

STRUCTURE AND ULTRASTRUCTURE OF THE EXTRAFLORAL NECTARIES OF *CROTON URUCURANA* BAILL. (EUPHORBIACEAE)

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Abstract - (Structure and ultrastructure of the extrafloral nectaries of *Croton urucurana* Baill., Euphorbiaceae). *Croton urucurana* bears two stalked and patelliform extrafloral nectaries (EFNs) on the petiole. We studied the structure and ultrastructure of the EFNs in relation to nectar secretory process. Three developmental stages of the EFN (pre-secretory, secretory and post-secretory) were recognized. The EFN is constituted by a secretory layer (epidermic and parenchymatic cells), subtended by ground parenchyma and sclerenchymatic cells. Vascular bundles cross the EFN stalk, terminating in the secretory parenchyma. Numerous plasmodesmata have been found in cell walls among parenchymatic cells and connecting these with epidermic cells. It suggests that pre-nectar transport occurs mainly by simplast. Some sugar is stored in starch grains inside chloroplasts and amyloplasts at the pre-secretory stage. Rough ER and dictyosomes seem to be involved in nectar accumulation and transport. Nectar secretion is carried out by vesicles, whose membranes fuse by the plasmalemma. Nectar is accumulated in the subcuticular space and flows outwards through cuticular channels. The most conspicuous features of the studied EFNs were the presence of both starch grains at the pre-secretory stage and ER profiles at the secretory stage.

Resumo - (Estrutura e ultra-estrutura dos nectários extraflorais de *Croton urucurana* Baill., Euphorbiaceae). *Croton urucurana* possui dois nectários extraflorais (EFNs) pateliformes e pedunculados no pecíolo. Nós estudamos a estrutura e ultra-estrutura dos EFNs, em relação ao processo de secreção do néctar. Três estágios de desenvolvimento do EFN (pré-secretor, secretor e pós-secretor) foram caracterizados. O EFN é constituído por uma camada secretora (células epidérmicas e parenquimáticas), por uma camada de preenchimento parenquimática e por células esclerenquimáticas. O sistema vascular do EFN atravessa o pedúnculo da glândula e alcança as células secretoras parenquimáticas. Numerosos plasmodesmas conectam as células parenquimáticas entre si e estas com as células epidérmicas. Isto sugere que o simplasto é a via principal de transporte de pré-néctar. No estágio pré-secretor, são observados muitos amiloplastos e cloroplastos com grãos de amido, os quais se hidrolisam durante a secreção. Retículo endoplasmático rugoso e dictiosomos parecem estar envolvidos no acúmulo e no transporte de néctar. A secreção do néctar é feita por vesículas, cujas membranas se fundem com a plasmalema. O néctar é acumulado no espaço subcuticular e é expelido através de canais cuticulares. As características mais destacadas dos EFNs estudados foram a presença de grãos de amido no estágio pré-secretor e de elementos do RE no estágio secretor.

Key words: nectar secretion, ultrastructure, structure, *Croton*.

Introduction

Some plant-animal interactions, as pollination and plant defense by ants, frequently comprise nectar secreted by plants and collected by the animals. Nectar is a watery solution constituted mainly by sugars, and secreted by glands named nectaries (Fahn 1988). The term extrafloral nectaries (EFNs) is applied to nectar-secreting glands not directly associated with pollination and generally situated on vegetative parts (Elias 1983).

Different taxa have a great variability in the nectary position, structure and ultrastructure, as well as in the ways of production, transport and secretion of nectar (Metcalfé & Chalk 1950, Fahn 1988). In relation to ultrastructural aspects for example, ER is the predominant cell compartment

(Rachmilevitz & Fahn 1973, Fahn & Benouaiche 1979, Sawids *et al.* 1989), but it may be absent (Mauseth 1982) or scarce (Zer & Fahn 1992). Moreover, ER and dictyosomes seem to be involved in the nectar secretion of different taxa (Fahn & Benouaiche 1979, Marginson *et al.* 1985, Fahn 1988).

The Euphorbiaceae family has 32 genera with EFNs (Koptur 1992), being *Croton* a well-known member with this trait (see Schnell *et al.* 1963, Koptur 1992). However, ultrastructural studies of nectaries in this genus are still unknown. *Croton urucurana* is a native tree widely distributed in the Brazilian gallery forests (Cordeiro 1985) and it has a pair of EFNs on the distal portion of the petiole, since seedling stages (Paoli *et al.* 1995).

The presence and the frequency of cell compartments and organelles in the nectaries can be correlated with the

nectar secretory process (Fahn 1988). In this way, the study of ultrastructural changes during nectary developmental stages are helpful for the understanding this process (Sawidis *et al.* 1989). In this paper we analyze the structure and the ultrastructure of *Croton urucurana* EFNs at three different developmental stages, to access the mode of nectar secretion.

Material and Methods

Leaves of adult plants were collected in a gallery forest in Rio Claro, São Paulo State, Brazil. Three developmental stages of the EFNs, distinguished by colour changing, were analyzed (green = pre-secretory, yellow to orange = secretory and brown or black = post-secretory).

Light microscopy - Fresh material was sectioned by hand and stained with Safrablau. Sections were temporarily mounted in 1:1 glycerol and distilled water. Histochemical tests were run with Lugol solution for starch, floroglucinol + HCl for lignin, hydrochloric and sulfuric acids for crystals, water solution of 10% iron chloride III for phenols, and Sudan IV for lipids (Johansen 1940, Sass 1951). Sections were observed with a Zeiss MC-100 light microscope.

