

STRUCTURE AND CYTOCHEMICAL FEATURES OF STIGMA AND STYLE OF *ORNITHOGALUM SIGMOIDEUM* FREYN&SINT, UNPOLLINATED AND POLLINATED PISTIL

ISIL İŞMAILOĞLU* AND MERAL ÜNAL

Department of Biology, Marmara University,
Goztepe 34722 Istanbul-Turkey

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The stigma of *Ornithogalum sigmoideum* is of dry and papillate type. The papillae are covered by a cuticle-pellicle layer, as revealed by staining. The activity of nonspecific esterase, acid phosphatase and peroxidase increases in the pellicle during the receptivity period. The style of *O. sigmoideum* is of the hollow type. Ultrastructural study of the cells lining the canal indicated that they are secretory cells and contain abundant endoplasmic reticulum, dictyosomes, mitochondria, plastids and ribosomes. After anthesis these organelles show degeneration at the end of the secretory phase. In canal cells, cytochemical tests showed the presence of acidic polyanions, insoluble and acidic polysaccharides, proteins and lipids. Before anthesis the canal cells are rich in polysaccharides, proteins and lipids. At maturity the cuticle is ruptured and secretory materials from the canal cells are released into the canal. In the unpollinated style of *O. sigmoideum* the exudates accumulated in the center of the canal; in pollinated pistils the same secretion materials were dispersed through the canal, which became wider.

Key words: *Ornithogalum sigmoideum*, stigma, style, unpollinated style, pollinated style, cytochemistry.

INTRODUCTION

Ornithogalum belongs to the group of species with a dry papillate stigma (Heslop-Harrison & Shivanna, 1977). Dry stigmas lacking copious surface secretions are covered by a continuous cuticle which is coated with a thin proteinaceous surface layer, and a pellicle is also detected indirectly by its strong nonspecific esterase and acid phosphatase activity (Hiscock et al., 2002).

The presence of esterase enzyme has been shown to demarcate the receptive side of stigmas in many plants (Mattsson et al., 1974) and this activity indicates the stigma's readiness to receive pollen grains (Kenrick and Knox, 1981; Kulloni et al., 2010). Acid phosphatase activity is intense in the subepidermal cell layers, reflecting their secretory function (Vithanage, 1984). Peroxidase activity is very high during maturity and pollen-receptive stages of angiosperm stigmas. Peroxidase is found in the composition of the pellicle, secreted by secretory papilla cells of the stigma epidermis (McInnis et al., 2006). Analysis of peroxidase activity is a useful method for determining phenomena such as pollen

receptivity/vitality in mature stigmas. Although there are various peroxidase tests, the exact biological function of stigmatic peroxidase is uncertain (Dafni and Motte Maues, 1998).

The style of the genus *Ornithogalum* is of hollow type. At the young stage the canal cells are lined with a layer of cuticle; then the cuticle is ruptured as development proceeds and secretory products formed in the canal cells pass to the canal (Shivanna and Johri, 1985). The secretory material in the stylar canal is rich in polysaccharides, glycolipids, glycoproteins and lipids (Kandasamy and Kristen, 1990; Jauh and Lord, 1996; Cheung 1996; Jauh et al., 1997; Tandon et al., 2001).

Canal cells contain abundant amounts of smooth and rough endoplasmic reticulum, plastids, mitochondria, active dictyosomes and ribosomes which are metabolically active (Raghavan, 1997).

Tilton & Horner (1980) made a detailed study of the structure of the dry stigma and hollow style of *O. caudatum* and divided the secretory phase in the style into periods. Hollow styles are found in only a few species, however, and not enough information is available about the secretory activities of the canal

*e-mail: isilismailoglu@gmail.com

TABLE 1. Nonspecific esterase, acid phosphatase and peroxidase activity in unpollinated and pollinated stigma papillae of *Ornithogalum sigmoideum*. +++ – intense reaction; ++ – positive reaction; + – weak reaction

Enzyme	Stage	Pellicle	Cytoplasm of the papillae	Bases of the papillae	Stigma tissue
Non-specific esterase	Unpollinated	+	+	+	++
	Pollinated	++	+++	++	++
Acid phosphatase	Unpollinated	+	+	+	+
	Pollinated	+	++ (Randomly distributed)	++	+++
Peroxidase	Unpollinated	+	+	+	+
	Pollinated	++	+++	++	++

cells. Here we investigated the structure of the stigma and style in pre- and post-anthesis stages and examined changes in cytochemistry.

