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Comparative anatomy of calyx and foliar glands of *Banisteriopsis* C. B. Rob. (Malpighiaceae)

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ABSTRACT

Banisteriopsis is considered one of the largest genera of Malpighiaceae with 58 species, of which 47 occur in Brazil. The typical calyx and leaf glands of *Banisteriopsis* are considered relevant to the adaptive success of Malpighiaceae. Comparative studies of anatomical and histochemical characteristics may reveal similarities and assist in the interpretation of the functions performed by such glands. The present study aimed to describe the anatomy of the calyx and leaf glands of 38 species of *Banisteriopsis* that occur in Brazil, and to analyze these structures histochemically in *B. campestris*, *B. laevifolia* and *B malifolia*, using standard methods. Calyx glands differ from leaf glands by possessing an irregular surface that is covered by a thick cuticle that is released from the epidermis by the accumulation of secretion; the glands are similar in all the other anatomical characteristics. Both types of glands produce secretions composed of a mixture of protein granules, lipids and polysaccharides. These findings reinforce the hypothesis that foliar glands have given rise to calyx glands in response to interactions with pollinators.

Keywords: *Banisteriopsis*, calyx glands, histochemistry, leaf glands, lipids, micromorphology, polysaccharides, protein

Introduction

Malpighiaceae, with ca. 1300 species, is widespread and is very diverse in the New World tropics and subtropics, where 80% of the species are endemic (Anderson 1990). The taxonomy of Malpighiaceae has changed as a result of recent molecular phylogenetic studies (Cameron *et al.* 2001; Davis *et al.* 2001; Davis & Anderson 2010). The former subfamilies, tribes (except Gaudichaudieae) and some genera were found to be polyphyletic, with some characters being recognized as homoplastic, especially regarding fruit morphology (Cameron *et al.* 2001; Davis *et al.* 2001; Davis & Anderson 2010).

Neotropical Malpighiaceae exhibit great diversity in habit, types of fruits, pollen morphology and chromosome number (Anderson 1979), however, the species have very similar floral structure. The flowers of Malpighiaceae have a calyx comprised of five free or basally connate sepals, of which four or all five usually bear two large multicellular glands (Anderson 1979). The five free petals are conspicuously clawed, and often reflex between the sepals in such a way that the calyx glands are readily accessible to insects that have landed in the middle of the flower (Anderson 1979). The flag petal stands at the back of the flower on the plane of symmetry and the eglandular sepal (if present) lies at the front of the flower on the plane of symmetry. When stamens or styles are heteromorphic they tend to remain symmetrical with respect to this front-to-back axis (Niedenzu 1928; Anderson 1977; 1979; 1990). The hypothesis for the conservation of floral morphology within the Malpighiaceae for tens of millions of years of evolution is based on the selection imposed by their specialist New World oil-bee pollinators (Davis *et al.* 2014).

Calyx glands are present in approximately 90% of the New World species of Malpighiaceae and 52% of the Old World species (Vogel 1990). The secretion produced by these glands may act as a reward to visitors or pollinators (Vogel 1990). The glands of Neotropical species produce non-volatile oils and are called elaiophores. On the other

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hand, in paleotropical Malpighiaceae flowers such structures produce nectar, and thus correspond to floral nectaries (Vogel 1974 cited in Anderson 1990; Vogel 1990).

Elaiophores are used as a diagnostic character for representatives of neotropical Malpighiaceae. In this group, oil and pollen produced by flowers attract visitors and/or pollinators specialized in collecting the oil, especially female bees of the tribe Centridini (Anderson 1979). The oil collected by the bees is used to feed their larvae and to line the nests and brood cells (Anderson 1979; Costa *et al.* 2006). These bees have replaced using nectar sugar for feeding larvae with flower oil because it contains more calories (Santos *et al.* 2007). On the other hand, the floral nectaries of paleotropical species are not related to the pollination syndrome (Vogel 1974 cited in Anderson 1990; Vogel 1990).

While floral nectaries function in flower pollination, extrafloral nectaries (EFNs) are involved in animal-mediated strategies, such as ecological interactions enabling plant protection, especially protection provided by ants with aggressive behavior that are attracted by these nectar sources (Schultz & McGlynn 2000; Cogni & Freitas 2002; Cogni *et al.* 2003).

In addition to calyx glands, leaf glands are common in Malpighiaceae (Elias 1983). Based on the anatomical similarity of calyx and leaf glands, Vogel (1990) comments that elaiophores have risen from the modification of nectaries in response to the mutualism between flowers and wild bees of the tribe Centridini.

Banisteriopsis is considered one of the largest genera of Malpighiaceae and is well represented in Brazil, where 47 of the 58 New World species are found (Gates 1982; Anderson & Davis 2006; Davis & Anderson 2010). Despite the importance of this genus and the involvement of calyx glands in the adaptive success of Malpighiaceae, there have been few studies that relate the anatomy of secretory structures occurring on the sepals and leaves to the chemical nature of their secretion. Such information would certainly be useful for ecological and taxonomic studies.

Thus, the present study aimed to describe and compare the anatomy of calyx and leaf glands of species of *Banisteriopsis* that occur in Brazil, and to histochemically analyze these glands in *B. campestris*, *B. laevifolia* and *B. malifolia*.

Materials and methods

Glands of 38 species (40 taxa) were analyzed, however, calyx glands of *B. adenopoda* (A. Juss.) B. Gates, *B. paraguariensis* B. Gates and *B. sellowiana* (A. Juss.) B. Gates were not examined because there were not enough flower samples. It was possible to make comparative evaluations of the calyx and leaf glands of 26 species (Tab. 1). The samples were obtained from different Brazilian herbaria (Tab. 1) and at least three specimens of each species were analyzed for replication, except for rare species or if only one exsiccate was available.

Leaves and flowers were obtain from herbarium material and subjected to the herborization reversion process which consists of boiling samples in distilled water for 10 min followed by submerging the samples in 2% potassium hydroxide solution for 2h at room temperature (Smith & Smith 1942). These samples were then rinsed in tap water five times, dehydrated in an ethanol series (30–70 %) and stored in 70 % ethanol.

