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Croceous glands in *Polygala adenophora* (Polygalaceae): structure, histochemical, and functional aspects

Augusto César da Silva JORGE[1,3,*], Joana Patrícia Pantoja Serrão FILGUEIRA[4], Layse Kristine Corrêa LOPES[1,3], Ana Cristina Andrade de AGUIAR-DIAS[2]

[1] Universidade Federal Rural da Amazônia, Av. Perimetral 2501, Universitário, 66077-901 Belém, PA, Brazil

[2] Universidade Federal do Pará, Instituto de Ciências Biológicas – Campus Básico, Rua Augusto Corrêa 1, Guamá, 66075-110 Belém, PA, Brazil

[3] Museu Paraense Emílio Goeldi, Coordenação de Botânica, Av. Perimetral 1901, Terra Firme,CP 6109,66077- 530 Belém, PA, Brazil

[4] Instituto Tecnológico Vale – ITV, Rua Boaventura da Silva 955, Nazaré, 66055-090 Belém, PA, Brazil

* Corresponding author: augustocesarjorge@gmail.com; https://orcid.org/0000-0002-0063-9846

ABSTRACT

Croceous glands are visible structures defined by their rounded shape and yellowish color. They have great taxonomic importance, being used to delimit the *Timutua* section, one of the 14 sections of the subgenera *Polygala*, genera *Polygala*, the most representative of the family Polygalaceae, with about 120 species recorded in Brazil. However, detailed information about the nature of croceous glands is still missing. Thus, this study aimed to characterize the origin, structure, and function of the croceous glands in the floral structures, fruits, leaf blades, and stems of *Polygala adenophora*. Samples of *P. adenophora* at different developmental stages (flower buds, fully developed flowers and fruits), as well as stems, and leaves collected from the 1st, 3rd, 6th, and 9th nodes were obtained from a coastal area in Pará state, Brazil. The samples were fixed in FAA_{50} or buffered neutral formalin and submitted to light microscopy and scanning electron microscopy. Histochemical tests were carried out for lipophilic and hydrophilic compounds. Our results showed that the croceous glands are secretory cavities and ducts of schizolysigenous origin, present since the beginning of the development of vegetative and reproductive organs. The cavities and ducts produce a lipophilic compound consisting of essential oils and lipids. We hypothesize that this exudate possibly has a protective function, acting against pathogen and herbivore attacks.

KEYWORDS: Fabales, plant anatomy, secretory cavities, essential oils

Glândulas cróceas em *Polygala adenophora* (Polygalaceae): estrutura, histoquímica e aspectos funcionais

RESUMO

Glândulas cróceas são estruturas visíveis, definidas por seu formato arredondado e coloração amarelada. Elas possuem grande importância taxonômica, sendo utilizadas para delimitar a seção *Timutua*, uma das 14 seções do subgênero *Polygala*, pertencente ao gênero *Polygala*, o mais representativo da família Polygalaceae, com cerca de 120 espécies registradas para o Brasil. Porém, as glândulas cróceas ainda carecem de informações mais detalhadas sobre sua natureza. Dessa forma, o presente estudo caracterizou a distribuição, estrutura e histoquímica das glândulas cróceas nos órgãos reprodutivos e vegetativos aéreos de *Polygala adenophora*. Amostras de *P. adenophora* em diferentes estágios de desenvolvimento (botões florais, flores totalmente desenvolvidas e frutos), assim como caule e folhas extraídas dos 1º, 3º, 6º e 9º nós, foram obtidas em uma área costeira no estado do Pará, Brasil. As amostras foram fixadas em FAA_{50} ou formalina neutra tamponada e submetidas a microscopia de luz e microscopia eletrônica de varredura. Foram realizados testes histoquímicos para substâncias lipofílicas e hidrofílicas. Os resultados mostraram que as glândulas cróceas são formadas por cavidades e ductos secretores, com formação de origem esquizolisígena, presentes desde o início do desenvolvimento dos órgãos vegetativos e reprodutivos. As cavidades e ductos secretam uma substância de natureza lipofílica, composta por óleos essenciais e lipídios. Hipotetizamos que esse exsudato possivelmente tenha uma função protetiva, atuando contra ataques de patógenos e herbívoros.

