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Author(s): Lia Ascensao, Natalia Marques, M. Salome Pais
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PELTATE GLANDULAR TRICHOMES OF *LEONOTIS LEONURUS* LEAVES: ULTRASTRUCTURE AND HISTOCHEMICAL CHARACTERIZATION OF SECRETIONS

LIA ASCENSÃO,[1] NATÁLIA MARQUES, AND M. SALOMÉ PAIS

Departamento de Biologia Vegetal, Faculdade de Ciências de Lisboa, Bloco C2, 1780 Lisboa Codex, Portugal

The histochemical characterization of the oleoresin produced by peltate trichomes of *Leonotis leonurus* revealed terpenoids and flavonoid aglycones. At the onset of secretion, glandular cells were more densely cytoplasmic than the other trichome cells. The lateral stalk wall underwent cutinization, and the cuticle over the glandular cells became thicker. During the active secretory stage, the most striking ultrastructural features of glandular cells were the hypertrophy of the leucoplastidome and the extensive proliferation of ER. The high development of these two cellular compartments was related with the biosynthesis and transport of the secretory product. A granulocrine secretion mechanism may operate alone or concurrently with an eccrine process. In the glandular head, the loosening of the outer wall fibrillar matrix, and the accumulation of secretion in the newly formed interfibrillar spaces led to a secretory cavity development by detachment of the cuticle and the outermost pectic layer of cell wall. Successive accumulation of secretion in the secretory cavity conferred the spherical shape, characteristic of a peltate gland, to the trichome. The interfibrillar spaces, initially small and elongated, enlarged, became roundish, and appeared as vesicles delimited by an electron-dense layer. These vesicle-like structures are interpreted as lipophilic secretion globules in a hydrophilic phase. It is suggested that pectic polysaccharide wall constituents may be the main components of this hydrophilic matrix. The dense layer around the vesicles may represent an interphase between lipophilic and hydrophilic compounds. The secretion seemed to remain trapped in the secretory cavity, since no cuticular disruption was observed.

Introduction

Many species of the Lamiaceae family have been used in folk medicine. The genus *Leonotis,* frequently associated with *Cannabis* through the term *dagga,* is often listed as a mild narcotic hallucinogen, although toxicity and hallucinogenic properties seem rather insignificant (Duke 1985).

Leonotis leonurus R. Br. (lion's ear or lion's tail), a perennial shrub widely distributed throughout South Africa and tropical regions of America, is reputed to possess a great variety of medicinal properties, being considered as emmenagogue, purgative, and vermifuge. Leaf decoctions are used by Africans (Zulus, Hottentots, and Xhosas) to treat asthma, fever, influenza, snakebite, epilepsy, skin diseases, and tapeworm. Dry leaves, alone or mixed with tobacco, may lead to habituation when smoked persistently (Watt and Breyer-Brandwigk 1962).

Phytochemical studies have revealed that *L. leonurus* produces labdane diterpenoids (Kaplan and Rivett 1968; Purushothaman and Vasanth 1988) and an essential oil rich in terpene hydrocarbons, mainly β-caryophyllene and α-pinene (Pedro et al. 1991).

The glandular trichomes that produce essential oils are a general feature of the mint family. In *L. leonurus* the vegetative and reproductive organs bear numerous glandular trichomes of two morphologically distinct types (peltate and capitate) that also seem to have different secretion processes, as indicated by SEM (Ascensão et al. 1995). It is, therefore, of interest to investigate the ultrastructure of these trichomes to verify whether morphological differences also are reflected in their cytology, their secretion process, and in the chemical nature of the secreted material.

Although a considerable number of studies deal with the chemical composition of the essential oils produced by Lamiaceae, the morphology and particularly the ultrastructure of secreting glandular trichomes have been examined only in a few species. Detailed studies were made on *Mentha piperita* (Amelunxen 1964), *Mentha spicata* (Gershenzon et al. 1989), *Salvia glutinosa* and *Salvia pratensis* (Schnepf 1972), *Salvia officinalis* (Venkatachalam et al. 1984), *Monarda fistulosa* (Heinrich 1973; Heinrich et al. 1983), *Origanum dictamnus* (Bosabalidis and Tsekos 1982), *Teucrium scorodonia* (Sevinate-Pinto and Antunes 1991), and *Nepeta racemosa* (Bourett et al. 1994).

Giving sequence to our work on *L. leonurus,* we describe in this article the ultrastructural development of glandular cells of peltate trichomes and their histochemistry. Special emphasis is given to the subcuticular space of these glandular trichomes where the essential oils accumulate.

Material and methods

Transmission electron microscopy (TEM)

Leaves of *Leonotis leonurus* R. Br., grown at the Lisbon Botanic Garden, were fixed at different stages of development with 3% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.2, at 4°C for 4–6 h, and postfixed for 2 h in 2% unbuffered osmium tetroxide (OsO_4). After dehydration in a graded acetone series, the material was embedded in Epon-Araldite resin. Thin sections were conventionally stained with uranyl and lead citrate and examined with a JEOL 100C electron transmission microscope at 80 kV.

For polysaccharide detection, ultrathin sections were collected on Formvar-coated gold grids and submitted to the PATAg test (Thiéry 1967).

Light microscopy (LM)

The majority of the histochemical tests used in this study have been referred to in a previous paper (Ascensão and Pais

[1]Author for correspondence and reprints.