Electron transmission microscopy - EFNs were fixed in a mixture of 2-3% glutaraldehyde and 2-2.5% formaldehyde in a potassium-phosphate buffer (0.1 M, pH 7.2) for a period of 4 h at 18°C and postfixed with 1% OsO₄ in the same buffer for 2 h (Hayat 1981). After dehydration in a graded acetone series, the material was embedded in epoxy resin (Spurr 1969). Ultrathin sections were contrasted with uranyl acetate and lead citrate and examined in transmission electron microscopes (Zeiss EM9-S2 and Phillips CM100).

Results

Structure

Croton urucurana has a pair of EFNs situated on the adaxial surface of the petiole distal portion. Viewed from above they resemble an elongated disk. They are epidermic, patelliform and stalked (Figure 1). Two or three similar nectaries can be found on the primary rib.

The EFN consists of a secretory epidermis subtended by sclerenchymatic cells and parenchymatous tissue (secretory and ground parenchyma, in the sense of Durkee *et al.* 1981) (Figure 1). The epidermis consists of a single layer of column-shaped cells covered by a thick cuticle (Figure 2). Intercellular spaces and stomates are lacking. The sclerenchymatic cells are brachysclereids with simple pits (Figure 2). They are mainly located in the EFN borders (Figures 1 and 2) and are absent in central parts of the gland, where secretory parenchyma is directly connected with secretory epidermis. The secretory parenchyma has 8-10 layers of isodiametric cells that stain

strongly with Safrablau. Idioblasts with calcium oxalate druses are frequent in those cells (Figure 2). The ground parenchyma comprises numerous layers (including the EFN stalk) of cells generally larger than those of the secretory parenchyma. They are highly vacuolate and stain poorly with Safrablau. The nectaries have their own vascular supply, derived from branches of the petiole vascular system. The collateral vascular bundles run into the EFN from the petiole in direction of the secretory parenchyma, crossing the ground parenchyma. Before they reach the secretory cells they are richly ramified (Figure 1).

Ultrastructure

Secretory tissue markedly differs from the ground parenchyma in which its cells are richer in cytoplasm. No ultrastructural changes were detected in the ground parenchyma among the three developmental stages. We paid close attention to the fine structure of the secretory cells (parenchyma and epidermis), since in these cells the phloem fluid is transformed and the nectar is secreted.

Pre-secretory stage - Epidermic cells showed similar ultrastructural features to the parenchyma cells at pre-secretory and secretory stage. The only difference was in the vacuolation. Epidermic cells presented numerous small peripheral vacuoles, and the central ones were fused into one central vacuole (Figure 3a). The parenchymatic secretory cells contained a large central nucleus and numerous small vacuoles (Figure 3b). The vacuoles can present different electron density (Figure 3a). Some mitochondria and several chloroplasts and amyloplasts with some plastoglobuli were observed (Figures 3b to 3e). Starch grains also occurred in many chloroplasts, which had ER profiles adjacent to them (Figure 4a). Rough ER cisternae were seldom observed close to the plasmalemma in these cells. During the pre-secretory stage, the conspicuous trait of secretory cells was the presence of starch grains inside chloroplasts and amyloplasts.

At pre-secretory and secretory stages, numerous plasmodesmata are found in cell walls between adjacent secretory parenchymatic cells and between them and epidermic cells (Figures 3b, 4d and 6d).

Secretory stage - At this point, both starch grains inside chloroplasts (Figure 4b) and amyloplasts were markedly reduced. Some amyloplasts were found inside vacuoles, where probably starch degradation took place. Ribosomes, mitochondria (Figure 4c) and rough ER profiles (Figure 4d) were profuse. Dilated rough ER cisternae were also abundant (Figure 5a). Dictyosomes with vesicles originated of them were seldom observed (Figures 4b and 5b). Peroxisomes were seen close to the plastids at the secretory stage and mainly at the post-secretory stage (Figures 5d and 6e). Numerous vesicles

and autophagic vacuoles could be observed in the cytoplasm (Figures 5c and 6b). The autophagic vacuoles comprised remnants of organelles, such as ER and mitochondria, as well as small vesicles (multivesicular bodies) (Figure 5c). The multilamellar bodies (myelin figures) are another trait related with autophagic process, which occurred near the plasmodesmata region in the epidermic cells (Figure 6d). The vesicles were found close to the plasmodesmata in the parenchymatic and epidermic cells (Figures 4d and 6d) and in fusion with the plasmalemma mainly in the epidermic cells (Figure 6a). The secreted material as well as intact vesicles were deposited in the extra-cytoplasmatic space of the epidermic cells (Figure 5c). Some vesicles transported

flocculated material (Figures 5c and 6a), which was observed in the secreted nectar (Figure 6b). The cuticle was forced up from epidermic cell walls under the pressure of the nectar to form a subcuticular space (Figure 6b). Then, the nectar was accumulated in the subcuticular space and flowed outwards through cuticular channels (Figures 6b and 6c).

Post-secretory stage - A disintegrative process was striking in the secretory cells; the cell walls presented conspicuous deposition of lignin and the degenerative cytoplasm was reduced to a thin parietal layer (Figure 6e). The cells accumulated phenols inside large vacuoles and some parenchymatic cells had electron-opaque plastids (Figure 6e).