For anatomical study, fragments of gland bearing blades were sectioned by freehand, cleared in 20% sodium hypochlorite, stained with basic fuchsin and astra blue (Kraus & Arduin 1997) and mounted on slides with glycerinated gelatin (Kraus & Arduin 1997). Since we could not obtain hand sections of sepals bearing glands, these samples were dehydrated through an ethanol series and embedded in methacrylate resin (Historesin Leica, Leica Microsystems Nussloch GmbH, Heidelberg, Germany) according to Meira & Martins (2003). Cross and longitudinal sections that were 7 microns thick were made using an automatic rotary microtome (Spencer 820, American Optical Corporation, Buffalo, NY) with a glass knife. The sections were stained with toluidine blue pH 4 (O'Brien & McCully 1981)

In order to perform histochemical tests *Banisteriopsis campestris* (Adr. Jussieu) E. L. Little, *B. laevifolia* (Adr. Jussieu) B. Gates and *B. malifolia* B. Gates were selected since these species had already been analyzed by Araújo *et al.* (2010), which made the identification and collection in the field easier. Samples of vegetative and floral branches of these three species were collected in Cerrado in the Floresta Nacional – FLONA (MG, Brazil) and samples of *B. campestris* were also collected in *campo rupestr*e in the Serra do Ouro Branco (MG, Brazil) State Park. Both leaf and calyx glands were fixed in glutaraldehyde. Sections of the samples were cut by freehand and submitted to the following histochemical tests: periodic acid–Schiff reagent (PAS) for general polysaccharides (McManus 1948); lugol reagent for starch (Johansen 1940); Coomassie blue for total proteins (Fisher 1968) and ninhydrin-Schiff (Yasuma & Ichikawa 1953); Sudan black B and neutral red under fluorescence for lipid compounds (Kirk 1970); Nadi reagent for essential oils and oleoresins (David & Carde 1964); phloroglucinol for lignins (Jensen 1962); and Wagner reagent for alkaloids (Furr & Mahlberg 1981). Controls were performed simultaneously.

Analysis and image capture were performed using an light microscope (model AX70TRF, Olympus Optical, Tokyo, Japan) that was equipped with a U-Photo System and an AxioCam HRc digital camera (Zeiss, Göttingen, Germany). Observations were also made using an epifluorescence microscope (Olympus Optical, Tokyo, Japan) with a mercury lamp (HBO 50-W), a BP 340-380 excitation filter, a dichroic mirror 459, and a LP-430 filter (Ushio USH-102D, Japan).

Micromorphology of flower buds and fragments of leaves from herbarium specimens were analyzed using a scanning electron microscope. These samples were mounted in holders and coated with gold (Bozzola & Russell 1992) using FDU 010 Balzers Sputter Coater equipment. The analyses were performed and documented using a Leo 1430 VP model SEM (Zeiss, Cambridge, England) belonging to the Centre for Electron Microscopy and Microanalysis of UFV.

Results

Calyx glands of the 35 evaluated species (Tab. 1) exhibited the same morphological pattern of being subsessile and having an oblong shape with an irregular surface (Fig. 1A-B). There is one pair of glands on each of four sepals while they are absent on the fifth, for a total of 8 glands (Fig. 1C). No differences were observed in the anatomical structure of the calyx glands among the analyzed species. In longitudinal section, the distal region consists of a thick cuticle, which in most individuals was not adhered to the epidermal surface (Fig. 1D-E), creating a subcuticular space in some regions of the epidermis (Fig. 1E). The epidermis is composed of palisade cells (Fig. 1F) with large nuclei and dense cytoplasm (Fig 1F). The secretory epidermal tissue has several transverse invaginations along the gland (Fig. 1D-F) forming an irregular surface (Fig. 1A-C), while the non-secreting epidermal cells are smaller (Fig. 1G). The parenchyma of the gland is made up of two distinct regions. The subepidermal tissue, especially in the portions between the invaginations, is composed of juxtaposed elongated cells with dense cytoplasm and reduced intercellular spaces (Figs. 1E-F). In the adjacent region, the parenchyma is composed of isodiametric shaped cells (Fig. 1G). Druses are commonly present in both regions (Fig. 1H). The vascularization of the glands is formed by highly branched bundles of xylem and phloem with a predominance of phloem (Fig. 1I).

Three morphological types of leaf glands were observed among the 29 species evaluated. Rounded, concave and stalked glands were observed (Fig. 2A-B) in *B. adenopada, B. arborea, B. argyrophylla* and *B. calcicola*. In *B. campestris*, *B. confusa*, *B. harleyi, B. martiniana, B. membranifolia, B. muricata, B. oxyclada, B. paraguariensis, B. parviflora, B. pulchra, B. schizoptera, B. sellowiana* and *B. stellaris* the glands were rounded, concave and sessile (Fig. 2C-D). In the remaining 12 species the glands are rounded, not concave, and sessile (Fig. 2E-F).

Leaf glands differed little from calyx glands. In most of the species analyzed, in the longitudinal plane the cuticle covering the region of the leaf secretory glands is thinner and adhered to the single-layered palisade epidermis (Fig. 3A-B). The secretory epidermal cells are density stained, with a thin wall and an evident nucleus. The secretory surface is smooth and without invaginations (Figs. 2B, D, F, 3A-B), and the subepidermal glandular parenchyma bears reduced spaces and isodiametric cells without chloroplasts, while the other parenchyma cells are elongated (Fig. 3A-B). Vascular xylem and phloem from accessory

Table 1. List of the analyzed species of *Banisteriopsis* with dried materials and presence of glands in the calyx and leaf.

Species	Sample materials	Calyx glands	Leaf glands
1. *B. adenopada* (Adr. Jussieu) B. Gates	Carcerelli, C. 30; Occhioni, P. 1215, Guillauman, J.R.; Constantino, D. 3; Bernacci, L.C. et al. 72; Salino, A e Morais, P.O. 4633; Proença, S. L. et al 11; Assis, L.C.S. 75	-	X
2. *B. andersonii* B. Gates	Martinelli, G.6269; Anderson, W.R. 11594; Pereira, E. 1720; Joly, A.B. et al s/nº, George Eiten e Liene T. Eiten, 11051; Giulietti, A.M. s/nº	X	X
3. *B. angustifólia* (Adr. Jussieu) B. Gates	Damazio, L. 1204; Martinelli, G. 5883; Carvalho, A.M.V. 3709; Wanderley M.G.L. 2665; Conceição A.A. 1723; Harley, R.M. 26943	X	X
4. *B. anisandra* (Adr. Jussieu) B. Gates	Prance, G.T. S/Nº; Farias, R. 606; Ragojino de Lima S/Nº; Nakajima, J.N. et al. 1329; Lino, M.R.D. et al. 2; Silveira, F.A. 17; Pirani, J.R. et al. 8271.	X	-
5. *B. arborea* B. Gates	Anderson, W.R. S/Nº; Anderson, W.R. 11585; Anderson, W.R. 35506; Irwin, H.S. 28271	X	-
6. *B. argyrophylla* (Adr. Jussieu) B. Gates	Brade, A.C. 17815; Correa Gomes, J. 240; Heringerm, E.P. 13197; Irwin, H.S. et al. 15811; Araújo, G.M. 263; Caliente, A.D. 140; Heringer, E.P. et al. 1199.	X	-
7. *B. byssacea* B. Gates	Romero, R. et al. 1417; Romero, R. et al. 1590; Nakajima, J.N. et al. 923; Romero, R. et al. 692.	X	-
8. *B. caapi* (Spruce ex. Grisebach) Morton	Ferreira, C.A.C. 3054; Policia Federal, S/Nº; Ferreira, C.A.C. 11554; Faria, R. S/Nº; Corrêa, M.A. 2; Handro, O. S/Nº.	X	X
9. *B. calcicola* B. Gates	Hatschbach, G. et al. 66068; Stannard, B.L. et al. 51585; Tameirão Neto, E. 2284; Guedes, M.F. et al. 7932.	X	X
10. *B. campestris* (Adr. Jussieu) E. L. Little	Campos Porto, P. 2881; Renato Becker 021; Gates, B.E. 153; Campos, S.M. 116; Cordeiro, I. et al. 1851.	X	X

Continues.

