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ULTRASTRUCTURE OF THE NECTARY SPUR OF *PLATANTHERA CHLORANTHA* (CUSTER) RCHB. (ORCHIDACEAE) DURING SUCCESSIVE STAGES OF NECTAR SECRETION

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In *Platanthera chlorantha*, nectar is secreted into the lumen of the nectary spur by numerous unicellular hairs. These hairs arise from epidermal cells lining the spur. The nectary was studied in the presecretory (closed bud, 4 days before anthesis), secretory (day 2 of anthesis) and resorption (days 14–16 of anthesis) stages to compare the ultrastructure of the cells during various stages of nectary activity. Plant tissue was fixed for conventional TEM or prepared by freeze-substitution. Secretory cell ultrastructure was observed to change significantly during the various stages of nectary activity. During the presecretory stage, the cells have a large nucleus, dense, granular cytoplasm with numerous mitochondria and ER, and several small vacuoles. Plastids within secretory epidermal cells or subepidermal parenchyma enclose starch, but starch is absent throughout nectar secretion and resorption. During the secretory stage, plastids with a dense stroma contain tubules enclosing osmiophilic material. Abundant dictyosomes and secretory vesicles occur in the cytoplasm, particularly near the plasmalemma, indicating that granulocrine secretion operates in this species. During the resorption stage, most cells still have dense cytoplasm with numerous mitochondria and ER, but dictyosomes are uncommon. Vesicles are present adjacent to the cell wall; they are similar in size and occur in similar numbers to those seen during the secretory stage. They are probably formed by endocytosis. The ultrastructure of the cells prepared by freeze-substitution compared favorably with that seen in cells subjected to conventional chemical fixation.

Key words: *Platanthera chlorantha*, Orchidaceae, nectary, nectar secretion, nectar resorption, ultrastructure, freeze-substitution.

INTRODUCTION

In *Platanthera chlorantha*, nectar is secreted into the lumen of the nectary spur, where it is presented to pollinators. Nectary spurs also occur in other orchid species such as *Limodorum abortivum* (L.) Sw (Figueiredo and Pais, 1992), *Platanthera bifolia* (L.) (Stpiczyńska, 1997) and *Gymnadenia conopsea* (L.) (Stpiczyńska and Matusiewicz, 2001). Nectar secretion begins in the bud stage (1–2 days before anthesis), and nectar can be found within the spur for more than two weeks thereafter. Nectar secretion in this species can be divided into four stages: (1) the beginning of anthesis, when secretion is slow and during which the volume of nectar within the spur gradually increases; (2) a stage of rapid secretion in older flowers, when the volume of nectar inside the spur suddenly increases; (3) cessation of secretory activity, when the volume of nectar within the spur has reached its maximum and remains constant; and (4) resorption, when the nectar accumulated within the spur is resorbed by the nectary tissue (Stpiczyńska, 2003a). As in *P. bifolia* (Stpiczyńska, 1997) and *G. conopsea* (Stpiczyńska and Matusiewicz, 2001), the epidermis lining the nectary spur of *P. chlorantha* develops numerous unicellular secretory hairs which are involved in nectar secretion. Autoradiography has shown that in *P. chlorantha* these cells are also involved in nectar resorption (Stpiczyńska, 2003b).

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Nectary cell ultrastructure during the consecutive stages of nectary activity has been studied by many researchers (Rachmilevitz and Fahn, 1973; Fahn and Benouaiche, 1979; Durkee et al., 1981; Durkee, 1982; Mauseth, 1982; Kronestedt et al., 1986; Sawidis et al., 1989; Zer and Fahn, 1992; Belmonte et al., 1994; Razem and Davis, 1999). However, few investigations have examined the structure of nectary cells during resorption (Nepi et al., 1996, 2001; Stpiczyńska, 2003b).

In the present study we examined nectary cells of *Platanthera chlorantha* during the presecretory stage and also during the secretory and resorption stages. Particular attention was paid to the secretory and resorption stages, to compare the structures involved in both processes. Freeze-substitution was used in order to reduce the presence of artefacts associated with conventional chemical fixation (Robards, 1991; Bourett et al., 1999; Ross et al., 2000). Despite the practical difficulties, freeze-substitution was chosen as the most advantageous method for preserving cell ultrastructure for examination of the dynamic process of nectar secretion and resorption.