PALAVRAS-CHAVE: Fabales, anatomia vegetal, cavidades secretoras, óleos essenciais

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INTRODUCTION

Polygala L. is the genus of Polygalaceae with the largest number of species, with about 580 widely distributed representatives (Pastore 2018), of which 120 occur in Brazil (BFG 2015). This genus is characterized by chlorophyllous herbs (annual to perennial) and shrubs, racemes (rarely with reduced axillary groups), flowers with crested keel, fruit in a bilocular capsule or drupe with a persistent calyx, capsule margins almost always entire (generally with emarginate apex, rarely crenate or wavy), and seeds without a crown of trichomes, but usually with short or glabrous trichomes (Mota *et al.* 2019).

The genus comprises two subgenera, *Polygala* L. and *Chodatia* Paiva (Pastore 2018). The subgeneric classification is based on phylogenetic studies by Abbott (2009), according to which *Polygala* is divided between Old and New World clades possibly distributed among 14 sections (*Brachytropis* DC., *Blepharidium* DC., *Chloropterae* (Chodat) Paiva, *Clinclinia* DC., *Conosperma* Paiva, *Leptaleae* (Chodat) Paiva, *Madecassa* Paiva, *Megatropis* Paiva, *Microlophium* Spach, *Monninopis* A. Gray, *Polygala* L., *Psychanthus* (Raf.) DC., *Tetrasepalea* (Chodat) Paiva, and *Timutua* DC).

The *Polygala* New World section *Timutua* is characterized by the presence of visible/croceous glands with an arched style terminating in an oblique cymbiform pre-stigmatic cavity, a posterior end with conspicuous crest appendage with abundant (sometimes absent) trichomes, an anterior globose stigma, a keel petal adorned by a crest, and a persistent calyx in the fruit. The section is distributed from Canada to Argentina, and 99 of its 175 species occur in Brazil (Pastore and Harley 2009; Pastore 2018).

Despite the importance of the croceous glands in delimiting *Timutua*, little information exists about their nature. Croceous glands were cited by Saint-Hilaire and Moquin (1828) and Chodat (1893), defined only by their yellowish coloration. Therefore more detailed anatomical studies are needed to identify the role of croceous glands in the plant's physiology, and describe their development, structure, and type of substance secreted in different plant organs. Thus the aim of this study was to characterize the distribution, structure, and histochemistry of croceous glands in a *Timutua* representative (*Polygala adenophora* DC).

MATERIAL AND METHODS

Polygala adenophora is a herbaceous species that occurs in several Brazilian phytogeographical domains (Amazon, *Caatinga*, *Cerrado* and Atlantic Forest), being distributed in all regions of Brazil, with confirmed occurrence in the states of Pará, Roraima, Bahia, Ceará, Maranhão, Paraíba, Pernambuco, Piauí, Federal District, Goiás, Mato Grosso, Minas Gerais, Rio de Janeiro, São Paulo and Paraná (BFG 2015; Pastore *et al.* 2015).

Fifteen specimens of *P. adenophora* were collected in an area of low vegetation on strips of sand (*restinga*) by Crispim Road, in the municipality of Marapanim, state of Pará (Brazilian Amazon) (00°36'19.3"S; 47°40'17.4"W) in an area of approximately 80 m². All the specimens were identified by Augusto César da Silva Jorge. A voucher specimen has been deposited in the João Murça Pires herbarium of Museu Paraense Emílio Goeldi (MG 231658). From this material, we sampled flower buds, flowers, fruits, floral rachis, leaf blades, and stem. For the anatomical analysis, samples of reproductive organs were considered at three different developmental stages (flower buds, fully developed flowers and fruits). Stem and leaf samples were taken from the 1st, 3rd, 6th, and 9th visible internodes (Figure 1).

