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Original article

Development, structure, and secretion of leaf colleters in *Clusia criuva* Cambess. subsp. *criuva* (Clusiaceae)

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ABSTRACT

Colleters produce a secretion composed of hydrophilic and/or lipophilic substances which lubricates and protects the shoot apical meristem against biotic and abiotic agents. Little attention has been given to these structures in Clusiaceae. In the present study, the structure and development of the leaf colleters of *Clusia criuva* subsp. *criuva* were described and variations in the exudate composition of the colleters at different stages of leaf development were identified. The samples were collected and processed according to techniques for light and scanning electron microscopy. Colleters are of the standard type and not vascularized, and during leaf development, changes in color, structure, and secretion abundance were observed. Asynchrony in the development was noticeable in the leaf primordia and young leaves, from colleters in early formation to those in early senescence. In early phases there was an abundance of polysaccharide, lipid, and protein secretions, whereas adult and senescent leaves revealed an accumulation of phenolic compounds and cell degradation. The secretion was released by the rupture of the cuticle. The structural changes and secretion composition during leaf development emphasize the role of colleters in protecting meristems and developing organs.

Keywords: exudate, glands, histochemistry, Malpighiales, morphoanatomy

Introduction

Throughout the evolution of terrestrial plants, protection strategies have been incorporated into the plant body that allow for greater competition and resistance to environmental adversities, thus ensuring the survival of plants in different environments (Paiva & Machado 2006; Paiva 2016; Prado & Demarco 2018). One of these strategies involves chemical defense through compounds produced by secretory structures (Prado & Demarco 2018). Plant secretions are often associated with defense strategies (Paiva 2016). They are constituted with different primary and/or secondary metabolites. The chemical predominance of certain compounds in the secretion may suggest specificity in the activity of the secretory cells and also on the ecological role played by such secretion (Castro & Machado 2006; Ascensão 2007; Paiva 2016).

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Colleters are external structures that produce a sticky secretion composed of mucilage, resins, or a mixture of both (Fahn 1979; Thomas 1991). The colleter secretion present on the surface of vegetative and reproductive organs, protects developing meristems and organs against desiccation (Thomas 1991), acts as an insect repellent (Smith 1963; Curtis & Lersten 1974) or herbivore and pathogen deterrent, and provides inhibition of fungi and bacterial growth through its antimicrobial properties (Castro & Demarco 2008; Calvo *et al.* 2010; Cardoso-Gustavson *et al.* 2014). The colleters have an early development and usually complete their differentiation and even their senescence long before the final development of the plant organ that surrounds them (Canaveze 2012).

Colleters are widely distributed among the angiosperms and were reported in about 60 families (Thomas 1991), including Clusiaceae (Malpighiales), a family known worldwide for the economic importance of its secretions (Judd *et al*. 2009). However, the structural and biological aspects of colleters are poorly investigated within the family being restricted to *Garcinia* and *Clusia*. Filiform colleters occur in the flowers of *Garcinia brasiliensis* (Leal *et al.* 2012); lachrymiform colleters (cone shaped) were found on the leaf axes of *Clusia fluminensis* and *C. lanceolata* (Silva *et al.* 2019) and on the petiole of *C. grandiflora* (Machado & Emmeric 1981). Recently, standard type colleters were found at the base of the petiole of *C. burchellii* (Teixeira *et al.* 2021).

Clusia is one of the largest genera of the Clusiaceae (300 - 400 spp.) with 68 species found in different Brazilian biomes (Lüttge 2007; BFG *et al.* 2015; Bittrich *et al.* 2015). During field expeditions in a fragment of Atlantic Forest located in the State of Espírito Santo (Brazil), the observation of copious secretions covering the apical meristems and young leaves of *Clusia criuva* subsp. *criuva* led us to hypothesize that such exudates were produced by colleters. In the present study we aim to confirm the structure identity responsible for the exudation of the secretion observed in the field. Additionally, we discussed the chemical composition of the secretion throughout the leaf development in a way to contribute to the understanding of the morphofunctionality of such leaf glands present in Clusiaceae.