MATERIALS AND METHODS

Nectary spurs of *Platanthera chlorantha* (Custer) Rchb. were investigated in the following stages of nectary activity: the presecretory stage (closed bud, about 4 days before anthesis), the secretory stage (day 2 of anthesis), and the resorption stage (days 14–16 of anthesis) when about half of the nectar is resorbed.

CHEMICAL FIXATION

Small pieces of the nectary spur (~1 mm thick) were rinsed in cacodylate buffer (0.066 M, pH 7.2) and fixed in 3% buffered glutaraldehyde at room temperature for 1 h. After 1 h of post-fixation in 1% OsO_4, the material was rinsed in buffer and then water, dehydrated in an ethanol series, and embedded in Spurr epoxy resin.

FREEZE-SUBSTITUTION

Material was prepared for TEM according to the method of Lancelle et al. (1986, 1987). Fresh nectary spurs were cut with a razor blade into small pieces ~5 mm thick, then collected on formvar-coated platinum loops (3 mm diam) in Brewbaker and Kwack medium (Brewbaker and Kwack, 1963) and rapidly quenched in liquid propane at -180˚C. Substitution was performed in 2 ml vials containing acetone at -80˚C over a period of 3 days. The temperature of the material was then increased 2˚C per h to 4˚C in a temperature-controlled substitution chamber (Heto CB 10 90). Conventional infiltration and embedding in Spurr epoxy resin was performed at room temperature.

Ultrathin sections were cut at 70 nm with a Reichert Ultracut S ultramicrotome, using a diamond or glass knife. The sections were stained with uranyl acetate for 20 min and lead citrate for 10 min, and viewed with a Philips Morgagni 268 D or TESLA BS 500 TEM. Micrometry of the nectaries was done using a Nikon Eclipse 600 microscope with Screen Measurement ver. 4.21 software.