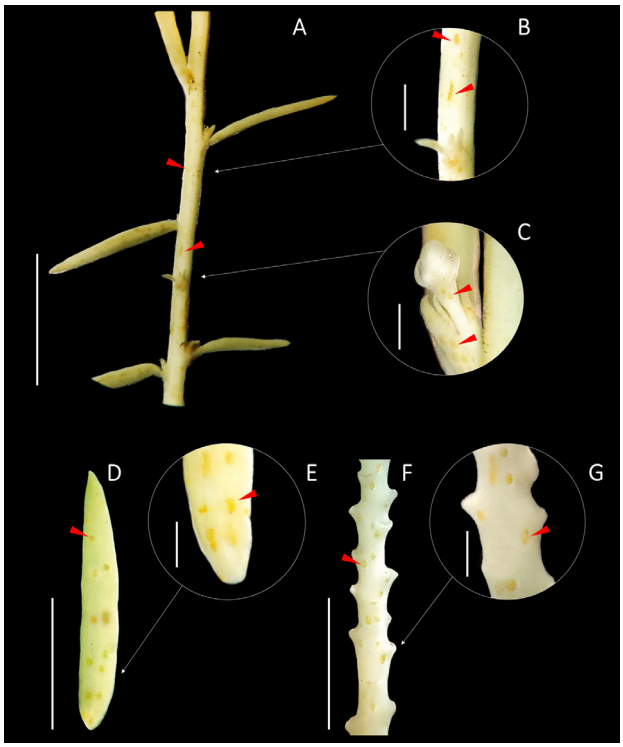

Figure 1. External morphology of the vegetative organs of *Polygala adenophora* DC. A – stem; B – detail of the croceous glands in the stem; C – detail of the croceous glands on the lateral bud; D – leaf blade; E – detail of the croceous glands on the leaf blade; F – floral rachis; G – detail of the croceous glands in the floral rachis. Arrows = cavities and ducts; Star = lumen; Ep = epithelium. Scale bars: A = 2 cm; B, C, E, G = 3 mm; D, F = 1 cm.

The samples were fixed in formaldehyde, glacial acetic acid, and ethyl alcohol 50% (FAA_{50}) for 24 hours (Johansen 1940) or in neutral buffered formalin (NBF) for 48 hours (Lillie 1965), kept under vacuum, and stored in ethanol 70%. Subsequently, the samples fixed in FAA_{50} were dehydrated in a tertiary ethylic series for inclusion in Historesin (hidroxietilmetacrilato, Leica® Biosystems, Heidelberg, Germany). Samples fixed in NBF were dehydrated in a tertiary butyl alcohol series followed by

an ethyl alcohol series, and included in *Paraplast®*. All samples were cross-sectioned and longitudinally sectioned using a rotary microtome (model RM 2245, Leica® Biosystems, Heidelberg, Germany). Sample sections in Historesin were stained in Toluidine Blue (Gerlach 1977) and blades were mounted in resin (Permount®, Fisher Scientific, New Jersey, EUA). A light microscope (model DM6 B, Leica® Biosystems, Heidelberg, Germany) equipped with a digital camera (model LAS V4.12, Leica® Biosystems, Heidelberg, Germany) was used for photographic documentation. Macro images were obtained using a stereomicroscope (model SteREO Discovery V8, ©Carl Zeiss, Jena, Germany) with an attached digital camera (model AxioCam ICc5, ©Carl Zeiss, Jena, Germany).

Histochemical analyses were made with the material included in *Paraplast®*. Four histochemical tests were applied to flower buds, flowers, fruits, floral rachis, leaf blades and stems. For each test, ten repetitions were performed. The samples were tested for the presence of lipophilic compounds using Sudan Black B (Pearse 1985) and Sudan III (Johasen 1940) for total lipids, Blue Nile Sulfate for acidic and neutral lipids (Cain 1947), and Nadi reagent for essential oils and oleoresins (David and Carde 1964). Concerning hydrophilic substances, PAS (Periodic-Acid-Schiff's reagent) was used for total polysaccharides (Jensen 1962), Lugol for starch (Johansen 1940), Dragendorff reagents (Svedsen and Verpoorte 1983) for alkaloids, Hydrochloric Vanillin for tannins (Mace and Howell 1974), and Ferric Chloride for total phenolic compounds (Johansen 1940). Control samples for lipophilic compound tests were stored for 24-48 hours in extracting solution (methanol/chloroform/water/HCl) (High 1984). Afterwards, the samples were fixed in NBF and subjected to the mentioned reagents and dyes. Six repetitions for each test were performed.