Table 1. Continuation.

Species	Sample materials	Calyx glands	Leaf glands
11. *B. cipoensis* B. Gates	Travassos, F.L. S/Nº; Duarte, A.P. 1958; Joly, A.B. S/Nº; Semir, J. et al. 546; Henrique, M.C. 5883; Joly, A.B. et al. 1128.	X	X
12. *B. confusa* B. Gates	Barbosa, E. 1968; Hatschbachii, G. 49208; Teixeira, L.O.A. 1050; Nee, M. 35022; Mendes Magalhães, G. S/Nº	X	-
13. *B. gardneriana* (Adr. Jussieu) Anderson & Gates	Macedo, A. S/Nº; Ratter, J.A. S/Nº; Mello-Silva, R. S/Nº; Saavedra et al 481; Forzza, R.C. et al 4489; Cavalcanti, T.B. et al 629.	X	X
14. *B. goiana* B. Gates	Macedo, A. S/Nº; Barroso, G.M. S/Nº; Barros, F. 2170; Cardovil Silva, S.P. et al 595; Rezende, J.M. 798.	X	X
15. *B. harleyi* B. Gates	Mori, S.A. S/Nº; Araújo, A.P.P. 21; Stannard, B.L. et al. 52128; Conceição, A.A. et al. 1713; Hind, D.J.N. et al. 50039.	X	X
16. *B. hatschbachii* B. Gates	Barroso, G.M. 608; Hatschbach, G. et al. 54784; Anderson, W.R. 11493; Estabrook,, G.F. 13; Paula-Souza, J. 3912	X	X
17. *B. hirsuta* B. Gates	Hatschbach, G. et al. 59521; Aparecida da Silva, M. et al. s/nº	X	X
18. *B. irwinii* B. Gates	Gates, B.E. 212; Estabrook, G.F. 7; Hatschbach, G. 36793; Vieira, R.F. et al. 726; Fonseca, M.L. et al. 759; Cavalcanti, T.B. et al. 1071.	X	-
19. *B. laevifolia* (Adr. Jussieu) B. Gates	Magnago, H. 56; Magnago, H. 02; Barroso, G.M. S/Nº; Ribas, O.S. 2608; Henrique, M.C. et al. 6858; Pott, A. et al. 12817; Tameirão Neto, E. et al. 329.	X	X
20. *B. latifolia* (Adr. Jussieu) B. Gates	Anderson, W.R. 10294; Macedo, A. 3673; Monteiro, R.F. 149; Gomes, B.M. 23; Cordovil Silva, S.P. 453; Pereira, B.A.S. et al. 2959; Henriger, E.P. 15805.	X	X
21. *B. malifolia* B. Gates	Harley R.M. 16855; Miranda, C.A. 321; Lisboa, M.A. 2490; Harley, R.M. 19963; Kirkbride Jr. J.H. 3510; Heringer, E.P. 13193; Irwin, H.S. 21837; Hensold, N. et al. 3189; Santos, E.R. 161; Ganev, W. 3088; Prado, J. et al. 12087; Freitas, G.P. 20; Pirani, J.R. et al. 12549.	X	X
22. *B. martiniana* B. Gates	Sandwith, N.Y. 143; Evans, R. 1891,Coelho, L. s/nº; Assunção, P.A.C.I. 236	X	X
23. *B. megaphylla* (Adr. Jussieu) B. Gates	Irwin, H.S. 14318; Damazio, L. 1714; Ratter, J.A. 896; Ratter, J.A. 1338; Duarte, A.P. 2441; Gates, B.E. 167; Domingos, N.S. et al. S/Nº; Vieira, R.F. et al. 783; Davidse, G. et al. 12273.	X	X
24. *B.membranifolia* (Adr. Jussieu) B. Gates	Sucre, D. 2661; Amorim, A.M.A. 5821; Duarte, A.P. 4256; Hatschabach, G. 47679; Amorim, A.M. et al. 3655; Mattos Silva, L.A. et al. 2669; Thomas, W.W. et al. 9866.	X	X
25. *B. multifoliolata* (Adr. Jussieu) B. Gates	Leite, K.R.B. 213; Jost, T. 434.	X	-
26. *B. muricata* (Cavanilles) Cuatrecasas	Teixeira, L.O.A. 326; Araújo, C.M. 73; Amorim, A.M.A. 22; Teixeira, L.O.A. 326; Coelho, L.F. 1739; Amaral, I.L. 1136; Davidson, C. 10268; Pastore, J.A. 159; Araújo, F. S. 43; Barreto, K.D. et al. 1769.	X	-
27. *B. nummifera* (Adr. Jussieu) B. Gates	Kuhlmann, J.G. 150; Frazão, A. 1918; Harley, R.M. 54681; Amorim, A.M. et al. 1993; Menezes, N.L. et al. 1283; Barreto, K.D. 2866.	X	-
28. *B. oxyclada* (Adr. Jussieu) B. Gates	Pereira, A.C. 54; Carvalho, A.M.V. 1675; Mendonça, R.C. 3578; Hoehne, W. S/Nº; Rezende, A.A. 424; Proença, S.L. et al. 10.	X	X
29. *B. paraguariensis* B. Gates	Hassler, E. 9832; Silva, J.M. 5238; Hatschbach, G. 38669; Hatschbach, G. 45881.	-	X
30. *B. parviflora* (Adr. Jussieu) B. Gates	Sekine, E.S. et al. 95;Hatschbach, G. 51980; Silvestre, M.S.F. 47; Gehrt, A. S/Nº.	X	X
31. *B. parviglandula* B. Gates	Queiróz, L.P. 1650; Tameirão Neto, E. 452; Lombardi, J.A. et al. 697; Kuhlmann, J.G. S/Nº	X	X
32. *B. prancei* B. Gates	Kuhlmann, J.G. 2058; Ferreira, C.A.C. 4489; Janssen, A.S. 449; Ferreira, C.A.C. 4393; Hoehne F. C. S/Nº.	X	X
33. *B. pulchra* B. Gates	Zardini, E.M. 50740; Hatschbach, G. et al. 73185; Hatschbach, G. et al. 73030; Hatschbach, G. 38646.	X	X
34. *B. schizoptera* (Adr. Jussieu) B. Gates	Irwin, H.S. et al. 19303; Amorim, A.M. et al. 2782; Hatschbach, G. et al. 70063; Fonseca, M.L. et al. 5623; Maury, C.M. 375.	X	X
35. *B. sellowiana* (Adr. Jussieu) B. Gates	Farney, C. 1695; Pereira, E. 4363	-	X
36. *B. stellaris* (Grisebach) B. Gates	Duarte, A.P. 2800; Denise, S.M. S/Nº; Martinelli, G. 9140; Ferreira, C.A.C. 4320; Teixeira, L.O.A. 1366; Fonseca, M.L. et al 5593; Carvalho, A.M. 2830.	X	X
37. *B. variabilis* B. Gates	Gomes 04; Sucre, D. 283; Cappellari, S.C. 1; Oliveira, P. 3002.	X	X
38. *B. vernoniifolia* (Adr. Jussieu) B. Gates	Shepherd, G. J. et al. 3674; Hatschbach, G. et al. 70734; Anderson, W.R. 7240; Santos, A.L. et al. 91; Braga, M.M.N. S/Nº; Anderson, W.R. et al. 7694.	X	X