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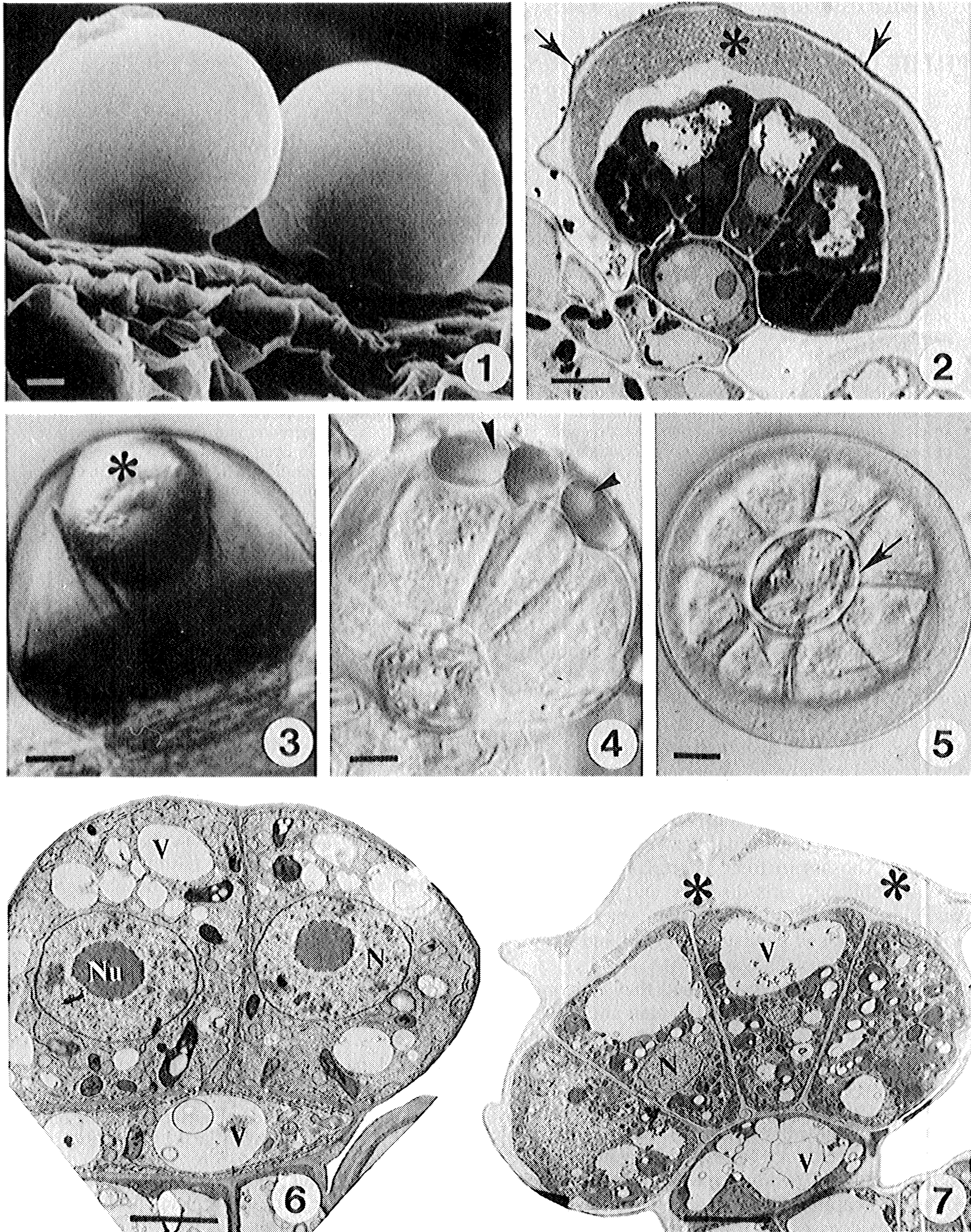

Figs. 1–7 Fig. 1, SEM micrograph of a peltate trichome with a swollen head, the result of accumulation of oleoresin in the subcuticular space. Figs. 2–5, Histochemical characterization of the glandular trichome secretion. Fig. 2, Semithin section stained with Sudan black B. Note at the secretory cavity the thick bounding dermal sheath (arrows) and the dark heterogeneous secretion product (asterisk). Figs. 3 and 4, Fresh sections stained, respectively, with Sudan IV and Nadi reagent. In fig. 3 the secretion accumulated in the subcuticular space stained red (asterisk) and in fig. 4 stained violet (arrowheads). Fig. 5, Control of the Nadi test. Top view of a trichome detached from epidermis. The stalk cell (arrow) can be seen in the center, overlapping the glandular disk cells. Figs. 6 and 7, TEM micrographs. In fig. 6 a developing peltate trichome at the four-celled head stage and in fig. 7 a mature trichome showing a large secretory cavity (asterisks). *N* = nucleus; *Nu* = nucleolus; *V* = vacuole. Bars = 10 μm.

1987). Sections of fresh material cut on a Reichert cryostat microtome were stained with Sudan black B, Sudan IV, and Nile blue A for total lipids; Nadi reagent and anthimonium trichloride for terpenoids; Wagner and Dittmar reagents for alkaloids; periodic acid–Schiff (PAS) reagent for polysaccharides with vicinal glycol groups; and mercuric bromophenol blue for proteins. The presence of phenolic compounds was tested with ferric trichloride (Johansen 1940), vanillin-hydrochloric acid (Gardner 1975), and Fast blue B (Ganter and Jollés 1969). Flavonoids were detected by induction of fluorescence with the fluorochromes aluminum chloride and Wilson reagent (Charrière-Ladreix 1973). Assays to demonstrate pectins were made by Ruthenium red (Johansen 1940). Standard control procedures were carried out simultaneously.

Semithin sections of material prepared for TEM were also stained for lipids with Sudan black B (Bronner 1975). Observations were made on a Leitz microscope using Nomarski optics and on a Leitz epifluorescence microscope equipped with a filter system A (exciter filter BP-340-380, dichroic mirror 450, barrier filter LP-430).

Scanning electron microscopy (SEM)

Leaves were fixed for 2 h at 4°C with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.2. After washing in the same buffer and dehydrating in acetone, the material was critical-point dried, sputter-coated with gold, and observed in a Jeol JSM T220 scanning electron microscope at 15 kV.

Results

Peltate trichomes are quite common on *Leonotis leonurus* leaves and flowers (Ascensão et al. 1995). They occur on both surfaces of leaf, calyx, and corolla, mixed with capitate trichomes, while on the stamens and gynoecium they are the only constituents of the indumentum. Fully developed peltate trichomes are about 60 μm (±15) in height and 50 μm (±10) in diameter at the spherical head (fig. 1). They consist of a short stalk cell and a large head with eight secretory cells arranged in a circle (figs. 2, 5).

