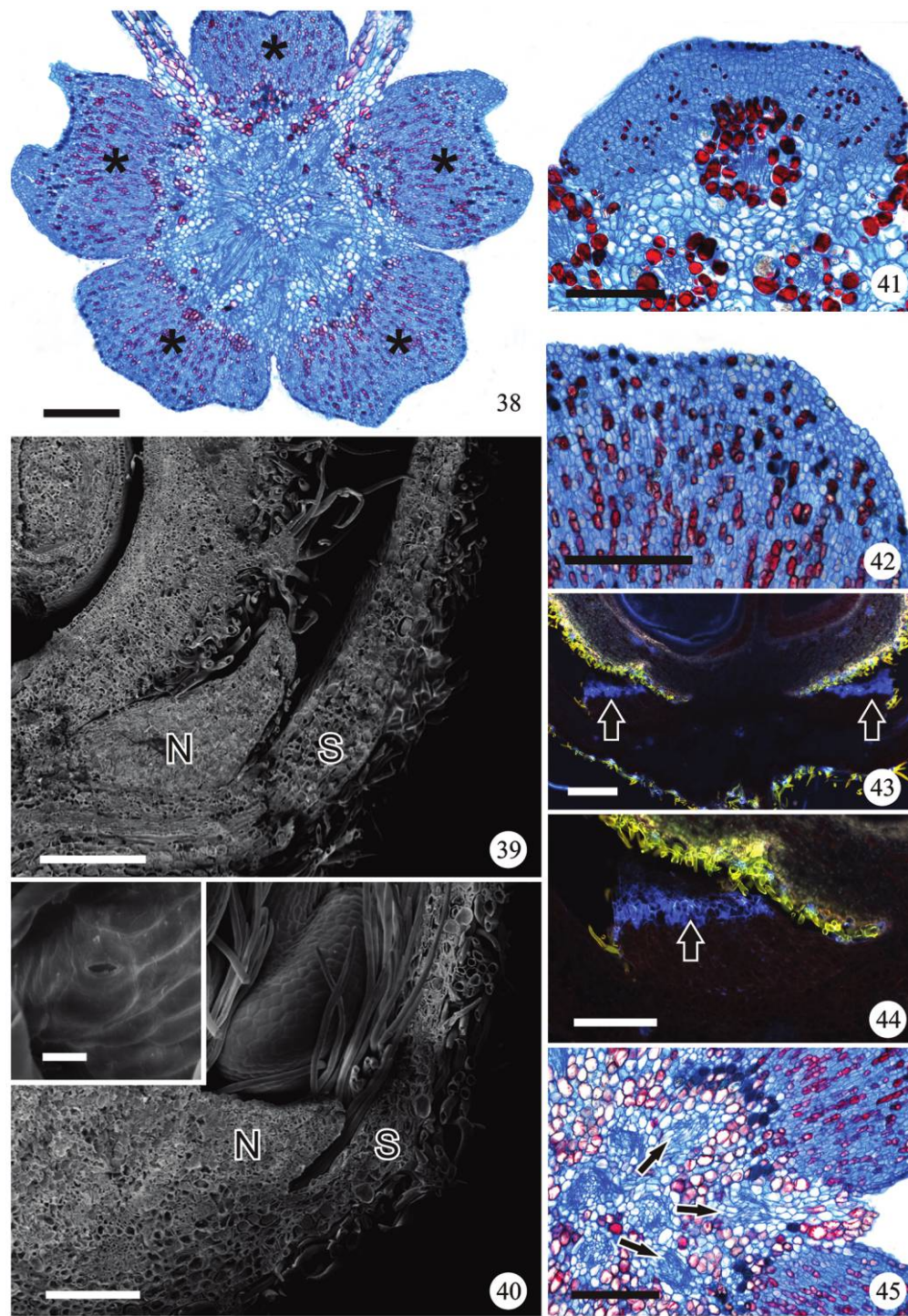
Figs. 38–45 Floral nectaries (FNs) of *Croton urucurana* (*38–40, 42, 45*) and *Croton echinocarpus* (*41, 43, 44*). *38*, Pistillate flower in cross section, showing the five-lobed FN (asterisks). *39, 40*. Horn-like FN lobe (N) opposite sepal (S). *41*, Convex surface of FN lobe. *42*, Detail of FN lobe, with epidermis not differentiated and thin cuticle. *43*, Fluorescent micrograph of FN, exhibiting layers of sclerenchyma in blue (arrow); see detail in *44* (arrow). *45*, General aspect of vascularization (arrows) derived from the receptacle. Bars = 50 μm (*40* inset), 100 μm (*40*), 200 μm (*39, 41*), 300 μm (*42*), 400 μm (*44, 45*), 500 μm (*38*), 600 μm (*43*).