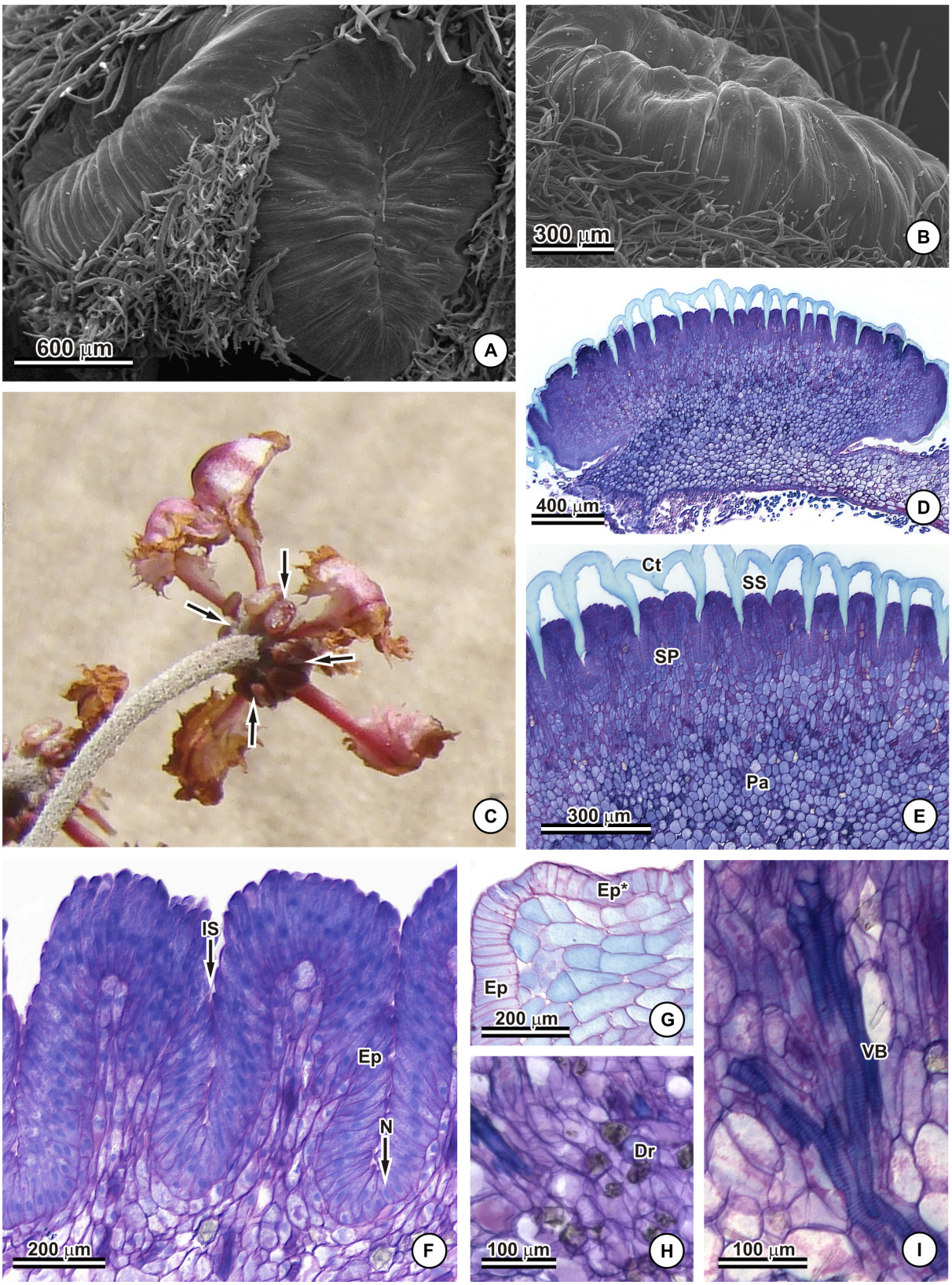

Figure 1. Anatomical description of calyx glands of *Banisteriopsis* species. Scanning electron microscope view (A-B) and longitudinal sections (D-I). (A) *B. argyrophylla* and (B) *B. harleyi*, note subsessile oblong shape with irregular surface. (C) *B. campestris* showing one pair of gland on each sepal (arrows). (D-E) *B. byssacea* highlighting the thick cuticle (Ct) and subcuticular space (SS). (F) *B. acerosa*, showing large nuclei, dense cytoplasm and irregular surface. (G) *B. anisandra*, note non-secretin epidermal cells (Ep*). (H-I) *B. adenopoda*, showing druses and vascular bundles. Ct, cuticle; Dr, druse; Ep, secretory palissade epidermis, Ep*, non-secretin epidermal cells; IS, irregular surface; N, nuclei; Pa, parenchyma; SS, subcuticular space; SP, subepidermal glandular parenchyma; VB, vascular bundle.