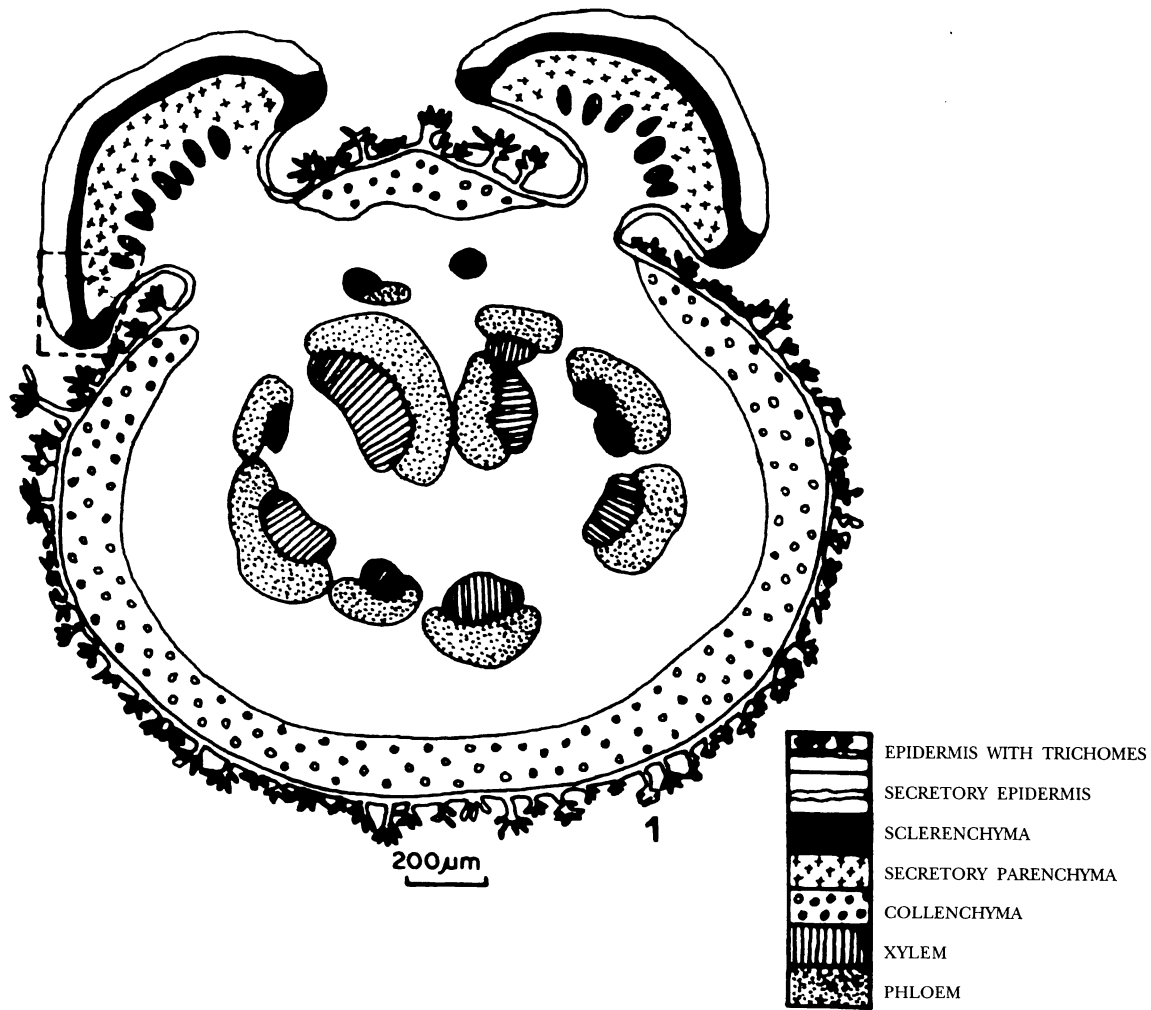


Figure 1. Diagram of the petiole with a pair of epidermic, patelliform and stalked EFNs of *Croton urucurana* (superior portion). Note many vascular bundles within the nectary.

Discussion

Croton urucurana EFNs are epidermal according to Wilkinson's (1979) classification. Other related species have EFNs with similar general morphology and position, such as *C. amabilis*, *C. aubrevillei*, *C. glandulosus* (Schnell *et al.* 1963) and *C. sarcopetalus* (Freitas 1997), as well as other Euphorbiaceae genus (e.g., *Hevea*, *Macaranga*, *Micrandra* and *Ricinus* - Metcalfe & Chalk 1950). The epidermal and patelliform nectary seems to be typical of Euphorbiaceae, as previously suggested by Schnell *et al.* (1963).

Distinctive structural traits of the *C. urucurana* EFNs are the sclerenchymatic cells, the oxalate calcium crystals in the parenchymatic cells and the phenols inside vacuoles, which probably promote protection of the secretory tissues against herbivores. These substances and structures for protection have been considered specialized features of the EFNs (Belin-Depoux 1989). Ultrastructural features of *C. urucurana* EFN, such as developed plasmodesmata, plastids, ER, mitochondria and numerous vesicles were reported for many nectaries (e.g., Durkee *et al.* 1981, Belmonte *et al.* 1994). In spite of the secreted material (e.g., nectar,

mucilages and gums) and the taxa singularities, the presence of several vesicles, mitochondria and plasmodesmata are general characteristics of the secretory tissues (see Fahn 1988).