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Histochemical Analysis

The results of histochemical tests are summarized in table 1 and figures 46–72. In laticifers, fatty acids were detected only in *C. urucurana*. The other metabolites (i.e., total lipids, acidic lipids, fatty acids, phenolic compounds, alkaloids, and proteins) were also detected in idioblasts of both species, except carbohydrates. EFNs, FNs, and colleters produce exclusively hydrophilic secretions, rich in carbohydrates and proteins.

Discussion

New Evidence Regarding Idioblasts and Laticifers

For species of tribe Crotoneae, secretory idioblasts have been described as containing lipophilic substances (Webster et al. 1996; Freitas et al. 2001; Sá-Haiad et al. 2009), and Vitarelli et al. (2015) indicated these structures as sites of lipophilic compounds; however, the above-mentioned studies tested only for total lipids and total polysaccharides. Our results show that in *Croton echinocarpus* and *Croton urucurana*, idioblasts secrete compounds of a mixed nature, including both lipophilic (fatty acids) and hydrophilic substances (table 1). We detected alkaloids in both idioblasts and laticifers in all tissues and external secretory structures. In fact, it is possible that part of the synthesis and storage of precursors of indolic, tropanic, and nicotinic alkaloids occurs in the vacuole of secretory idioblasts, as observed in Apocynaceae (DeLuca and Cutler 1987; DeLuca and St-Pierre 2000), and that these structures thus act as transition sites.

The type of laticifer in the two studied species, i.e., branched and nonarticulated, agrees with the pattern described in the literature for section *Cyclostigma* (Rudall 1987, 1989; Wiedenhoeft et al. 2009). In contrast, in *Croton* sections *Alabamenses*, *Lamprocroton*, and *Cleodora*, laticifer type varies (between articulated and nonarticulated), even within the same section (Vitarelli et al. 2015). These authors suggested that articulated laticifers might be the most common and widespread in the entire tribe Crotoneae; however, their taxon sampling was limited, considering the size of *Croton*, and section *Cyclostigma* was among the unsampled clades.

The latex and the cork of dragon's blood *Croton* are used for medicinal purposes throughout the Neotropics (Meza 1999*a*, 1999*b*; Jones 2003; Salatino et al. 2007). Phytochemical studies have reported bactericidal and antifungal (Peres et al. 1997; Gurgel et al. 2005) activities for *C. urucurana*, and both activities are related to the presence of phenolic compounds, alkaloids, and diterpenes. Although diterpenes have not been detected for *C. echinocarpus*, this species also showed bactericidal activity as well as antioxidant and anti-HIV action (Athayde 2013).

Distinguishing Colleters from Nectaries

Colleters and nectaries (EFNs and FNs) in *C. echinocarpus* and *C. urucurana* present hydrophilic secretion, but sugar was investigated only in nectaries. Even if both colleters and nectaries have similar histochemical results, the other two criteria, anatomical characterization and period of secretory activity, allow us to make a distinction between the two structures. When nectaries are still differentiating and unable to produce secretion, colleters are fully developed and active in the shoot meristems. On the other hand, when nectaries become active, colleters become senescent or fall off, showing the asynchronous

Table 1

Results of Histochemical Tests Applied to the Secretion of Different Structures Present in *Croton echinocarpus* and *Croton urucurana*

