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RESEARCH PAPER

Leaf blade structure of *Verbesina macrophylla* (Cass.) F. S. Blake (Asteraceae): ontogeny, duct secretion mechanism and essential oil composition

L. D. A. Bezerra[1], P. A. O. Mangabeira[1], R. A. de Oliveira[1], L. C. D. B. Costa[1] & M. Da Cunha[2]

1 Centro de Microscopia Eletrônica, Universidade Estadual de Santa Cruz, Ilheus, Brazil

2 Laboratório de Biologia Celular e Tecidual, Centro de Biociências e Biotecnologia, Universidade Estadual do Norte Fluminense Darcy Ribeiro, Campos dos Goytacazes, Brazil

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Correspondence
M. Da Cunha, Laboratório de Biologia Celular e Tecidual, Centro de Biociências e Biotecnologia, Universidade Estadual do Norte Fluminense-Darcy Ribeiro (UENF), Avenida Alberto Lamego, Parque Califórnia, n° 2000/ CBB, Campos dos Goytacazes, RJ 28013-602, Brazil.
E-mail: maurauenf@gmail.com

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ABSTRACT

- Secretory structures are common in Asteraceae, where they exhibit a high degree of morphological diversity. The species *Verbesina macrophylla*, popularly known as assa-peixe, is native to Brazil where it is widely used for medicinal purposes. Despite its potential medical importance, there have been no studies of the anatomy of this species, especially its secretory structures and secreted compounds. This study examined leaves of *V. macrophylla* with emphasis on secretory structures and secreted secondary metabolites.
- Development of secretory ducts and the mechanism of secretion production are described for *V. macrophylla* using ultrastructure, yield and chemical composition of its essential oils.
- *Verbesina macrophylla* has a hypostomatic leaf blade with dorsiventral mesophyll and secretory ducts associated with vascular bundles of schizogenous origin. Histochemistry identified the presence of lipids, terpenes, alkaloids and mucopolysaccharides. Ultrastructure suggests that the secretion released into the duct lumen is produced in plastids of transfer cells, parenchymal sheath cells and stored in vacuoles in these cells and duct epithelial cells. The essential oil content was 0.8%, and its major components were germacrene D, germacrene D-4-ol, β-caryophyllene, bicyclogermacrene and α-cadinol.
- Secretory ducts of *V. macrophylla* are squizogenous. Substances identified in tissues suggest that both secretions stored in the ducts and in adjacent parenchyma cells are involved in chemical defence. The essential oil is rich in sesquiterpenes, with germacrene D and its derivatives being notable components.

INTRODUCTION

The family Asteraceae is cosmopolitan and includes about 23,600 species and approximately 1,620 genera (Stevens 2012). In Brazil, the family is represented by about 2,000 species. The family contains morphological and anatomical diversity, associated with very effective mechanisms for pollination and dispersal, as well as biologically active secondary metabolites (Devore & Stuessy 1995). The Asteraceae genus *Verbesina* is one of the largest in the tribe Heliantheae. Species of this genus have economic importance due to their chemical properties, and although they have been targets of several taxonomic studies (Olsen 1986) using morphological and molecular data (Panero & Jansen 1997), anatomical studies are scarce.

Phytochemistry of the genus *Verbesina* has revealed many terpenoids, including eudesmanes, germacrenes and elemenolides as major constituents. Other potentially bioactive compounds isolated from species of *Verbesina* include guanidine alkaloids, flavonoids and aromatics, along with several other minor components (Lobitz *et al.* 1998). Members of the genus have been used in folk medicine for the treatment of diabetes, hypertension, external wounds and inflammation. For example, *V. turbacensis* and *V. virginica* are used to treat gastrointestinal problems, whereas *V. encelioides* is used for treatment of spider bite symptoms and haemorrhoids (Lobitz *et al.* 1998).

Verbesina macrophylla (Cass.) F. S. Blake, popularly known as 'assa-peixe' (Moreira *et al.* 2002; Agra *et al.* 2007), is native to Brazil, Argentina and Bolivia (Panero & Jansen 1997). It occurs along the Brazilian coast and is most abundant in the northeast, especially in the state of Bahia. It is a small tree reaching up to 3 m in height, with white aromatic inflorescences that are very attractive to pollinators (Milet-Pinheiro & Schlindwein 2008). There are reports from southeast Bahia of its medicinal value in a tea made from the leaves and used to treat kidney and urethra infections due to its anti-inflammatory and antipyretic properties (Moreira *et al.* 2002), while tea from the flowers is used to treat inflammation (Agra *et al.* 2007). Medicinal plants are a rich source for discovery of molecules that can be exploited therapeutically. Thus, the study of secretory structures and secondary metabolites of medicinal plants is of interest to the pharmaceutical industry, which

conducts pharmacological and toxicological tests during the development of new herbal medicines. Furthermore, essential oils obtained from aromatic herbs can have broad application in the cosmetic, pharmaceutical and chemical industries (Cordell *et al.* 2001).

There is great morphological diversity of secretory structures among species of the Asteraceae, *e.g.* trichomes, cavities, idioblasts, hydathodes, ducts, latex, extra floral glands and appendages (Castro *et al.* 1997). Ducts are mostly schizogenic in origin and secrete oleoresins rich in lactones, polyacetylenes and sesquiterpenes (Ascensão & Pais 1988). The distribution of secretory structures is an important diagnostic feature for some species (Metcalfe & Chalk 1950; Fahn 1979). In addition, identification of the main classes of compounds present in secretions helps to understand the functional and ecological roles of such structures. Secretory structures have already been investigated in several genera of Asteraceae (Fahn 1979; Ascensão & Pais 1988; Monteiro *et al.* 1999; Pagni & Masini 1999; Werker 2000; Cornara *et al.* 2001; Pagni *et al.* 2003; Bartoli *et al.* 2011); however, there have been few ultrastructural studies, particularly on the secretion mechanisms of such cells and organelles.

Research into the location, development, structure, ultrastructure and histochemistry of secretory structures can provide insight into their functions. Despite the potential medicinal importance of leaves and flowers of *V. macrophylla*, there have been no anatomical studies of this species, especially with regard to the secretory structures or composition of the exudates. Thus, the aim of the present research was to study the anatomy of the leaves of *V. macrophylla* with emphasis on secretory structures and secreted secondary metabolites. In particular, we investigated the development of secretory ducts and the mechanism of secretion production by examining the anatomy and identifying the main groups of secondary metabolites. In addition, we discuss the ecological roles of the secretory structures and their exudates. We also evaluated the chemical composition of the essential oil of *V. macrophylla* in order to better characterize the phytochemical profile of this species.