The secretory cells showed numerous amyloplasts and chloroplasts with starch grains before the nectar secretion. The phloem sugar probably is the main source of this starch (in according to Durkee *et al.* 1981, Figueiredo & Pais 1992). The presence of both amyloplasts and chloroplasts with starch in the secretory cells seems to indicate that at least some nectar sugar would be first stored as starch grains. Then, during the secretory stage the starch hydrolysis to sugar would occur. A similar hypothesis has been suggested for floral nectaries of some unrelated species (Figueiredo & Pais 1992, Zer & Fahn 1992, Belmonte *et al.* 1994). In another *Croton* species (*C. sarcopetalus*), a similar feature was observed in both floral and extrafloral nectaries (Freitas 1997). The stored starch can allow a rapid allocation of sugar during the secretory stage.

In spite of the role of plastids, the ER seems to be the most important cell compartment involved in nectar accumulation and transport in *C. urucurana* EFNs, as reported for many nectaries (Fahn 1988, Kronstedt-Robards & Robards 1991). The abundance of rough ER

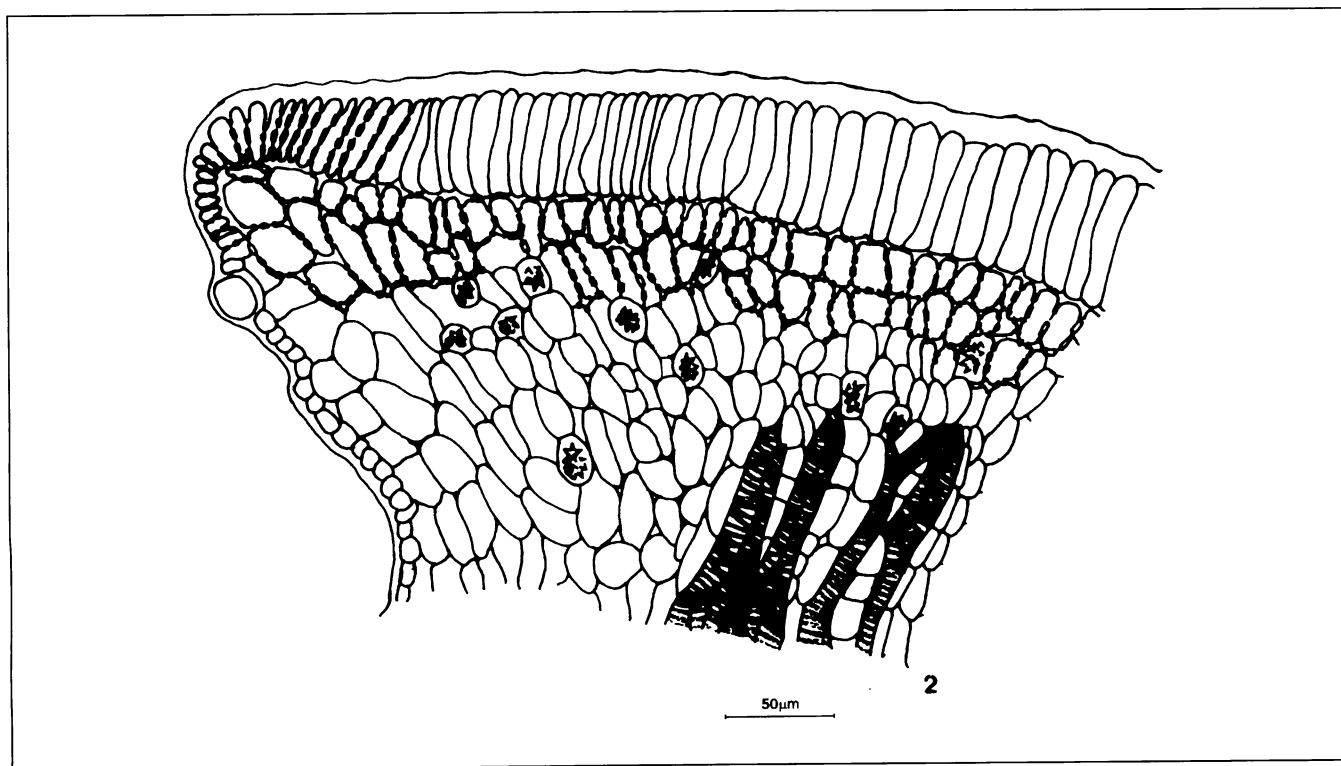


Figure 2. Detail of Figure 1 showing lateral portion of EFN with thick cuticle covering the epidermis, brachysclereids with simple pits and parenchyma. Note idioblasts with druses in the secretory parenchyma.