| | Species, secretory structures | | | | | | | | | |
| | *C. echinocarpus* | | | | | *C. urucurana* | | | | |
Tests	Col	Id	EFN	FN	Lat	Col	Id	EFN	FN	Lat
Carbohydrates:										
PAS	+	−	+	+	+	+	−	+	+	+
Ruthenium red	+	−	+	+	+	+	−	+	+	+
Tannic acid/ferric chloride	+	−	+	+	−	+	−	+	+	−
Lipids/terpenoids:										
Sudan black B	−	+	−	−	+	−	+	−	−	+
Neutral red	−	+	−	−	+	−	+	−	−	+
NADI	−	−	−	−	−	−	−	−	−	−
Copper acetate/rubeanic acid	−	+	−	−	−	−	+	−	−	+
Nile blue	−	+	−	−	+	−	+	−	−	+
Phenolic compounds:										
FSF	−	+	−	−	+	−	+	−	−	+
Vanillin/hydrochloric acid	−	−	−	−	−	−	−	−	−	−
Alkaloids:										
Wagner's reagent	−	+	+	+	+	−	+	+	+	+
Proteins:										
Xylidine ponceau	+	+	+	+	+	+	+	+	+	+
Ninhydrin/Schiff's reagent	+	+	+	+	+	+	+	+	+	+

Note. A plus sign denotes a positive result; a minus sign denotes a negative result; Col = colleter; Id = idioblast; EFN = extrafloral nectary; FN = floral nectary; Lat = laticifer; PAS = periodic acid–Schiff reagent; FSF = ferrous sulfate in formalin.
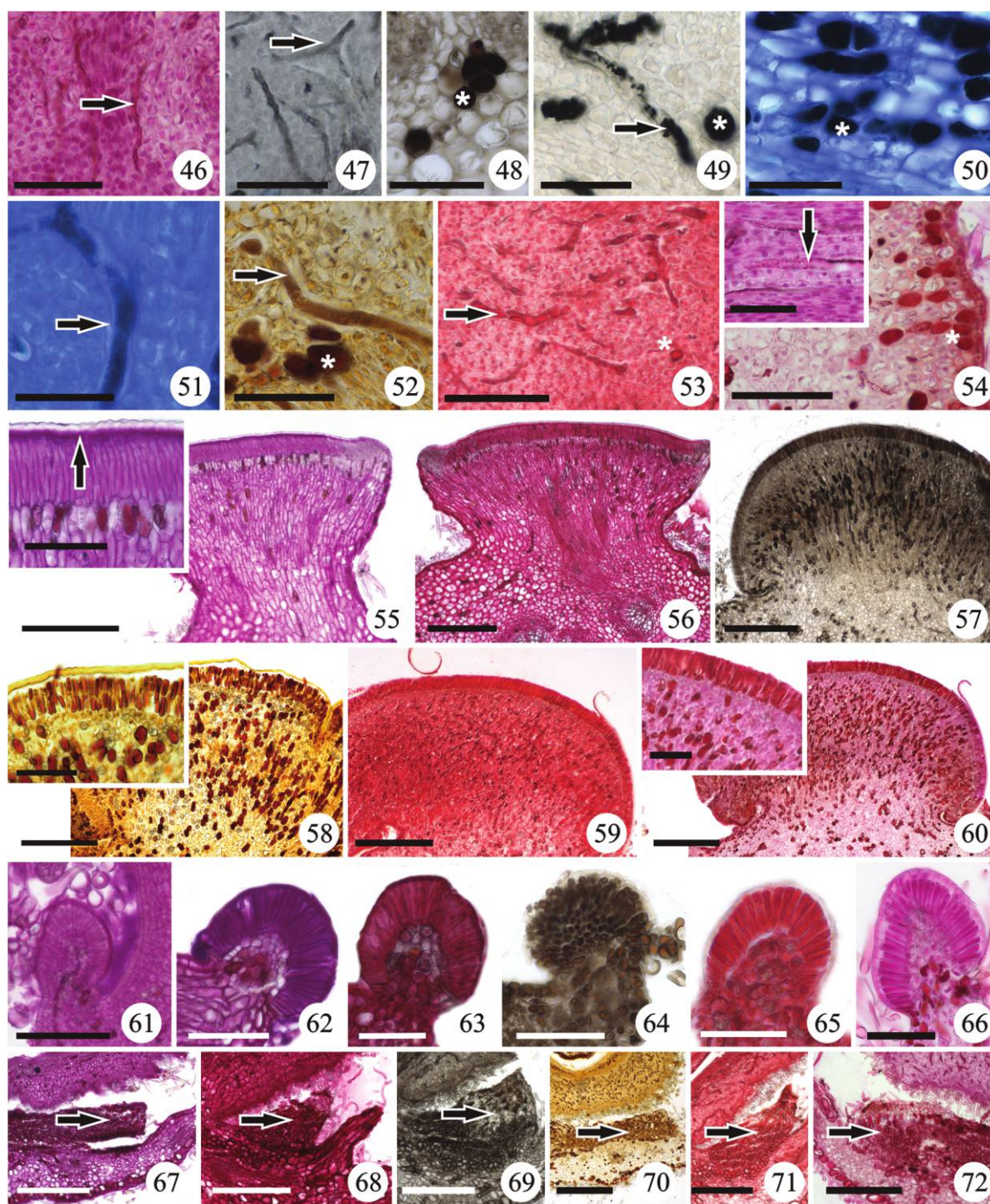
Figs. 46–72 Positive histochemical results on the secretions of the secretory structures in *Croton echinocarpus* (*46*, *48*, *50*, *53*, *54*, *57*, *59*, *60*, *62*, *64*, *65*, *69*, *71*) and *Croton urucurana* (*47*, *49*, *51*, *52*, *55*, *56*, *58*, *61*, *63*, *66–68*, *70*, *72*). *46–54*, Idioblasts (white asterisks) and laticifers (arrows). *55–60*, Extrafloral nectaries. *61–66*, Colleters showing the reaction in the secretory epidermis. *67–72*, Floral nectaries (arrows): *67*, *70*, *72*, pistillate flower; *68*, *69*, *71*, staminate flower. *46*, *55*, *61*, *62*, *67*, Periodic acid–Schiff reaction showing total polysaccharides; note the inset in *55* showing strong reaction below cuticle (arrow). *56*, *63*, *68*, Ruthenium red: acidic mucilage. *57*, *64*, *69*, Tannic acid/ferric chloride: neutral mucilage. *47*, Sudan black B: total lipids. *48*, Copper acetate/rubeanic acid: fatty acids. *49*, Ferrous sulfate in formalin: phenolic compounds. *50*, *51*, Nile blue: acidic lipids. *52*, *58*, *70*, Wagner's reagent: alkaloids; note the inset in *58* showing the reaction on secretory epidermis and idioblasts. *53*, *59*, *65*, *71*, Xilidine ponceau: total proteins. *54*, *60*, *66*, *72*, Ninhydrin/Schiff's reagent: proteins; note the inset in *60* showing secretory epidermis and idioblasts. Bars = 100 μm (*46–52*, *54*, *54* inset, *61–66*), 150 μm (*55* inset), 200 μm (*58*, *60* insets), 250 μm (*53*), 500 μm (*58*), 600 μm (*67–69*, *71*, *72*), 800 μm (*55–57*, *59*, *60*, *70*).