The anatomical analysis was done with the aid of a scanning electron microscopy. The samples were dried at the critical point of CO_2 affixed to metal supports (stubs) using double-sided carbon tape, and metalized with a 20 nm-thick gold layer for 150 seconds in a current of 25 mA. Secondary electron micrographs were obtained using a scanning electron microscope (model Tescan, Mira 3, Tescan® Orsay Holding, Czech Republic), at an acceleration voltage of 15 Kv and high vacuum. The analyses were done in the scanning electron microscopy laboratory at Museu Paraense Emílio Goeldi (Belém, Pará).

RESULTS

Morphology and distribution

The croceous glands were located on the stems, leaves, floral rachis, lower external sepals, keel, ovary, and fruit capsules (Figures 1 and 2). The glands were present throughout the vegetative and reproductive development, from the initial stages of the organs. Externally, the glands had a yellowish color and their cells were slightly different from the surrounding epidermal cells, with no opening for exudation. This indicates that the stomata do not function as a route for exudate release.

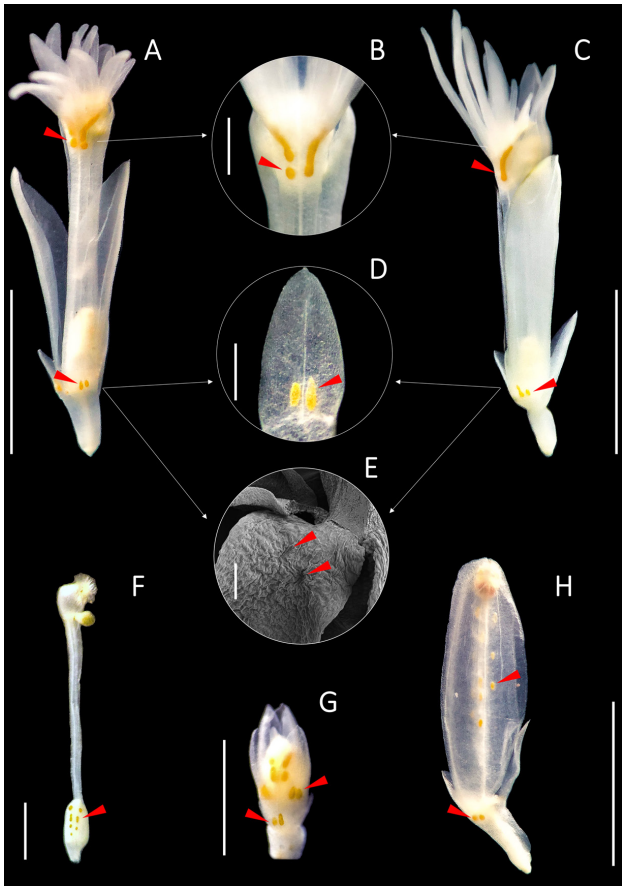

Figure 2. External morphology of the reproductive organs of *Polygala adenophora* DC. A – front view of the flower; B – detail of the croceous glands in the carina; C – lateral view of the flower; D – detail of the croceous glands in the inferior external sepal; E – detail of the croceous glands in the inferior external sepal by scanning electron microscopy; F – gynoecium; G – flower bud; H – capsule-type fruit. Arrows = cavities and ducts; Star = lumen; Ep = epithelium. Scale bars: A, C, H = 3 mm; B, D, F = 1 mm; G = 2 mm; E = 100 µm.

Anatomy and development

In the tissue cross-sections, all glands were structurally similar, with flattened, multiseriate epithelial cells and an oval to round lumen (Figures 3–7). In all vegetative and reproductive organs, cell differentiation and formation of secretory glands began in a single parenchyma cell with dense cytoplasmic content and a well-evidenced nucleus, denoting intense cell division and forming a spherical or oval cluster (Figure 3a,b). The central cells of the cluster increased their cytoplasmic volume and their walls become extremely thin (Figure 3c). From then on, the lumen formation began through cell lysis and separation of the central cells, leading to the appearance of intercellular spaces (Figure 3d–h) until the