MATERIALS AND METHODS

Whole pistils of *Ornithogalum sigmoideum* Freyn&Sint were removed from flowers 4 days before anthesis and 4 days after anthesis, fixed in 2% glutaraldehyde in phosphate buffer (pH 8.0) for 1 h at room temperature and then placed in fresh 3% glutaraldehyde (same buffer) at 4°C overnight. Following dehydration in an ethanol series the material was embedded in Epon. Semi-thin sections (1µm) were stained with 0.01% Auramine O (Heslop-Harrison and Shivanna, 1977) for cuticle, periodic acid-Schiff (PAS) reagent (Feder and O'Brien, 1968) for total insoluble polysaccharides, 1% Alcian blue in 3% acetic acid (Heslop-Harrison, 1979) for pectinaeous material and acidic polysaccharides, Coomassie brilliant blue in a mixture of water, methanol and acetic acid (v:v:v, 87:10:3) (Heslop-Harrison, et al., 1973) for proteins, and Sudan black B in 70% ethanol (Pearse, 1961) for lipoidal material. Transverse sections were cut from the top and bottom of the style.

Fresh material was used to determine enzyme activity in the stigma papillae. The stigmas were collected at pre- and post-anthesis and the methods for determining nonspecific esterase activity (Gomori, 1950), acid phosphatase activity (Gomori, 1941) and peroxidase activity (Birecka et al., 1973) were applied. Squash preparations were made from the stigmas.

For light microscopy the material was photographed through an Olympus BH-2 microscope with an Evolution LC color camera and the images were analyzed with Image-Pro Express ver. 6.0.

For transmission electron microscopy (TEM), samples were fixed in 2% glutaraldehyde for 1 h and then in 3% glutaraldehyde overnight and in

1% OsO₄ for 4 h, after which they were blocked in Epon. Cross sections were contrasted with uranyl acetate-lead citrate and examined with a Jeol Jem 1011 electron microscope. Then they were transferred to the imaging system and photographed.

For scanning electron microscopy (SEM), both fresh and fixed stigmas and styles were mounted directly on stubs, coated with gold particles and studied with a Jeol 5910 L.V. SEM.