development and activity of these structures only in the early stages of development. This asynchrony between colleters and nectaries was also observed by Riina et al. (2015) and Vitarelli et al. (2015) for other species of *Croton*. Although we did not conduct systematic measurements of colleters and nectaries at maturity, our field observations suggest that size and external shape might be useful characters to distinguish these two structures. In fact, the reason we could not conduct the Glicofita Plus test on the secretion of colleters (marginal glands) was that the colleters were minute and smaller than nectaries. Future studies should look at the size and external shape of colleters relative to nectaries, using an overall sampling of the genus to corroborate these observations.

Sá-Haiad et al. (2009) studied several species of tribe Crotoneae, including *C. echinocarpus* and *C. urucurana*; however, the leaf marginal glands described by these authors as EFNs are actually colleters of the standard type (Riina et al. 2014, 2015; Vitarelli et al. 2015). Our results show that colleters are also present in the reproductive organs of *C. echinocarpus* and *C. urucurana*, where they alternate with the lobes of the FN, and a recent study documented colleters along the inflorescence axis of *Croton glandulosus* L. (Machado et al. 2015).

In the vegetative organs of *C. echinocarpus* and *C. urucurana*, colleters occur only along the leaf margin and at the base and margin of stipules, which is similar to what was found for *Croton amentiformis* Riina, another species of section *Cyclostigma* (Riina et al. 2015). These leaf marginal colleters distinguish species of section *Cyclostigma* from other *Croton* groups, where such structures alternate with EFNs along the leaf margin, as in section *Cupreati* (A. C. Feio, R. M. S. A. Meira, and R. Riina, unpublished manuscript) and section *Cuneati* (Riina et al. 2010, their fig. 3*F*). In taxonomic descriptions of *Croton*, leaf marginal colleters have been called "ovoid glands" (Riina et al. 2010) or simply "glands" (e.g., Caruzo and Cordeiro 2013). None of these terms is incorrect; however, "gland" is a generic term used just to recognize a structure usually protruding and secretory.