MATERIAL AND METHODS

Plant material

Leaf samples were collected from five adult specimens of *V. macrophylla* grown in the Garden of Medicinal Plants of the Universidade Estadual de Santa Cruz (UESC), Ilhéus, Bahia. Specimens were deposited in the Herbarium at UESC with the registration number 19101.

Microscopy investigations

For anatomical analysis, samples from the middle region of completely expanded leaves were fixed in an aqueous solution of 2.5% glutaraldehyde in 0.5 M sodium cacodylate buffer, pH 7.2, dehydrated in an ethanol series and embedded in Historesina®. Transverse sections (0.70 μm) made with a sliding microtome (SM2010 R, Leica, Germany) were stained with 0.05% toluidine blue (modification of O'Brien *et al.* 1964). Slides were sealed with Entellan, observed under a light microscope (Axioplan; Carl Zeiss, Oberkochen, Germany) and photographed with a Cannon Power Shot A640 camera and analysed with the software LINK/ISIS/Zeiss (Oxford, UK). For ontogenetic study, the first two leaves in the first stage of formation were fixed in historesin. Sections were made in series (0.30 μm) and observed and photographed in sequence using light microscopy.

To evaluate leaf micromorphology, leaf samples were fixed in a solution of 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, dehydrated in an acetone series and subjected to critical point drying (Bal-Tec Critical Point Dryer CPD 030). The samples were adhered to a metal substrate, affixed with metallic silver and covered with a thin (20 nm) layer of gold (Bal-Tec SCD Sputter Coater 050). Samples were observed and documented using a Quanta 250 (FEI Company) scanning electron microscope (SEM). The presence of epicuticular wax was evaluated by washing leaves in chloroform for 1 min and making comparisons with unwashed leaves under SEM (Moraes *et al.* 2011).

For ultrastructural analysis, leaf fragments were fixed as described for SEM and post-fixed in 1% osmium solution in 0.1 M sodium cacodylate buffer. After dehydration, the material was infiltrated, embedded in LR White resin and polymerised in an oven at 60 °C. Ultrathin sections (80 nm) were then produced using an ultramicrotome (Leica EMFC6) with a diamond knife (Diatome). The sections were collected on copper mesh grids (300) and contrasted with uranyl acetate in 1.0% alcohol followed by 5.0% aqueous lead citrate (Reynolds 1963). The sections were observed using a Morgagni 268D (FEI Hillsboro, OR, USA) transmission electron microscope with a voltage of 80 kV.

Histochemistry

The major classes of chemical compounds present in the secretions of the secretory structures were investigated in hand-cut transverse and longitudinal sections of fresh material using the following: Sudan black and Sudan IV (Pearse 1980) for total lipids; Nadi (David & Carde 1964) reagent for terpenoids; sulphuric acid (David & Carde 1964) and Abraham reagent (Caniato *et al.* 1989) for sesquiterpene lactones; Wagner (Furr & Mahlberg 1981) and Dragendorff (Costa 1962) reagents for alkaloids; periodic acid Schiff (PAS) reagent (Jensen 1962) for polysaccharides; vanillin hydrochloric acid reagent (Jensen 1962) for tannins; Ruthenium red (Johansen 1940) for pectins and mucopolysaccharides; and tannic acid/ferric chloride reagent (Pizzolato 1977) for phenolic compounds. Standard control procedures were performed simultaneously. Observations and images of sections were made using an Axioplan Zeiss optical microscope with a Cannon Power Shot A640 camera and the software as above.

Essential oil steam distillation

To calculate the essential oil content of *V. macrophylla* and identify its chemical components, leaves of four specimens were collected in the early morning hours while the plants were in the reproductive stage. The plant material for phytochemical investigation was collected during the same phenological stage as that for micromorphological analysis. The leaves were dried at 40 °C and essential oil steam distillation was performed in quadruplicate by hydrodistillation for 3 h using a Clevenger apparatus with 30 g dry biomass of leaves. Essential oil yield was determined as the amount extracted per 100 g dry weight

of leaves (% w/v). Chemical composition was quantitatively measured with gas chromatography coupled to a flame ionization detector (GC-FID) in a Varian Saturn 3800 gas chromatograph (Palo Alto, CA, USA) equipped with a VF5-ms fused silica capillary column (0.25 mm × 30 mm) with stationary phase 5% phenyl–95% dimethyl polysiloxane (0.25 μm film thickness), with helium gas to 5.0 and drag flow of 1.2 ml·min^{-1} (10 psi). Injector and detector temperatures were 250 and 280 °C, respectively. A solution of 1.0 μl in $CHCl_3$was injected in splitless mode (1:10). The column temperature started at 50°C and was increased by 4°C min^{-1} to 180°C and 15°C min^{-1} to 280 °C , and maintained at this temperature for 5 min for a total time of 44.17 min. Quantification of the components was obtained through electronic integration of the peaks detected with FID regulation. Qualitative analysis was performed on a Varian Saturn mass spectrometer 2000, using the electron impact 70 eV method, and a temperature of 280 °C transfer line, 120 °C manifold,and 240 °C trap. The column temperatures and conditions were identical to those used in the GC-FID analysis. Identification of essential oil components was performed through analysis of fragmentation patterns from the mass spectra, and confirmed by comparison with mass spectra in the database provided with the equipment (NIST 08), and comparison with retention indices of known compounds obtained by injection of a mixture of standards containing a homologous series of alkanes C8–C26 (Sigma-Aldrich, St. Louis, MI, USA) and with data from Adams (2007).

RESULTS

Leaf anatomy

Adult leaves of *V. macrophylla* have epicuticular wax without ornamentation on the adaxial surface (Fig. 1A). The outer periclinal cell wall of the adaxial epidermis is convex and the anticlinal cell wall is straight, in frontal view (Fig. 1A). Ordinary epidermal cells of the abaxial surface are flat with an unorganised striated cuticle and with the limits of the anticlinal cell wall not visible in frontal view (Fig. 1B and C). Among the specialised cells of the epidermis, abundant trichomes on the abaxial surface stand out; trichomes are less abundant on the adaxial epidermis (Fig. 1A). Trichome size varies, possibly due to different stages of development. The leaf blade is hypostomatic with anomocytic stomata enveloped by a variable number of cells that do not differ in shape, size or level from the other epidermal cells.

In transverse section, the epidermis is uniseriate, with cells of the abaxial and adaxial surfaces differing from each other; the adaxial cells are rounded whereas the abaxial cells are smaller in size and irregular in shape. The epidermis possesses a thin cuticle covering the outer cell wall (Fig. 1D). The mesophyll is dorsiventral with palisade cells forming a single cell layer and spongy parenchyma forming three to five cell layers. The vascular bundles have a parenchymal sheath composed of large cells. Secretory ducts are observed associated with the vascular bundles, positioned close to the xylem and distinguished by a lumen surrounded by a single layer of epithelial secretory cells (Fig. 1D).

