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Histolocalization of chemotaxonomic markers in Brazilian Vernonieae (Asteraceae)

MAKELI G. LUSA[1,2]*, FERNANDO B. DA COSTA[3] and BEATRIZ APPEZZATO-DA-GLÓRIA[1]

[1]*Departamento de Ciências Biológicas, Escola Superior de Agricultura 'Luiz de Queiroz', Universidade de São Paulo, Piracicaba, São Paulo 13418-900, Brazil*
[2]*Plant Biology Postgraduate, Instituto de Biologia, Universidade Estadual de Campinas, CP 6109, Campinas, São Paulo 13083-970, Brazil*
[3]*Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, 14040-903 Ribeirão Preto -SP, Brazil*

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Vernonieae are well represented in South America by subtribe Lychnophorinae, with 104 Brazilian species, some of them rare and endangered. Lychnophorinae are well known for producing metabolites of high pharmaceutical and chemotaxonomic value. Despite the importance of secondary metabolites in Lychnophorinae, there is still little evidence regarding the secretory structures responsible for producing these compounds. Therefore, in this study we investigated 15 species representative of the main lineages of Lychnophorinae, with the following objectives: to identify where the chemotaxonomic markers of Lychnophorinae are secreted and stored and in which developmental stage of the organ these metabolites are found. Samples of stems and leaves were processed according to the usual techniques in plant anatomy. It was found that the main sites of secondary metabolite biosynthesis are glandular trichomes, epidermal cells and parenchyma tissues. Metabolites from glandular trichomes, especially sesquiterpene lactones (STL), are prevalent in the early developmental stages of organs. The metabolite compounds stored in parenchyma tissues are mainly terpenoids, flavonoids and other phenolic compounds; young and expanded leaves are equally rich in metabolites. Thus, the information obtained in this study is essential for conducting chemotaxonomic studies in this group, helping to promote selective collection and conservation of species. © 2016 The Linnean Society of London, *Botanical Journal of the Linnean Society*, 2016, **182**, 581–593

ADDITIONAL KEYWORDS: epidermal cells – flavonoids – glandular trichomes – histochemistry – parenchyma tissues – secretory endodermis – sesquiterpene lactones.

INTRODUCTION

Asteraceae include many species of economic interest, many of these are used therapeutically, and consequently their morphology and anatomy have been studied, including the structures responsible for the production, storage and secretion of secondary metabolic compounds (Werker & Fahn, 1981; Ascensão & Pais, 1987; Figueiredo & Pais, 1994; Ascensão *et al.*, 2001; Andreucci *et al.*, 2008; Camilotti *et al.*, 2014). These secretory structures in Asteraceae take the form of glandular trichomes, canals, cavities, laticifers, idioblasts, hydathodes and glandular appendices (Metcalfe & Chalk, 1950; Carlquist, 1958; Lestern & Curtis, 1985; Castro, Leitão Filho & Monteiro, 1997). In the tribe Vernonieae, glandular trichomes, idioblasts, cavities and secretory channels have all been described (Castro *et al.*, 1997; Appezzato-da-Glória *et al.*, 2008; Favi *et al.*, 2008; Cury & Appezzato-da-Glória, 2009; Appezzato-da-Glória *et al.*, 2012), although the presence of secretory endodermis is less frequently reported (Luque, Menezes & Semir, 1997; Appezzato-da-Glória *et al.*, 2008).

Lychnophorinae are one of the most representative subtribes of Vernonieae in Brazil (Keeley & Robinson, 2009), comprising 18 genera and 104 species including shrubs, subshrubs, trees and treelets, which are nearly all restricted to the Cerrado

*Corresponding author. E-mail: makelilus@yahoo.com.br

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Domain, occurring predominantly in campos rupestres (Loeuille *et al.*, 2015a) and exhibiting morphological and anatomical adaptations to these arid zones (Lusa *et al.*, 2014, 2015). Findings of recent phylogenetic analysis for Lychnophorinae based on molecular and morphological data show that the subtribe is monophyletic (Loeuille, Keeley & Pirani, 2015b). However, questions remain concerning the relationships of some taxa in this phylogenetic hypothesis and additional characteristics, such as chemical and morphological data, may prove useful for a better understanding of the evolution of the group.

It is well know that Lychnophorinae produce metabolites of therapeutic importance, such as terpenoids (mono-, sesqui-, di- and triterpenes and sesquiterpene lactones) and phenolic compounds (flavonoids and cinnamic acid derivatives) (Vichnewski & Gilbert, 1972; Vichnewski *et al.*, 1976; Herz *et al.*, 1980; Dias Barros *et al.*, 1985; Oliveira *et al.*, 1996; Grael *et al.*, 2000; Dos Santos *et al.*, 2005; Kanashiro *et al.*, 2006; Taleb-Contini *et al.*, 2008; Silvério *et al.*, 2008; Keles *et al.*, 2010; Dos Santos *et al.*, 2010; Baldin *et al.*, 2012; De Souza *et al.*, 2012; Abreu *et al.*, 2013; Silvério *et al.*, 2013; Gouvea *et al.*, 2014; Saúde-Guimarães, Raslan & Oliveira, 2014). In addition, many studies have investigated the chemotaxonomy of Lychnophorinae because of the chemical richness that characterizes the subtribe (Bohlmann *et al.*, 1980a, b; Herz *et al.*, 1980; Bohlmann *et al.*, 1981; Zdero *et al.*, 1981; Bohlmann *et al.*, 1982; Bohlmann & Jakupovic, 1990; Borella *et al.*, 1992; Lunardello *et al.*, 1995; Borella *et al.*, 1998; Vichnewski *et al.*, 1999; Sacilotto, Sartori & Vichnewski, 2002; Sartori *et al.*, 2002; Sakamoto *et al.*, 2003, 2005; Keles *et al.*, 2010; Sakamoto *et al.*, 2010; Souza *et al.*, 2012; Lusa *et al.*, 2016). In regard to the secretory structures of Lychnophorinae, the presence of glandular trichomes has been reported by Luque, Menezes & Semir (1999) in leaves and by Luque & Menezes (2003) in stems of *Lychnophora* Mart. *s.l.* Silva *et al.* (2014) recently reported the presence of flavonoid metabolites in palisade parenchyma cells in the leaves of six *Lychnophora* spp. Despite these efforts, the secretion sites of the major classes of secondary metabolites for the group are not well established, especially in view of its new circumscription (Loeuille *et al.*, 2015b).