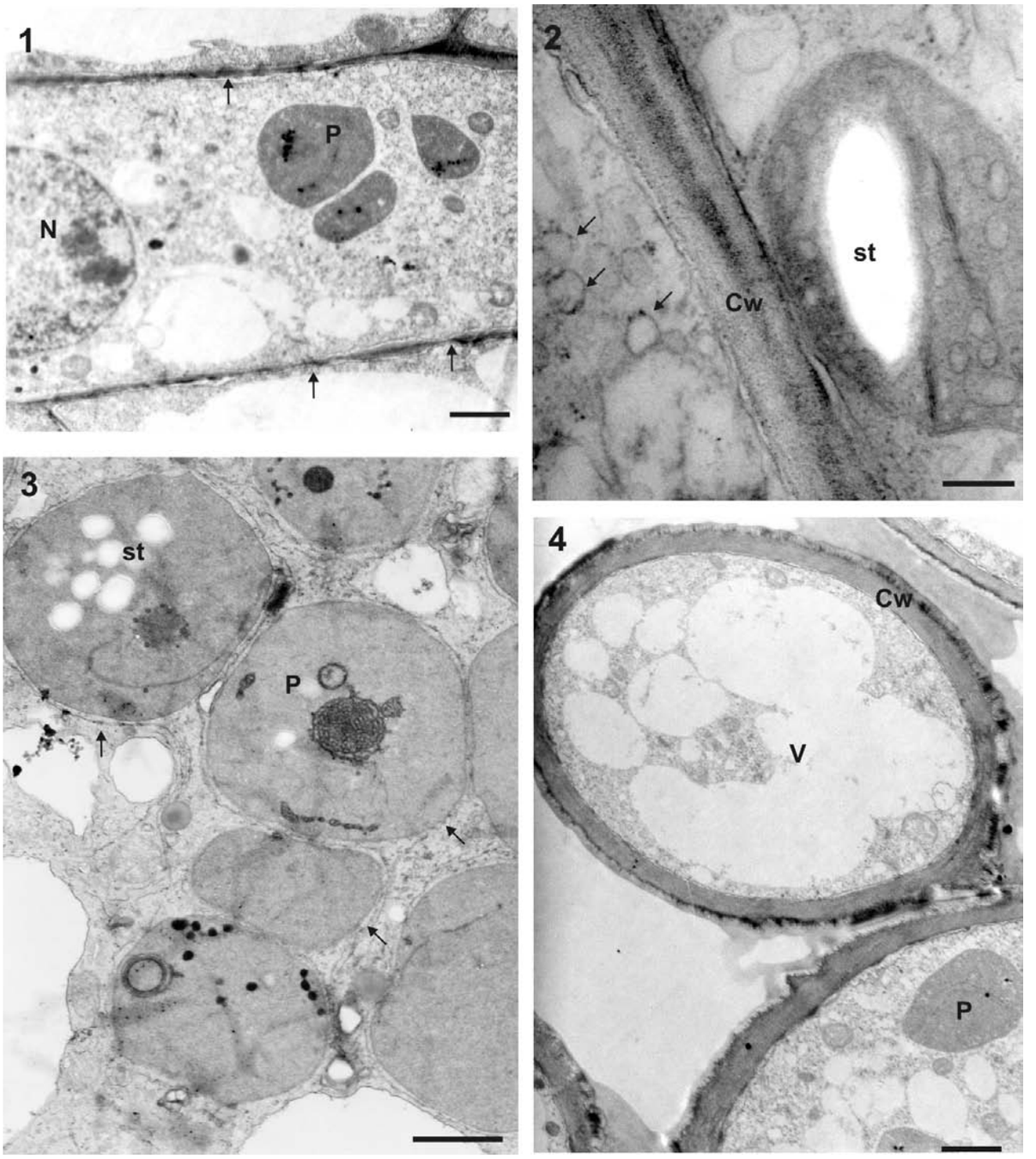
RESULTS

CHEMICAL FIXATION

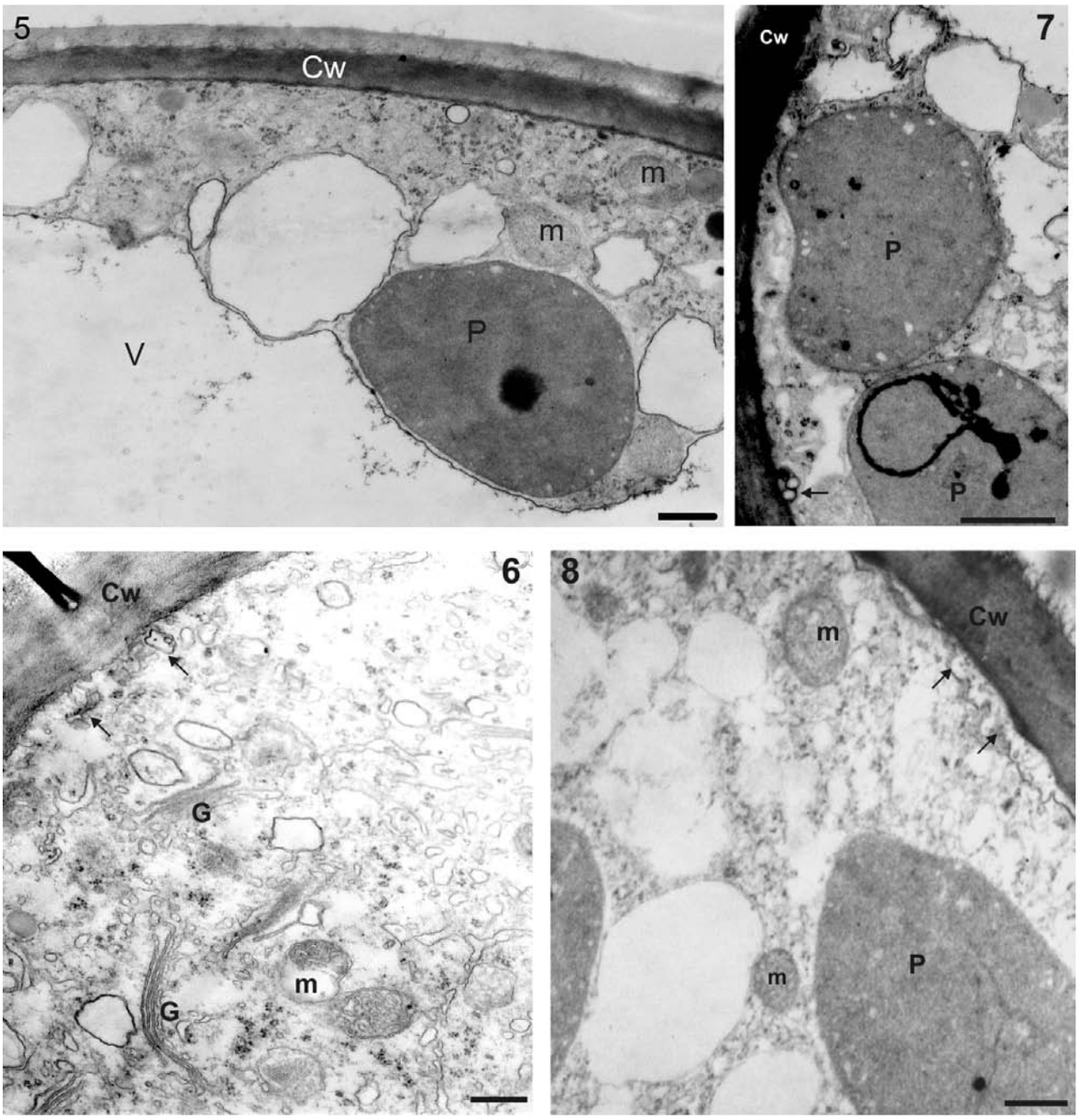
The ultrastructure of the secretory cells changes significantly during the sequential stages of nectary activity. In the presecretory stage, the cells contain a relatively large, centrally positioned nucleus and dense, granular cytoplasm with numerous mitochondria, ER and several small vacuoles (Fig. 1). Secretory vesicles occasionally occur close to the cell wall. The plastids of the secretory epidermis and subepidermal parenchyma cells contain starch grains (Figs. 2, 3). These plastids are spherical and surrounded by extensive profiles of predominantly smooth ER (Fig. 3). They enclose a network of tubular internal membranes resembling prolamellar bodies or ring-like profiles, and contain small plastoglobuli. Growing secretory hairs have several small vacuoles or a large central vacuole (Fig. 4).