The secretion contained in the subcuticular space stained red with Sudan IV (fig. 3, asterisk) and dark blue with Sudan black B (fig. 2, asterisk), indicating lipids. The Nadi reagent (fig. 4, arrowheads) and antimonium trichloride gave, respectively, a violet and a red color to the secretion, revealing terpenoids. The controls of these tests were negative. Only the control for Nadi reagent is shown (fig. 5). Glandular secretion reacted (bright red) for phenolics only when tested against Fast blue B.

Secretion showed a blue autofluorescence under ultraviolet light, indicating phenolic compounds. Fluorochromes for flavonoids, such as aluminum chloride and Wilson reagent, induced a bright yellow fluorescence that suddenly decayed by acidification of the mounting medium. The histochemical tests performed to detect pectins, polysaccharides, proteins, and alkaloids gave negative results with the secretion.

The trichome cell walls stained strongly with PAS. However, the outer layer of glandular cell walls stained lightly with PAS and intensely with Ruthenium red, whereas in the inner layer, the staining intensity observed was the reverse. The thick cuticle on the stalk lateral walls stained heavily with Sudan IV.

The ultrastructure of the glandular cells underwent dramatic changes during peltate trichome development, going through three different stages: a presecretory, a secretory, and a postsecretory phase.

Presecretory phase. In the presecretory phase, which corresponds to the differentiation of the trichome, the ultrastructure of the glandular cells was similar to the other meristematic cells of the leaf and flower. They contained a large nucleus with a prominent nucleolus and a dense cytosol, rich in ribosomes (figs. 6, 8). The multishaped plastids had dense stromas, scarce internal membranes, and few starch grains (figs. 8, 9, 10). Occasional dictyosomes and a few cisternae of endoplasmic reticulum, dispersed throughout the cell, were observed (figs. 8, 9). Plasmodesmata were frequent on the periclinal stalk walls and on the anticlinal glandular cell walls. At this stage glandular cells had a thin cuticle (fig. 9, arrow), comparable to the one found in epidermal cells.

At the later presecretory phase, the stalk cell and the basal cells developed central vacuoles. In addition, the gland cuticles began to thicken, while cuticles of epidermal cells remained thin. This cuticle-thickening process was first detected on the stalk lateral walls (fig. 8).

Secretory phase. In the beginning of the secretory phase, the increase in thickness of gland cuticles

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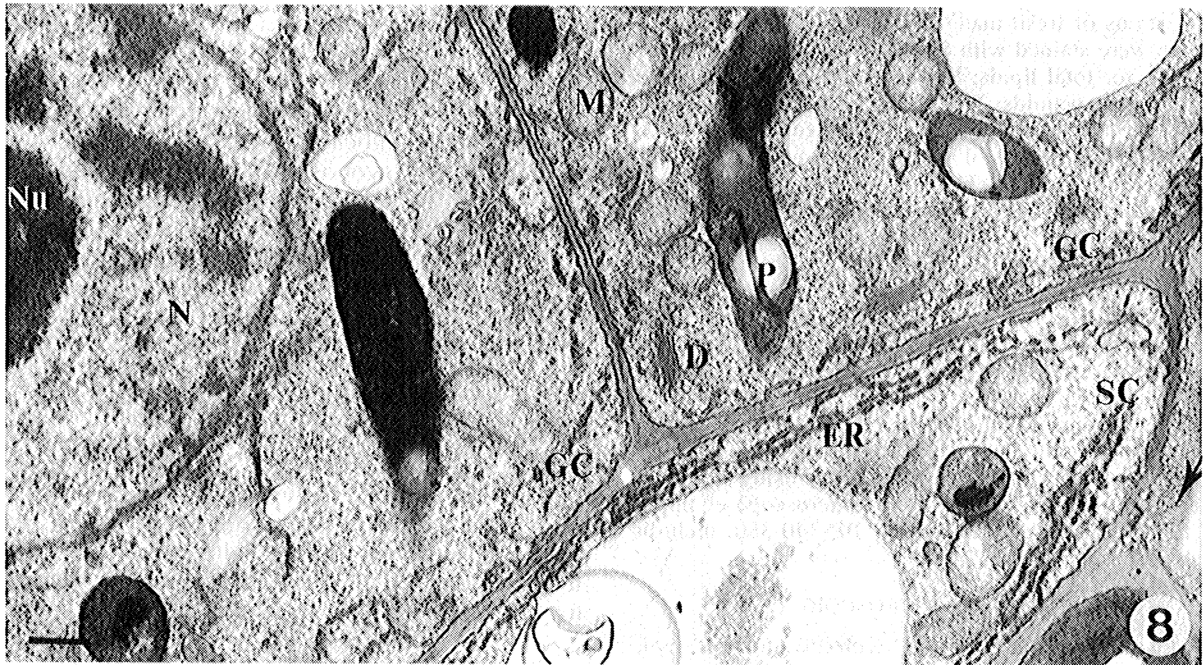
Figs. 8–12 TEM micrographs of peltate trichomes in the presecretory phase. Fig. 8, Glandular cells (*GC*) showing a cytosol rich in ribosomes, few cisternae of *ER*, and scarce dictyosomes (*D*). A large nucleus (*N*), various plastids (*P*), and mitochondria (*M*) can also be seen. On the stalk cell (*SC*), the thickening of the cuticle becomes evident (arrow). Fig. 9, A thin cuticle (arrow) is present over the glandular head wall (*CW*). Fig. 10, Pleomorphic plastids (*P*), with poorly developed internal membranes and without thylakoids, are observed. Figs. 11 and 12, TEM micrographs of peltate trichomes in the beginning of the secretory phase. Fig. 11, Detail of the well-developed reticulate cuticle (*Cu*). The fibrillar network extending into the cuticle and its continuity with the pectocellulosic layer underneath is evident (arrows). In fig. 12, note the cutinized lateral wall of the stalk cell and the different cuticle thickness of glandular (arrows) and epidermal cells (arrowheads). *SC* = stalk cell; *GC* = glandular cell; *EP* = epidermal cell. Bars = 0.5 μm.