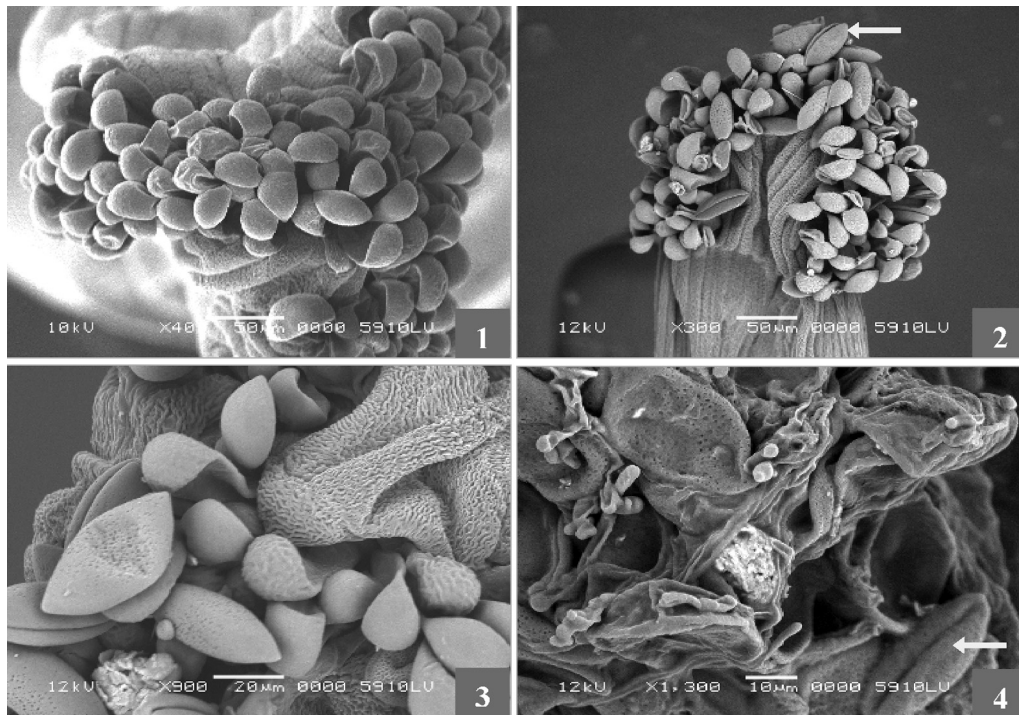
RESULTS

STIGMA

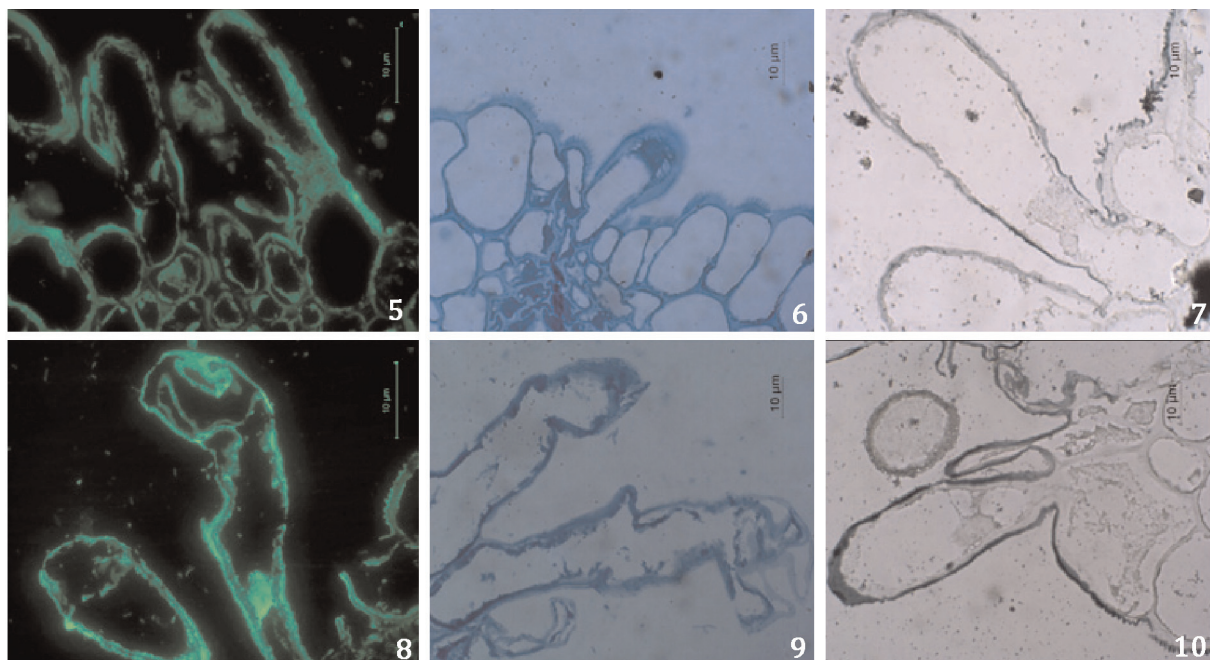
The three lobes of the stigma are of dry and papillate type (Figs. 1–4). The densely arranged papillae are unicellular, uniseriate and club-shaped. Elongated unicellular papillae are $289.39 \pm 2 \mu\text{m}$ in length.

In unpollinated pistils (pre-anthesis stage) the papillae have centrally located nuclei and dense cytoplasm. They are covered by a thin cuticle-pellicle layer, as revealed by staining with Auramine O, Sudan black B and Coomassie brilliant blue (Figs. 5–7). In pollinated pistils (post-anthesis stage) the nuclei become basally situated and the cells contain large vacuoles. The walls of the papillae increase in thickness and show strong fluorescence with Auramine O. At the receptive stage the cuticle becomes disrupted in places (Figs. 8–10), particularly at the base of papillae, through which the exudate is released. As papillae mature their cytoplasm becomes denser.