The midrib has an average of five large collateral vascular bundles, and two ducts associated with the phloem (Fig. 1E and F). In addition to parenchyma, angular collenchyma are also present (Fig. 1G). The presence of an accumulation of crystals was not observed in leaf tissues.

Histochemistry

The Sudan IV test for total lipids revealed the presence of lipophilic droplets in parenchyma cells of the mesophyll (Fig. 2A). These droplets also reacted with Nadi reagent, indicating the presence of a mixture of terpenes and acid resin (Fig. 2H). The cells of the duct and the secretion tested positive for lipids (Fig. 2B–D), and intense blue staining in the interior of the ducts in response to Nadi reagent indicates the presence of terpenes (Fig. 2I). Alkaloids (Fig. 2E) and mucopolysaccharides (Fig. 2F and G) were also present inside the duct cells. Tests for detection of phenolic compounds, polysaccharides, tannins and sesquiterpene lactones were all negative for the duct secretion.

Mesophyll duct ontogeny

The development of the secretory duct of *V. macrophylla* was observed in the first leaf node from the meristem. The secretory ducts of the mesophyll are formed prior to differentiation of the palisade parenchyma, similarly to protodermal cells. In the leaf primordium the mesophyll is composed of meristem cells characterised by the presence of prominent, spherical and large nuclei. Among meristem cells, those that give rise to vascular bundles (procambium) and the duct separate immediately (Fig. 3A and B). These cells have dense differentiated content, with division of the remaining cells giving rise to palisade and spongy parenchyma (Fig. 3A and B). The formation of the duct starts with four cells at the start of dissolution of the middle lamella, which is ultimately removed (Fig. 3C and D). New cell divisions give rise to epithelial cells, and the increasing separation among these leads to formation of a large intercellular space, *i.e.* the lumen (Fig. 3E); new epithelial cells have nuclei at this stage (Fig. 3F and G). After development of secretory ducts, these cells become vacuolated (Fig. 3H). Sometimes the cells surrounding the secretory epithelium arrange themselves so as to form the parenchyma of the sheath. The development of the ducts does not occur simultaneously in the leaf primordia; cavities were observed at different stages of development in the primordia.

Secretion mechanism

In the mesophyll, ducts always appear associated with a vascular bundle and close to its xylem. The vascular bundles are surrounded by several large vacuolated parenchymatous cells that form a sheath (Fig. 4A). A tissue composed of transfer cells was found in the region of the phloem (Fig. 4B and D), characterised by the presence of invaginations of the cell walls (Fig. 4E) to increase efficiency in mobile exchange. These cells have active cytoplasm with mitochondria and plastids and many vesicles containing oil (Fig. 4F). Oil droplets from plastids are released into the cytoplasm and transferred *via* adjacent cells to the vascular bundle (Fig. 4C). These droplets are stored in vacuoles (Fig. 5A and B) or transferred to epithelial cells of the ducts. Oil was frequently observed exuding from discharge cells (Fig. 5C) with large vacuoles (Fig. 5A and B). These cells are located between the transfer cells and the