Figs. 13–16 TEM micrographs of trichome glandular cells in the active secretory stage. Figs. 13 and 14, Amoeboid leucoplastids (*P*), closely stacked. Note the association of *ER* cisternae with the plastid envelope (arrows) and the presence of an electron-dense content within plastid tubules (arrowheads). Fig. 15, A high proliferation of *SER* is visible. Fig. 16, Sinuous plasmalemma (arrows) seems to suggest a granulocrine secretion. Bars = 0.5 μm.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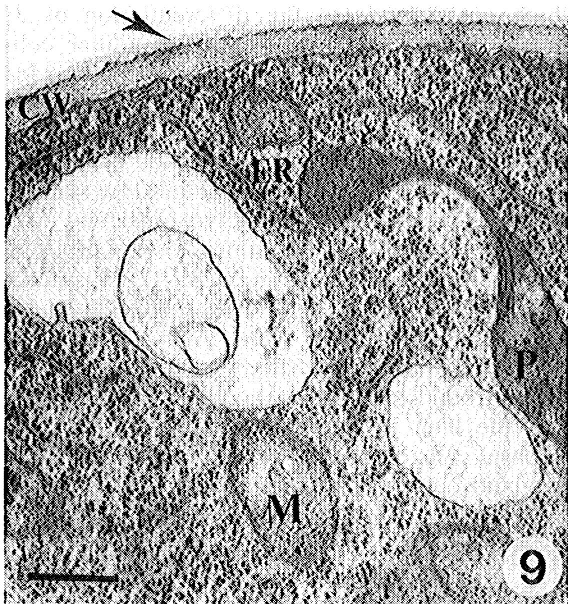
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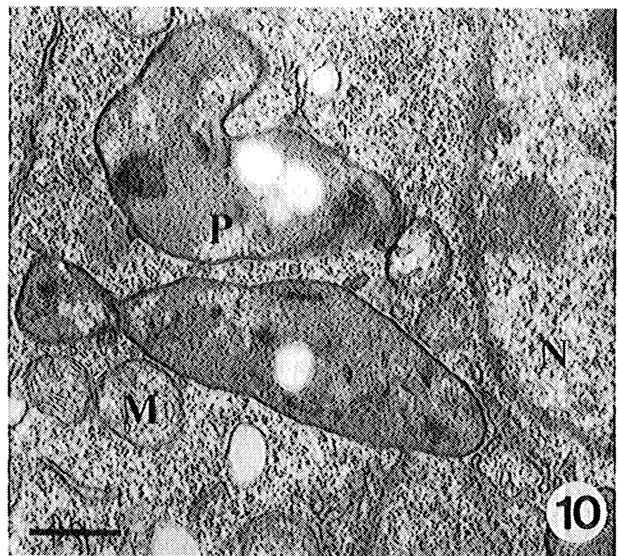
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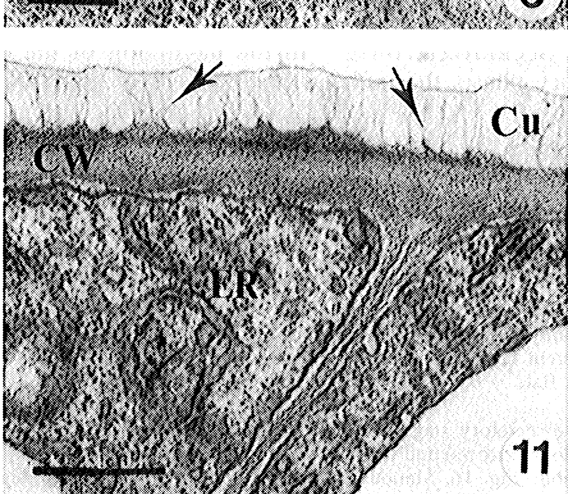
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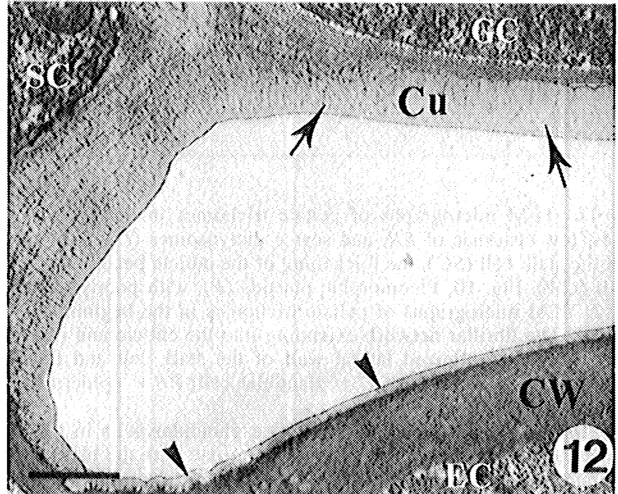
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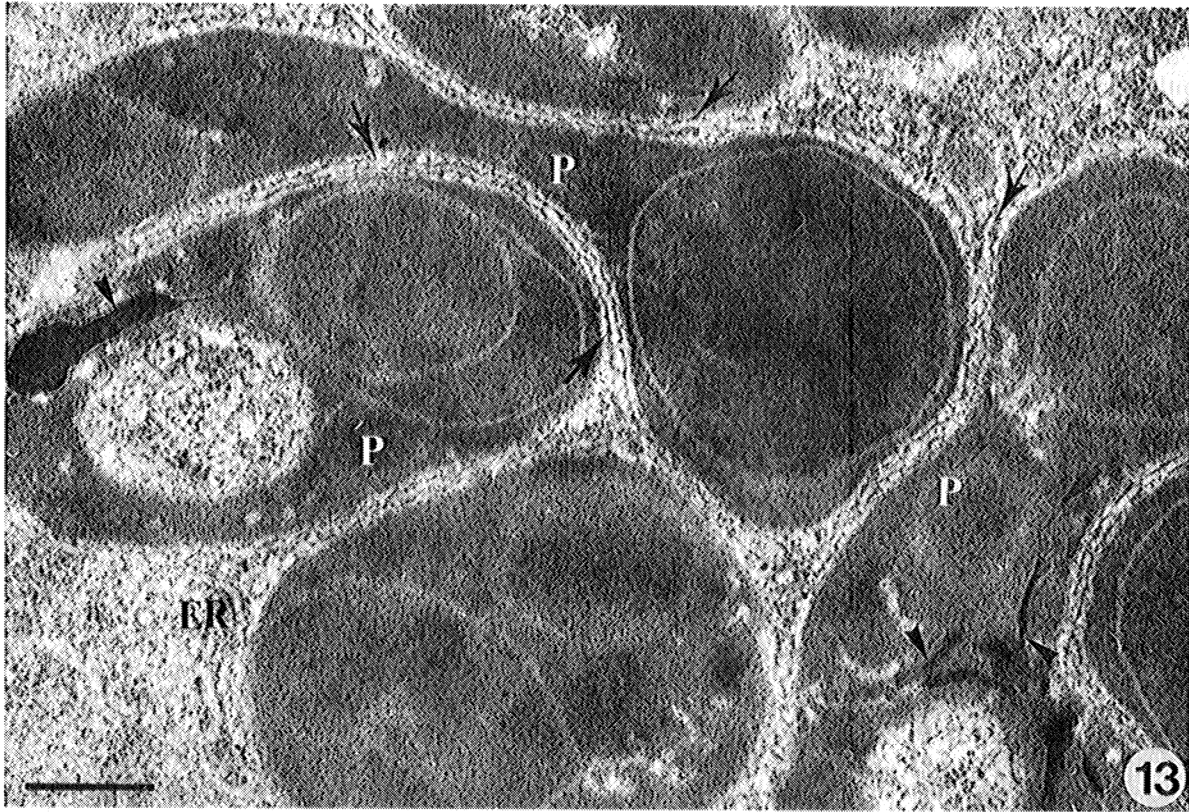
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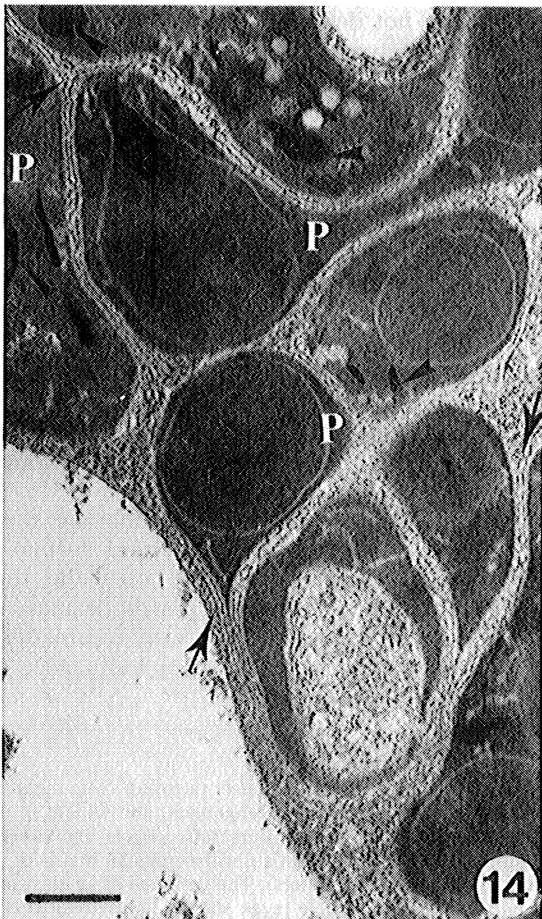
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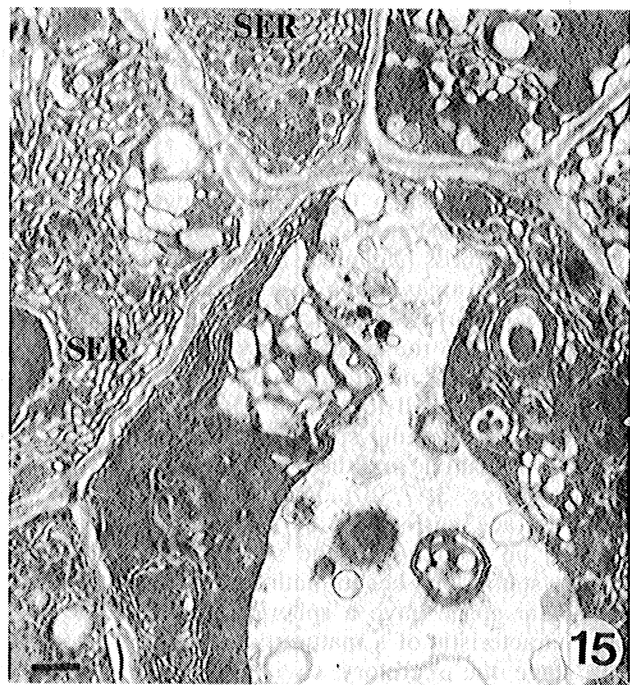
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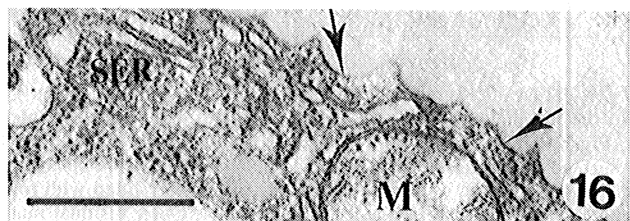
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was evident (figs. 11, 12). Cuticles appeared reticulate due to a fibrillae network that permeated through an apparently amorphous matrix. The fibrillae seemed to have some continuity with the structural elements of the pectocellulosic layer beneath (fig. 11, arrows). During this stage, several ultrastructural changes occurred in glandular cells, e.g., densification of cytoplasm, increase in ribosome number, and an extensive development of the plastid compartment that was accompanied by ER proliferation. Most of the cell volume was occupied by numerous large and amoeboid plastids. They were closely stacked and were separated by only a thin layer of cytoplasm, containing ER cisternae associated with the outer membrane of the plastidial envelope (figs. 13, 14, arrows). These polymorphic plastids, frequently bell-shaped, were typically leucoplasts characterized by a very dense stroma and a poorly developed system of internal membranes. Leucoplasts did not contain thylakoids; they had only short membrane tubules connected with the envelope inner membrane. The tubules had dilated portions that very often were filled with an electron-dense content (figs. 13, 14, arrowheads).