Material and methods

Collection and sampling location

Samples of the (i) shoot apical meristem with leaf primordia and the base of (ii) young (first and second nodes), (iii) completely expanded (third and fourth nodes) and (iv) senescent leaves (sixth node on) were collected (Fig. 1A - D). The samples were collected from three individuals of *Clusia criuva* Cambess. subsp. *criuva* in the Parque Estadual da Pedra Azul (20°24' S, 41°01' W), in the municipality of Domingos Martins, located in the state of Espírito Santo, Brazil (Fig. 1E). The area is an Upper Montane Rain Forest with rupestrian vegetation (Fig. 1F) which belongs to the Atlantic Forest Domain. Female (Fig. 1G) and male (Fig. 1H) individuals were collected in a transition region from forest to rupestrian area, growing on a rocky outcrop in full sun (Fig. 1I). Reproductive branches were dried and deposited in the collection of the herbarium at the Instituto Federal Goiano, campus Rio Verde (IFRV): Dalvi VC 111, Dalvi VC 112 and Dalvi VC 113. The species identification was confirmed by Dr. José Elvino do Nascimento Júnior.

Light microscopy

In the field, the samples were fixed in FAA (formalin, acetic acid, and 70 % ethanol 1:1:18 v/v) (Johansen 1940) for structural characterization, as well as in neutral-buffered formalin solution (phosphate buffer: formalin, 9:1 v/v) (Lillie 1965) for histochemical tests. After 48 h, all samples were subjected to dehydration in an ethanol series until their storage in 70% ethyl alcohol.

Subsequently, the samples were embedded in methacrylate resin (Historesin Leica; Leica Microsystems, Heidelberg, Germany), transversely and longitudinally sectioned using a rotary microtome (1508R; Logen Scientific). Sections at 5 µm thickness were stained with toluidine blue (pH 4.6) (O'Brien *et al.* 1964), mounted on slides with synthetic resin (Permount; Fisher Scientific, NJ, USA) and anatomically characterized.

Samples fixed in neutral-buffered formaldehyde solution were included in methacrylate resin, as described above, and subjected to histochemical tests including: periodic acid and Schiff's reagent (McManus 1948); ruthenium red (Johansen 1940); potassium dichromate (Gabe 1968) and ferric chloride (Johansen 1940); Sudan III (Pearse 1985); and Coomassie brilliant blue (Fisher 1968) for detection of total polysaccharides, pectins/mucilage, phenolic compounds, total lipids, and proteins, respectively. The sections with no reagent (control) were also observed under a light microscope. Material analysis and photographic documentation were performed using an Olympus (model BX61) light microscope equipped with an image capture system, a DP-72 camera.

Scanning Electron Microscopy

For scanning electron microscopy, leaf samples of all four developmental stages were fixed in Karnovsky's fixative (0.1 M sodium phosphate buffer, pH 7.2) (Karnovsky 1965) for 48 h. After dehydration in an ethanol series, the specimens were critical point-dried using CO_2 (Autosamdri®, 815, Séries A) (Bozzola & Russel 1992), mounted on stubs with double-sided adhesive tape, and sputter-coated with gold (Denton Vacuum, Desk V). Photographs were taken using a scanning electron microscope (Jeol, JSM – 6610), equipped with Energy-Dispersive X-Ray Spectroscopy (EDS) and NSS Spectral Imaging (Thermo Scientific, USA).

Results

Variations in the morphology and secretory activity of the colleters

The colleters were located on the adaxial surface of the leaves, in the region of insertion with the stem, and they presented different coloration according to the leaf developmental stages (Fig. 2). We observed a large accumulation of translucent secretion (Fig. 2A-C) covering the leaf primordia. The young leaves displayed three to four rows of colleters with a whitish color and a brownish apical portion (Fig. 2D). In these two stages we observed asynchrony in the development of the colleters and the beginning of senescence was noticeable by the wilting