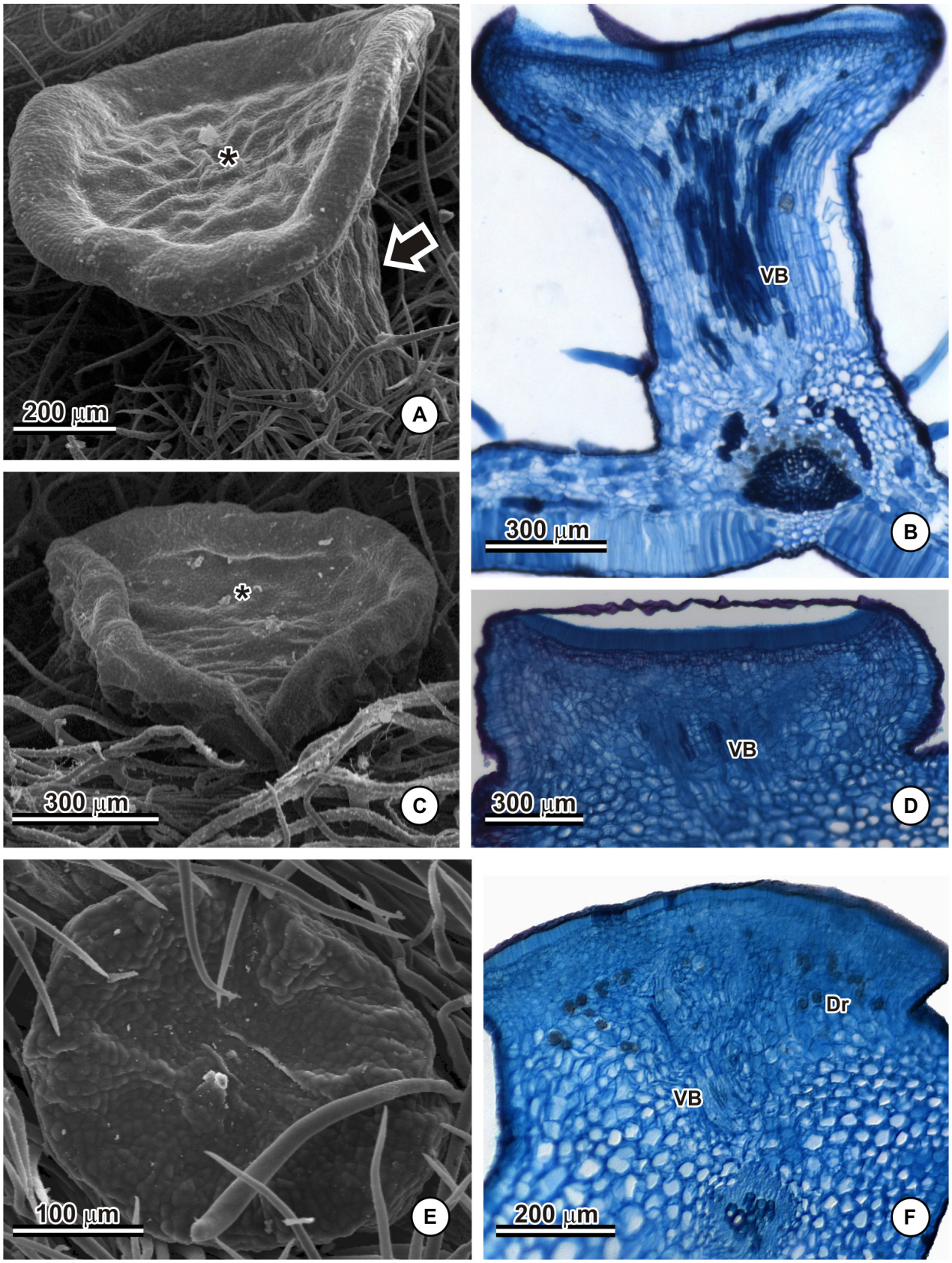

Figure 2. Anatomical description of leaf glands of *Banisteriopsis* species, under scanning electron microscope view (A, C, E) and longitudinal sections (B, D, F). (A, B) Rounded, concave and stalked glands observed in *B. calcicola* and *B. adenopoda*, respectively. (C, D) Rounded, concave and sessile gland in *B. campestris* and *B. pulchra*, respectively. (E, F) Rounded glands without concavity in *B. goiana* and *B. caapi*, respectively. Asterisco, concave; arrow, stalk; Dr, druse; VB, vascular bundle.

bundles or from secondary lateral veins, converge and end in this region, and are responsible for vascular supply to the gland (Fig. 3B). Most often the cells of phloem reach into the subepidermal parenchyma until two secretory cells layers below the epidermis (Fig. 3A). The presence of druse is common in the glandular parenchyma cells of all the analyzed species (Fig. 2F).

The results of the histochemical tests of *Banisteriopsis campestris*, *B. laevifolia* and *B. malifolia* are summarized in Tab. 2. The two types of glands of the three species analyzed showed an absence of essential oil/resin, starch and alkaloids since the reagent NADI, lugol reagent and Wagner tests showed negative results, respectively. The presence of conductive elements in the xylem of the two types of glands was confirmed by the positive phoroglucinol reaction (Fig. 3C-F).

Tests for lipids in the calyx gland showed them to be present in the cuticle and as conspicuous drops in the cy-