Many species of Lychnophorinae are endemic with small populations and 15 species considered rare (Nakajima *et al.*, 2009). Due to the peculiar environmental conditions under which most species of the subtribe live (Lusa *et al.*, 2014), their restricted distribution and the destruction of natural habitats, 46 species of Lychnophorinae are endangered (Nakajima, Dematteis & Loeuille, 2013). This situation must be taken into account when planning to collect biological material, especially for endangered species. With the advent of new and modern analytical (Marston, 2007) and spectrophotometric techniques (Eisenreich & Bacher, 2007) for phytochemical studies, much less plant material is required for compound isolation and substance identification (Sumner, Mendes & Dixon, 2003). If the research focuses on the detection of substances located in specific sites (trichomes, organs, secretions etc.), the amount of biological material required is even smaller, as little as a few grams or milligrams (Sumner *et al.*, 2003; Allwood & Goodacre, 2010; Chagas-Paula *et al.*, 2015). To implement these new approaches, it is essential to have exact knowledge of the structures in which the compounds of interest are secreted or stored and in which organ and at which developmental stage of the organ the key substances are accumulated. In this study, we investigated 15 species of Lychnophorinae representative of the main lineages in the group (Loeuille *et al.*, 2015b). The main objectives were to verify where the main secondary metabolites or chemotaxonomic markers of Lychnophorinae are secreted and stored and to determine at which developmental stage of the organ these substances are found.

MATERIAL AND METHODS

The plant material analyzed was collected in the states of Minas Gerais and Bahia, Brazil (Table 1). Vouchers were deposited at the Universidade de São Paulo Herbarium (SPF) (Table 1). Samples of stems (from apical to woody regions in early thickening stages) and young and fully expanded leaves were collected from adult plants.

Micromorphological characterization

For structural analyses, samples were fixed in Karnovsky solution (Karnovsky, 1965; modified to incorporate phosphate buffer pH 7.2), subjected to vacuum conditions to remove the air in the tissues, dehydrated using ethyl alcohol and infiltrated with plastic resin hydroxyethyl methacrylate (Leica Historesin; Heraeus Kulzer, Hanau, Germany) according to the manufacturer's instructions. The samples were sectioned at 5–7 μm thickness using a rotary microtome (Model RM 2245; Leica Microsystems Nussloch GmbH, Nussloch, Germany) and then stained with 0.05% toluidine blue in citrate-phosphate buffer, pH 4.5 (Sakai, 1973), and mounted in Entellan synthetic resin (Merck, Darmstadt, Germany). In addition, fresh material from all species was cut with a razor

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Table 1. Plant material used in this study

Species	Locality	Geographic coordinates	Elevation (m)	Voucher
Anteremanthus hatschbachii H.Rob.	Grão Mogol, MG	S16°35′37.6″ W42°54′07.7″	673	*Loeuille et al. 533*
Blanchetia heterotricha DC.	Dom Macedo Costa, BA	S12°54′11.1″ W39°12′06.6″	193	*Loeuille et al. 680*
Chronopappus bifrons DC.	Santo Antônio do Itambé, MG	S18°23′49.9″ W43°21′21.0″	1819	*Lusa et al. 63*
Eremanthus leucodendron Mattf.	Rio de Contas, BA	S13°31′29′′ W41°57′39.9′′	1691	*Loeuille et al. 669*
Eremanthus polycephalus (DC.) MacLeish	Diamantina, MG	S18°11′52.0″ W43°37′33.0″	1141	*Loeuille et al. 531*
Heterocoma gracilis Loeuille, J.N.Nakaj. & Semir.	São Gonçalo do Rio Preto, MG	S18°07′42.1″ W43°22′40.5″	1039	*Lusa et al. 61*
Lychnophora diamantinana Coile & S.B.Jones	Diamantina, MG	S18°11′52.5″ W43°37′33.1″	1141	*Loeuille et al. 530*
Lychnophora granmogolensis (Duarte) D.J.N.Hind	Mucugê, MG	S12°59′52.7′′ W41°20′41.2′′	924	*Loeuille et al. 665*
Lychnophora santosii H.Rob.	Rio de Contas, BA	S13°31′27.8′′ W41°57′40.3′′	1693	*Loeuille et al. 668*
Lychnophora tomentosa Sch.Bip.	Diamantina, MG	S18°12′52.9″ W43°35′44.1″	1362	*Loeuille et al. 528*
Lychnophora triflora (Mattf.) H.Rob.	Palmeiras, BA	S12°27′25.5″ W41°28′17.3″	1092	*Loeuille et al. 658*
Minasia scapigera H.Rob.	Diamantina, MG	S18°12′52.9″ W43°35′44.1″	1362	*Loeuille et al. 529*
Paralychnophora bicolor (DC.) MacLeish	Rio de Contas, BA	S13°31′33.7″ W41°57′56.7″	1876	*Loeuille et al. 672*
Piptolepis monticola Loeuille	Santo Antônio do Itambé, MG	S18°23′53.8″ W43°19′33.0″	1630	*Lusa et al. 62*
Prestelia eriopus Sch.Bip.	Santana do Riacho, MG	S19°17′28.1″ W43°36′01.5″	1131	*Loeuille et al. 524*