Following the hypertrophy of the leucoplastidome, a high proliferation of smooth endoplasmic reticulum (SER) occurred. A large number of long and sinuous cisternae were parallel or around the cell organelles (fig. 15). Although neither direct connections between ER membranes and the plasmalemma nor extrusion vesicles containing the secretory product were detected, a very sinuous plasmalemma was observed (fig. 16, arrows).

Concurrently with the production of the secretory material, an extracellular space developed over the glandular cells. It began in the outer cell wall by a loosening of the fibrillar matrix (fig. 17, arrows). The gaps between the fibrils began as small and elongated electron-translucent areas, often in a tandem arrangement (figs. 18, 19, arrows), indicating their aggregation and conferring to the outer cell wall the appearance of a loose fibrillar mesh (figs. 21, 22).

As a result of the wall loosening and of secretion accumulation, a subcuticular space was formed by the detachment of the cuticle and the outermost pectic layer of cell wall (figs. 3, 7, 20). Vesicle-like structures of irregular surface, surrounded by a fibrillar-granular matrix, could be seen within the subcuticular space (figs. 21, 22, stars). The accumulation of secretion in the subcuticular space gave a spherical shape to the trichome, characteristic of a mature peltate gland.

At this stage the secretory cavity had numerous round vesicle-like structures of different sizes. These vesicles, with an electron-light content, appeared delimited by an electron-dense layer and apparently increased in size by coalescence (fig. 23). The inner layer of glandular cell wall stained heavily by PATAg test, while the residual matrix of outer wall and the bounding vesicle layer stained lightly (fig. 24).

POSTSECRETORY PHASE. During this phase, an increase in vacuolation was observed. Vacuoles containing a flocculent material and membrane debris occupied the greatest volume in the glandular cells and limited the cytoplasm to a thin parietal layer. Secretions that accumulated in the subcuticular space compressed the glandular disk cells, inducing their anticlinal walls to fold up. There was no indication of spontaneous cuticle rupture. A progressive degradation of the cellular constituents was observed.

Discussion

In spite of the poor specificity of the histochemical tests, they can be useful to localize in situ the main chemical classes of metabolites present in plant secretions. Our histochemical results indicate that the secretion of *Leonotis leonurus* peltate trichomes is an oleoresin containing terpenoids (essential oils and resiniferous acids) and flavonoid aglycones as its main constituents. Proteins, polysaccharides, alkaloids, and tannins were not detected in the secretion. These results are consistent with those obtained by GC and GC/MS. Mono- and sesquiterpenes were identified in the essential oil of *L. leonurus* (Pedro et al. 1991), and the presence of labdane diterpenoids was also reported (Kaplan and Rivett 1968; Purushothaman and Vasanth 1988). Tannins appear to be absent in Lamiaceae, and only a few minor alkaloids seem to occur in this family (Richardson 1992). Small amounts of polysaccharides were histochemically detected in the secretion of the peltate trichomes of some Lamiaceae species (Werker et al. 1985). After our TEM studies of the secretory cavity development, we are convinced that the polysaccharides detected may be cell wall components, corresponding probably to the loose fibrillar mesh of the outer cell wall, where the secretory products accumulated.

At the ultrastructural level, the trichome stalk cell of *L. leonurus* showed little structural specialization besides the cutinization of lateral cell walls, the lack of chloroplasts, and numerous plasmodesmata in the periclinal walls. Similar features were reported by several authors for trichomes of other Lamiaceae (Ame-