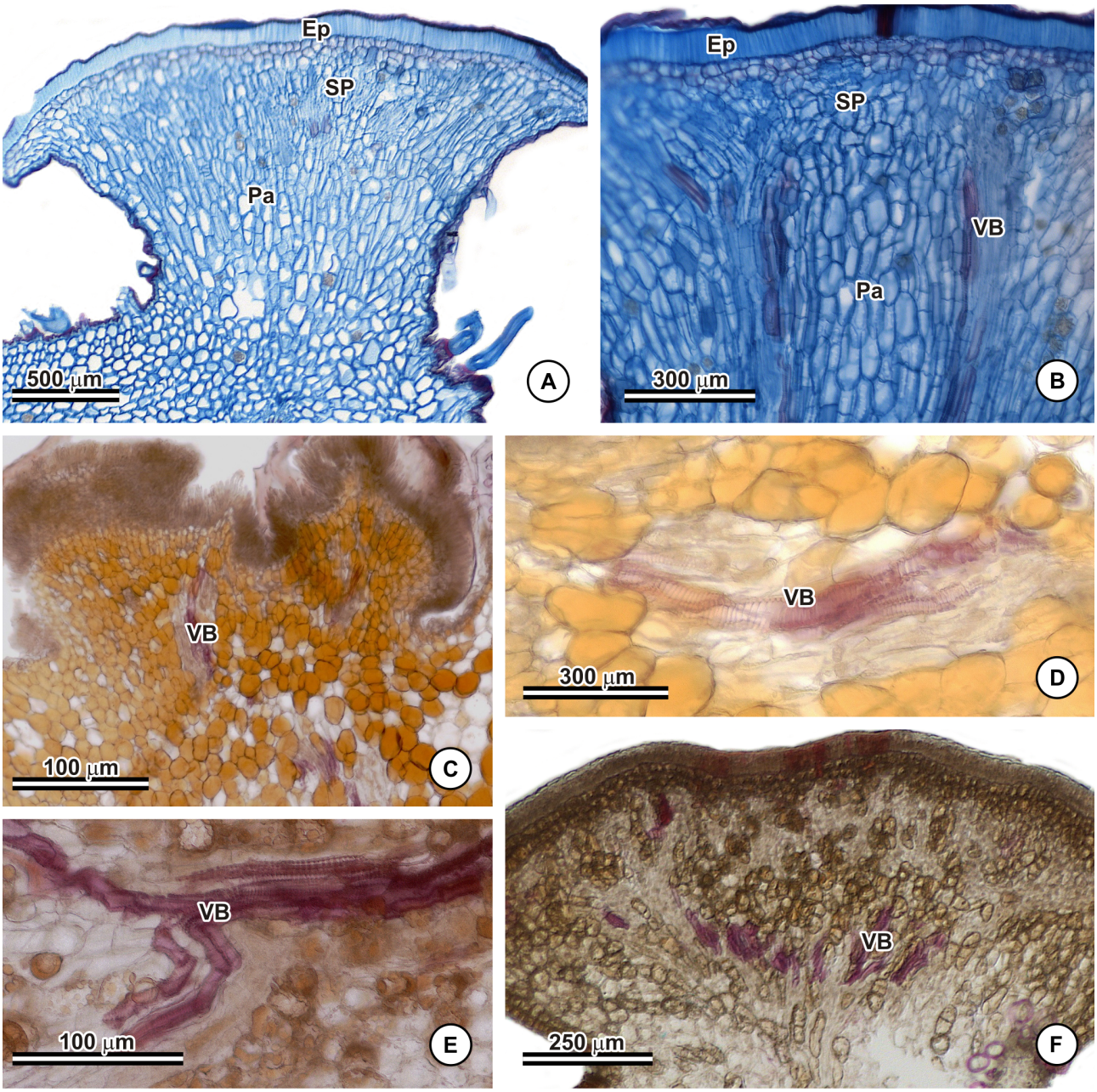

Figure 3. Anatomical description of leaf glands (A-B) and histochemical results of tests carried out in longitudinal sections of calyx (C, D) and leaf (E, F) glands of *Banisteriopsis* species. (A-B) Gland with smooth secretory surface without invaginations in *B. parviglandula*. (C-F) Xylem cells detected by phoroglucinol reaction in *B. malifolia* (C-D) and *B. campestris* (E-F). Ep, palissade epidermis, VB, vascular bundle, Pa, parenchyma; SP, subepidermal glandular parenchyma. Please see the PDF version for color reference.

Table 2. Results of histochemical test carried out on the section of leaf gland (LG) and calyx gland (CG).

Test		*B. campestris*	*B. laevifolia*	*B. malifolia*
Periodic acid–Schiff reagent (PAS)	LG	+	+	+
	CG	+	+	+
Lugol	LG	-	-	-
	CG	-	-	-
Coomassie blue	LG	+	+	+
	CG	+	+	+
Ninhydrin-Schiff	LG	+	+	+
	CG	+	+	+
Sudan Black B	LG	-	-	-
	CG	+	+	+
Neutral Red	LG	+	+	+
	CG	+	+	+
NADI	LG	-	-	-
	CG	-	-	-
Wagner Reagent	LG	-	-	-
	CG	-	-	-
Fhoroglucinol	LG	+	+	+
	CG	+	+	+

(+) positive reaction; (-) negative reaction

toplasm of epidermal cells and subepidermal parenchyma (Fig. 4A), whereas the in the leaf glands they were only present in the cuticle (Fig. 4B). However, under induced neutral red fluorescence seen in UV light, lipid droplets were observed in epidermal cells and in subcuticular spaces of calyx glands (Fig. 4C), but in leaf glands droplets were observed only in epidermal cells (Fig. 4D).

The Coomassie blue test (Tab. 2) demonstrated the presence of protein in the secretory epidermis in calyx glands (Fig. 5A) and in subepidermal parenchyma of leaf glands (Fig. 5B). The ninhydrin test also showed the presence of protein in the subepidermal parenchyma of the both leaf and calyx glands (Fig. 5C-D). Total polysaccharides were detected by PAS test in the secretory epidermis and subepidermal parenchyma of calyx (Fig. 5E) and leaf glands (Fig. 5F).

Discussion

The anatomical similarity of the glands of 26 of the 38 species of *Banisteriopsis* evaluated in this study expands the database available for future investigations into the evolution of this character in Malpighiaceae. These data agree with the literature that has emphasized anatomical similarities between the glands of species in other genera of Malpighiaceae such as *Galphimia brasiliensis, Byrsonima sericea, Heteropterys chrysophylla, Peixotoa hispidula* and *Diplopterys pubipetala* (Castro *et al.* 2001; Vieira 2005; Possobom *et al.* 2015).

Regarding morphology, the calyx glands observed in the studied species of *Banisteriopsis* are sessile, similar to the glands of *Diplopterys pubipetala* (Possobom *et al.* 2015), but differ from those of *Dinemandra ericoides,* which are stalked (Cocucci *et al.* 1996). The distance between the secretory surface of the gland and the insertion site in the sepal may be related to the interaction between the pollinator and the gland and should be considered in future ecological studies. Anatomically, calyx glands of the 35 species of *Banisteriopsis* studied in this work are similar to the descriptions of *Diplopterys pubipetala* (Possobom *et al.* 2015) and *Galphimia brasiliensis* (Castro *et al.* 2001), except for the presence of transverse invaginations along the gland. Such invaginations increase the secretory surface and the location where the exudate is accumulated, making available a larger amount of this resource to pollinators.

In the analyzed species, the presence of vascularization in leaf and calyx glands was detected, similar to what was observed by Possobom *et al.* (2010; 2015) in *Diplopterys pubipetala* and Mamede (1993) in eight species of *Camarea*. According to Elias *et al.* (1975), nectaries that are vascularized are highly specialized and characteristically remain active for only for a short period of time, usually only a few weeks when the leaves are expanding.

The histochemical analysis of calyx and leaf glands of the species *Banisteriopsis* studied detected the presence of lipids, proteins and polysaccharides, which agrees with that found for the glands of *Diplopterys pubipetala* (Possobom *et al.* 2010; 2015). In additional, these results are partially similar to that found for *Galphimia brasiliensis,* which secrete a mixture of lipids and sugars (Castro *et al.* 2001). On the other hand, a hydrophilic secretion composed by sucrose and glucose, and without lipid compounds, was detected in the calyx glands of *Hiptage benghalensis* (Ren *et al.* 2013). These results indicate the need for complementary analyses in order to determine the similarity and differences that can exist between secretions produced by calyx and leaf glands of species of Malpighiaceae.