blade or sliding microtome (Model SM 2000R; Leica Microsystems Nussloch GmbH).

Histochemical reactions

Histochemical reactions were chosen based on the chemical classes of higher occurrence in Lychnophorinae (Keles *et al.*, 2010) and were performed on fresh material and/or material embedded in plastic resin. The following chemical reactions on fresh material were carried out immediately after collection: Sudan IV (Jensen, 1962) and neutral red under fluorescence (blue excitation filter, 450–490 bandpass filter) (Kirk, 1970) for lipophilic compounds; NADI reagent (David & Carde, 1964) for terpenoids; sulphuric acid (Geissman & Griffin, 1971) for STL terpenoids; ferric chloride 3% (Johansen, 1940) for phenolic compounds; and aluminum chloride under induced fluorescence (UV excitation filter, 340–380 bandpass filter) (Charrière-Ladreix, 1976) for flavonoid phenolic compounds. The sections were examined immediately after each reaction. Two histochemical tests were used for lipophilic substances, because different reactions occurred in the same tissue when both tests were used. For the tests conducted under fluorescence, the sections were observed under a microscope (Model DM LB; Leica Microsystems Wetzlar GmbH, Wetzlar, Germany) in UV light produced by an HBO 100 W mercury vapour lamp. Control sections were subjected simultaneously to the histochemical tests, in accordance with standard procedure. Untreated sections were assembled and observed to investigate the natural appearance of the organs and secretions.

On the material embedded in plastic resin, tests using 3% ferric chloride (Johansen, 1940), Sudan IV (Jensen, 1962) and NADI reagent (David & Carde, 1964) were performed. The results were documented by capturing images from the sections, using a video camera (Modelo DC 300F; Leica Microsystems (Schweiz) AG, Heerbrugg, Switzerland) coupled to the microscope previously cited.

RESULTS

Histochemical identification of secretion phases in leaves and stems

Histochemical tests showed that the secondary metabolites related to the main chemotaxonomic markers of Lychnophorinae are secreted and accumulated in leaf and stem glandular trichomes and

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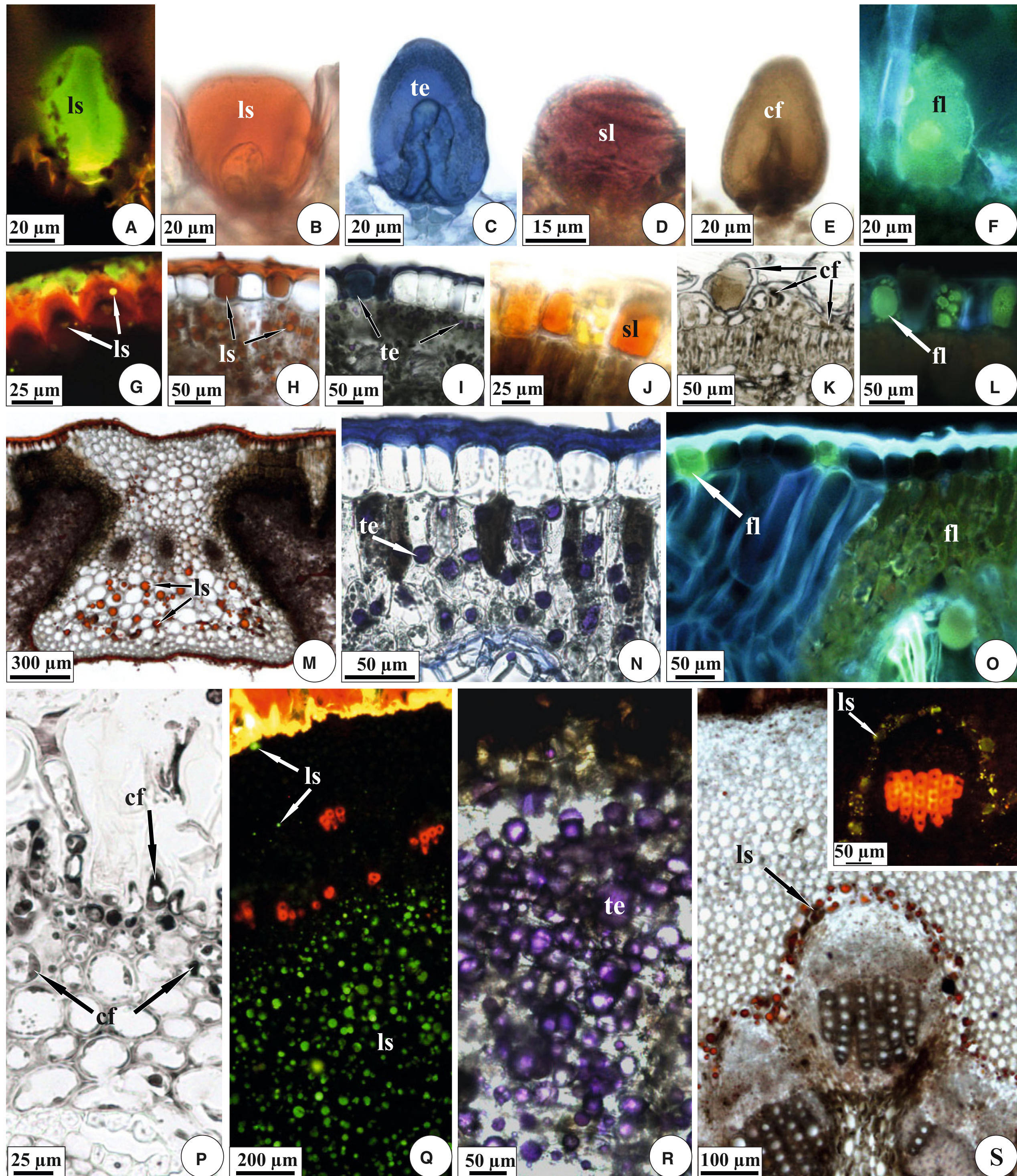