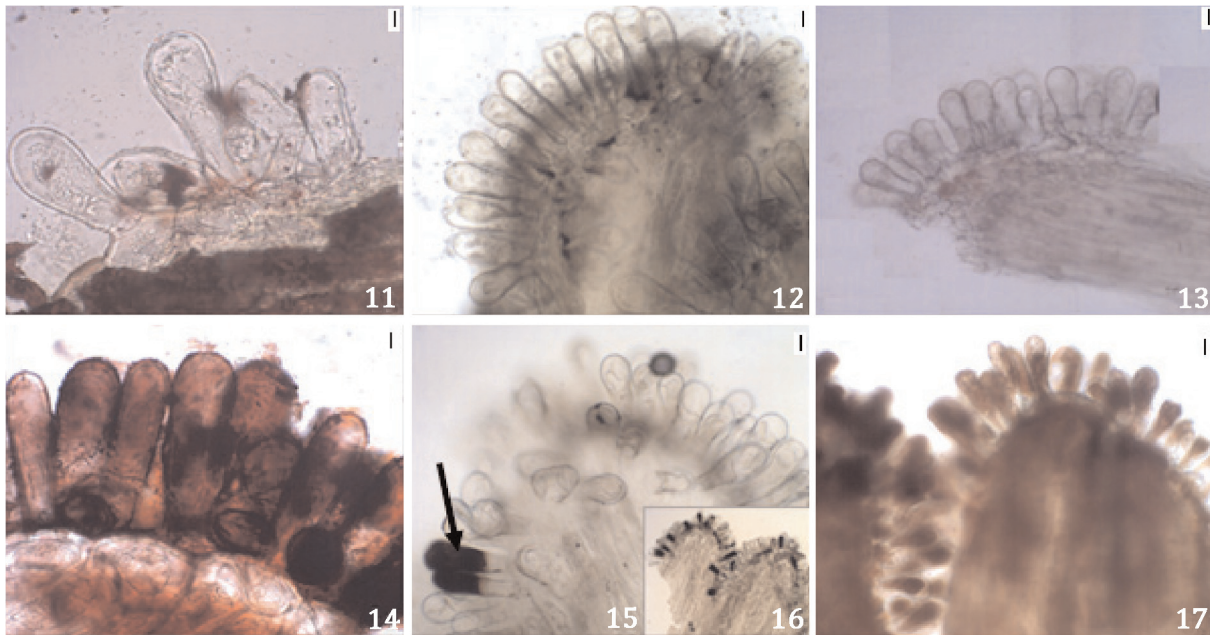
In the pre-anthesis stage (unpollinated stigma), nonspecific esterase, acid phosphatase and peroxidase activity were detected on the cuticle, which was a continuous layer (Figs. 11–13). There was weak staining in the cytoplasm and base of the papillae (Tab. 1). Staining was denser on the cuticle, cytoplasm and base of the papillae in the post-anthesis stage (pollinated stigma) (Figs. 14–17). Acid phosphatase activity was much more intense



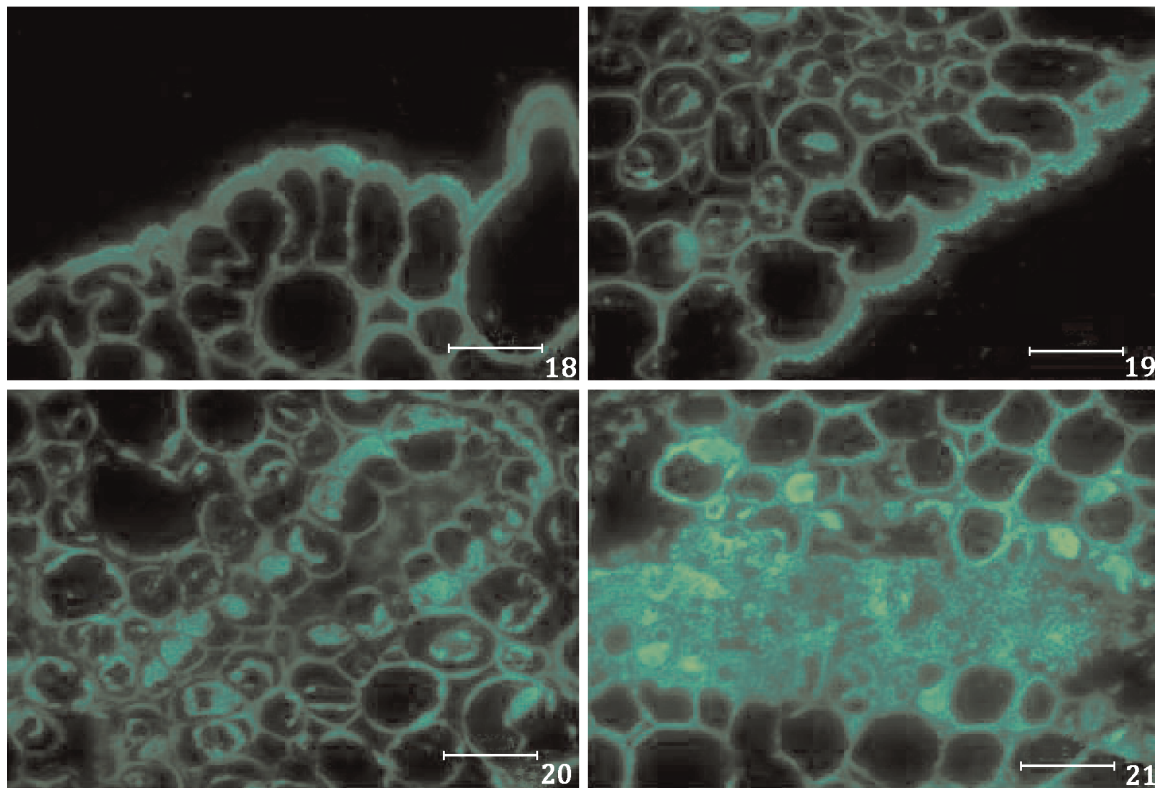
Figs. 1–4. SEM of the stigma in *Ornithogalum sigmoideum*. **Fig. 1.** Unpollinated papillate, dry stigma. **Fig. 2.** Pollinated, mature stigma. Pollen grains (arrow) stick to the tip regions of the papillate stigma cells. **Fig. 3.** Higher magnification of pollinated stigma surface. **Fig. 4.** Oldest stigma surface, with adhering pollen grains (arrow). Bars in Figs. 1,2 = 50 μ m, Fig. 3 = 20 μ m, Fig. 4 = 10 μ m.