The accumulation of lipid substances in subcuticular space of calyx glands generated the detachment of the cuticle from the epidermis, as was observed by use of neutral red under UV light. Subcuticular space with lipid content seems to be common since it has also been reported for the calyx glands of species of other genera of Malpighiaceae, such as *Dinemandra ericoides, Malpighia coccigera, Byrsonima sericea, Heteropterys chrysophylla,* and *Peixotoa hispidula* (Cocucci *et al.* 1996; Seipold *et al.* 2004; Vieira 2005). In these species, the pollinator breaks the cuticle during its visit and the exposed secretion is collected (Cocucci *et al.* 1996; Seipold *et al.* 2004; Vieira 2005).

Lipids were also detected in the leaf glands of the species of *Banisteriopsis* studied herein, which is in agreement with the reports of Possobom *et al.* (2010) for the leaf glands of *Diplopterys pubipetala*. However, these authors

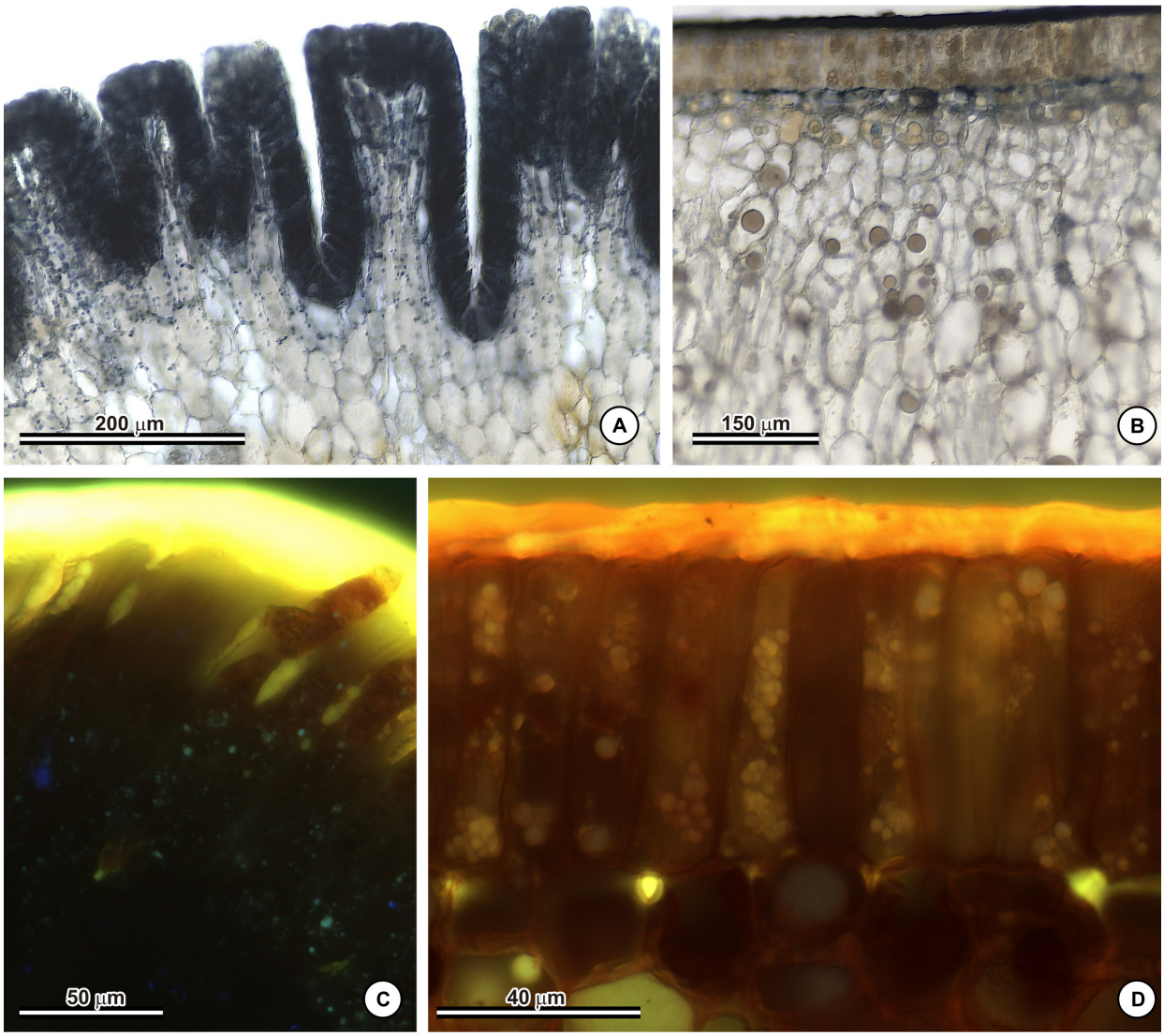

Figure 4. Results of the histochemical tests carried out on longitudinal sections of calyx (A, C) and leaf (B, D) glands of *Banisteriopsis* species. (A) Positive reaction for Sudan black B showing the cuticle and conspicuous drops in the cytoplasm of epidermal cells and subepidermal parenchyma calyx glands of *B. laevifolia*. (B) Reaction with Sudan black B highlighting only the cuticle of leaf glands of *B. malifolia*. (C) *B. campestris* and (D) *B. malifolia* showing drops only in epidermal cells and cuticle using neutral red under UV light. Please see the PDF version for color reference.

opted to call such glands extrafloral nectaries (EFN) based on ultrastructural analyses and detection of sugar in the secretion. Anatomically comparable secretory structures have been described for *Hymenaea stigonocarpa* (Paiva & Machado 2006), *Passiflora foetida* (Durkee *et al.* 1984) and *Prockia crucis* (Thadeo *et al.* 2008), which produce different compounds. In such cases it is necessary to clarify the nature of the secretion. For example, when sugars are present the structure is nectary. It is possible that the presence of lipids in secretion is related to the resources required by visitors, as suggested by Real (1983). On the other hand, existence of lipid compounds in the EFN nectar, as well as the presence of sugars in the secretion of elaiophores have been used to establish phylogenetic inferences on Malpighiaceae, and also to clarify coevolution with polarizations (Cocucci *et al.* 1996). Such inference can be made for the genus *Banisteriopsis*, as demonstrated by our data. Interestingly, selection for plant-pollinator mutualism (extrinsic factors) has been used to explain the origin and conserved floral morphology of Malpighiaceae (Davis *et al.* 2014). Such hypothesis is based on clades that have lost interaction oil-bee pollination display major evolutionary shifts in floral characters related with this interaction (Davis *et al.* 2014).