epidermal cells (Fig. 1A–L), in the palisade and spongy parenchyma (chlorenchymal cells) and ground parenchyma of the leaf midvein (Fig. 1K, M–O) and in the cortical, medullar and phloem parenchyma and secretory endodermis in stems (Fig. 1P–S). The histochemical tests performed on leaves and stems, classes of compounds detected, and secretion sites in the species evaluated are indicated in Tables 2 and 3. In general, lipophilic compounds are correlated with the occurrence of terpenoids, i.e.

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Figure 1. Histochemical reactions in cross-sections of leaves and stems of Lychnophorinae. A, F, G, L, O, Q, T, fluorescence microscopy. B–E, H–K, M, N, P, R, S, light microscopy. A, F, S, *Anteremanthus hatschbachii*; B, L, *Lychnophora santosii*; D, N, *Chronopappus bifrons*; C, E, J, *L. diamantinana*; G, *L. granmogolensis*; H, M, O, R, *Prestelia eriopus*; I, *L. triflora*; K, P, *Eremanthus polycephalus*. Q, *Piptolepis monticola*. A–F, Glandular trichomes. G–L, epidermal cells of leaves. K, N, O, accumulation of metabolites in the epidermis and/or chlorophyll parenchyma of the mesophyll. M, midvein with accumulation of metabolites in ground parenchyma. P, detail of the epidermis and stem cortical parenchyma with secretion. Q, stem cortical and medullar cells with secretion. R, detail of secondary phloem of stems, with secretion in parenchyma cells. S, T, detail of secretory endodermis in the stem. The figures show positive reactions for lipophilic substances (ls), highlighted by neutral red (A, G, N, Q, T) and Sudan IV (B, H, M, S); terpenoids (te), indicated by the NADI (C, I, N, R); sesquiterpene lactones (sl), evidenced by sulphuric acid (D, J); phenolic compounds (pc) evidenced by ferric chloride (E, K, P); and flavonoids (fl) indicated by aluminum chloride (F, L, O).

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Table 2. Histochemical reactions and respective classes of substances for secretions in leaves of species of Lychnophorinae

Species	Histochemical reactions Metabolic classes	Sudan IV and neutral red Lipophilic substances	NADI reagent Terpenoids	Sulphuric acid Sesquiterpene lactones	Ferric chloride Phenolic compounds	Aluminum chloride Flavonoids
Anteremanthus hatschbachii		GT, CL, MV	CL, MV	GT	GT, CL, MV	GT, CL, MV
Blanchetia heterotricha		GT, CL, MV	GT, CL, MV	GT	GT, EC, CL	GT, EC, CL
Chronopappus bifrons		GT, CL, MV	GT, CL, MV	GT, EC	GT, EC, CL, MV	GT, EC, CL, MV
Eremanthus polycephalus		GT, CL, MV	GT, CL, MV	GT	GT, EC, CL, MV	GT, MV
Eremanthus leucodendron		GT, MV	MV	GT	CL	CL
Heterocoma gracilis		GT, CL, MV	CL, MV	GT	GT, EC, CL, MV	GT, EC, CL MV
Lychnophora diamantinana		GT, EC, CL, MV	GT, EC, CL, MV	GT, EC	GT, EC, CL	GT, EC, CL
Lychnophora granmogolensis		GT, EC, CL, MV	EC, CL, MV	GT, EC	EC, CL	EC, CL
Lychnophora santosii		GT, EC, CL, MV	GT, EC, CL, MV	GT	GT, EC	GT, EC
Lychnophora tomentosa		CL, MV	CL, MV	–	GT, EC, CL, MV	EC, CL, MV
Lychnophora triflora		GT, CL, MV	GT, EC, CL, MV	EC	GT, EC, CL	EC, CL
Minasia scapigera		MV	MV	GT	GT, CL	CL
Paralychnophora bicolor		GT, CL, MV	GT, CL, MV	–	EC, CL, MV	EC, CL, MV
Piptolepis monticola		GT, CL, MV	GT, CL, MV	GT	GT, EC, CL, MV	GT, EC, CL MV
Prestelia eriopus		GT, EC, CL, MV	GT, CL	–	GT, EC, CL	GT, EC, CL

CL, chlorenchymal cells; EC, epidermal cells; MV, midvein; GT, glandular trichome; '–', negative.

organ regions with positive reactions for lipophilic compounds have predominantly positive reactions to terpenoids.