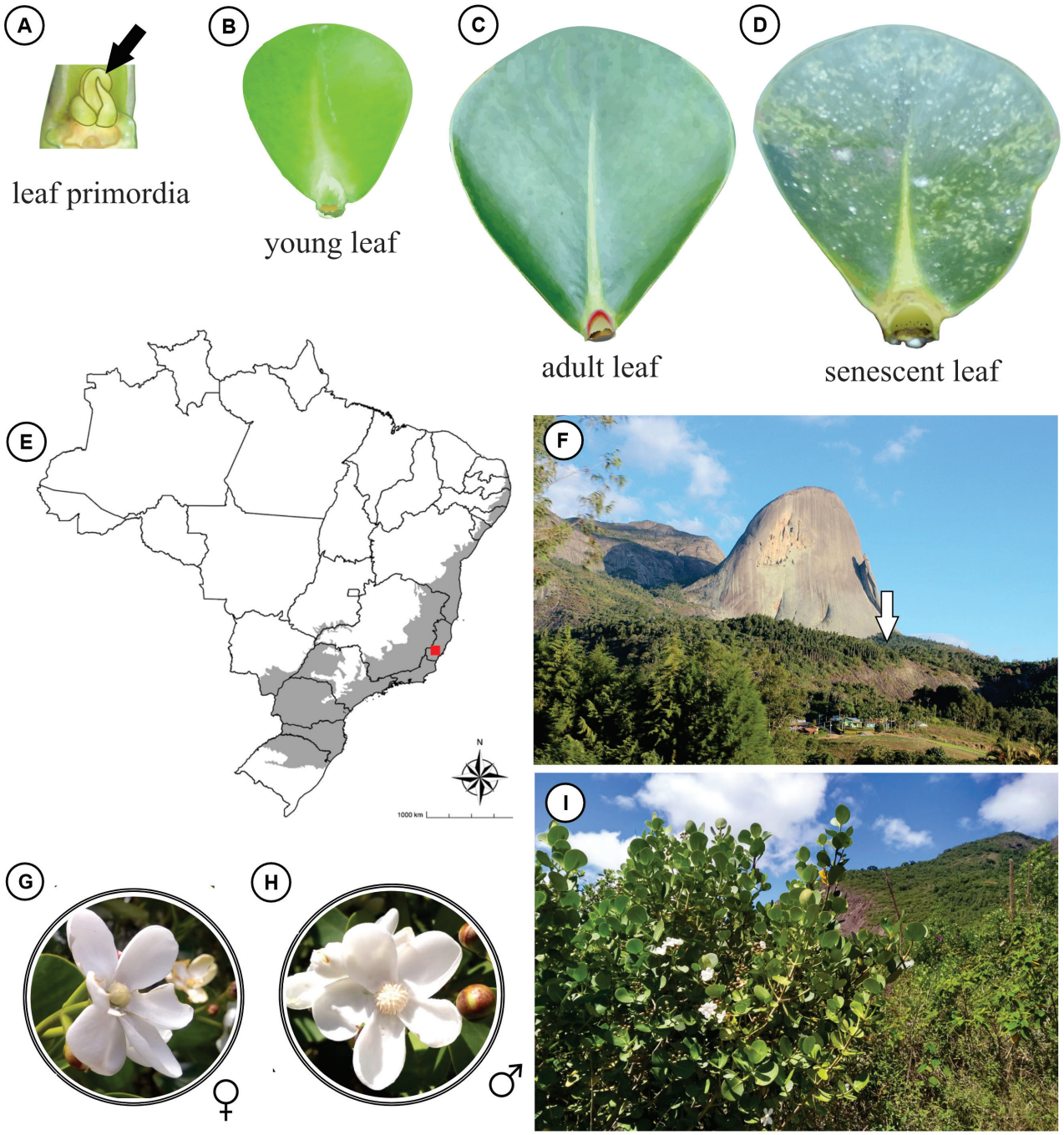

Figure 1. Map with the geographic distribution of the Atlantic Forest in Brazil, collection site of *Clusia criuva* Cambess. subsp. *criuva* and sampled material. **A-D.** Leaves at different developmental stages. **A.** Stem apical meristem with leaf primordia (arrow). **B.** Young leaf. **C.** Adult (completely expanded) leaf. **D.** Senescent leaves. **E.** Map for the sampling area, Domingos Martins, State of Espírito Santo, Brazil. **F.** Collection site (arrow) near the Parque Estadual da Pedra Azul. **G.** Female flower. **H.** Male flower. **I.** Details of individual (arrow) growing in a rocky outcrop area.