The presence of protein granules in the calyx glands of *Banisteriopsis* may be related to the energetic demands of pollinators, as proposed by Nicolson & Thornburg (2007) who considered amino acids as an important resource for visitors. Pollination of species of Malpighiaceae is primarily accomplished by female bees of the tribe Centridini

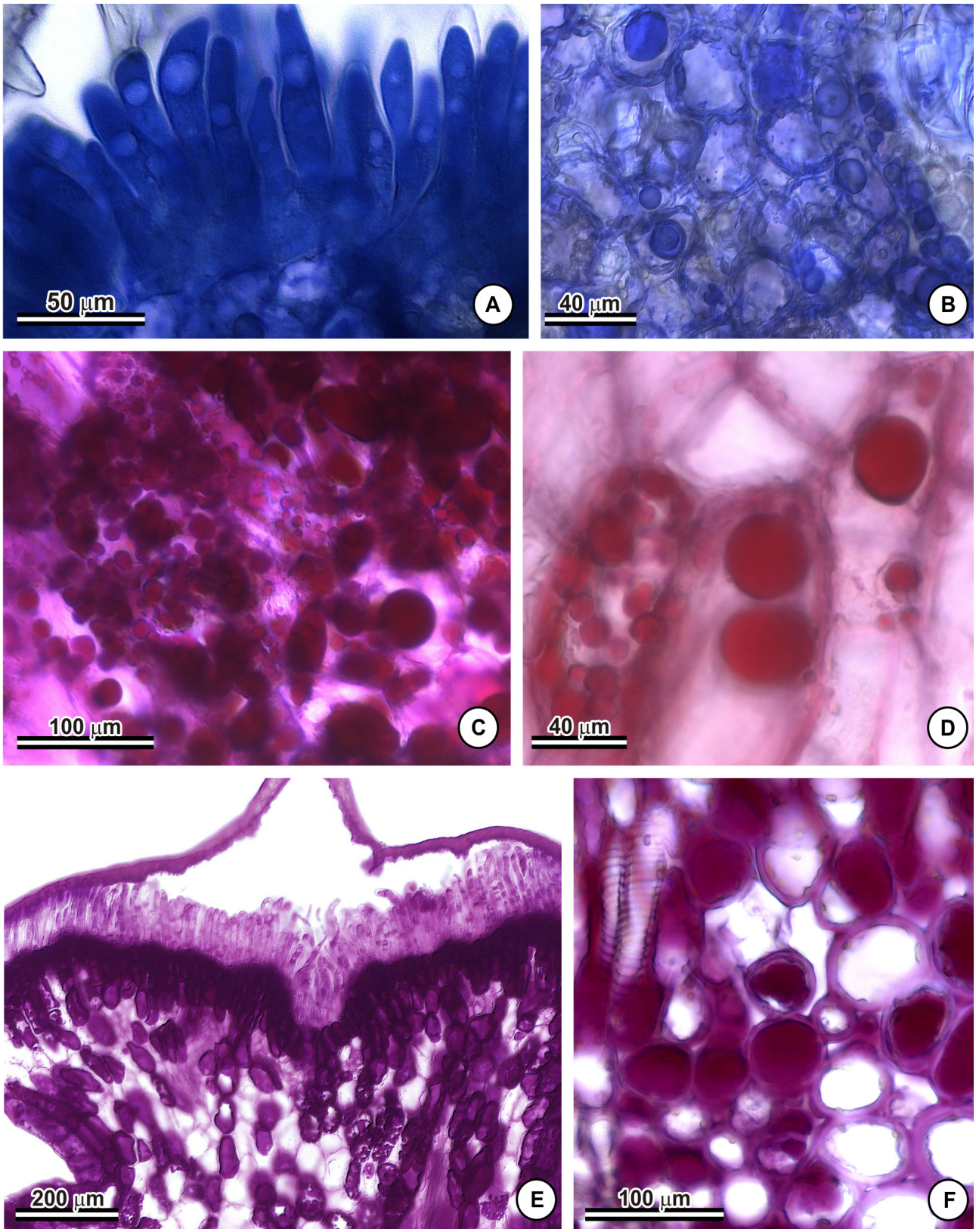

Figure 5. Results of the histochemical tests carried out on longitudinal sections of calyx (A, C, E) and leaf (B, D, F) glands of *Banisteriopsis* species. (A, B) Glands submitted to Coomassie blue in *B. malifoliae* and *B. campestris*, respectively, note presence of proteins in the secretory epidermis and subepidermal parenchyma. (C-D) Gland of *B. malifolia* submitted to ninhydrin-Schiff showing protein in the subepidermal parenchyma. (E-F) Positive reaction with PAS in *B. campestris*. Please see the PDF version for color reference.

(Anderson 1979), and these insects are considered to be deficient at producing amino acids (Baker 1977). Hence, a protein-rich exudate may have influenced the selection for this specific interaction in Malpighiaceae.

In the present work, proteins detected in leaf glands strengthen the case of a mutualistic interaction between these structures and ants. Ants feeding on nectar were observed in EFNs of *Byrsonima intermedia, Diplopterys pubipetala* and *Heteropterys pteropetala* (Réu 2005, Possobom *et al.* 2010). These authors verified the presence of greater quantities of secretion when the plants were flowering and no injuries to organs under development were observed. The detection of protein in the leaf glands of the studied species of *Banisteriopsis* indicates one more characteristic selected for in the interaction of these species with ants, by conferring an adaptive advantage. This argument was also used to explain the grain protein observed in extrafloral nectaries of *Chamaecrista* (Leguminosae) by Coutinho *et al.* (2012).

The anatomical similarity observed between leaf and calyx glands of species of *Banisteriopsis*, in addition to the presence of lipids and polysaccharides, reinforces the hypothesis of Vogel (1990) that calyx glands originated from leaf glands. According to Vogel (1990), the transformation of nectaries into elaiophores must have happened in response to the mutualism between flowers of species of Malpighiaceae and wild bees of tribe Centridini. Subramanian *et al.* (1990) studying *Hiptage sericea* and Castro *et al.* (2001) analyzing *G. brasiliensis* both reached the same conclusion and considered that these structures are homologous. In the same way, Durkee *et al.* (1984) suggested that the EFNs of *Passiflora foetida* represent a transition to lipid-secreting glands. In additional, Davis *et al.* (2014) consider the conserved floral morphology in Malpighiaceae as a result of extrinsic factor, in especially the plant-pollinator mutualism. The complexity of secretions produced by the leaf glands of the species studied here shows that the metabolism of these glands can be altered, which provides additional evidence of the importance of plant-animal interactions in the diversification of Malpighiaceae.

The results of the histochemical tests performed on calyx and leaf glands of *Banisteriopsis* reveal that the glands are of a mixed nature. It is believed that the presence of proteins, lipids and polysaccharides is directly related to plant-insect interactions given the nutritional value of these compounds. This is true for plant-pollinator interactions and calyx glands, as well as for plant-defense and leaf glands.

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