The outer cell walls of the secretory epidermal cells and secretory hairs are on average 0.8 µm thick and are covered with thick (0.33 µm) cuticle with numerous microcanals. The anticlinal walls of the secretory epidermis and underlying parenchyma cells have a mean thickness of 0.3 µm and do not develop ingrowths. They are traversed by numerous plasmodesmata (Fig. 1). The structure of the cell wall remains constant throughout the subsequent stages of nectary activity.

During the secretory stage, the epidermal cells and subepidermal parenchyma are larger and show varying degrees of vacuolation (Fig. 5). In the cytoplasm of the epidermal cells and secretory hairs, dictyosomes and secretory vesicles are plentiful, and the vesicles measure 110 nm on average. The vesicles are visible close to the cell wall (Figs. 6, 7). At this stage, the plasmalemma has an irregular profile with numerous invaginations (Figs. 6, 8). Mitochondria are abundant and associated with plastids and the outer cell wall (Figs. 5, 6, 8). A significant difference between this stage and the presecretory stage is the absence of starch from the plastids of the secretory epidermal cells. Some starch remnants were noted only in the subepidermal parenchyma. The plastids contain a dense, homogeneous stroma with prominent tubular peripheral membranes. Intraplastidal tubules frequently contained dark, osmiophilic material (Figs. 5, 7).