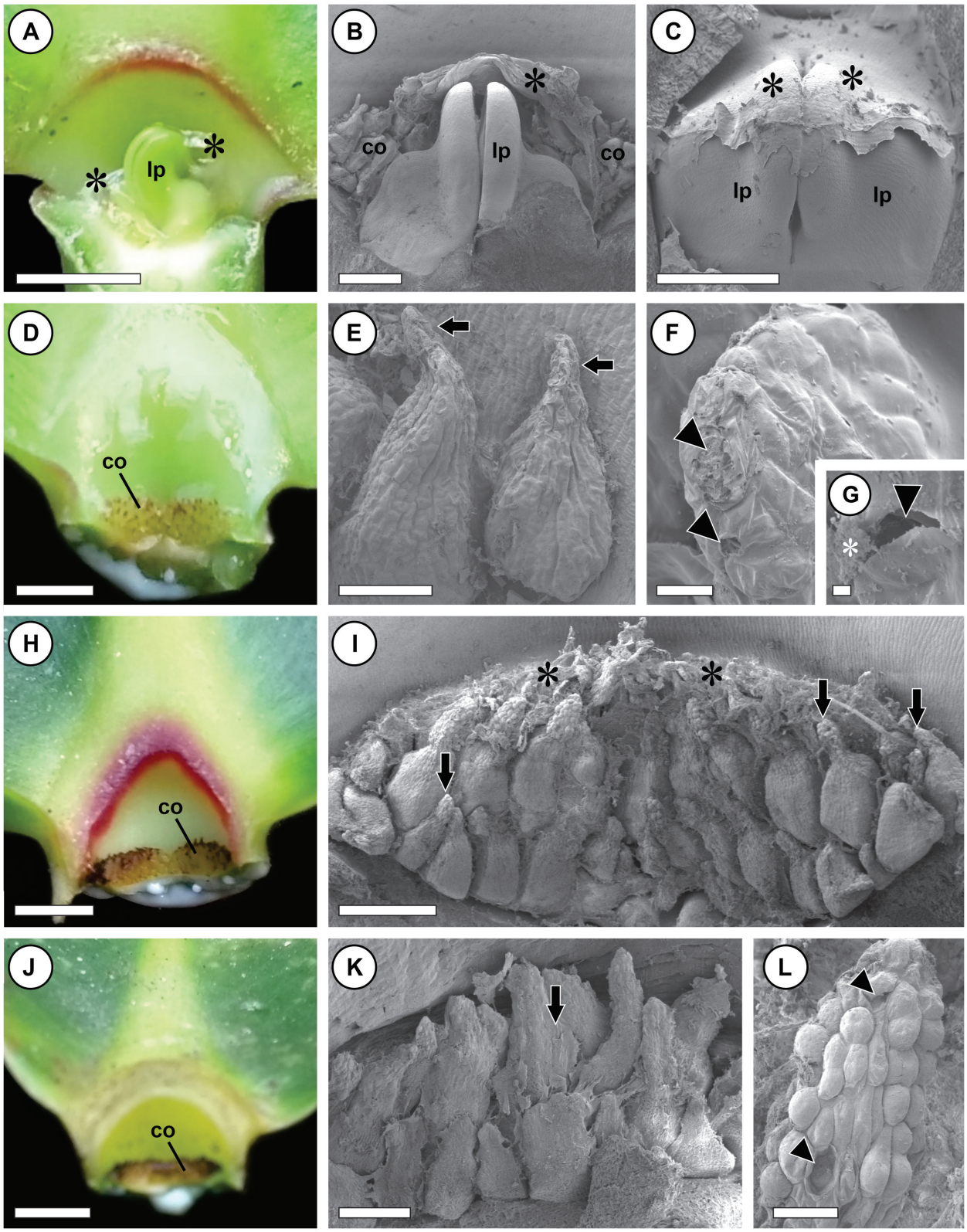

Figure 2. Colleters in *Clusia criuva* Cambess. subsp. *criuva*. **A-C.** Abundant secretion (asterisk) covering the leaf primordia (lp). **D-F.** Colleters on young leaves. **D.** Brownish color at the apex of the colleters. **E.** Constriction at the apical portion (arrows). **F.** Cuticle rupture (arrowheads). **G.** Extravasated secretion (asterisks). **H, I.** Colleters on adult leaves. **H.** Accentuated brown color. **I.** Collapsed cell, reduced secretion (asterisks) and constriction at the apex of the colleter (arrows). **J-L.** Colleters in senescent leaves. **J.** Blackened colleters. **K.** Collapsed colleters. **L.** Cuticle rupture (arrowheads). Abbreviation: co = colleters. Bars: A, D, H = 2 mm; B, C, I = 500 µm; E, L = 100 µm; F = 20 µm; G = 2 µm; J = 2 mm; K = 200 µm.

of cells at the apical portion (Fig. 2E). Intense production and release of secretion via cuticular rupture (Fig. 2F-G) was observed.

In the adult leaves, the brown color of the colleters was intensified and a reduction in the amount of secretion was observed both in the field (Fig. 2H) and with SEM (Fig. 2I). In the apical portion of the colleter, a constriction was noted that separates the brownish apex, where wilting and cell disruptions were common, from the lower region (Fig. 2I). Finally, colleters acquired a blackish color (Fig. 2J), coinciding with the senescence of the leaves. In this phase little or no secretion was observed and the colleters were withered (Fig. 2K). Cellular disruptions, especially in the secretory portion, were common (Fig. 2L).