Figs. 5–10. Stigmatic papillae of *Ornithogalum sigmoideum*. **Figs. 5–7.** Papillae in unpollinated pistil. **Figs. 8–10.** Papillae in pollinated pistil. **Figs. 5,8.** Fluorescence micrographs of papillae stained with Auramine O to show cuticle. **Figs. 6,9.** Stained with Coomassie brilliant blue to show pellicle. **Figs. 7,10.** Stained with Sudan black B to show cuticle. Bars in Figs. 5–10 = 10 μ m.



Figs. 11–17. Localization of enzyme activity in papillae of unpollinated and pollinated stigma of *Ornithogalum sigmoideum*. **Figs. 11–13.** Papillae in unpollinated pistil. **Figs. 14–17.** Papillae in pollinated pistil. **Figs. 11, 14.** Nonspecific esterase activity. **Figs. 12, 15, 16.** Acid phosphatase activity, randomly distributed on papillar surface (arrow). **Figs. 13, 17.** Peroxidase activity. Bars in figs. 11–17 = 10 μm .



Figs. 18–21. Stilar epidermis and stilar canal stained with Auramine O. **Fig. 18.** Stilar epidermis covered by cuticle in unpollinated pistils. **Fig. 19.** Stilar epidermis covered by thick rugose cuticle in pollinated pistils. **Figs. 20, 21.** Stilar canal stained. **Fig. 20.** Canal cells covered by continuous cuticle in unpollinated pistils. **Fig. 21.** Remnants of cell content and cuticle in canal of pollinated pistils. Bars in Figs. 18–21 = 10 μm .

in the cytoplasm of some papillae on the receptive surface. It was striking that the papillae showing a very intense reaction were randomly distributed on the surface (Fig. 16).

STYLE

The style of *O. sigmoideum* is of the hollow type. It comprises an epidermis with cuticle, a stylar cortex, and a wide stylar canal bordered by canal cells. The stylar canal opens directly into the cleft between the stigma lobes.

The epidermis is composed of a single layer of columnar cells. In unpollinated pistils the cuticle of the stylar epidermis is thin. The outer wall, which is relatively smooth in unpollinated pistils, becomes undulate and rugose in a pollinated pistil, as revealed by Auramine O staining (Figs. 18, 19). The stylar cortex is composed of 3 to 6 layers of parenchymal cells. Vascular tissue in the style is found within the cortex and consists of one collateral bundle in each lobe.

In the pre-anthesis stage the opposing sides of the canal cells at each arm of the stylar canal are very close yet not in contact with each other (Fig. 20). The cell wall produces numerous ingrowths at both lateral and canal-facing sides of the canal cells, which also indicates that these cells are transfer cells (Fig. 23). From this point, secretion material is released via exocytosis. An examination of their fine structure reveals that the cell wall has a thick, dense fibrillar structure, exhibits uneven thickening, and is thicker on the side facing the canal than in the other areas (Fig. 25). There is a continuous smooth cuticle layer on the cell wall.

Although regularly shaped nuclei were observed in the canal cells, some of the cells contained nuclei with irregular boundaries and membranes usually protruding towards the center. The cytoplasm around the nucleus is dense and rich in organelles, which usually are concentrated on the side of the cell facing the canal (Fig. 22). At this stage, ER (mainly rough endoplasmic reticulum, and ribosomes are the most commonly found organelles. Rough endoplasmic reticulum (RER) is seen as closely packed lamella parallel to each other and to the plasma membrane, particularly on the side of the cytoplasm facing the canal (Figs. 26, 27). Ribosomes are found individually or as polyribosomes. The number of active dictyosomes in the cytoplasm is high and there are numerous electron-dense vesicles varying in volume around the dictyosomes. Some of these vesicles, surrounded by a single membrane, contain electron-dense material; some contain less of it. The cytoplasm abounds in mitochondria (Fig. 24). Plastids are numerous, of different sizes, and contain starch grains (Fig. 26). At the pre-anthesis stage the vacuoles in canal cells

are larger and fewer than in the post-anthesis stage. Large vacuoles are often divided after anthesis, forming smaller vacuoles which spread out inside the cell.