The presence of metabolites was found to vary between young and fully expanded leaves, and between the apical and middle portions of branch stems. All species evaluated share the same secretory structures and metabolic classes in young and fully expanded leaves. However, the density of glandular trichomes with secretion is noticeably higher

Table 3. Histochemical reactions and respective classes of substances for secretions in stems of species of Lychnophorinae

Species	Histochemical reactions Metabolic classes	Sudan IV and Neutral red Lipophilic substances	NADI reagent Terpenoids	Sulphuric acid Sesquiterpene lactones	Ferric chloride Phenolic compounds	Aluminum chloride Flavonoids
Anteremanthus hatschbachii		GT, EN	EN	GT	GT	GT
Blanchetia heterotricha		GT, CP, MP	CP, MP	GT	GT, EC, CP	GT, CP
Chronopappus bifrons		CP, PP, MP	CP, PP, MP	–	CP	CP
Eremanthus polycephalus		GT, EN, MP	GT, EN, MP	GT	GT, EC, CP, PP	GT, CP, PP
Eremanthus leucodendron		GT, CP, MP	CP, MP	GT	EC, CP	CP
Heterocoma gracilis		CP, PP, MP	CP, PP, MP	EC, PP	EC, CP, PP	CP, PP
Lychnophora diamantinana		GT	GT	GT	GT, EC, CP, PP	GT, CP, PP
Lychnophora granmogolensis		GT, CP, MP	GT, CP, MP	GT	EC, CP, PP	CP, PP
Lychnophora santosii		GT, CP, PP, MP	CP, PP, MP	GT	GT, EC, CP, PP	GT, CP
Lychnophora tomentosa		CP	CP	–	–	–
Lychnophora triflora		PP, MP	MP	–	GT, EC, CP, MP	CP, MP
Minasia scapigera		PP, MP	PP, MP	–	–	–
Paralychnophora bicolor		CP, PP, MP	GT, EC, CP, PP, MP	EC	GT, EC, CP, PP, MP	CP, PP, MP
Piptolepis monticola		GT, CP, PP, MP	CP, PP, MP	GT	GT, EC, CP, PP, MP	GT, CP, PP, MP
Prestelia eriopus		PP, MP	PP, MP	–	–	–

EN, endodermis; EC, epidermal cells; CP, cortical parenchyma; PP, phloem parenchyma; MP, medullar parenchyma; GT, glandular trichome; '–', negative.

in the young leaves of most species (Fig. 2A) compared with expanded leaves (Fig. 2B). In *Blanchetia heterotricha* DC., *Eremanthus polycephalus* (DC.) MacLeish, *Lychnophora diamantinana* Coile & S.B.Jones and *L. santosii* H.Rob., fully expanded leaves also have abundant glandular trichomes with secretion (Fig. 2C, D). At the other secretion sites (epidermal cells and parenchymal tissues), no difference was found in the accumulation of metabolites between adult and young organs.

In regard to stems, species have glandular trichomes with secretion in apical portions of the branch stems (Fig. 2E, F), with the exception of *Minasia scapigera* H.Rob. and *Prestelia eriopus* Sch.Bip., which have stems with short internodes and no glandular trichomes. In the other species, during the secondary thickening of the branches, the epidermis containing glandular trichomes is replaced by a secondary coating and, therefore, the terpenoids, sesquiterpene lactones and flavonoids restricted to glandular trichomes and epidermal cells cease to occur in middle portions of branches.

Structure of glandular trichomes

The glandular trichomes in leaves and stem branches of Lychnophorinae are capitate and biseriate, with a short peduncle and secretory head (Fig. 3A). The ontogenesis of glandular trichomes was studied for all species. The process is similar throughout and is illustrated in this study only for *Prestelia eriopus*. The development of glandular