Development and histology of colleters

Colleters were formed during the first stage of leaf development (Fig. 3). In early development, most cells

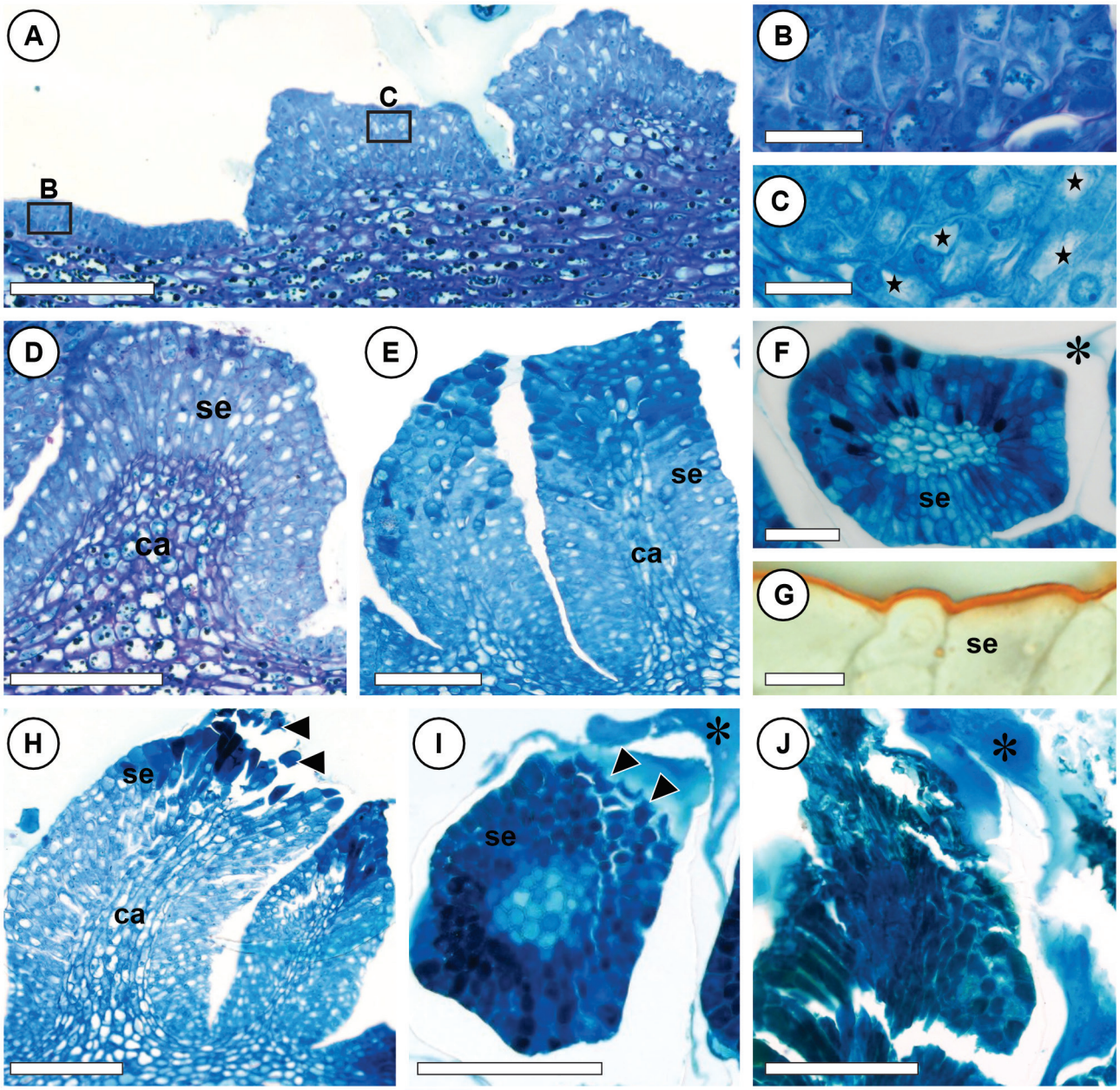

Figure 3. Anatomical sections of the leaf colleters of *Clusia criuva* Cambess. subsp. *criuva*. **A-F.** Colleters in early leaf. **A.** Asynchronous development from protodermal and subepidermal cells. **B.** Detail of the undifferentiated cells of the colleters, which have an evident nucleus and nucleolus. **C.** Intense vacuolization (stars). **D.** Colleter with central axis and secretory epidermis. **E.** Standard type colleters, sessile with multiseriate epidermis. **F.** Non-vascularized colleter. **G.** Cuticle stained with Sudan III. **H, I.** Colleters starting senescence (arrowheads). **J.** Senescent colleter. Asterisks indicate secretion. Abbreviations: ca = central axis; se = secretory epidermis. Bars: A, D, I, J = 500 µm; E, F, H = 200 µm; B, C = 20 µm; G = 50 µm.