The cytoplasm contains abundant lipid droplets varying in electron density and size and not enclosed by membranes (Fig. 23). The canal also contains lipid droplets as well as lipoidal substances in irregular clusters.

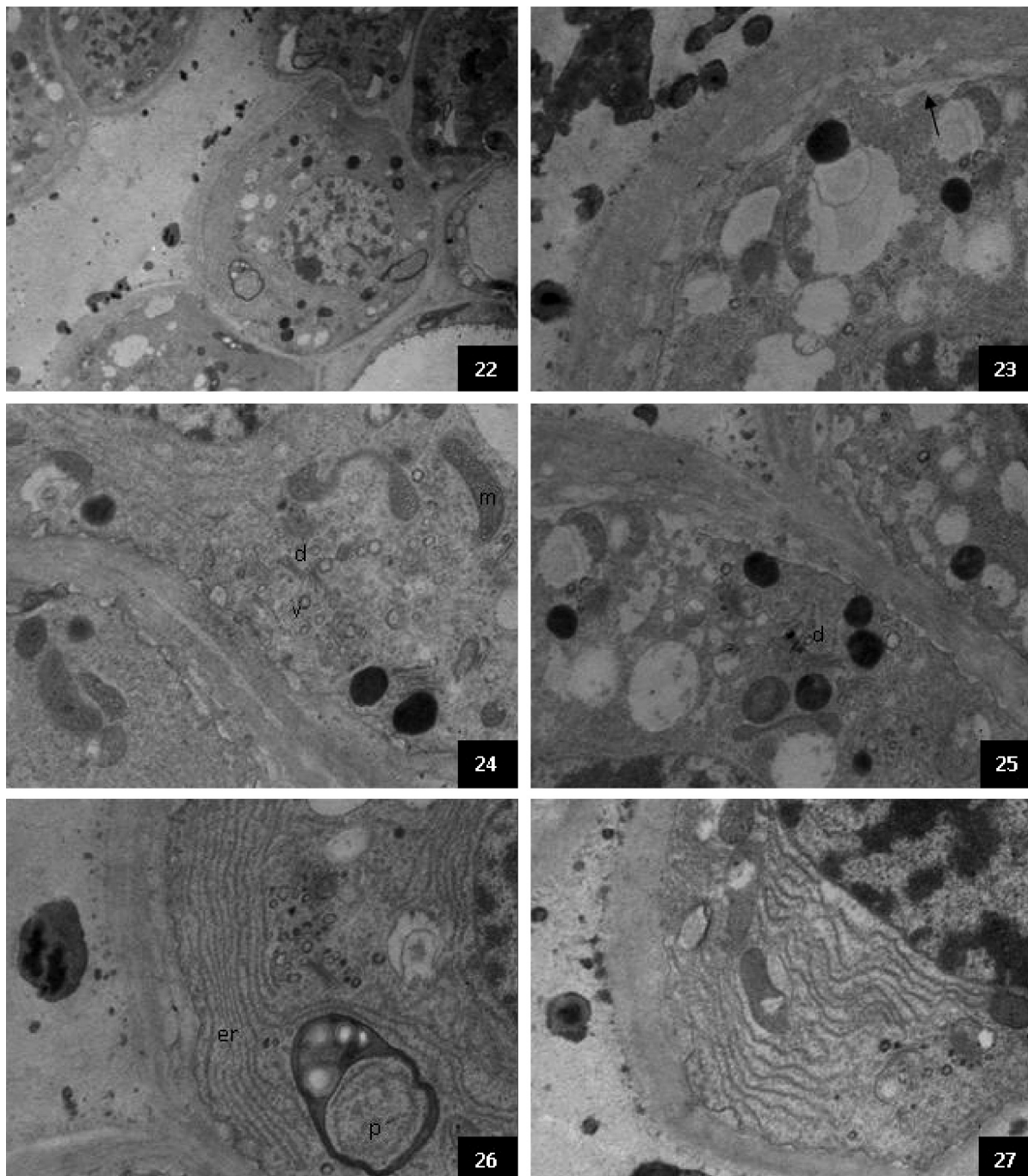
At the post-anthesis stage the opposing sides of the canal cells are very close to each other but not in contact (Fig. 21). The walls of canal cells are thicker than in the pre-anthesis stage. The cuticle becomes disrupted in places and there are cuticle particles on the surface of the wall and inside the canal. Secretory material fills the canal. SEM showed pollen tubes inside the canal at this stage (Fig. 33).