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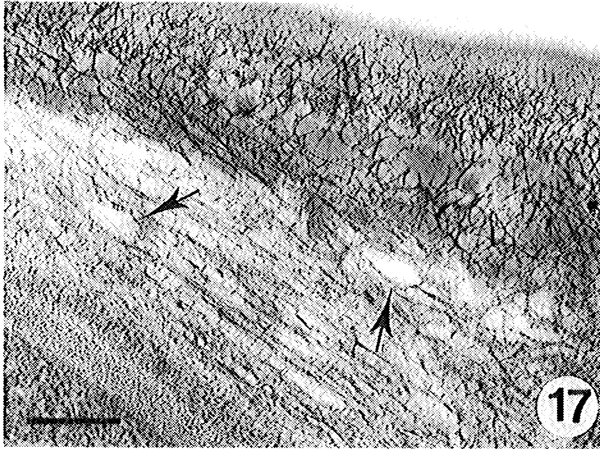
Figs. 17–24 TEM micrographs of secretory cavity development. Fig. 17, Loosening of the outer wall matrix (arrows). Note the inner wall pressed to the plasmalemma. Figs. 18 and 19, Small and elongated interfibrillar spaces, in a tandem arrangement, are evident in the wall (arrows). Fig. 20, Early formation of secretory cavity (asterisk). Fig. 21, The loose wall matrix and numerous vesicle-like structures of irregular surface (stars) can be seen. Fig. 22, Detail of fig. 21. Vesicle-like structures (stars) embedded in a fibrillar-granular matrix are clearly observed. Fig. 23, Portion of a secretory cavity completely filled with round vesicle-like structures (stars). The presence of an electron-dense layer around the vesicles is evident (arrows). Fig. 24, Outer residual matrix and the bounding vesicle layer, stained lightly with PATAg test for polysaccharide. Bars = 0.5 μm.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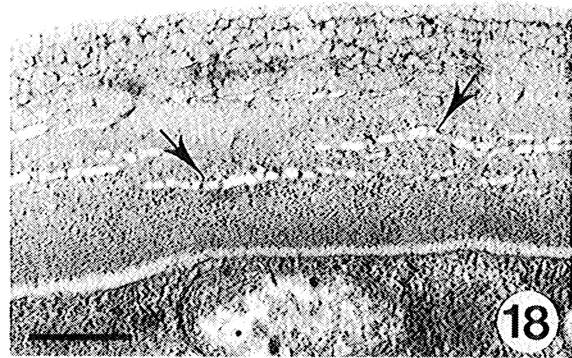
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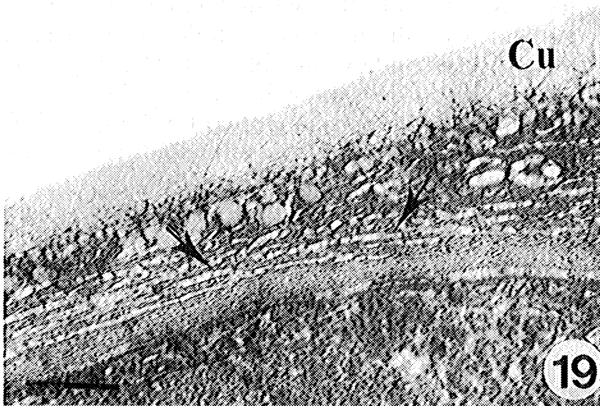
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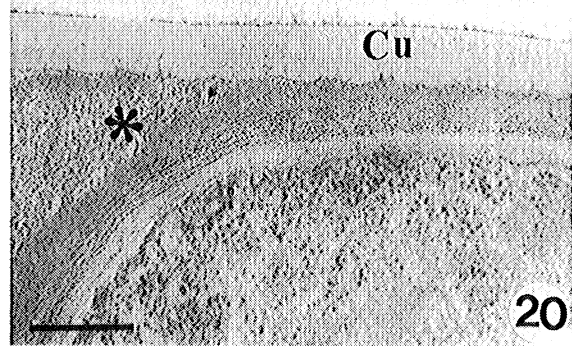
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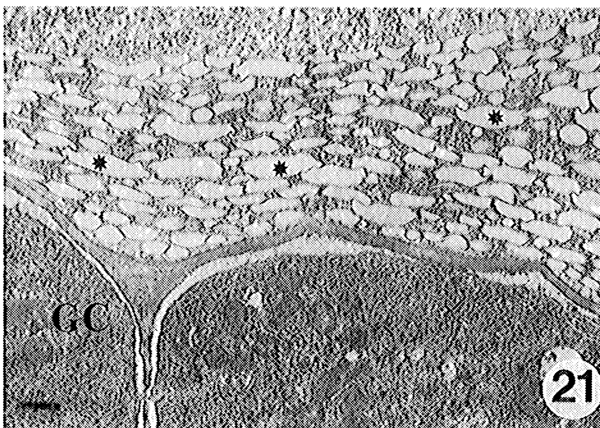
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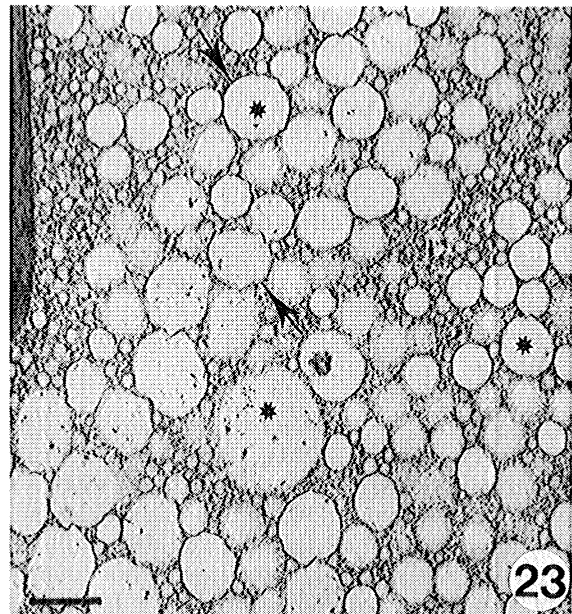
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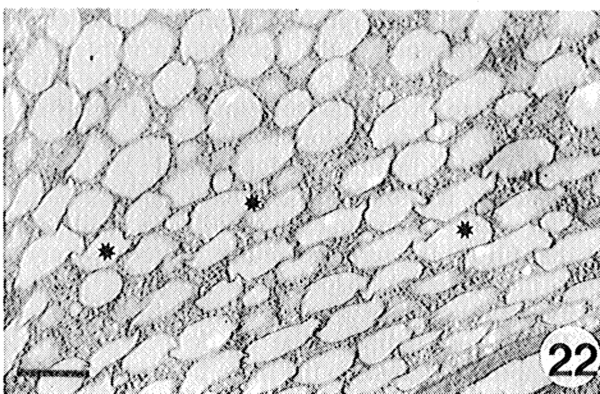
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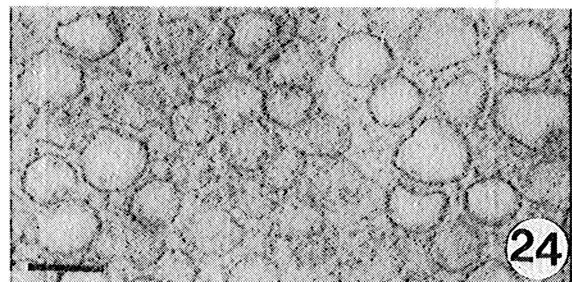
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lunxen 1964; Schnepf 1974; Bosabalidis and Tsekos 1982; Fahn 1988; Bourett et al. 1994).