of the protoderm elongated (Fig. 3A) and successive anticlinal divisions resulted in a projection formed by the multiseriate epidermis (Fig. 3A). The newly divided cells presented dense cytoplasm, voluminous nuclei and nucleoli, and high nucleus/cytoplasm ratio (Fig. 3B). The subepidermal cells then divided and formed the central axis made up of parenchymal cells (Fig. 3A, D), and at this stage, the vacuolization process was observed throughout the cytoplasm of the parenchymal cells (Fig. 3C). Divisions were intensified, culminating in the formation of colleters (Fig. 3D).

Colleters were sessile and non-vascularized with a truncated or tapered apex (Fig. 3D-E). They presented a multicellular head formed by a central axis of parenchymal cells and a secretory epidermis (Fig. 3D-F) coated with a thick cuticle (Fig. 3G), following into the standard-type colleters. The secretory epidermis had commonly more than two layers of columnar juxtaposed cells which tended to palisade (Fig. 3E).

In adult leaves, the degradation of cells in the apical portion was accentuated (Fig. 3H). In senescent leaves, the secretory epidermis of most colleters was completely degraded with collapsed and/or ruptured cells (Fig. 3I-J). Although senescent, *Clusia criuva* subsp. *criuva* colleters did not come into abscission.

Histochemical characterization

Histochemical tests showed polysaccharides as the main components of the secretion produced by *Clusia criuva* subsp. *criuva*. Total polysaccharides (Fig. 4A-C) and pectins/mucilage (Fig. 4D-F) were observed in the exudates of the colleters at all leaf development stages. In the primordia, young, and adult leaves, accumulation of these compounds was observed in the cytoplasm of secretory cells (Fig. 4A, B, D, E), in the subcuticular space (Fig. 4B, E), and in extravasated secretion (Fig. 4A, C, F).

Proteins were observed in the exudate of the colleters from the leaf primordia (Fig. 4G) through to the senescent leaves (Fig. 4I). However, the accumulation of proteins in secretory cells was evident only in colleters from leaf primordia and young leaves (Fig. 4G). Lipid droplets were observed within the secretory cells as well as in the cells from the central axis in the young leaves. In adult and senescent leaves, lipids were detected only in secretory epidermal cells (Fig. 4H), and structural lipids impregnated in cell walls in cells of the central axis (Fig. 4H).

Phenolic compounds were registered in the colleters present in all leaf development stages. However, in leaf primordia and young leaves, these compounds were restricted to the apical portion of the colleters (Fig. 4J), whereas in adult and senescent leaves, phenolics were observed throughout the entire structure of the colleter, particularly in the secretory cells of the epidermis (Fig. 4K, L).

Discussion

The abundant secretion observed in the field in the leaf primordia and young leaves of *Clusia criuva* subsp. *criuva*, the distribution as well as the structural and chemical nature of the secretion confirm our hypothesis for the presence of colleters on the leaves of this species. The colleters of *C. criuva* subsp. *criuva* are derived from the protoderm and the ground meristem and are classified as emergencies according to Evert (2006). In *Ilex* species, González (1998) reported that a group of protodermal cells undergoes radial enlargement and anticlinal division and some subepidermal cells divide to form a protuberance. Thus, the anticlinal division gives rise to the secretory epidermis and the subepidermal cells produce the parenchymal axis of the colleter, and the same pattern has been observed here. Other studies report that the most common occurrence is that of colleters forming only by the protoderm from periclinal and anticlinal divisions, without no involvement of the ground meristem (Paiva & Machado 2006; Paiva 2009; Paiva & Martins 2011; Rocha *et al*. 2011). As pointed out by our results, the involvement of the protoderm and the ground meristem in the process for colleter was reported in *C. fluminensis*, *C. lanceolata* (Machado & Emmerich 1981; Silva *et al*. 2019) and *C. burchellii* (Teixeira *et al*. 2021), which suggests that it may be a conservative characteristic in the group, although studies on colleters in the genus are still scarce.