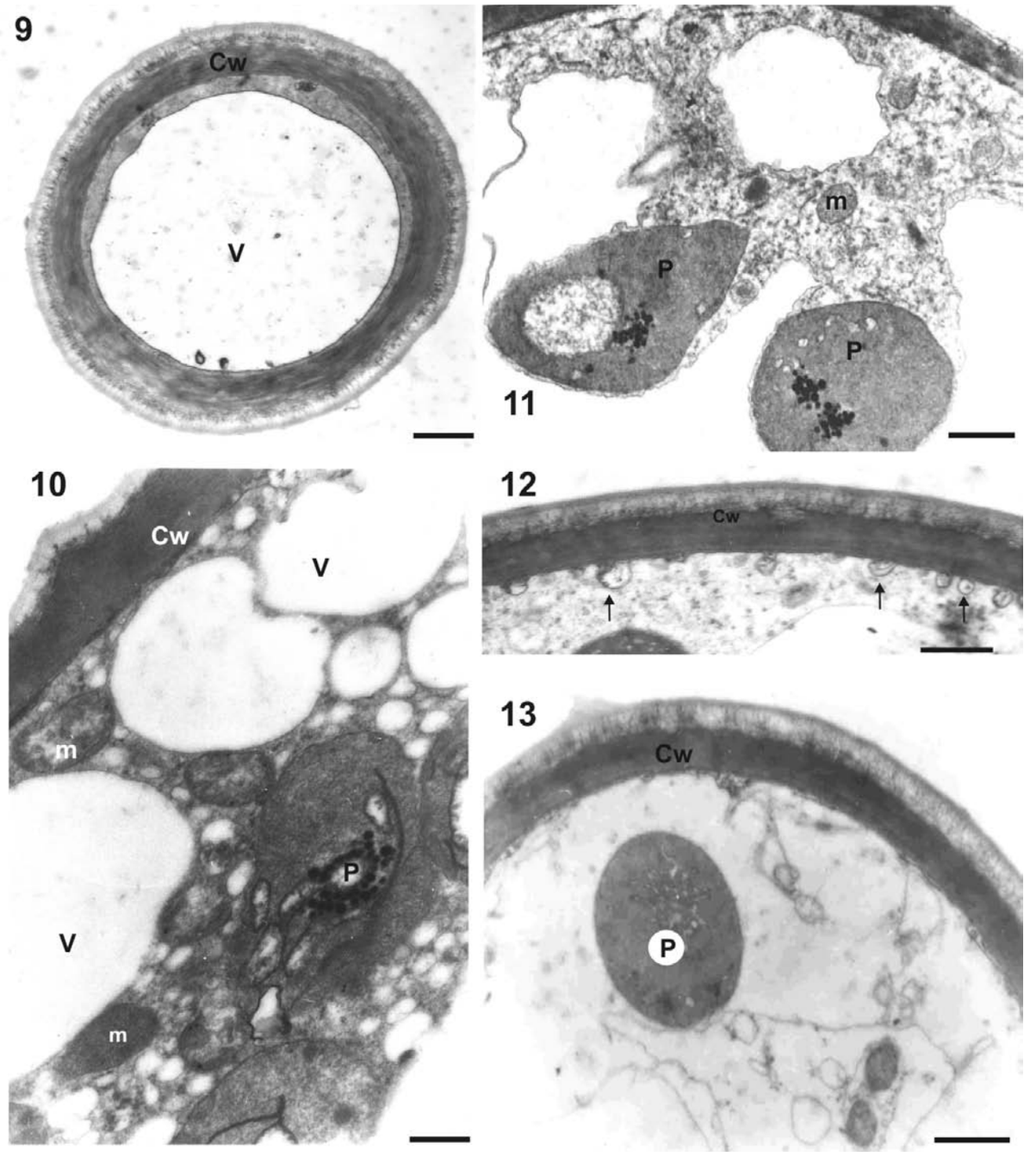


Figs. 1–13. Nectary cells prepared by chemical fixation. **Figs. 1–4.** Nectary cells in presecretory stage. **Fig. 1.** Nectary epidermis cell with large, centrally positioned nucleus and dense cytoplasm. Plasmodesmata (arrows) occur in cell wall. Bar = 15 µm. **Fig. 2.** Amyloplast close to cell wall. Secretory vesicles indicated by arrows. Bar = 0.3 µm. **Fig. 3.** Plastids with starch grains and sparse tubular network. Plastids encircled by numerous profiles of ER (arrows). Bar = 2 µm. **Fig. 4.** Transverse section of secretory hair with dense cytoplasm and several small vacuoles. Numerous microcanals occur in cuticle. Bar = 15 µm. Cw – cell wall; P – plastid; st – starch; N – nucleus; V – vacuole.



Figs. 5–8. Nectary cells in secretory stage. **Fig. 5.** Secretory epidermis cell with starchless plastid and numerous mitochondria close to outer cell wall. Bar = 1 μm. **Fig. 6.** Parietal cytoplasm of secretory hair with numerous dictyosomes and secretory vesicles (arrows). Bar = 0.3 μm. **Fig. 7.** Secretory epidermal cell. Intraplastidal tubules contain dark osmiophilic substance. Secretory vesicles occur close to anticlinal cell wall (arrow). Bar = 2 mm. Fig. 8. Starchless plastid, mitochondria and secretory vesicles near cell wall of secretory epidermal cell. Some vesicles visible between plasmalemma and cell wall (arrows). Bar = 0.5 mm. Cw – cell wall; P – plastid; m – mitochondrion; V – vacuole; G – Golgi apparatus.