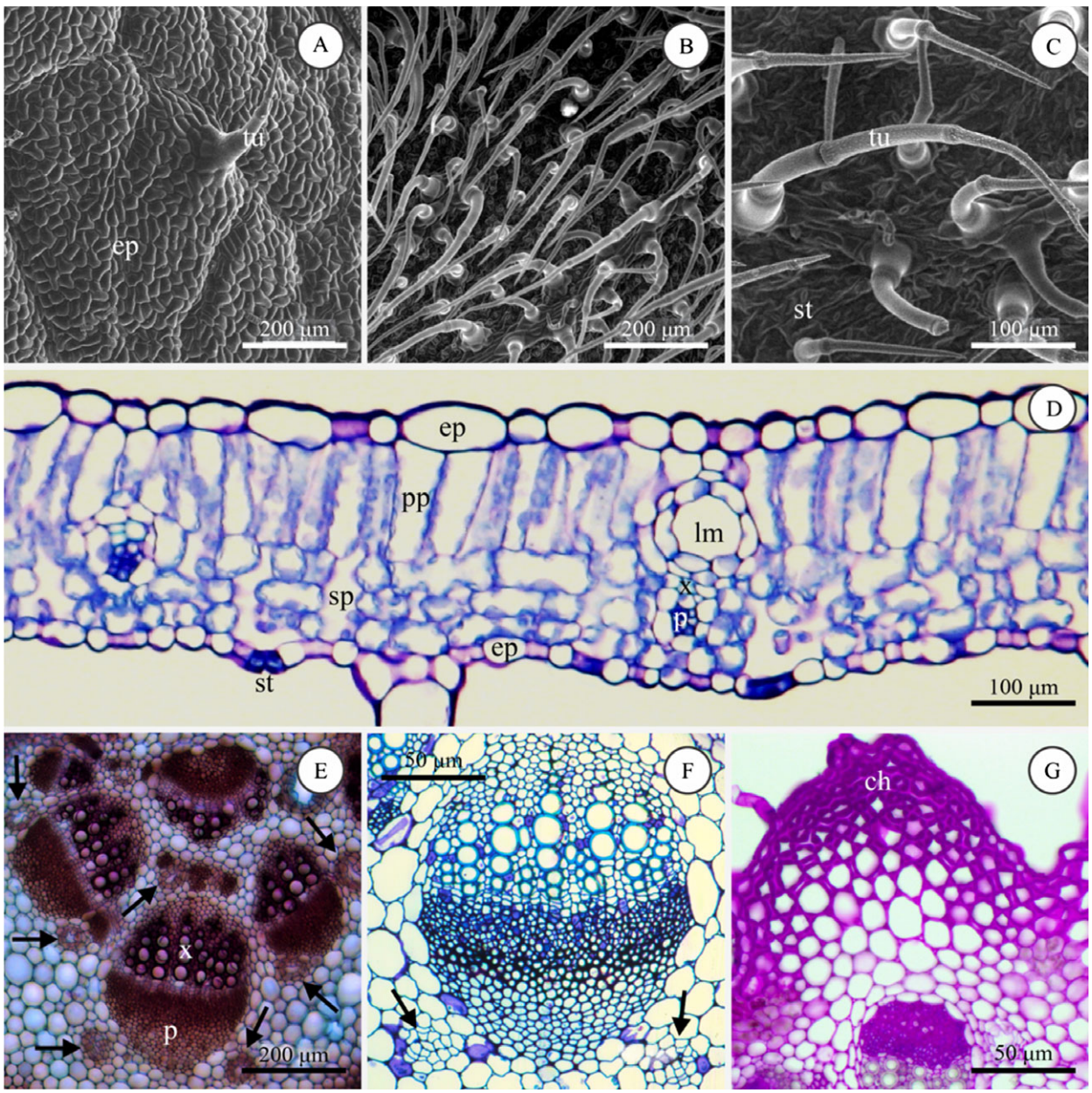

Fig. 1. Anatomy of the leaf blade *V. macrophylla*. A: Adaxial surface of the epidermis with smooth epicuticular wax and trichomes. B: Abaxial surface of the epidermis with numerous uniseriate trichomes. C: Detail of uniseriate trichomes, adaxial surface of the epidermis showing unorganised streaks of epicuticular wax and stomata. D: Leaf transverse section. E: Cross-section of the midrib. The presence of two ducts (arrow) can be observed on the side of each vascular bundle. F: Details of the vascular bundle and ducts. G: Transverse section of the midrib; note the angular collenchyma. A–C: Scanning electron microscopy. D–G: Light microscopy. ep: epidermis; st: stomata; tu: uniseriate trichomes; pp: palisade parenchyma; sp: spongy parenchyma; lm: lumen of the duct; x: xylem; p: phloem; ch: collenchyma.

epithelial cells of the duct, with stored granular material and oil droplets (Fig. 5B).

Essential oil

The essential oil content obtained from samples of dried leaves was 0.8%. Results of analysis of the composition of essential oil are provided in Table 1.

Thirty compounds were identified in the essential oil of *V. macrophylla*, of which 99.2% were sesquiterpenes (55.1%) and oxygenated sesquiterpenes (44.1%). The chemical composition is rich in sesquiterpene derivatives (63.2%). The major component was germacrene D (37.3%), followed by germacrene D-4-ol (17.0%); β-caryophyllene (5.9%) and α-cadinol (4.5%) were also present in significant quantities.

DISCUSSION

In general, the anatomical and micromorphological study of the leaves of *V. macrophylla* revealed characters common in the

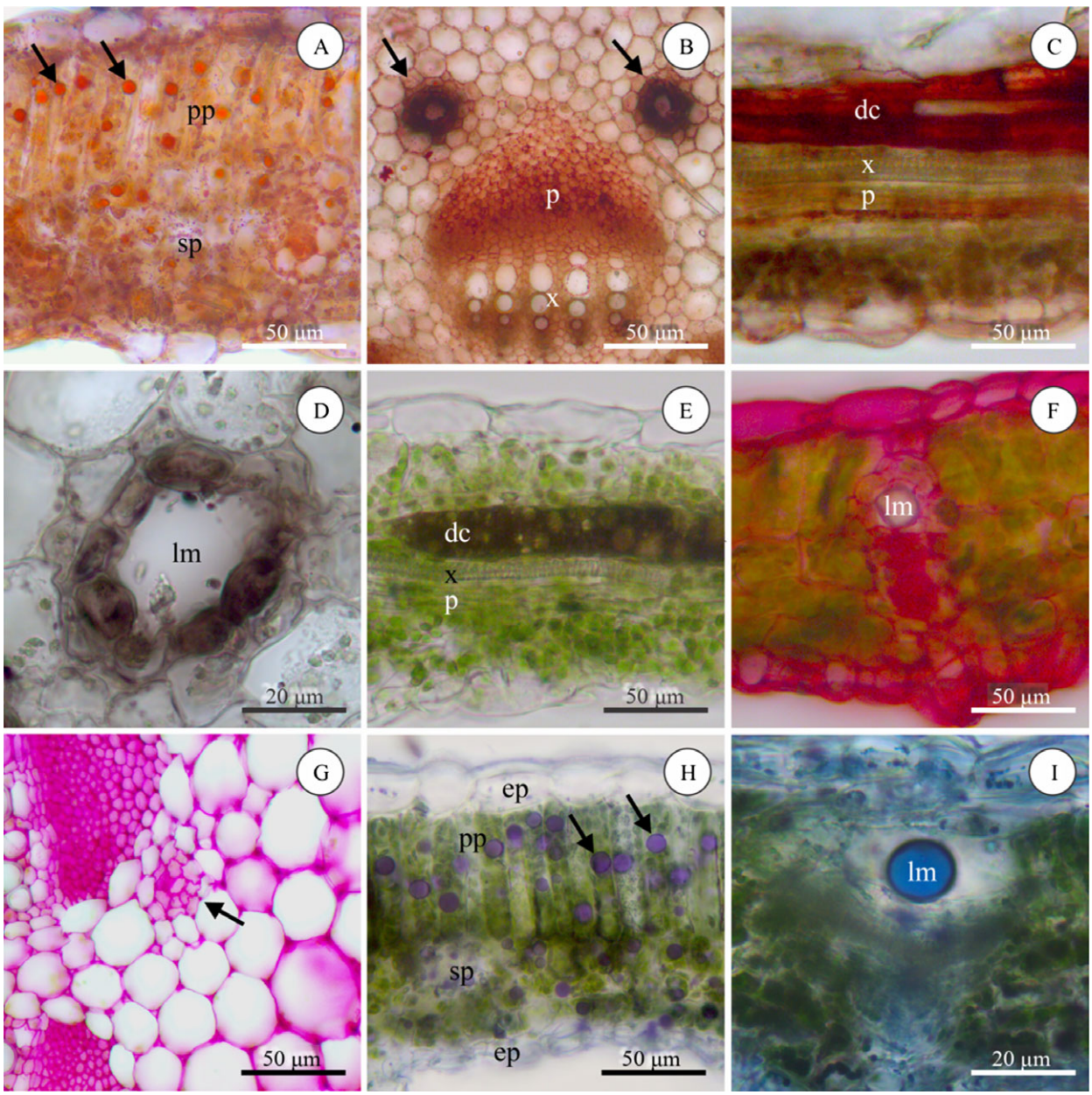

Fig. 2. Histochemical tests on the leaf blade of *V. macrophylla*. A: Lipid droplets (arrow) stained with Sudan IV in parenchyma of the mesophyll, in transverse section. B: Duct of the midrib in cross-section. Note the reaction to Sudan IV for total lipids in the two ducts (arrow) located next to the vascular bundle. C: Mesophyll duct in longitudinal section. Note the reaction to Sudan IV for total lipids. D: Duct of the midrib in cross-section. Note the reaction to Sudan black for total lipids. E: Duct mesophyll in longitudinal section. Note Wagner test results for alkaloids. F: Mesophyll duct in cross-section. Note the reaction to Ruthenium red for mucopolysaccharides. G: Duct (arrow) of the midrib in transverse section. Note the reaction to Ruthenium red for mucopolysaccharides. H: Drops of resin oil (arrow) stained with Nadi reagent in the mesophyll parenchyma in cross-section. I: Duct mesophyll in transverse section. Note the Nadi reaction for terpenes. dc: duct; lm: duct lumen; x: xylem; p: phloem; ep: epidermis; pp: palisade parenchyma; sp: spongy parenchyma.