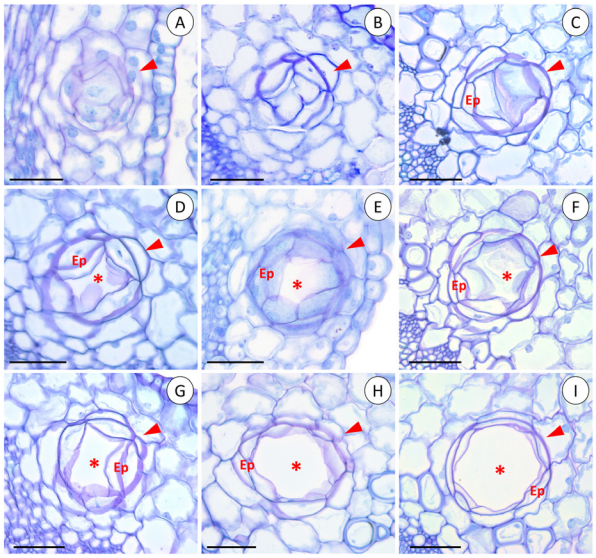

Figure 3. Development of croceous glands in the floral rachis of *Polygala adenophora* DC. in cross section. A–C – gland in the initial stage, showing intense cell division; D–F – gland with secretory epithelium in formation; G,H – formation of cavity lumen by spreading apart developing cells; I – cavity in the final stage of development, with a multiseriate secretory epithelium of thin-walled cells, flattened, and concentrically elongated, delimiting the isodiametric lumen. Arrows = cavities and ducts; Star = lumen; Ep = epithelium. Scale bars: A–I = 50 µm.

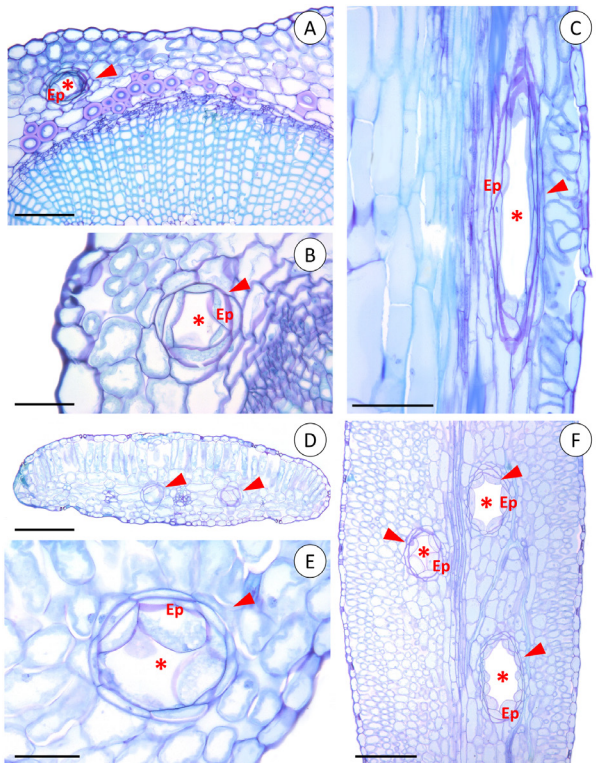

Figure 4. Croceous glands in the vegetative organs of *Polygala adenophora* DC. A,B – stem in cross-section; B – setail of the croceous glands in the stem; C – stem in longitudinal section showing secretory duct; D,E – leaf blade in cross-section; E – detail of the croceous glands on the leaf blade; F – leaf blade in longitudinal section. Arrows = cavities and ducts; Star = lumen; Ep = epithelium. Scale bars: A,C = 100 µm; B,E = 50 µm; D,F = 200 µm.