De-Paula et al. (2011) observed secretory structures in flowers of *Croton* (including *C. urucurana*) with the same colleter position found here; however, they described them as filamentous structures instead of as colleters. Likewise, structures on the surface of sepals were described as secretory trichomes instead of as colleters, even if the authors recognized their similarity to colleters. To date, the absence of colleters in the flowers of *Croton* was one important difference separating this genus from its close relative, the genus *Astraea* (De-Paula et al. 2011); however, our results suggest that colleters in flowers appear not to be exclusive to *Astraea* and could be widespread in *Croton*, given the relatively conserved floral morphology across the genus (van Ee et al. 2011).

We found sclerenchyma cells forming a continuous layer in active EFNs and senescent FNs, under the secretory epidermis, which was also observed by Freitas and Paoli (1999) in EFNs of *C. urucurana*. Sclerenchyma cells in nectaries are considered a specialized feature that may be related to the protection of the parenchymatic secretory tissue (Belin-Depoux 1989). Another characteristic regarded as highly specialized is the vascularization of nectaries (Elias 1983), as observed in *C. echinocarpus* and *C. urucurana*. The vascularized nectaries found here support the findings of Vitarelli et al. (2015) regarding EFNs and De-Paula et al. (2011) for FNs.

The role of FNs in *Croton* is still unclear and requires further investigation with better taxon sampling across the genus. The few pollination studies of *Croton* available report entomophilous and anemophilous species, such as *C. urucurana* (Pires et al. 2004) and *Croton sarcopetalus* Müll. Arg. (Freitas et al. 2001), respectively, as well as several ambophilous species (Bullock 1994; Webster 1994). On the other hand, Narbona and Dirzo (2010) described FNs that possess extranuptial and nuptial functions in *Croton suberosus* Kunth (i.e., not related to pollination). In *C. urucurana*, it has been hypothesized that the FNs are not essential for pollination because of the amount of nectar produced and the morphology of the flowers, which better fit the anemophilous syndrome (Pires et al. 2004). The anemophilous syndrome has also been suggested for *Croton floribundus* Spreng. and *Croton priscus* Croizat (Passos 1995).

Histochemical Evidence

The histochemical tests detected proteins in the secretions of EFNs, FNs, colleters, laticifers, and idioblasts. The presence of proteins in laticifers can be due to the fact that the latex blends with the cytoplasmic contents of the laticifer cell, where proteins are usually found (Demarco et al. 2006). On the other hand, the proteins present in nectaries can be related to the energetic demands for nitrogen of flower visitors and pollinators (Nicolson and Thornburg 2007), since many insects have deficiencies in the production of proteins (Baker 1977).

In the case of colleters, of which the primary function is lubrication and protection against desiccation of organs in early development (Fahn 1979), proteins are also important for protection against herbivores and pathogens (Klein et al. 2004; Miguel et al. 2006). In addition, the presence of mucilage in these structures also indicates their classification as colleters. Mucilage is important for the protection of developing organs, water retention, and defense against herbivores (Fahn 1979; Gregory and Baas 1989).

The classes of secondary metabolites found in this study (table 1) support the biological activities described in the literature for *C. urucurana* and *C. echinocarpus*, especially antifungal, anti-inflammatory, and antioxidant activities (Gurgel et al. 2005; Salatino et al. 2007; Simionatto et al. 2007). These properties are related to substances primarily derived from phenolic compounds such as catechin, terpenes such as acetyl aleuritic acid (Gurgel et al. 2005), and alkaloids such as taspine (Salatino et al. 2007).

Conclusion

The great similarity in morphoanatomy and position of secretory structures between *Croton echinocarpus* and *Croton urucurana* is in agreement with their phylogenetic proximity (Riina et al. 2009) and habitat affinities. We detected the presence of colleters in *Croton* flowers, a structure that was overlooked or misinterpreted in previous studies dealing with floral anatomy of *Croton* and may be a unifying character between this genus and the closely related genus *Astraea*. The diversity of secretory structures and the chemical compounds detected confirm the potential of these species for bioprospection.