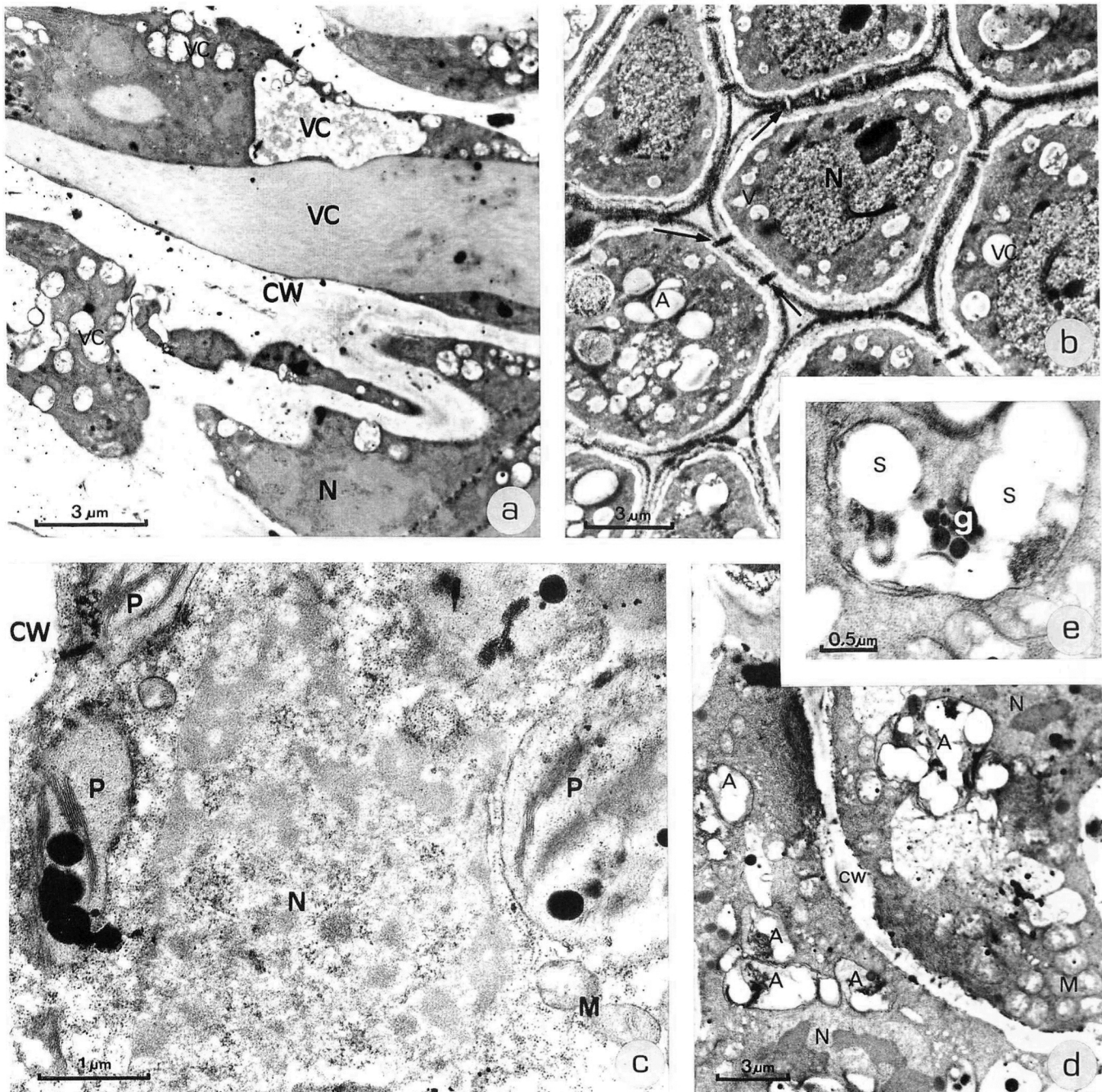


Figure 3. Electron-micrographies (EM) of the EFN secretory cells at the pre-secretory stage. 3a. Epidermic cells with a large central vacuole and numerous small peripheral vacuoles. Note distinct electron density among the vacuoles. 3b. Parenchymatic cells with a large central nucleus and several small vacuoles. Note plasmodesmata connecting the adjacent cells (arrows) and amyloplasts. 3c. Parenchymatic cell with a large nucleus, several chloroplasts and some mitochondria. Note plastoglobuli inside chloroplasts. 3d. Parenchymatic cells with several amyloplasts and mitochondria. Note prominent heterochromatin in the nucleus. 3e. Detail of an amyloplast with starch grains and plastoglobuli. (A - amyloplast; CW - cell wall; g - plastoglobuli; M - mitochondria; N - nucleus; P - chloroplast; S - starch grain; VC - vacuole)