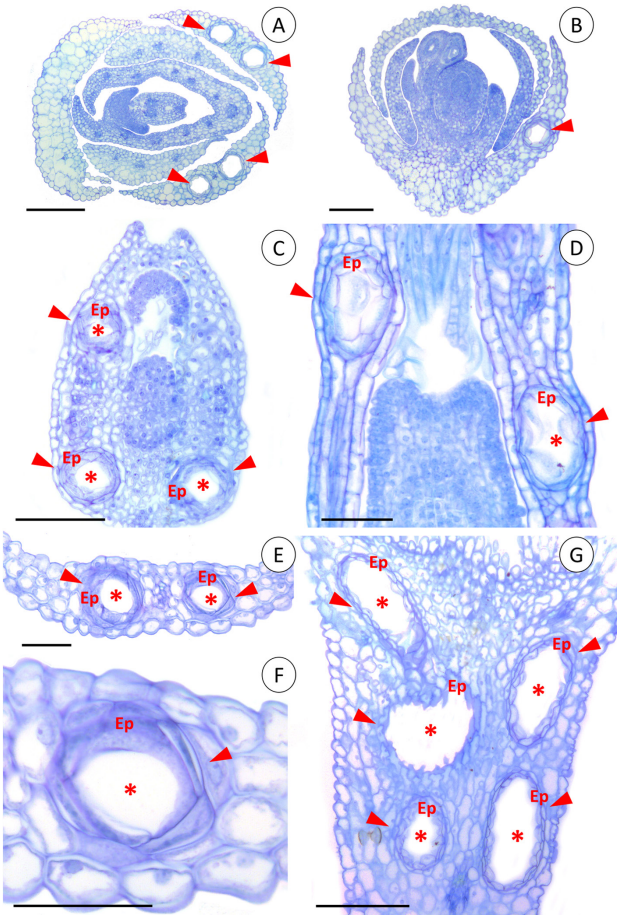

Figure 5. Croceous glands in the reproductive organs of *Polygala adenophora* DC. A – flower bud in cross-section; B – flower bud in longitudinal section; C – ovary in cross-section; D – ovary in longitudinal section; E,F – external lower sepal in cross-section; F – detail of the secretory cavity in the inferior external sepal; G – carena in longitudinal section. Arrows = cavities and ducts; Star = lumen; Ep = epithelium. Scale bars: A,G = 200 µm; B,C = 100 µm; D–F = 50 µm.

lumen formation was complete (Figure 3i). This was observed in all vegetative organs in the most apical internodes, and in the reproductive organs, except in the fruit. This process has a schizolysigenous ontogeny (separation of adjacent cell walls), concomitant with the lysigenic process (cell autolysis).

In the vegetative organs, the glands were distributed on the stems, leaves, and floral rachis (Figure 1a–g). Internally, they were in the cauline cortex and spongy parenchyma (Figures 4 and 6a–c). The cross-sections of these three organs revealed spherical cavities with multiseriate secretory epithelium consisting of thin-walled, flattened, and concentrically elongated cells, delimiting the isodiametric lumen (Figure 4a,b,d,e). In longitudinal section, the leaf glands had a round to oval lumen, being classified as cavities (Figure 4f), while the glands of the stem and floral rachis exhibited a narrow and elongated lumen, probably formed by anastomosis of two or more cavities, forming ducts (Figure 6c). The presence of secretory ducts was also observed in the floral rachis and stems

(Figure 4c), formed from the anastomosis of two or more contiguous cavities during their development.

In the reproductive organs, the cavities were observed in the outer lower sepals, carina, and ovary, being persistent until the development of the fruit (Figures 5 and 6d–f). Cross-sections revealed that the cavities, like in the vegetative organs, also had a rounded contour, a multiseriate secretory epithelium with a thin cell wall, flattened, and elongated cells in a concentric shape, delimiting a isodiametric lumen. An exception was the cavities of the fruits, which had an oval lumen. In longitudinal section, the cavities had an oval to round lumen (Figures 5b,d,g and 6e–f).

Exudate composition

The histochemical tests showed positive results only for lipophilic compounds. Hydrophilic and phenolic compounds were not detected, as evidenced by the negative reaction to PAS, Lugol, Dragendorff, hydrochloric vanillin, and ferric chloride (Table 1). The cavities in both vegetative and reproductive organs produced exudates of an exclusively lipidic nature, and they reacted positively to all reagents of this compound class (Figure 7), except for Nadi, which reacted positively only for essential oils, with no oleoresins being observed. The exudate was present only inside the epithelial cells and was not observed in the lumen (Figure 7), which may be due to the processing of the material, probably by the