Despite having similar histochemical compositions, nectaries and colleters can be distinguished on the basis of their

structure, function, and period of activity. Our results show that the use in combination of the three criteria applied here (anatomical structure, histochemistry, and period of activity) allows a more accurate classification and homology assessment of secretory elements in a genus exceptionally rich in this type of structures.

Acknowledgments

Literature Cited

Athayde NR 2013 Perfil químico e atividades biológicas de *Croton echinocarpus Baill*. e *Croton vulnerarius* Müll.Arg. MSc diss. Instituto de Biociências, Universidade de São Paulo.

Baker HG 1977 Non-sugar chemical constituents of nectar. Apidologie 8:349–356.

Barreiro DP, SR Machado 2007 Coléteres dendróides em *Alibertia sessilis* (Vell.) K. Schum., uma espécie não-nodulada de Rubiaceae. Rev Bras Bot 30:387–399.

Belin-Depoux M 1989 Des hydathodes aux nectaires foliaires chez les plantes tropicales. Bull Soc Bot Fr 136:151–168.

Bentley BL, TS Elias 1983 The biology of nectaries. Columbia University Press, New York.

Bukatsch F 1972 Bermerkungen zur Doppelfärbung Astrablau-Safranin. Mikrokosmos 61:255.

Bullock SH 1994 Wind pollination of Neotropical dioecious trees. Biotropica 26:172–179.

Cain AJ 1947 The use of Nile blue in the examination of lipids. Q J Microsc Sci 33:383–392.

Caruzo MBR, I Cordeiro 2007 Sinopse da tribo Crotoneae Dumort. (Euphorbiaceae s.s.) no Estado de São Paulo, Brasil. Hoehnea 34:571–585.

——— 2013 Taxonomic revision of *Croton* section *Cleodora* (Euphorbiaceae). Phytotaxa 121:1–41.

Caruzo MBR, RF dos Santos 2015 First record of *Croton echinocarpus* (Euphorbiaceae: Crotoneae) in São Paulo state, Brazil. Check List 11 (4):1684. doi:10.15560/11.4.1684.

Cordeiro I, R Secco, JM Cardiel, V Steinmann, MBR Caruzo, R Riina, LR de Lima, et al. 2015 *Euphorbiaceae. In* Lista de Espécies da Flora do Brasil. Jardim Botânico do Rio de Janeiro. http://floradobrasil .jbrj.gov.br/jabot/floradobrasil/FB17497. Accessed April 9, 2015.

Cruz MAL, VM Gomes, KVS Fernandes, OLT Machado, J Xavier-Filho 2002 Identification and partial characterization of a chitinase and a β-1,3-glucanase from *Copernicia cerifera* wax. Plant Physiol Biochem 40:11–16.

David R, JP Carde 1964 Coloration différentielle des inclusions lipidique et terpéniques des pseudophylles du *Pin maritime* au moyen du réactif Nadi. CR Acad Sci Paris 258:1338–1340.

De Bary A 1884 Comparative anatomy of the vegetative organs of the phanerogams and ferns. Clarendon, Oxford.

DeLuca V, AJ Cutler 1987 Subcellular localization of enzymes involved in indole alkaloid biosynthesis in *Catharanthus roseus*. Plant Physiol 85:1099–1102.

DeLuca V, B St-Pierre 2000 The cell and developmental biology of alkaloid biosynthesis. Trends Plant Sci 5:168–173.

Demarco D, LS Kinoshita, MM Castro 2006 Laticíferos articulados anastomosados—novos registros para Apocynaceae. Rev Bras Bot 29:133–144.

De-Paula OC, MG Sajo, G Prenner, I Cordeiro, PJ Rudall 2011 Morphology, development and homologies of the perianth and floral nectaries in *Croton* and *Astraea* (Euphorbiaceae-Malpighiales). Plant Syst Evol 292:1–14.

Elias TS 1983 Extrafloral nectaries: their structure and distribution. Pages 174–203 *in* B Bentley, T Elias, eds. The biology of nectaries. Columbia University Press, New York.

Fahn A 1979 Secretory tissues in plants. Academic Press, London.

Freitas L, G Bernardello, L Galetto, AAS Paoli 2001 Nectaries and reproductive biology of *Croton sarcopetalus* (Euphorbiaceae). Bot J Linn Soc 136:267–277.