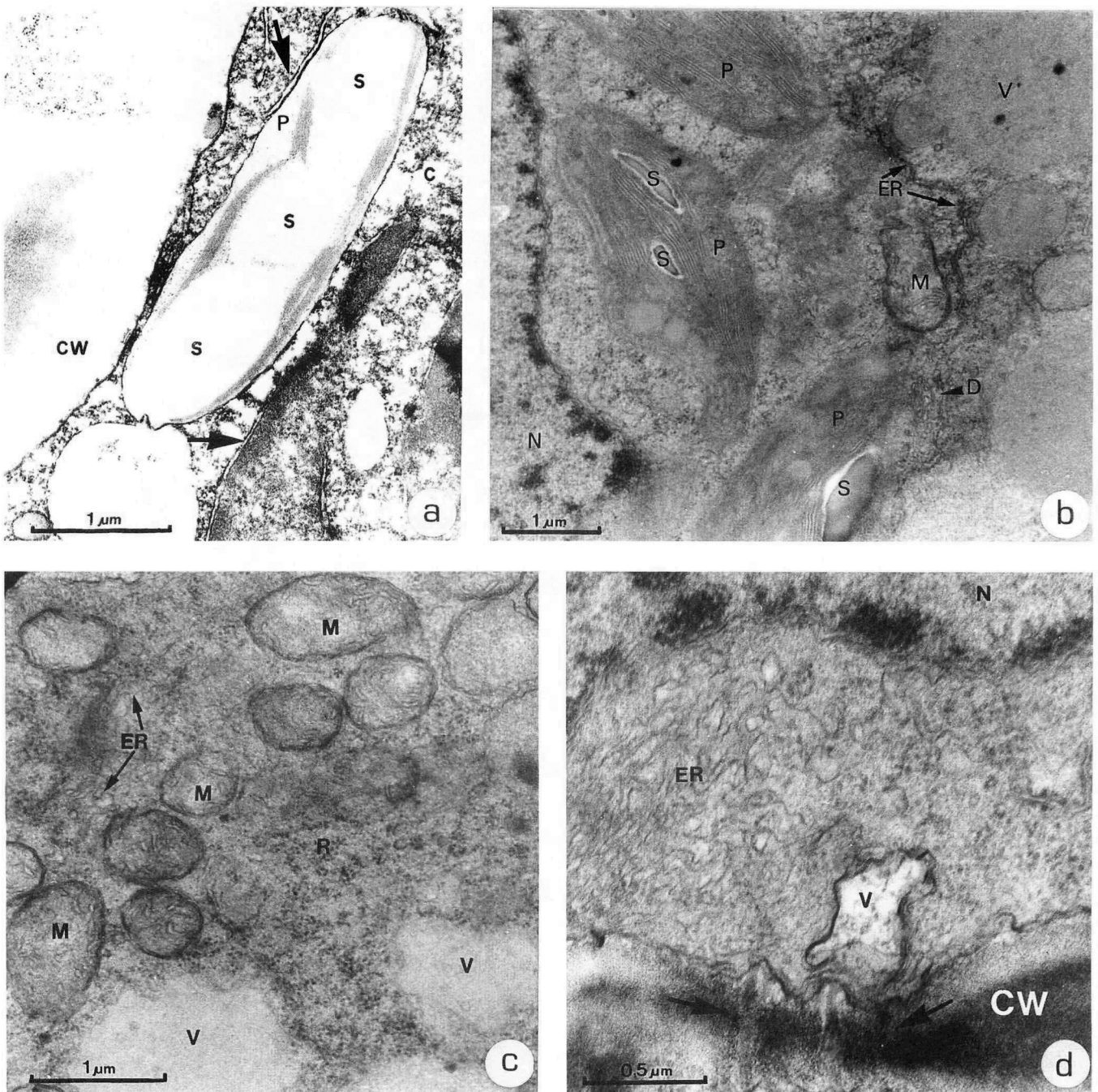


Figure 4. EM of the EFN secretory cells at the pre-secretory stage (4a) and at the secretory stage (4b to 4d). 4a. Parenchymatic cell with a chloroplast with starch grains. Note rough ER profiles adjacent to plastid (arrows). 4b. Parenchymatic cell with chloroplasts, mitochondria, ER profiles and dictyosome. Note the reduction of starch inside chloroplasts (see Figure 4a). 4c. Parenchymatic cell with several mitochondria and free ribosomes. 4d. Epidermic cell with numerous rough ER profiles located between the nucleus and the plasmalemma. Note a vesicle near the plasmodesmata region (arrows). (C - cytoplasm; CW - cell wall; D - dictyosome; ER - endoplasmatic reticulum; M - mitochondria; N - nucleus; P - chloroplast; R - free ribosomes; S - starch grain; V - vesicle)