TEM indicated a reduction in volume and deformation of the canal cells' nuclei, which were dumb-bell-shaped with nodes and lobes (Fig. 28). Following anthesis the cytoplasm of canal cells begin to atrophy and diminish. There were very few plastids, mitochondria and other organelles in the cytoplasm, and only ER was detected in some places. Along with the reduction in number, the structure of organelles was degraded. The fewer plastids did not contain any starch (Fig. 30). Instead of the few initially large vacuoles, much of the cytoplasm was occupied by many small vacuoles at this stage (Fig. 29). The lipid droplets in the cells were few and small, but there were more lipid droplets on the secretory surface of canal cells and inside the canal than in the pre-anthesis stage. Cytochemical staining clearly showed lipid droplets dispersed in the reticulate protein matrix, surrounded by a lighter-colored area. This area suggested centripetal catabolism, which was not observed in the pre-anthesis stage.

There were cell wall ingrowths containing material that is similar, in terms of electron density, to the cell wall material on the secretory surface of canal cells (Fig. 31).

In canal cells at pre-anthesis there was a positive reaction for acidic polyanions, insoluble acidic polysaccharides, proteins and lipids. We took sections from the upper stylar area close to the stigma and from the lower stylar area close to the ovary, and examined them to compare and detect differences. The canal cells in the upper area stained more intensely with all reagents in the pre-anthesis stage; the canal cells in the lower area stained more intensely at post-anthesis than at pre-anthesis, indicating that secretion started in the upper area and then moved downward. This finding is confirmed by the following observations regarding the canal.

The upper canal region contained less secretion material at pre-anthesis than at post-anthesis. The combined data on canal cells indicate that much of the secretory material found in the canal