Freitas L, AAS Paoli 1999 Structure and ultrastructure of the extrafloral nectaries of *Croton urucurana* Baill. (Euphorbiaceae). Bol Bot Univ São Paulo 18:1–10.

Furr M, Mahlberg PG 1981 Histochemical analyses of laticifers and glandular trichomes in *Cannabis sativa*. J Nat Prod 44:153–159.

Ganter P, G Jollés 1969–1970 Histologie normale et pathologique. 2 vols. Gauthier-Villars, Paris.

Gregory M, P Baas 1989 A survey of mucilage cells in vegetative organs of the dicotyledons. Isr J Bot 38:125–174.

Gurgel LA, JJC Sidrim, DT Martins, V Cechinel Filho, VS Rao 2005 In vitro antifungal activity of dragon's blood from *Croton urucurana* against dermatophytes. J Ethnopharmacol 97:409–412.

Johansen DA 1940 Plant microtechnique. 2nd ed. McGraw-Hill, New York.

Jones K 2003 Review of sangre de drago (*Croton lechleri*)—a South American tree sap in the treatment of diarrhea, inflammation, insect bites, viral infections, and wounds: traditional uses to clinical research. J Altern Complement Med 9:877–896.

Kaiser E 1880 Verfahren zur herstellung einer tadellosen glyceringelatine. Bot Zentbl 180:25–26.

Karnovsky MJ 1965 A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. J Cell Biol 27:137A.

Kirk PW Jr 1970 Neutral red as a lipid fluorochrome. Stain Technol 45:1–4.

Klein DE, VM Gomes, SJ Silva-Neto, M da Cunha 2004 The structure of colleters in several species of *Simira* (Rubiaceae). Ann Bot 94:733–740.

Kropp U 1972 Leitbündel. Mikrokosmos 61:342–345.

Lersten NR, JD Curtis 1996 Survey of leaf anatomy, especially secretory structures, of tribe Caesalpinieae (Leguminosae, Caesalpinioideae). Plant Syst Evol 200:21–39.

Lillie RD 1965 Histopathologic technic and practical histochemistry. 3rd ed. McGraw-Hill, New York.

Mace ME, CR Howell 1974 Histochemistry and identification of condensed tannin precursors in roots of cotton seedlings. Can J Bot 52:2423–2426.

Machado SR, LM Paleari, EAS Paiva, TM Rodrigues 2015 Colleters on the inflorescence axis of *Croton glandulosus* (Euphorbiaceae): structural and functional characterization. Int J Plant Sci 176:86–93.

McManus JFA 1948 Histological and histochemical uses of periodic acid. Stain Technol 23:99–108.

Meza EN 1999*a* Cosecha de sangre de grado (*Croton* spp.) y factores que influyen en su abundancia. Pages 45–76 *in* EN Meza, ed. Desarrollando nuestra diversidad cultural: "sangre de grado" y el reto de su producción en el Perú. Fondo Editorial, Universidad Nacional Mayor de San Marcos.

——— 1999*b* Nombres aborígenes peruanos de las especies de *Croton* que producen el latex denominado "sangre de grado." Pages 25–44 in EN Meza, ed. Desarrollando nuestra diversidad cultural: "sangre de grado" y el reto de su produccion en el Peru. Fondo Editorial, Universidad Nacional Mayor de San Marcos.

Miguel EC, VM Gomes, MA Oliveira, M Cunha 2006 Colleters in *Bathysa nicholsonii* K. Schum. (Rubiaceae): ultrastructure, secretion protein composition, and antifungal activity. Plant Biol 8:715–722.

Narbona E, Dirzo R 2010 A reassessment of the function of floral nectar in *Croton suberosus* (Euphorbiaceae): a reward for plant defenders and pollinators. Am J Bot 97:672–679.

Nicolson SW, Thornburg RW 2007 Nectar chemistry. Pages 215–264 *in* SW Nicolson, M Nepi, E Pacini, eds. Nectaries and nectar. Springer, Dordrecht.

O'Brien TP, N Feder, ME McCully 1965 Polychromatic staining of plant cells walls by toluidine blue O. Protoplasma 59:368–373.

O'Brien TP, ME McCully 1981 The study of plant structure: principles and selected methods. Termarcarphi, Melbourne.

Passos L 1995 Fenologia, polinização e reprodução de duas espécies de *Croton* (Euphorbiaceae) em mata semidecídua. MSc diss. Universidade de Campinas.