Asteraceae, such as the presence of epicuticular wax without any ornamentation on the adaxial surface, as in *Mikania glomerata* Spreng. (Milan *et al.* 2006). a hypostomatic leaf blade, as found in other species of the tribe Heliantheae (Camilotti *et al.* 2014), and hypostomatic and dorsiventral mesophyll (Milan *et al.* 2006; Duarte *et al.* 2011; Oliveira *et al.* 2011; Souza *et al.* 2011, 2013). In *Vernonia sessilifolia* Less. and *V. linearis* Spreng., Sajo & Menezes (1994) noted that the dorsiventral mesophyll has palisade parenchyma near the adaxial epidermis and three to four layers of spongy parenchyma; in *Vernonia psilophyta*, however, the mesophyll is isobilateral. Our study also revealed characters that can be interpreted as the result of adaptation to the environment, such as the presence of a cuticle and collenchyma in the central midrib (Ribeiro & Walter 2008).

Metcalfe & Chalk (1950) reported that due to the diversity of habitats occupied by species of Asteraceae, they exhibit a large variety of anatomical structures, some of which are

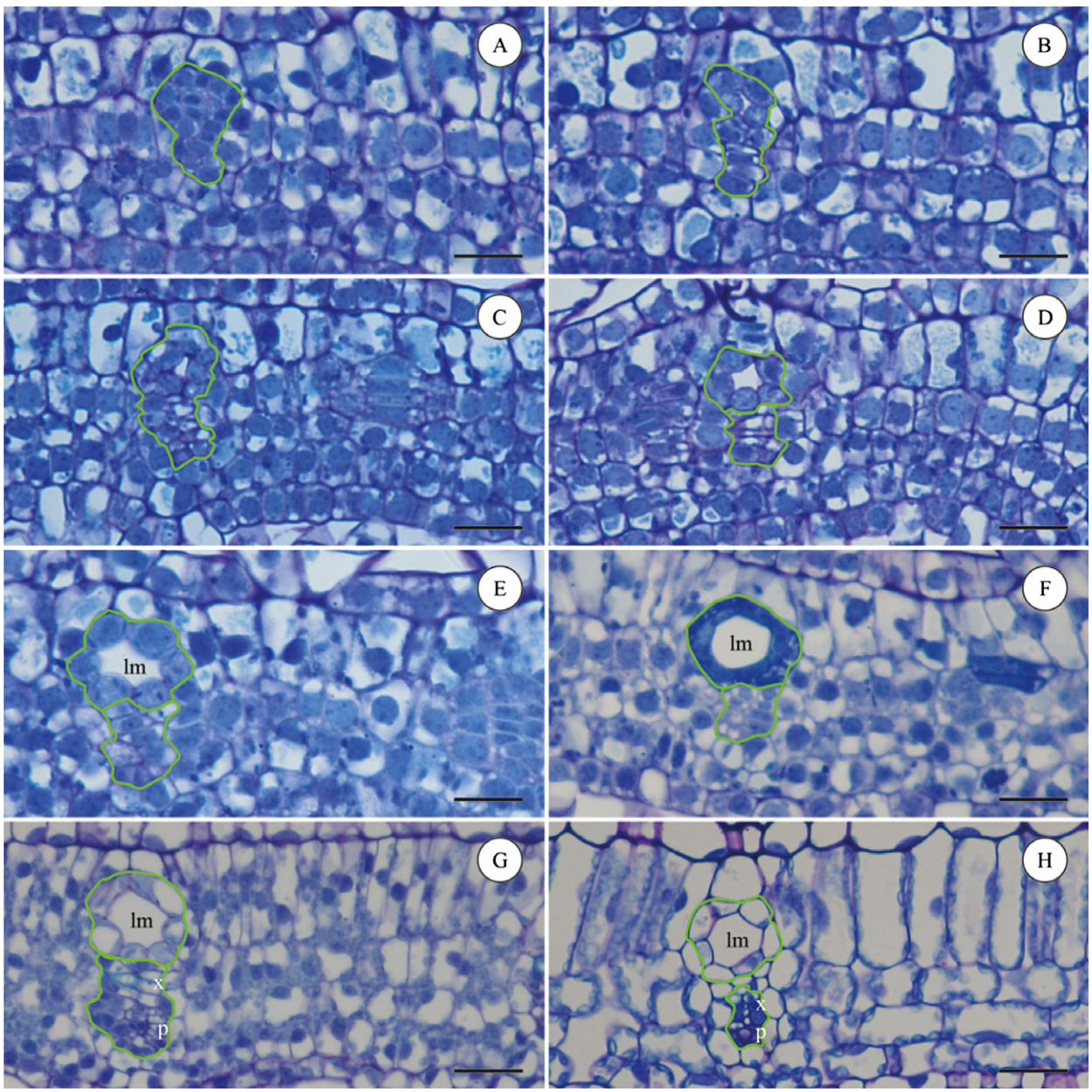

Fig. 3. Transverse section of the leaf blade of *V. macrophylla*. Mesophyll duct ontogeny. Digital marking of cells during duct ontogeny and vascular bundle development. lm: duct lumen; x: xylem; p: phloem. Bar = 50 μm.

ecological specialisations. Certain anatomical features may have favoured the adaptive success of *V. macrophylla* in rain forest environments, which are hot and humid, with high temperatures and a very irregular distribution of rainfall. The hypostomatic leaves and large number of abaxial trichomes prevent the humid microclimate from interfering with leaf temperature control and transpiration rate (Sajo & Menezes 1994). Moreover, *V. macrophylla* has other characteristics that are considered xeromorphic, such as the presence of a thick cuticle and epicuticular layer protecting the epidermis, and collenchyma in the central midrib (Ribeiro & Walter 2008).

The secretory structure of ducts located near or associated with vascular bundles in *V. macrophylla* is common to several species of Asteraceae (Schnepf 1974; Ascensão & Pais 1988; Corsi & Nencioni 1995; Castro *et al.* 1997; Pagni & Masini 1999; Pagni *et al.* 2003; Milan *et al.* 2006; Andreucci *et al.* 2008; Bartoli *et al.* 2011; Budel *et al.* 2012). Castro *et al.* (1997) identified various types of secretory structure among species of Asteraceae from the Cerrado, and proposed an identification key for the genera. In fact, the criteria they used to classify the genus *Verbesina* are the presence of ducts located near the xylem of vascular bundles in leaves, and glandular trichomes. However, the latter criterion does not apply to *V. macrophylla* because it does not possess glandular trichomes.