During resorption, the vacuoles within the epidermal cells and secretory hairs are still enlarged, and the cytoplasm generally has a parietal distribution (Fig. 9). Many small new vacuoles appear in the cytoplasm. In the majority of cells, the cytoplasm remains dense and contains numerous mitochondria and ER (Figs. 10, 11). Dictyosomes were observed infrequently, unlike in previous stages of nectary activity. The plastids are similar to those seen during the secretory stage, but some of them have an irregular profile (Fig. 11). Vesicles



Figs. 9–13. Nectary cells in resorption stage. **Fig. 9.** Transverse section of secretory hair with large central vacuole and parietal cytoplasm. Bar = 2 µm. **Fig. 10.** Dense cytoplasm of secretory hair with numerous mitochondria and small vacuoles formed near cell wall. Bar = 0.5 µm. **Fig. 11.** Parietal cytoplasm of secretory epidermis with mitochondria and plastids (one has an irregular profile). Bar = 10 µm. **Fig. 12.** Invaginations of plasma membrane of secretory hair (arrows). Bar = 1.5 µm. **Fig. 13.** Secretory hair with sparse cytoplasm undergoing degradation. Bar = 1.5 µm. Cw – cell wall; P – plastid; m – mitochondrion; V – vacuole; G – Golgi apparatus.

similar in size and frequency to those present in the secretory stage were observed in close proximity to the cell wall. Deep invaginations of the plasmalemma were also observed (Fig. 12). However, it is obvious that during this stage the protoplasts of some cells undergo autolysis and that the cytoplasm becomes less dense (Fig. 13).

FREEZE-SUBSTITUTION

Although the protoplasts of some cells were destroyed by ice crystals, others were well preserved. Generally, the ultrastructure of cells prepared by freeze-substitution closely resembled that of cells that had been chemically fixed, in that the cytoplasm retained its dense, granular appearance (Figs. 14–16). During the presecretory stage the plastids have well-preserved internal tubules and starch grains (Fig. 14), whereas during the secretory or post-secretory stages they have dark, osmiophilic contents (Fig. 15). During both the secretory and resorption stages, numerous vesicles occur in the parietal cytoplasm (Figs. 16, 17). The plasmalemma remains intact, and it too is closely associated with the cell wall (Figs. 14, 17). The cuticle covering the outer cell wall of the secretory hair has numerous microcanals and does not show any cracks (Fig. 17).

DISCUSSION

In the spur of *Platanthera chlorantha*, ultrastructural changes occur throughout the various stages of nectary activity. These include changes in plastid structure, the presence of starch, the degree of vacuolation and the presence of dictyosomes. Similar changes have been observed in nectary cells of other species (Sawidis et al., 1989; Razem and Davis, 1999). The presence of starch in the presecretory stage and its absence during the secretory and post-secretory period indicates that this carbohydrate is utilized as a source of nectar sugars and/or is involved in providing energy for the secretory process. Degradation of starch in active nectaries has been observed in numerous plant species (Rachmilevitz and Fahn, 1973; Durkee et al., 1981; Zer and Fahn, 1992; Pais and Figueiredo, 1994; Galetto et al., 1997; Razem and Davis, 1999; Fahn and Shimony, 2001; Radice and Galati, 2003; Pacini et al., 2003). In contrast to *Platanthera*, however, starch reappears in the nectaries of *Cucurbita pepo* during the resorption stage (Nepi et al., 1996). Sucrose derived from nectar resorbed by *Platanthera* nectaries is transported to developing fruits and incorporated into starch (Stpiczyńska, 2003c).