The standard type colleters present in *C. criuva* subsp. *criuva* is constituted by a central parenchymal axis surrounded by a layer of palisade-secreting epidermal cells, as previously reported by Lersten (1974). The standard type colleter also was found in *C. grandiflora* (Machado & Emmerich 1981) and *C. burchelli* (Teixeira *et al*. 2021). In *C. fluminensis* and *C. lanceolata*, lachrymiform cone-shaped colleters were described (Silva *et al*. 2019). However, we understand that the lachrymiform refers to the external morphology of the colleter, which is anatomically organized as the standard type, according to Lersten (1974). Studies on the morphological differences of colleters can be important to offer contributions to taxonomy studies and to establish phylogenetic relationships within the Clusiaceae. Our results clearly show cuticle ruptures in the apical portion of the colleters, which is a common way to secrete substances by colleters (Paiva & Machado 2006; Silva *et al*. 2017).

The chemical composition of the colleter exudates in *C. criuva* subsp. *criuva* is diverse as it is composed of a mixture of hydrophilic and lipophilic compounds, that is, polysaccharides, proteins, lipids, and phenolic compounds. However, variations in the composition of the secretion were observed depending on the leaf development stage. Mucilage consisting mainly of polysaccharide polymers with high molecular weight (Fahn 1988) together with pectin may act as a water absorber due to its hygroscopic properties (Hall 1981; Nobel *et al*. 1992), which may protect

meristem and young leaves of *C. criuva* subsp. *criuva* against desiccation. This corroborates the primary function of colleters in producing secretions that protect young organs during their development (Lersten & Horner 1968; Lersten 1974; Fahn 1979) and/or that promote the lubrication of the stem meristem, minimizing friction of the developing leaf tissues and preventing dehydration (Fahn 1979; Thomas 1991; Mayer *et al.* 2011; Silva *et al.* 2012; Mayer *et al.* 2013).