The present ontogenetic study suggests that the ducts of *V. macrophylla* are schizogenous in origin, as described in other species of Asteraceae (Andreucci *et al.* 2008; Silva *et al.* 2015). Observations of tissue in early stages of development revealed

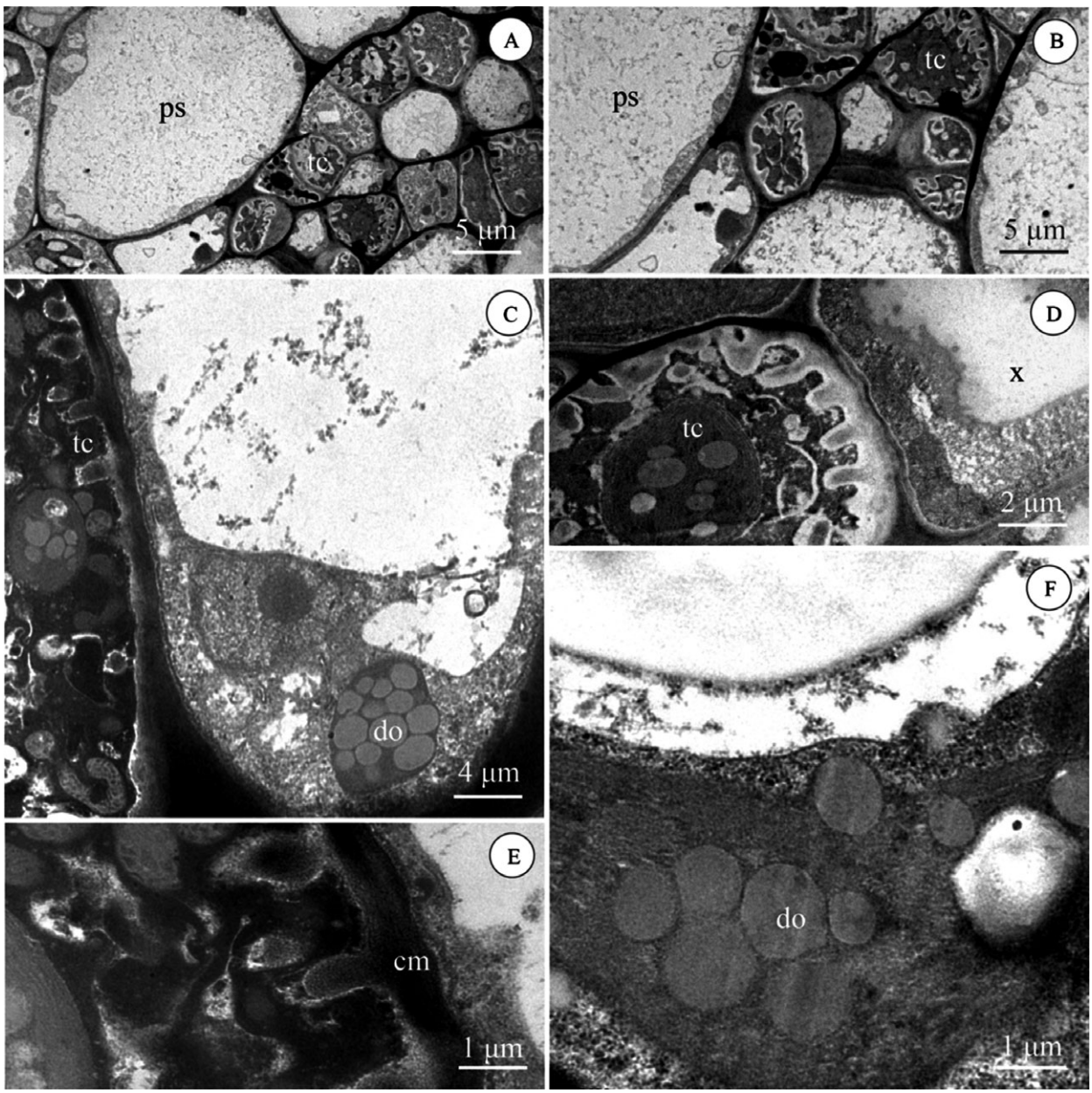

Fig. 4. Ultrastructure of cells of the vascular bundle and duct in the mesophyll of *V. macrophylla*. A: Phloem region. Note parenchyma cells and transfer sheath surrounding the vascular bundle. B: Transfer cells. C: Transfer cell adjacent to a parenchyma cell, with oil droplets, several of which are close to the cell membrane. D: Transfer cell and part of an adjacent xylem cell. E: Detail of invaginations of transfer cells. F: Detail of plastid containing oil droplets. ps: parenchyma sheath; tc: transfer cell; x: xylem; do: oil droplet; cm: cell membrane.

the presence of preserved epithelial cells without damaged membranes and a well-defined squizogenous lumen, which suggest that the discharge in ducts originates through a granulocrine process (Delbón *et al.* 2007). The cells in the mesophyll that give rise to the ducts are derived from the same cells that give rise to the vascular bundles, thus suggesting that the ducts are of procambial origin.

Histochemical analysis revealed the presence of a mixed secretion within the ducts, with the presence of lipids, terpenes, alkaloids and mucopolysaccharides. Terpenes, lipids and alkaloids in duct secretion have been reported for other species of Asteraceae (Ascensão & Pais 1988; Milan *et al.* 2006). Mucopolysaccharides in the *V. macrophylla* ducts may be related to their capacity to store water, since the main water storage substance is attributed to this group of polysaccharides (Abreu *et al.* 2002; Souza *et al.* 2015). These substances may also be involved in the attraction of pollinators (Waterman 1992), but the actual roles of these compounds in leaf ducts remain unclear.