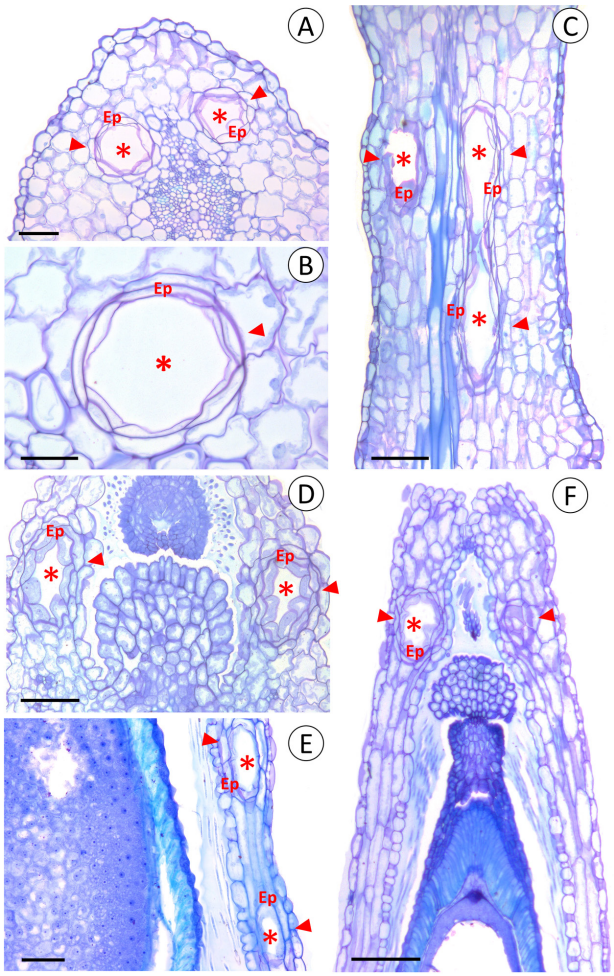

Figure 6. Croceous glands in the floral rachis and reproductive organs of *Polygala adenophora* DC. A,B – floral rachis in cross-section; B – detail; C – floral rachis in longitudinal section; D – fruit in cross-section; E,F – fruit in longitudinal section. Arrows = cavities and ducts; Star = lumen; Ep = epithelium. Scale bars: A, D, E = 100 µm; B = 50 µm; C, F = 200 µm.

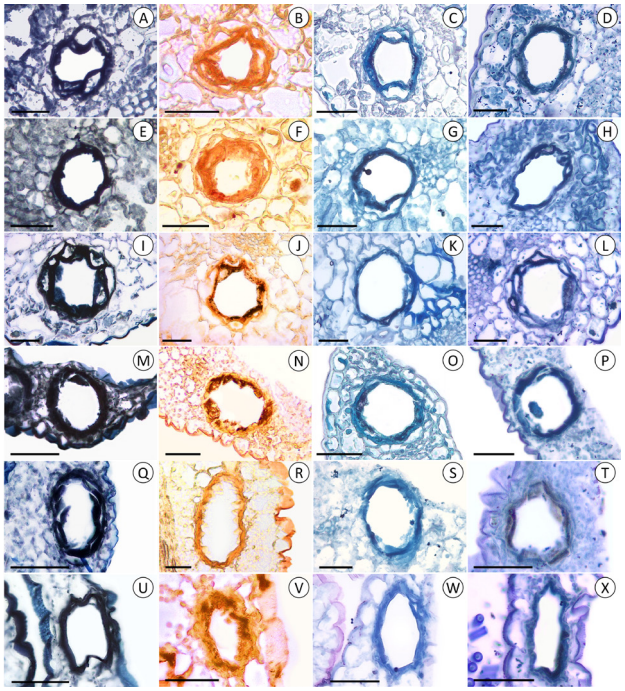

Figure 7. Histochemical reactions of the croceous glands in the vegetative and reproductive organs of *Polygala adenophora* DC. A–D – stem; E–H – leaf blade; I–L – floral rack; M–P – flower bud; Q–T – flower; U–X – fruit. A, E, I, M, Q, U – Sudan Black B; B, F, J, N, R, V – Sudan III; C, G, K, O, S, W – Blue Nile sulfate; D, H, L, P, T, X – Nadi reagent. Arrows = cavities and ducts; Star = lumen; Ep = epithelium. Scale bars: A–X = 50 µm.

Table 1. Histochemical tests performed on croceous glands of *Polygala adenophora* included in *Paraplast*®. Ten repetitions were made for each test.