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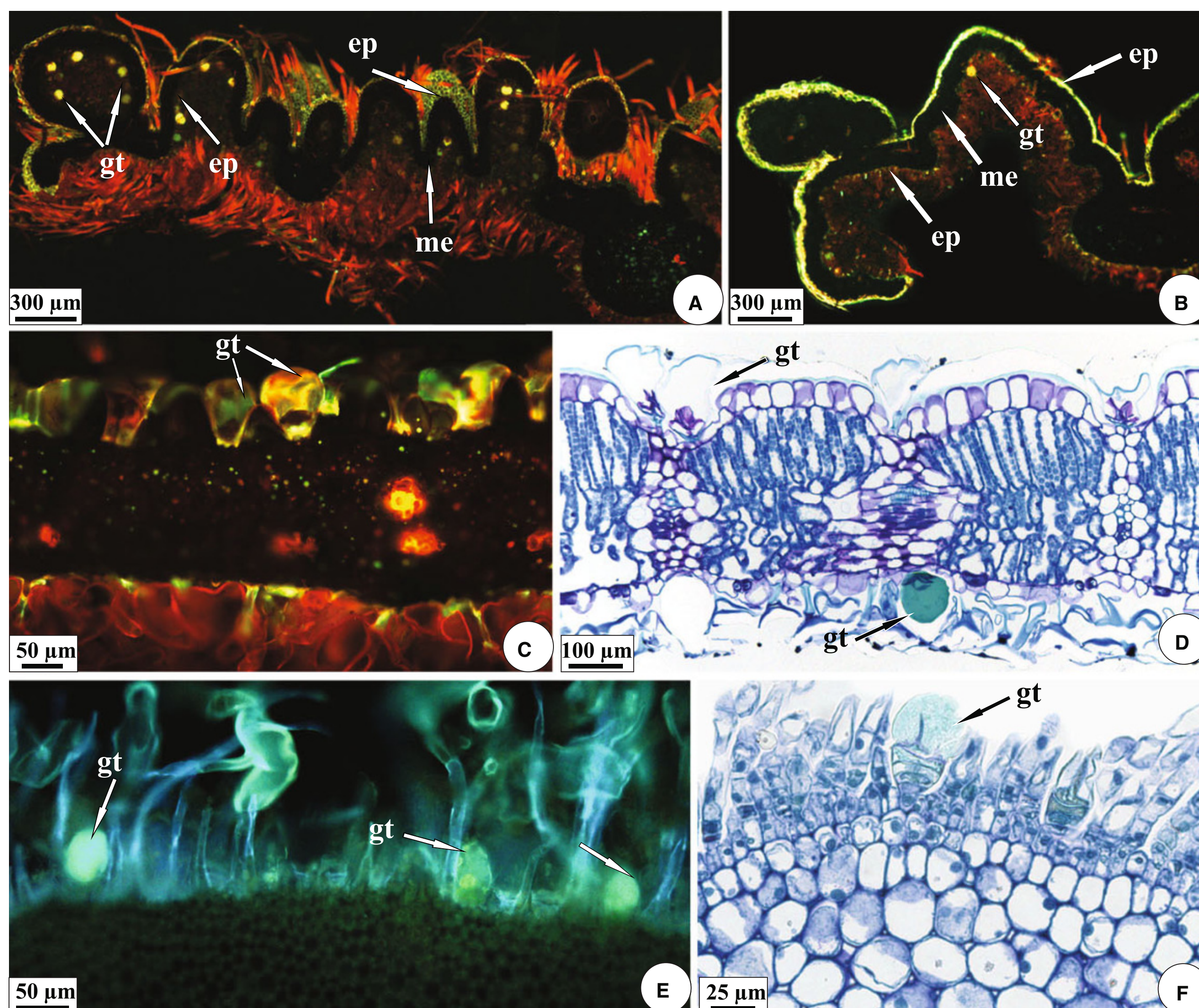

Figure 2. Secretory phases associated with the presence of metabolites in glandular trichomes of leaves and stems of species of Lychnophorinae. A–C, E, fluorescence microscopy (A–C, reactions with neutral red; E, reaction with aluminum chloride). D, F, light microscopy (staining with toluidine blue). A, B, leaves with wavy edges, young (A) and fully expanded (B), of *Chronopappus bifrons*. Density of trichomes with secretion is visually higher in young leaves. C, fully expanded leaf of *Lychnophora santosii* with glandular trichomes on the adaxial face. D, fully expanded leaf of *Eremanthus polycephalus* with capitate glandular trichome showing secretion on the abaxial face. E, F, surface of the apical portion of stems of *Anteremanthus hatschbachii* and *L. diamantinana* with glandular trichomes, respectively. ep, epidermis; me, mesophyll; gt, glandular trichome.

trichomes is not synchronous, i.e. trichomes at different stages are found side by side (Fig. 3A). This process begins in leaf primordia (Fig. 3B, C) and on the axes of shoot buds, and ends in young leaves (Fig. 3D) and the apical portions of branches in which meristematic tissues are no longer observed. Trichomes with secretion are found in fully expanded leaves and the middle portions of the branches, but trichomes in differentiation are never found, whereas the senescent leaves and basal and thickened portions of the stem no longer have any glandular trichomes.

When glandular trichomes begin to develop, a protodermic cell with evident nucleus and dense cytoplasm extends anticlinally until its apical end acquires a capitated shape (Fig. 3E–G). The first division is anticlinal (Fig. 3H) and is followed by another periclinal division, originating four cells (Fig. 3I). The two basal cells differentiate into the peduncle pair and the two apical cells divide