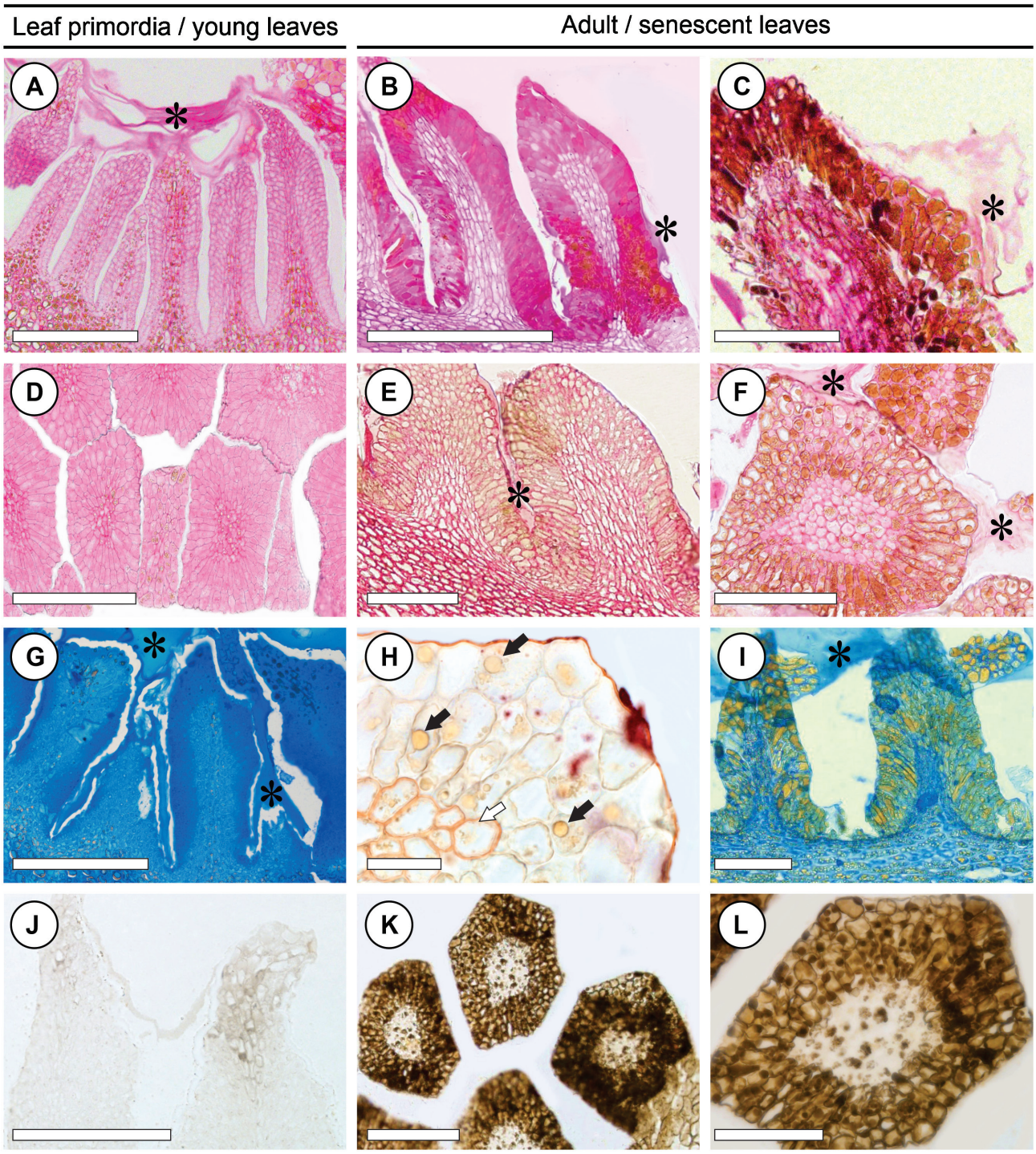

Figure 4. Histochemical tests of *Clusia criuva* Cambess. subsp. *criuva*. **A-C.** Reaction with PAS showing the presence of polysaccharides. **D-F.** Presence of pectin confirmed by the red ruthenium test. **G, I.** Reaction with Coomassie brilliant blue showing proteins in the cytoplasm and extravasated secretion. **H.** Lipid droplets (black arrow) and impregnation of lipids in the cells of the central axis (white arrow), evidenced by Sudan III. **J-L.** Ferric chloride test demonstrates the increase in the amounts of phenolic compounds in the colleters during leaf development. Asterisks indicate extravasated secretion. Bars: A, D, G, J = 500 µm; B, C, E, F, I, K, L = 100 µm; H = 20 µm.

Proteins are involved in the defense against fungal activity and attack of pathogens (Klein *et al*. 2004; Miguel *et al*. 2006) and are commonly reported in the secretion of colleters (González & Tarragó 2009; Coelho *et al*. 2013; Dalvi *et al*. 2014; Lacchia *et al*. 2016; Pinheiro *et al*. 2019).

The phenolic compound production was intensified, in colleters, of adult and senescent leaves of C. *criuva* subsp. *criuva*. Phenolics have been reported for other species of *Clusia* both in the secretion present in the meristem and in the adult leaves (Silva *et al*. 2019). According to Coelho *et al*. (2013), phenolic compounds and lipids are the final products of the secretion of colleters in a senescence stage. The cuticle wrinkling, the formation of a constriction at the base of the colleters and cell disruption also indicate the senescence of the colleters (Pinheiro *et al*. 2019), which were also shown here for C. *criuva* subsp. *criuva* colleters even they did not come into abscission.

The color of colleters changes in the field during the development of *C. criuva* subsp. *criuva* leaf, as reported for *C. burchellii* (Teixeira *et al*. 2021) and species from other families, including Apocynaceae (Thomas & Dave 1989; Appezzato-da-Glória & Estelita 2000; Rio *et al*. 2002), Gentianaceae (Dalvi *et al*. 2013; 2014), and Rubiaceae (Lersten 1974; Miguel *et al*. 2006; 2009; Vitarelli & Santos 2009; Klein *et al*. 2010; Pinheiro *et al*. 2019). These authors generally associate changes in color with the presence of phenolic compounds, which was confirmed in colleters of *C. criuva* var. *criuva*. The accumulation of phenolic compounds in colleters is also related to a change in the function of these glands, being produced in a second stage of the secretory phase (Ribeiro *et al*. 2017), which would act to avoid predation (Castro & Demarco 2008). However, the position of colleters in this species and others would not preclude the predation of leaves by herbivores.

In summary, we report here the occurrence of standard-type colleters in *C. criuva* subsp. *criuva* as well as the morphoanatomical variations of the structure and chemical composition of the exudate throughout the leaf development of this species. Our results contribute to a better understanding of the morphofunctionality of colleters in Clusiaceae, structures that are scarcely studied in this group of plants. Comparative studies with a larger number of Clusiaceae species are also needed in order to understand the evolution of colleter for this family and their correlation with the biomes where such species occur.

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