Reagent	Metabolites	Organs					
		Stem	Leaf	Floral rack	Flower bud	Flower	Fruit
SuB	total lipids	+	+	+	+	+	+
SuIII	total lipids	+	+	+	+	+	+
SAN	acidic and neutral lipids	+	+	+	+	+	+
Nadi	essential oils and oleoresins	+	+	+	+	+	+
PAS	total polysaccharides	–	–	–	–	–	–
Lu	starch	–	–	–	–	–	–
Drgff	alkaloids	–	–	–	–	–	–
VCl	tannins	–	–	–	–	–	–
ClFe	total phenolic compounds	–	–	–	–	–	–

SuB = Sudan black B; SuIII = Sudan III; SAN = Blue Nile sulfate; Nadi = Nadi reagent; PAS = periodic acid Schiff; Lu = Lugol; Drgff = Dragendorff reagent; ClFe = ferric chloride. + indicates a positive result; – indicates a negative result.

inclusion medium used (FAA and NBF). In fixed material the secretion is washed, only being observed in the lumen when a freehand cut is made in fresh material. The glands can be classified as oleiferous, as they presented exudate composed of lipids (Sudan Black B, Sudan III, and Blue Nile Sulfate), specifically essencial oils (Nadi).

DISCUSSION

The croceous glands observed in several taxonomic studies are actually cavities and ducts. However, we will continue to refer them as croceous glands, in order to standardize the taxonomic term that is characteristic of the Polygalaceae family.

The croceous glands found in both vegetative and reproductive organs of *P. adenophora* predominantly showed an isodiametric lumen, except for the stems and floral rachis, which had cavities with a slightly longitudinally elongated lumen, described by Thadeo *et al.* (2009) as "tubular cavities". These cavities are formed by the coalescence of two contiguous cavities originating from various transitional shapes between cavities and ducts (Fernandes *et al.* 2018). Only structures that are at least three times as long as they are wide should be called ducts (Fernandes *et al.* 2018), corresponding to some of the structures identified in the floral rachis and stems of *P. adenophora*. Therefore, anatomically the croceous glands in *P. adenophora* can be characterized as ducts in addition to cavities.

Aguiar-Dias *et al.* (2012) reported the occurrence of lysigenous cavities in the mesophyll of *Polygala paniculata* L. These structures were also cited in leaves of *Polygala* species, highlighting the taxonomic importance of the glands for Polygalaceae (Chodat 1893; 1896; Solereder 1908; Metcalfe and Chalk 1950; Eriksen 1993a;1993b). The formation of the lumen of the croceous cavities through a schizolysigenous process (lysis and distancing of developing cells) differs from the cavitiy-forming process in *P. paniculata*, which occurred only through cell lysis (Aguiar-Dias *et al.* 2012).

The croceous cavities and ducts in our study were present and secreted exudates throughout the ontogenetic development of vegetative and reproductive organs. We hypothesized that the exudate produced by the croceous glands, with a lipid composition, has a protective function, both because of its composition and the regular occurrence of the glands in the vegetative and reproductive organs, possibly acting against pathogen and herbivore attacks for self-preservation (Dudareva *et al.* 2006; Dudareva *et al.* 2013).

Substances of a lipophilic nature, as found to compose exclusively the croceous-gland exudate in *P. adenophora*, are common in this type of structure (Fanh 1979). The exudate is composed of essential oil. Based on these results, the croceous glands can be classified as oil glands in the form of cavities and ducts. Although the absence of accumulated secretion in the lumen of the glands may be a methodological artifact, the presence of the secretion of the glands inside the epithelial cells, with no exudate in the lumen, was also observed by Aguiar-Dias *et al*. (2011) in the mucilaginous ducts of the stem of *Polygala angulata* DC. The croceous glands do not have any openings for exudation and the stomata present do not function as an exudate release route, which corroborates our hypothesis that the croceous glands can have a protective function against pathogen attacks and herbivory.

CONCLUSIONS

The croceous glands observed in the vegetative and reproductive organs of *P. adenophora* were identified as essential oil-secreting cavities and ducts. The glands were present throughout the vegetative and reproductive development of the plant. The exudate is retained in the lumen of the structure with no release route. Given its constant occurrence throughout the plant, we hypothesized that the glands can have a protective function, with the exudate acting as a defense against pathogen and herbivore attacks. Further studies should address the structure, anatomy and exudate composition of these glands in other *Timutua* taxa to better understand their function.

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DATA AVAILABILITY

The data that support the findings of this study are available, upon reasonable request, from the corresponding author, Augusto César da Silva Jorge.