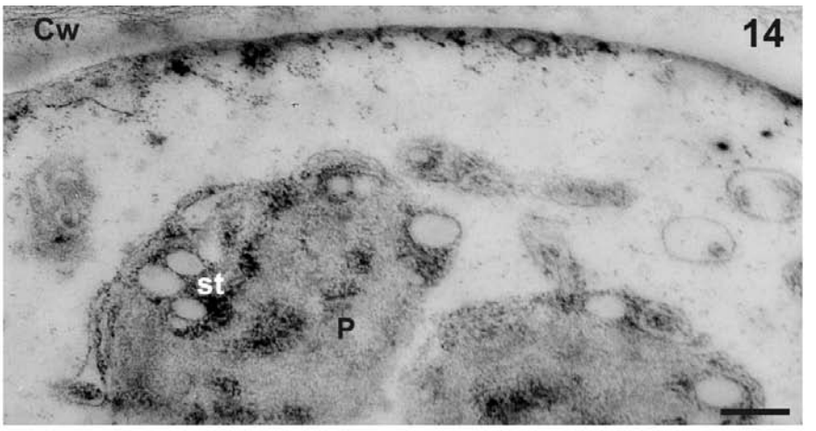
The plastid lamellae of *P. chlorantha* contain dense osmiophilic material. A similar substance has been reported from plastids occurring in the nectaries of other orchid species (Pais and Figueiredo, 1994; Stpiczyńska and Matusiewicz, 2001; Stpiczyńska et al., 2004) and from glandular trichomes involved in terpenoid synthesis (Cheniclet and Carde, 1985; Turner et al., 2000), where plastids are also in close association with ER. However, in *P. chlorantha* the presence of plastoglobuli and osmiophilic substance during the secretory and resorption stages is probably related to plastid development, since these appear in mature cells following depletion of starch. In *Platanthera*, unlike in *Mentha* (Turner et al., 2000), osmiophilic material was visible in both chemically fixed and freeze-substituted material, indicating that in these species it may have a different chemical composition.

Numerous microcanals occur in the cuticle covering the secretory cells of *P. chlorantha*. Almost certainly they facilitate the release and resorption of nectar, all the more so as cracks are absent in the cuticle. Similar cuticular layer structure has been reported from nectary cells of *Abutilon* (Kronestedt et al., 1986), *Leonotis* (Ascensão and Pais, 1998) and *Mentha* (Turner et al., 2000). Some authors interpret the microcanals in the cuticle as a cellulosic wall material having continuity with the inner layer of the wall. Such continuity was observed in *P. chlorantha*, particularly in freeze-substituted cells.

During the secretory stage, numerous vesicles gather near the walls of the nectary epidermal cells of *P. chlorantha*. These vesicles are presumably derived from dictyosomes, abundant in the secretory stage. They are present both in chemically fixed and in freeze-substituted samples. This indicates that granulocrine nectar secretion operates in *Platanthera*. Similarly, secretory vesicles derived from dictyosomes occur in the nectaries of *Diplotaxis* and *Helleborus* (Eymé, 1966), *Vicia* (Figier, 1971), *Cynoglossum* (Tacina, 1973) and some species of Bromeliaceae (Benner and Schnepf, 1975). However, most studies report that ER (or ER together with Golgi apparatus) is the most common organelle involved in the production of secretory vesicles in nectary cells (Kronestedt-Robards and Robards, 1991; Fahn, 2000).

In *Platanthera chlorantha*, following the process of nectar secretion, the nectary cells prolong metabolic