Cutinization of the side walls of stalk cell was frequently observed in glandular trichomes. It is generally assumed that cutinized walls may block the back flow of secretions stored in the subcuticular space, preventing the intoxication of mesophyll cells. The lack of chloroplasts in the trichome cells together with the presence of a barrier to apoplastic flow in the stalk indicate that precursors of secretion components might come from the mesophyll. Plasmodesmata in the periclinal walls of stalk cells may provide transport of photosynthates into the glandular cells, contributing to secretion rate regulation.

At the secretion stage, the most striking ultrastructural features of the peltate trichome glandular cells were the extensive development of the leucoplastidial compartment and the high proliferation of SER. The hypertrophied leucoplastidome in close association with ER is characteristic of various glandular structures secreting essential oils and resins (Dell and McComb 1978; Charon et al. 1987; Fahn 1988; Kleinig 1989; Wagner 1991; Duke and Paul 1993).

The involvement of leucoplasts and ER in terpene secretion has been biochemically demonstrated. For monoterpenes, plastids are clearly implicated as the exclusive site of synthesis. In fact, the biosynthesis of monoterpene hydrocarbons in vitro was achieved by a leucoplast-enriched fraction (Gleizes et al. 1983; Pauly et al. 1986), and the biosynthesis of geranyl pyrophosphate was recently localized in plastids (Soler et al. 1992). The synthesis of farnesyl pyrophosphate and its derived sesquiterpenes occurred in the cytosol/SER (Gleizes et al. 1980; Belingheri et al. 1988; Hugueney and Camara 1990) as the synthesis of triterpenes (Goodwin 1979). On the other hand, correlation studies, performed in a large number of species, indicated a constant relationship between the expansion of leucoplastidome and the ratio of monoterpenes in the oil and the extension of SER and the rate of sesquiterpene or oxygenated compounds (Cheniclet and Carde 1985).

The essential oil of *L. leonurus,* analyzed by GC and GC/MS (Pedro et al. 1991), showed that the sesquiterpenes were the major fraction of the oil (59.4%), followed by monoterpenes (30.4%). In this species, apparently, the ultrastructural features and the results of the essential oil analysis of *L. leonurus* did not confirm the correlation found by Cheniclet and Carde (1985); however, it is necessary to consider that on *L. leonurus* organs two kinds of glandular trichomes occur and that probably both are involved in essential oil production.

In *L. leonurus* peltate trichomes, the transport of terpenes to the cellular surface may be via ER, since dictyosomes were not common in the glandular cells and, therefore, the participation of the Golgi apparatus in the synthesis and transport of essential oils seemed to be negligible; moreover, proteins and polysaccharides were absent in the secretion, as shown by histochemical tests.

The elimination of secretory product to the subcuticular space is not fully understood. Although extrusion vesicles were not detected, the sinuous aspect of the plasmalemma suggests fusion of vesicles. One may speculate that a granulocrine secretion occurs via ER vesicles or through transient ER-plasmalemma fusion, which implies the existence of an alternative pathway of membrane flow. Some components of the oleoresin, such as β-pinene, must be sequestered within a membrane compartment. It was proved that this compound inhibits the mitochondrial and chloroplastidial electron transport (Douce et al. 1978; Pauly et al. 1981). Otherwise, Gleizes et al. (1980) have assumed that sesquiterpenes as steroids, for instance, may act as integral components of the membrane structure. The difficulties showing anastomosis between the exocytotic vesicles and the plasmalemma by TEM may be explained by the massive secretion of terpenoids in a very short period of time. Nevertheless, an eccrine pattern of secretion cannot be excluded for some compounds. This view is supported by Stern et al. (1987), who suggested that volatile terpenoids apparently cross the plasmalemma as single molecules.

Our observations on the peltate trichomes of *L. leonurus* indicate that the secretion released in the cellular surface passes the inner cell wall, does not accumulate in the periplasmic space, and fills up the interfibrillar spaces of the outer cell wall. The thickening of the apical glandular cell wall seems to result largely from the loosening of the outer wall fibrils rather than by deposition of wall material, since only scarce dictyosomes are found.

The wall loosening, presumably the result of enzymatic action of endoglycanases, and the accumulation of secretory products may contribute to the subcuticular space development. Secretory cavities are common in many types of exotropic glands that secrete lipophilic compounds (Schnepf 1974; Fahn 1988).

In most of the secretory cavities described, accumulation of the secretory product occurs between the cuticle and the wall of the glandular cells. On the contrary, in *L. leonurus* peltate trichomes, the bounding dermal sheath of the secretory cavity consists of cuticle and a portion of the pectic outermost cell wall layer. The presence of a cuticular sheath in some Lamiaceae peltate trichomes was reported by Werker (1993). This wall reinforcement along the cuticle may give resistance to the secretory cavity when large amounts of secretion are stored. To the best of our knowledge, a similar secretory cavity was described by TEM and an analogous formation mechanism was suggested (Kim and Mahlberg 1991, 1995) only in the glandular trichomes of *Cannabis sativa.*

It seems likely that the round vesicle-like structures that fill up the large secretory cavity may correspond to the secretion, dispersed as lipophilic globules in a hydrophilic phase. The histochemical reactions and the Thiéry test indicate that pectic polysaccharide wall components must be the main constituents of this hydrophilic residual matrix and the single dense layer

around the vesicles may represent an interface between lipophilic and hydrophilic compounds. Vesicle-like structures within secretory cavities were also reported in *Humulus lupulus* (Oliveira and Pais 1990) and in *C. sativa* (Mahlberg and Kim 1992). According to these authors, the dense layer delimiting the secretory vesicles may contain proteinaceous components such as those that occur at the oil bodies in plant cells or at low density lipoprotein bodies in animal cells (Kim and Mahlberg 1995).

In *L. leonurus* peltate trichomes, the secretion remains trapped in the intact subcuticular space unless external factors, such as extreme climatic conditions or grazing, cause its disruption. However, we still believe that highly volatile secretory products can escape through microchannels at the reticulate cuticle.

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