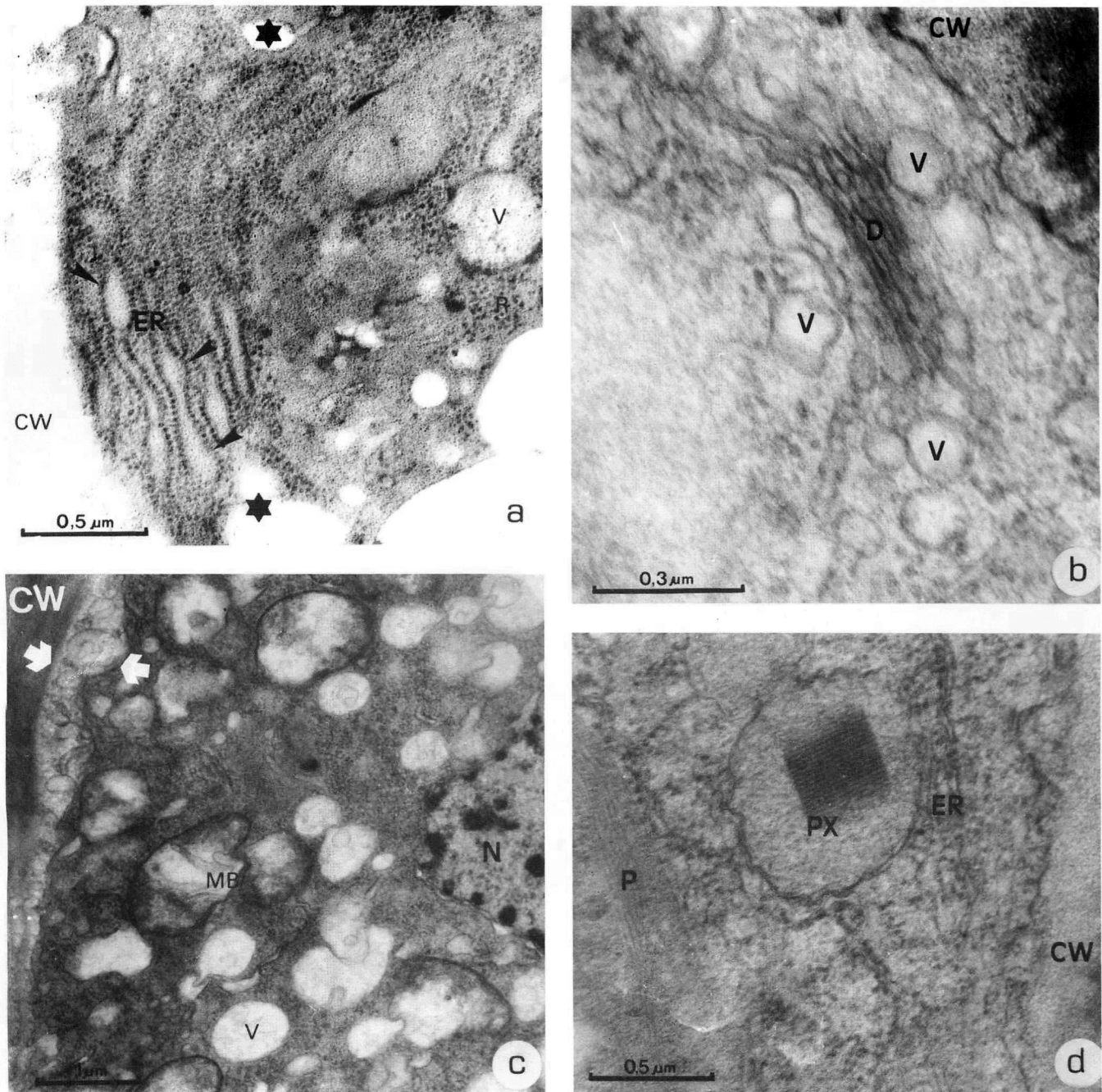


Figure 5. EM of the EFN secretory cells at the secretory stage. 5a. Epidermic cell showing rough ER like cisternae with dilations (arrowheads). Observe vesicles near the ER edges (stars). 5b. Epidermic cell with a dictyosome near the plasmalemma. Note vesicles in both trans- and cis-face of the dictyosome. 5c. Epidermic cell with many vesicles and multivesicular bodies (autophagic vacuoles). Note vesicles and secreted material in the extra-cytoplasmic space (arrows), located between the cell wall and the plasmalemma. 5d. Parenchymatic cell with a peroxysome adjacent to ER profiles and a chloroplast. (CW - cell wall; D - dictyosome; ER - endoplasmatic reticulum; MB - multivesicular body; N - nucleus; P - chloroplast; PX - peroxysome; R - free ribosomes; V - vesicle)