The production of essential oil and alkaloids in the ducts may be related to chemical defence since these substances are toxic to various insects (Corsi *et al.* 1982; Werker *et al.* 1985). Furthermore, several species devoid of alkaloids have leaves showing evidence of herbivory, such as *Protium icicariba* (DC)

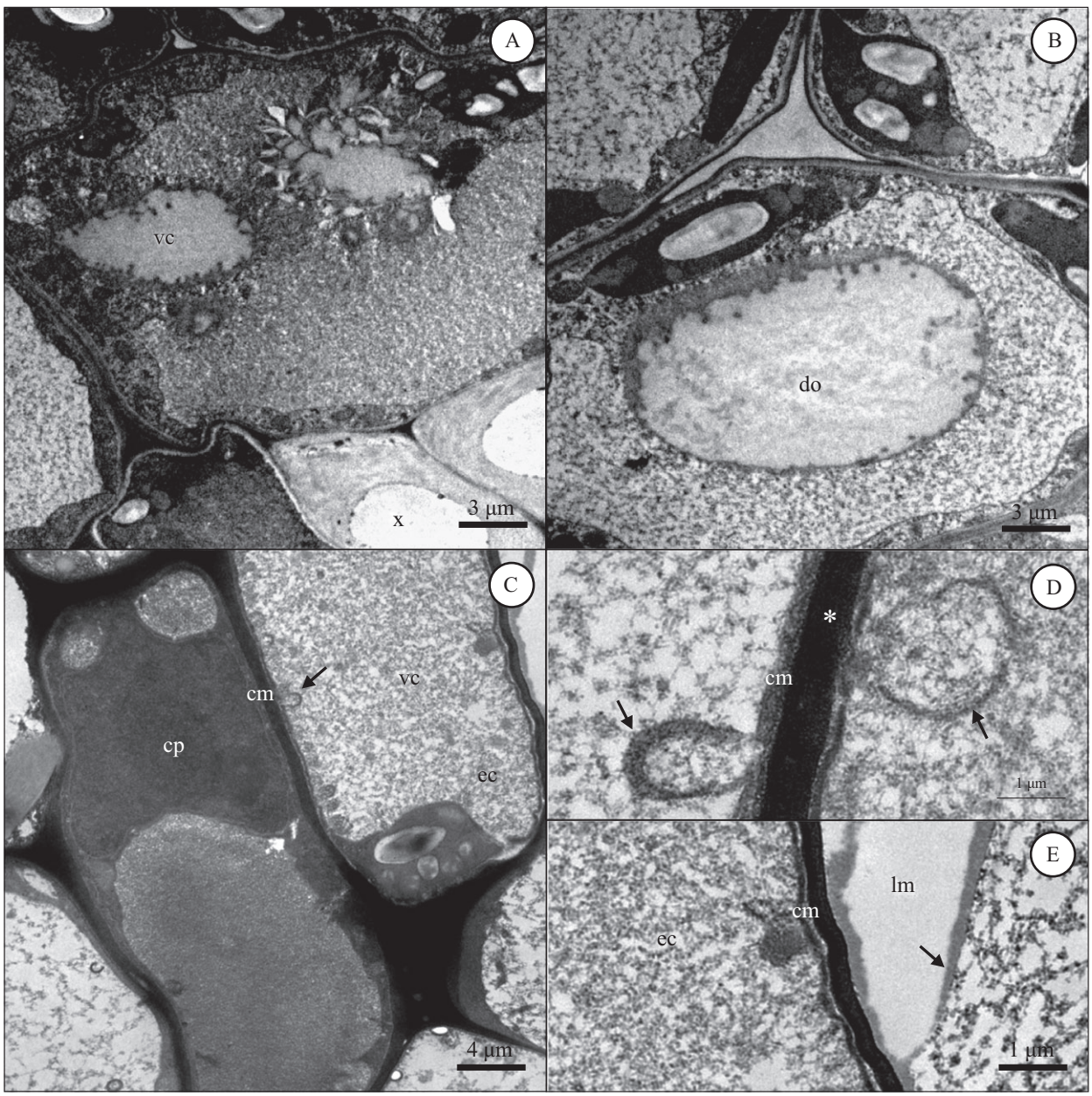

Fig. 5. Ultrastructure of cells of the vascular bundle and duct in mesophyll of *V. macrophylla*. A: Parenchyma cell between xylem and a duct, with vacuole containing secretions. B: Cell parenchyma, adjacent to epithelial cells of the duct, with large vacuole containing an oil droplet. C: Epithelial cell with vacuole in which the secretion is stored. Details of secretion transfer through the cell membrane (arrow). D: Granulocrine mechanism of transport of secretions. Details of secretion (arrow) transfer through the cell wall (*). E: Duct epithelial cell and lumen with secretion (arrow). vc: vacuole; do: oil droplet; lm: lumen; ec: epithelial cell; pc: parenchyma cell; cm: cell membrane.

March. (Burseraceae), which is more vulnerable to herbivores than other species of the same genus that possess this secondary metabolite (Souza *et al.* 2015).

The functions of lipids and lipid metabolism as a defence response have been extensively studied (Rojas *et al.* 2014), especially the genes associated with enzymes for biosynthesis of fatty acids and sphingolipids (Berkey *et al.* 2012). The activities of these enzymes regulate the spatial and temporal production of lipid metabolites that mediate specific responses to biotic stimuli (Wang 2004; Zhao *et al.* 2013). In *V. macrophylla*, lipid droplets, as seen in secretion from the ducts, were also observed in mesophyll cells. These results suggest that both the secretion stored in ducts and in the parenchyma cells may be involved in plant defence.

The ducts of *V. macrophylla* showed a positive blue reaction to Nadi reagent in the lumen, indicating the presence of terpenes. Furthermore, the lipid droplets observed in mesophyll cells produced a blue-violet colour, indicating secretion of oleoresin in this species. Essential oils can serve to attract pollinators, provide defence against herbivory or regulate the rate of decomposition of organic matter in the soil by acting as antimicrobial agents (Castro *et al.* 2004).

Table 1. Chemical composition of the essential oil in leaves of *V. macrophylla*.

Components	RI[a]	RI[b]	%[c]
α-Copaene	1374	1376	0.7
β-Cubebene	1387	1390	2.4
β-Elemene	1389	1391	2.4
β-Caryophyllene	1418	1418	5.9
β-Gurjunene	1428	1432	1.0
α-Humulene	1455	1454	1.5
β-Chamigrene	1473	1475	0.7
γ-Muurulene	1477	1477	1.1
Germacreno D	1484	1480	37.3
β-cis-Guaiene	1488	1490	0.7
Biciclogermacrene	1495	1494	5.3
γ-Cadinene	1510	1513	1.5
δ-Cadinene	1518	1524	5.7
Selina-3,7 (11)-diene	1550	1542	0.7
Germacrene B	1558	1556	2.6
Germacrene D-4-ol	1580	1574	17.0
Cariophyllene oxide	1584	1581	0.5
Isolongefolan-7-α-ol	1621	1619	0.9
1-epi-Cubenol	1629	1627	0.5
γ-Eudesmol	1633	1630	0.6
epi-α-Muurolol	1645	1641	1.7
α-Muurolol	1648	1645	0.5
α-Cadinol	1656	1653	4.5
Patchoulialcohol	1658	1659	0.8
Khusinol	1686	1680	1.1
8-cedren-13-ol	1691	1688	0.5
Eudesm-7(11)-en-4-ol	1694	1700	1.1
Total identified			99.2
Sesquiterpenes			55.1
Oxygenated sesquiterpenes			44.1

[a]RI = retention index calculated against C_8–C_{26} n-alkanes on the VF-5 ms column (30 m × 0.25 mm × 0.25 μm ID).
[b]RI lit = Adams (2007).
[c]Percentages obtained after FID-peak normalisation.