Figs 14–17. Nectary cells prepared by freeze-substitution. **Fig. 14.** Vesicles aggregating near cell wall of secretory hair during presecretory stage. Plastids contain small starch grains. Bar = 0.3 µm. **Fig. 15.** Dense granular cytoplasm of secretory epidermal cell during secretory stage. Intraplastidal tubules contain osmiophilic material. Bar = 2 µm. **Fig. 16.** Secretory vesicles in parietal cytoplasm in secretory stage. Bar = 0.25 µm. **Fig. 17.** Transverse section of secretory hair at resorption stage, with vesicles close to cell wall. Cuticle with evident reticular layer. Bar = 0.3 µm. C – cuticle; Cw – cell wall; P – plastid; st – starch; m – mitochondrion; G – Golgi apparatus.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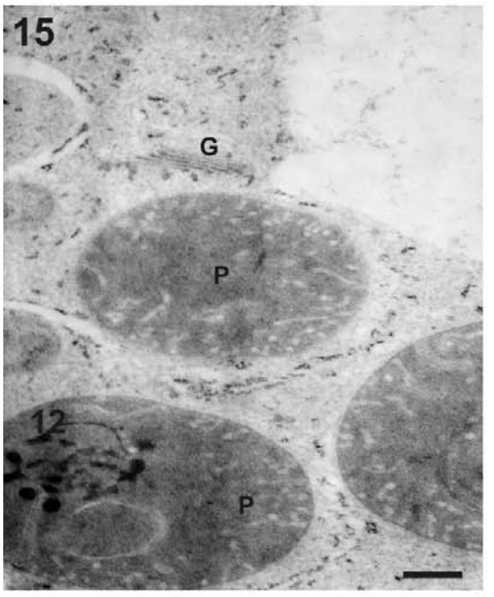
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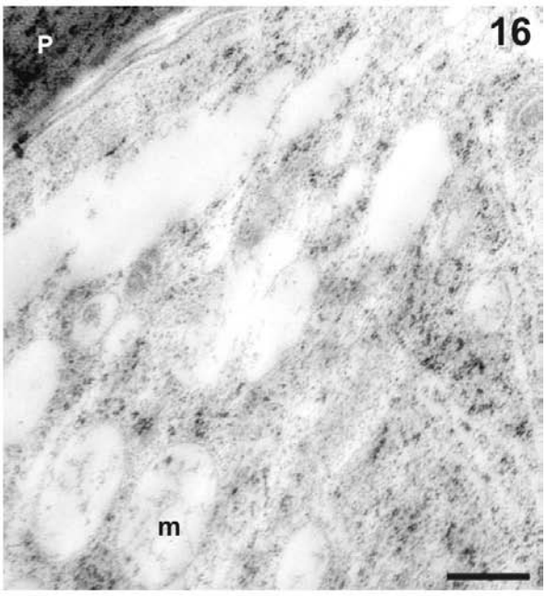
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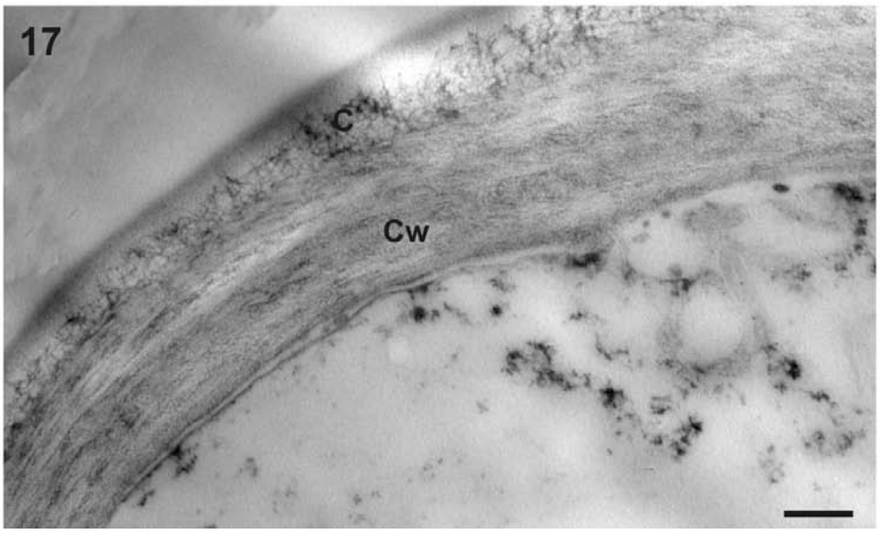
17
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activity and thereby resorb the remaining nectar. Numerous vesicles become associated with the plasmalemma during the resorption stage. In view of the clear decline in the frequency of dictyosomes during this stage, it is likely that the vesicles are formed by endocytosis. Since vesicles are abundant during nectar resorption, it is probable that they participate in this process. In plant cells, turgor pressure can significantly prevent endocytosis, but evidence derived from studies on the uptake of membrane-impermeable molecules such as lipophilic styryl (FM) support the operation of endocytosis in plants (Carroll et al., 1998; Kubitscheck et al., 2000) and thus argue for the hypothesis that vesicles are produced by this process here. In *P. chlorantha* there is no difference in mitochondrial frequency between the secretory and resorption stages, and cell wall ingrowths characteristic of transfer cells were not observed. The large numbers of mitochondria observed during the resorption stage can be explained in terms of the energy requirement of cells during the formation of endocytotic vesicles, since metabolic energy is essential for maintenance of endocytosis in turgid cells (Battey et al., 1999; Fricke et al., 2000; Marcote et al., 2000).

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