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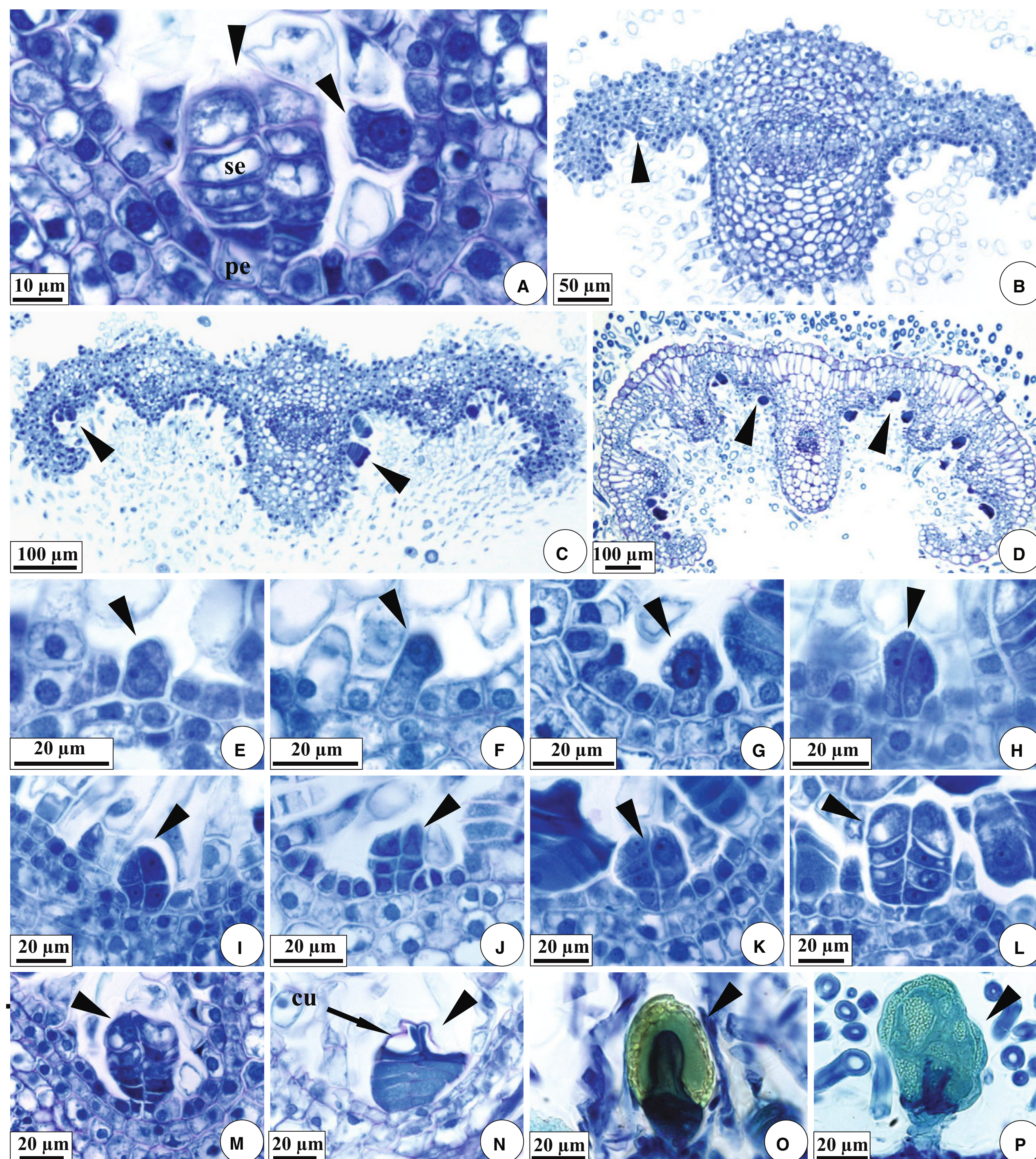

Figure 3. Characterization and development of capitate glandular trichomes (arrowheads) of species of Lychnophorinae. A–N, *Prestelia eriopus*. B–D, cross-sections of leaves of *P. eriopus*. O and P, glandular trichomes of *Lychnophora diamantinana*. A, trichomes at different developmental stages (arrowheads). Glandular trichome showing peduncle (pe) and secretory cells (se). B, early leaf primordium with young trichomes only. C, D, late leaf primordium (C) and young leaf (D) with trichomes at different developmental stages. E–M, ontogeny of glandular trichomes. N, glandular trichome with distended cuticle. O, P, glandular trichome with secretion in the subcuticular portion, with whole secretory cells in the early secretory phase (O) and with degenerate secretory cells, at the end of the secretion process (P).

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periclinally generating four to seven pairs of secretory cells (Fig. 3J–M). When the secretion is released, the external periclinal wall of the last pair of cells retracts (Fig. 3M, N). The cuticle distends (Fig. 3N), forming the subcuticular space in which the secretion is deposited (Fig. 3O–P) until it ruptures to release the secretion. In general, the cuticle is small, thick (Fig. 3N) and ruptures easily.

Therefore, for most species examined, only a small number of glandular trichomes keep the secretions stored until the fully expanded leaf stage and in subapical portions of the branches. However, in the glandular trichomes of *Blanchetia heterotricha*, *Lychnophora santosii* (Fig. 2C), *Eremanthus polycephalus* (Fig. 2D) and *Lychnophora diamantinana* (Fig. 3O–P), the cuticle does not rupture easily and the secretion remains in trichomes on the fully expanded leaves, even after the degradation of the secretory cells of the trichome (Fig. 3P).

DISCUSSION

The histochemical analyses carried out in this study on 15 species of Lychnophorinae representative of the main lineages in the group confirm the presence of chemical classes of secondary metabolites that are important chemotaxonomic markers for the subtribe (Keles *et al.*, 2010; Lusa *et al.*, 2016), including phenolic compounds, such as flavonoids, and terpenoids, such as sesquiterpene lactones.