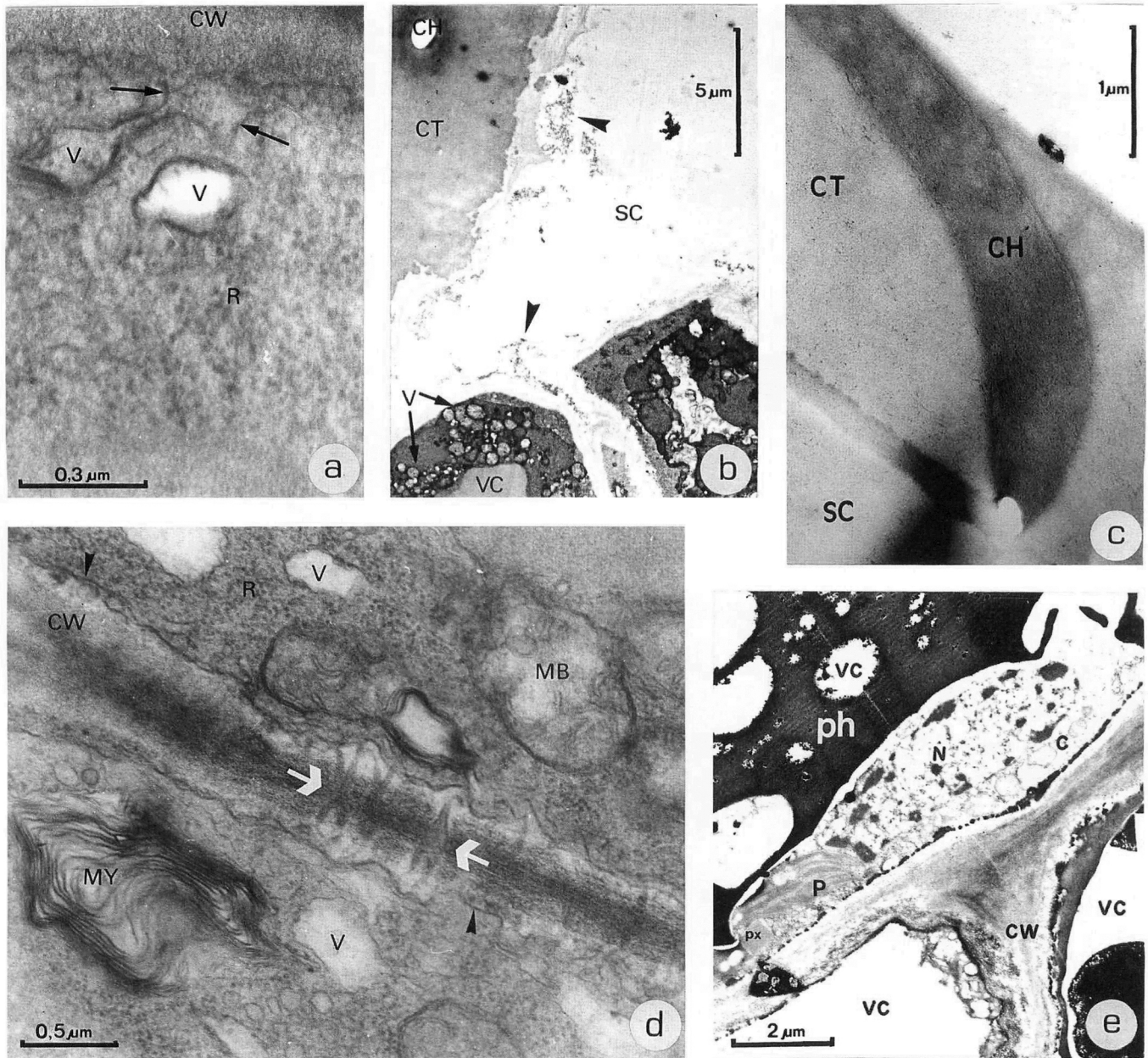


Figure 6. EM of the EFN secretory cells at the secretory stage (6a to 6d) and at the post-secretory stage (6e). 6a. Epidermic cell with vesicle, whose membrane are in fusion by the plasmalemma (arrows). Note flocculated material inside vesicle. 6b. Superior portion of the EFN showing thick cuticle with a channel transversally sectioned, subcuticular space with nectar accumulation and epidermic secretory cells with numerous vesicles near the plasmalemma (arrows). Note flocculated material in the subcuticular space (arrowheads). 6c. Detail of the cuticle with a channel in longitudinal view. 6d. Epidermic cells showing myelin figures, multivesicular body and vesicles near the plasmodesmata region (arrows). Note plasmalemma not attached to cell wall (arrowheads). 6e. Parenchymatic cells with large vacuoles accumulating phenols, cytoplasm limited to a thin parietal layer, electron-opaque chloroplast and peroxysome. (C - cytoplasm; CH - cuticular channel; CT - cuticle; CW - cell wall; MB - multivesicular body; MY - myelin figure; P - chloroplast; px - peroxysome; R - free ribosomes; SC - subcuticular space; V - vesicle; VC - vacuole)

profiles, which were frequently observed associated with plastids and plasmodesmata, as well as the ER-originated vesicles, reinforce this assumption to *C. urucurana*.

Some fluid transport could be carried out by multivesicular bodies, as suggested for the EFNs of *Ricinus communis* (Euphorbiaceae), by Kálmán and Gulyás (1974). These authors proposed that vesicles of multivesicular bodies contain the untransformed phloem fluid (saccharose rich pre-nectar), which is converted to a sugary fluid with a different composition (glucose and fructose rich nectar). These vesicles with pre-nectar would transverse the plasmodesmata to reach the adjacent secretory cells. In *C. urucurana* EFNs, the multivesicular bodies seem to be related with autophagic process and the vesicles located inside these bodies probably had an ER- or dictyosome-origin.

In summary, the presence of vesicles, autophagic vacuoles and ER profiles associated with plasmodesmata seem to indicate that the pre-nectar transport among secretory cells is mainly via the symplast in *C. urucurana*, as happens in most of the studied plants (Fahn 1988, Marginson *et al.* 1985, Zer & Fahn 1992, Belmonte *et al.* 1994). However, the apparent fusion between ER-origin vesicles and the plasmalemma, observed seldom in the parenchymatic cells during the secretory stage, could indicate that some part of the pre-nectar transport would be done by endocytosis and exocytosis, as previously reported in other nectaries (Findlay & Mercer 1971, Zer & Fahn 1992).

The nectar elimination from the secretory cells occurs by granulocrine secretion in many taxa (Eleftheriou & Hall 1983, Sawidis *et al.* 1989). Vesicles in fusion with the plasmalemma of epidermic cells indicate that nectar is probably eliminated in this way in *C. urucurana*. Then, the nectar is accumulated in the subcuticular space and exudated through cuticular structures like channels. Similar channels were observed only in other Euphorbiaceae taxa, *C. sarcopetalus* (Freitas 1997) and *Ricinus communis* (Kálmán & Gulyás 1974).

The degenerative aspect shown by the nectariferous cells has been observed in many nectaries. At the post-secretory stage the vacuole increases, reducing the cytoplasm to a thin parietal layer (Sawidis *et al.* 1989, Belmonte *et al.* 1994); the cells accumulate phenols (Dave & Patel 1975, Marginson *et al.* 1985) and peroxysomes and electron-opaque plastids are found (Kálmán & Gulyás 1974, Zer & Fahn 1992).

Cell compartments (*e.g.*, ER, multivesicular bodies and plastids) frequently observed in *C. urucurana*, and their probable involvement in nectar production, transport and secretion, have been reported in nectaries of many unrelated taxa. However, these processes are as yet incompletely understood. For example, there is a little knowledge about the possible relationship between the organelles that participate in the process of nectar

secretion and nectar features like chemical composition and sugar concentration.

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