Pearse AGE 1985 Histochemistry theoretical and applied. Vol 2. 4th ed. Livingstone, Edinburgh.

Peres MTLP, F Delle Monache, AB Cruz, MG Pizzolatti, RA Yunes 1997 Chemical composition and antimicrobial activity of *Croton urucurana* Baillon (Euphorbiaceae). J Ethnopharmacol 56:223–226.

Pires MMY, LA Souza, Y Terada 2004 Biologia floral de *Croton urucurana* Baill. (Euphorbiaceae) ocorrente em vegetação ripária da ilha Porto Rico, Porto Rico, Estado do Paraná, Brasil. Acta Sci Biol Sci 26:209–215.

Pizzolato TD, RD Lillie 1973 Mayer's tannic acid-ferric chloride stain for mucins. J Histochem Cytochem 21:56–64.

Riina R, PE Berry, BW van Ee 2009 Molecular phylogenetics of the dragon's blood *Croton* sect. *Cyclostigma* (Euphorbiaceae): a polyphyletic assemblage unraveled. Syst Bot 34:360–374.

Riina R, N Cumbicus, AC Feio, CE Cerón, RMSA Meira, PE Berry 2015 A new species of dragon's blood *Croton* (Euphorbiaceae) from South America with singular inflorescences. Webbia 70:187–192.

Riina R, BW van Ee, AC Wiedenhoeft, A Cardozo, PE Berry 2010 Sectional rearrangement of arborescent clades of *Croton* (Euphorbiaceae) in South America: evolution of arillate seeds a new species, *Croton domatifer*. Taxon 59:1147–1160.

Riina R, MA Vigo, CE Ceron 2014 *Croton condorensis*: an enigmatic new species of Euphorbiaceae from southern Ecuador. Phytotaxa 164:154–158.

Rudall PJ 1987 Laticifers in Euphorbiaceae—a conspectus. Bot J Linn Soc 94:143–163.

——— 1989 Laticifers in vascular cambium and wood of *Croton* spp. (Euphorbiaceae). IAWA Bull 10:379–383.

Sá-Haiad B, ACC Serpa-Ribeiro, CN Barbosa, D Pizzini, DO Leal, L Senna-Valle, LDR Santiago-Fernandes 2009 Leaf structure of species from three closely related genera from tribe Crotoneae Dumort. (Euphorbiaceae *s.s.*, *Malpighiales*). Plant Syst Evol 283:179–202.

Salatino A, MLF Salatino, G Negri 2007 Traditional uses, chemistry and pharmacology of *Croton* species (Euphorbiaceae). J Braz Chem Soc 18:11–33.

Shobe WR, NR Lersten 1967 A technique for clearing and staining gymnosperm leaves. Bot Gaz 128:150–152.

Simionatto E, VFL Bonani, AF Morel, NR Poppi, JLR Júnior, CZ Stuker, GM Peruzzo, MTLP Peres, SC Hessa 2007 Chemical composition and evaluation of antibacterial and antioxidant activities of the essential oil of *Croton urucurana* Baillon (Euphorbiaceae) stem bark. J Braz Chem Soc 18:879–885.

Thomas V 1991 Structural, functional and phylogenetic aspects of the colleter. Ann Bot 68:287–305.

van Ee BW, R Riina, PE Berry 2011 A revised infrageneric classification and molecular phylogeny of new world *Croton* (Euphorbiaceae). Taxon 60:1–33.

Vitarelli NC, R Riina, MBR Caruzo, I Cordeiro, J Fuertes-Aguilar, RMSA Meira 2015 Foliar secretory structures in Crotoneae (Euphorbiaceae): diversity, anatomy, and evolutionary significance. Am J Bot 12:1–15.

Webster GL 1994 Synopsis of the genera and suprageneric taxa of Euphorbiaceae. Ann Mo Bot Gard 81:33–144.

Webster GL, MJ Del-Arco-Aguilar, BA Smith 1996 Systematic distribution of foliar trichome types in *Croton* (Euphorbiaceae). Bot J Linn Soc 121:41–57.

Wiedenhoeft AC, R Riina, PE Berry 2009 "Ray-intrusive" laticifers in species of *Croton* section *Cyclostigma* (Euphorbiaceae). IAWA J 30:135–148.