Based on what was known of the extraction of the main classes of metabolites of Asteraceae and in particular for subtribe Heliantheae, glandular trichomes were considered the main sites of biosynthesis and accumulation of chemotaxonomic markers for the family, especially sesquiterpene lactones (Spring, Benz & Ilg, 1989; Spring, 1991; Spring *et al.*, 2001; Da Costa, Terfloth & Gasteiger, 2005; Sakamoto *et al.*, 2005; Appezzato-da-Glória *et al.*, 2012) and flavonoids (Gobbo-Neto, Gates & Lopes, 2008). For subtribe Lychnophorinae, our results confirm that leaf glandular trichomes are indeed the main sites of secretion and accumulation of sesquiterpene lactones. However, the histochemical analyses carried out in this study show that flavonoids and phenolic compounds are found mainly in the epidermal cells, chlorophyll parenchyma in leaves and fundamental parenchyma in stems. Terpenoids are found in the glandular trichomes and parenchymal tissues of stems and leaves. The presence of secretions at these sites has also been reported for widely distributed species of Asteraceae belonging to tribes Eupatorieae, Heliantheae, Mutisieae, and Vernonieae by Castro *et al.* (1997). However, the authors reported idioblasts distributed in the epidermis, mid- and lateral veins and chlorophyll parenchyma. In this study, we did not observe idioblasts, but the metabolites were well distributed in most cells of the aforementioned tissues.

Unlike other sites at which metabolites occur, observed in most species of Lychnophorinae, the secretory endodermis is restricted to *Anteremanthus hatschbachii* Rob. and *Eremanthus polycephalus*. Although not commonly described in the literature, the secretory endodermis has already been documented in underground systems of Vernonieae by Luque *et al.* (1997), Hayashi & Appezzato-da-Glória (2005, 2007) and Cury & Appezzato-da-Glória (2009).

In all the secretory structures identified in Lychnophorinae, the secreted content can be considered mixed due to the presence of more than one class of secondary metabolites, usually accumulated at the same time. This mixed metabolite composition has already been reported in species of tribe Heliantheae (Heinrich *et al.*, 2002; Aschenbrenner, Horakh & Spring, 2013). Heinrich *et al.* (2002) observed lipophilic compounds and flavonoids in the glandular trichomes of *Sigesbeckia jorullensis* Kunth and Aschenbrenner *et al.* (2013) observed terpenoids and sesquiterpene lactones in the glandular trichomes of *Helianthus* L. This suggests that mixed secretions are common in Asteraceae.

Glandular trichomes are important biosynthesis sites in Lychnophorinae, since they are present in the leaves of all species evaluated herein and in the stems of most of them, and are responsible for the biosynthesis of all the classes of metabolite evaluated. The structure of these trichomes is similar to that of other glandular trichomes already described for Eupatorieae and Vernonieae (Monteiro *et al.*, 2001; Appezzato-da-Glória *et al.*, 2012). However, ontogenetic studies on the glandular trichomes of Lychnophorinae show that development occurs only at leaf primordia and is asynchronous, i.e. trichomes at different developmental stages are found in the same portion of the organ, which could mean that the secretion phase lasts longer. The number of secretory cells in the glandular head varies from one species to another and even within a single species. However, no relationship was observed between the number of secretory cells in the glandular head and the permanence of trichomes in adult organs.

For most of the species evaluated, the phase during which secreting trichomes are more abundant coincides with initial developmental phases of leaves and stems, which may indicate a protective role for young organs, as reported by Siebert (2004) and Machado, Gregório & Guimarães (2006), mainly related to the inhibition of herbivores and pathogens through the release of metabolite repellents (Werker,

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2000). In the species *Blanchetia heterotricha*, *Eremanthus polycephalus*, *Lychnophora diamantinana* and *L. santosii*, abundant trichomes were observed with secretion in fully expanded leaves. Amrehn, Heller & Spring (2014) observed that the increase in cuticle thickness of the glandular trichome of *Helianthus annuus* L. as it develops was a prerequisite for preventing premature cuticle rupture. The retention of secretion products in the trichomes of *B. heterotricha*, *E. polycephalus*, *L. diamantinana* and *L. santosii* might be the result of cuticle resistance to rupture.

No differences were found in the epidermal cells, secretory endodermis and parenchymal tissues of leaves and stems of Lychnophorinae regarding the accumulation of metabolites when young organs were compared to developed organs, despite the fact that thickened branch stems cease to exhibit metabolites in trichomes. The presence of phenolic compounds (mainly flavonoids) in epidermis and chlorophyll parenchyma tissues is probably related to the protection of the cell mechanism against damaging solar radiation (Kofidis, Bosabalidis & Moustakas, 2003; Lusa *et al.*, 2014; Silva *et al.*, 2014). This would explain the presence of these phenolic compounds in early and developed organs exposed to intense sunlight.

CONCLUSIONS

In this study, we found that the classes of secondary metabolites related to the main chemotaxonomic markers of Lychnophorinae are secreted and stored in glandular trichomes, epidermal cells and parenchyma tissues of leaves and stems. The glandular trichomes on the leaves are the main site of secretion and accumulation of sesquiterpene lactones. Flavonoids and other phenolic compounds are found mainly in the epidermis and chlorophyll parenchyma in the leaves and in ground parenchyma in the stems. Terpenoids are found both in glandular trichomes and in parenchyma tissues of stems and leaves. The classes of metabolites of interest located in glandular trichomes are prevalent in the early developmental stages of the organs (young leaves and young branches). However, young and expanded organs are equally rich in metabolites stored in parenchyma tissues, except for thickened portions of branches. Therefore, the information obtained in this study on the histolocalization and abundance of compounds of interest provides a basis for the selective collection of botanical material in Lychnophorinae, so that phytochemical investigations of the group can be carried out more effectively and species conservation enhanced.

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