Ultrastructure analysis revealed a special tissue associated with the secretory ducts and the vascular bundles. Apparently, this tissue is involved in the secretion mechanism and transport of substances to epithelial cells of the ducts. Transfer cells, with invaginations, were observed in these tissues; these invaginations are often associated with more efficient transport mechanisms (Gunning & Pate 1969). Moreover, the presence of abundant lipid globules in dense cytoplasm strongly suggests that these cells are involved in the synthesis of the secretion. It can be hypothesised that this secretion is produced in plastids of the transfer cells and of cells in the parenchymatous sheath. The secreted material is carried by a granulocrine process to epithelial cells of the ducts (Fig. 5C and D), which probably function mainly to store the material until it is released into the lumen (Fig. 5E). A granulocrine process is probably also responsible for secretion release from the epithelial cells into the lumen of the duct.

Bartoli *et al.* (2011) and Kromer *et al.* (2016) also reported the presence of a particular tissue type associated with secretory ducts in *Grindelia pulchella* Dunal (Asteraceae), where this tissue has vacuoles containing material of a different appearance, comprising globules with electron density similar to that of lipid droplets. In *G. pulchella,* discharge started in transfer cells and then transferred to epithelial cells of the duct, where it accumulated in vacuoles (Bartoli *et al.* 2011).

In studying the secretory structures of *Arnica*, Kromer *et al.* (2016) observed that strands of phloem parenchyma cells included in the vascular bundles of rhizomes accumulate large amounts of lipids and give rise to the secretory system. These strands run in longitudinal arrays located outside the sieve tube cells and are surrounded by an endodermis in the youngest rhizome segments. The walls of epithelial cells develop structurally specific acidic lipid invaginations, characteristic of transfer cells, which could facilitate a high rate of apoplasmic/symplasmic transport. In the middle of these lipid storage cells, reservoir formation is initiated through cell loosening or cell lysis giving rise to oil bodies. During essential oil synthesis, the triacylglycerols enclosed in the oil bodies are released and become the substrate for terpene synthesis, as well as the medium in which essential oils dissolve.

Oil bodies present in tissues of leaves and other organs are little explored. Perhaps in plants, as with lipid droplets in animals, they perform various functions related not only to long-distance transport of triacylglycerols, but also to transport of bound protein molecules and various particles (Kromer *et al.* 2016). Several studies have found plastids containing dark material in thylakoids, between the membranes and stroma (Ascensão & Pais 1988; Monteiro *et al.* 1999; Bartoli *et al.* 2011), as well as the presence of plastids with oil droplets in secretory tissue (Lacchia & Carmello-Guerreiro 2009; Bartoli *et al.* 2011). These oil droplets are released into the cytoplasm and transported to cells surrounding the vascular bundles, where they are stored in vacuoles or transferred to the epithelial cells of the duct. These observations suggest that the secretion released into the lumen of the duct in *V. macrophylla* is produced in plastids of transfer cells and parenchyma cells of the sheath. The main function of the epithelial cells of the duct is probably to store the secretion produced in the special tissue associated with the duct and carried by a granulocrine process to the epithelial cells.

The essential oils extracted from samples of dried leaves of *V. macrophylla* accounted for 0.8%. Oil content can be affected by many factors, including the plant parts analysed, the methodology used for steam distillation, physiological variation inherent to the plant, *e.g.* development phase, seasonal variation, environmental conditions and geographic variation (Figueiredo *et al.* 2008). About 70% of the essential oil of *V. macrophylla* is composed of germacrene D, followed by germacrene D-4-ol, β-caryophyllene, α-bicyclogermacrene and cadinol. The composition of the essential oil from our samples was similar to that found for *Verbesina diversifolia* DC., which included β-caryophyllene, bicyclogermacrene, germacrene D and germacrene D-4-ol as major components (Albuquerque *et al.* 2006).

Germacrene D is common in the genus *Senecio* (Asteraceae; Mozuraitis *et al.* 2002; Murari *et al.* 2008) and is a precursor to biosynthesis of many other compounds of sesquiterpene structure (Bülow & Wilfried 2000). This constituent is widely distributed in nature and is unstable (Kraker *et al.* 1998). Data from the literature indicate that high temperatures, such as those used in steam distillation of essential oils, may lead to degradation of sesquiterpenes or induce molecular rearrangement, affording other compounds a sesquiterpenoid nature,

which are actually artefacts (Radulović *et al.* 2007). With respect to biological activity, sesquiterpenes have been reported to interact with insects and other organisms (Rostelien *et al.* 2000; Mozuraitis *et al.* 2002; Stranden *et al.* 2002). β-caryophyllene and germacrene D can be biosynthetically related, and so the flow of precursors toward biosynthesis of one these components can lead to a reduced concentration of the other (Bülow & Wilfried 2000).

Most sesquiterpenes have insecticidal or antibiotic properties (Dey & Harborne 1997). The essential oil of *Croton pullei* Lanj. (Euphorbiaceae) contains germacrene D and caryophyllene and has anti-inflammatory and anti-nociceptive activity (Rocha *et al.* 2008). It would be interesting to investigate whether the high concentration of germacrene D in the essential oil of *V. macrophylla* is related to the popular medicinal use of this plant. Further investigation is needed to understand how concentrations of these compounds in *V. macrophylla* vary according to season and different phenological stage. Biological tests are also needed to better understand the biological and ecological function of these chemical compounds of the essential oil of *V. macrophylla*.

CONCLUSION

Substances identified in leaves of *V. macrophylla* suggest that both the secretion stored in ducts and in adjacent parenchyma cells is probably involved in chemical defence. It appears that this secretion released into the lumen of the duct in *V. macrophylla* is produced, at least in part, in plastids of transfer cells and cells of the parenchymatous sheath. The main function of epithelial cells of the ducts, on the other hand, is probably producing and storing the secretion produced in the special tissues associated with the duct and carried by a granulocrine process to the epithelial cells. The essential oil of *V. macrophylla* is rich in sesquiterpenes, the most significant components being germacrene D and its derivatives. This work contributes to the identification and description of a diversity of classes of metabolites present in the studied species.

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