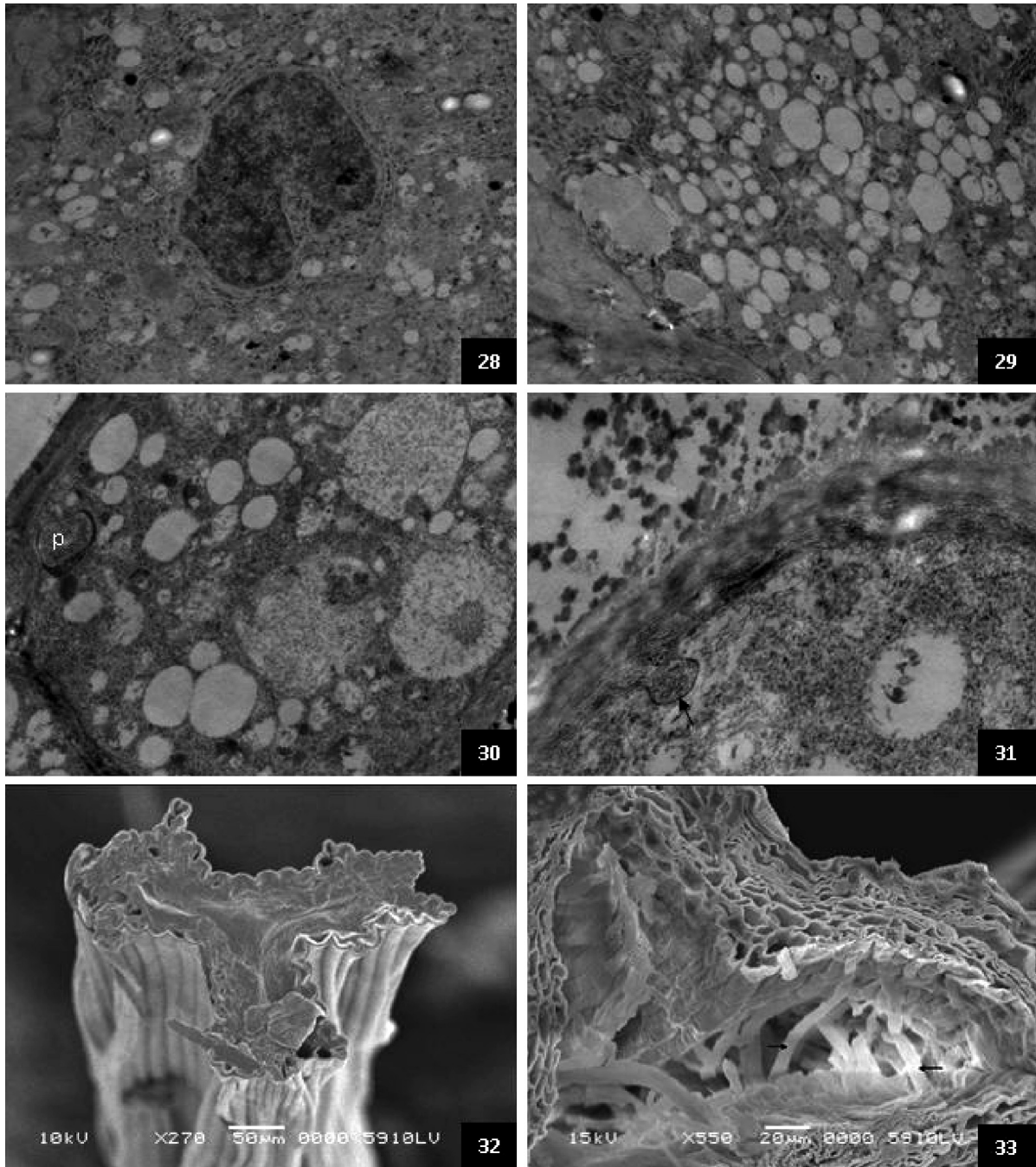


Figs. 22–27. *Ornithogalum sigmoideum* canal cells in unpollinated pistil (TEM). **Fig. 22.** Canal cells during peak secretory activity, $\times 5000$. **Fig. 23.** Lipid droplets in canal and on wall. Wall invaginations (arrow) along inner surface of this wall, $\times 20,000$. **Figs. 24,25.** Wall between two canal cells. Dictyosomes (d) are actively producing vesicles (v), mitochondria (m) also present, $\times 20,000$. **Fig. 26.** Starch present in large plastids (p), dictyosomes with large vesicles and ER (er) in cytoplasm, $\times 20,000$. **Fig. 27.** Extensive ER close to secretory face, $\times 20,000$.

cells passed into the canal between pre- and post-anthesis.

In the upper region, nutrients in the canal at pre-anthesis were dispersed throughout the canal, which

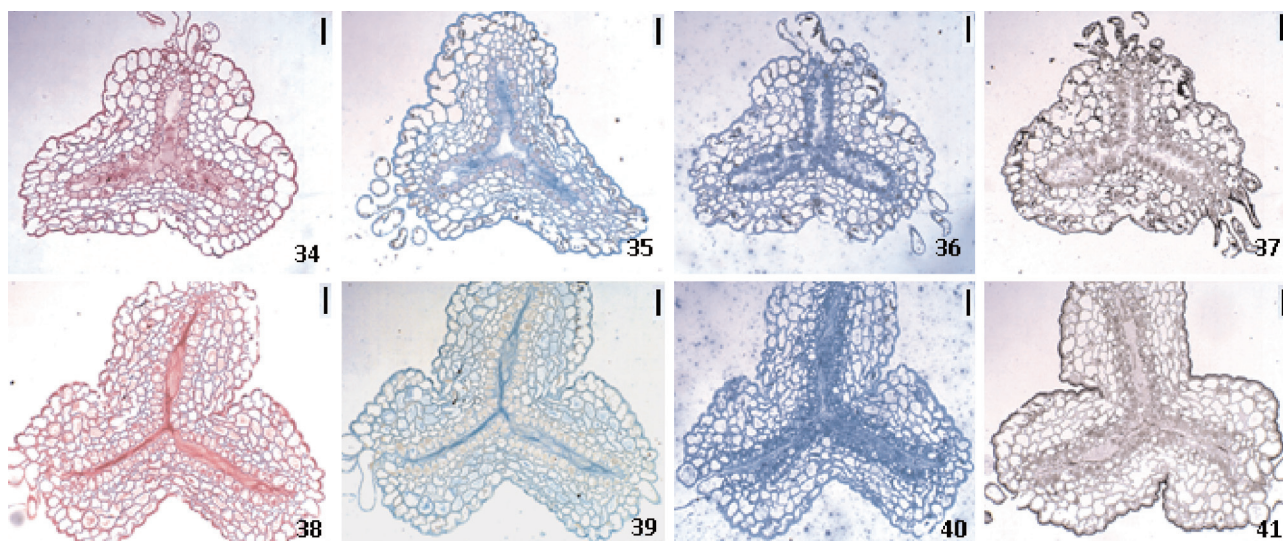
was approximately six times wider at post-anthesis ($55.41 \pm 9 \mu\text{m}$ pre-anthesis, $355.80 \pm 18 \mu\text{m}$ post-anthesis). At pre-anthesis, secretory materials were concentrated in the center of the canal rather than the



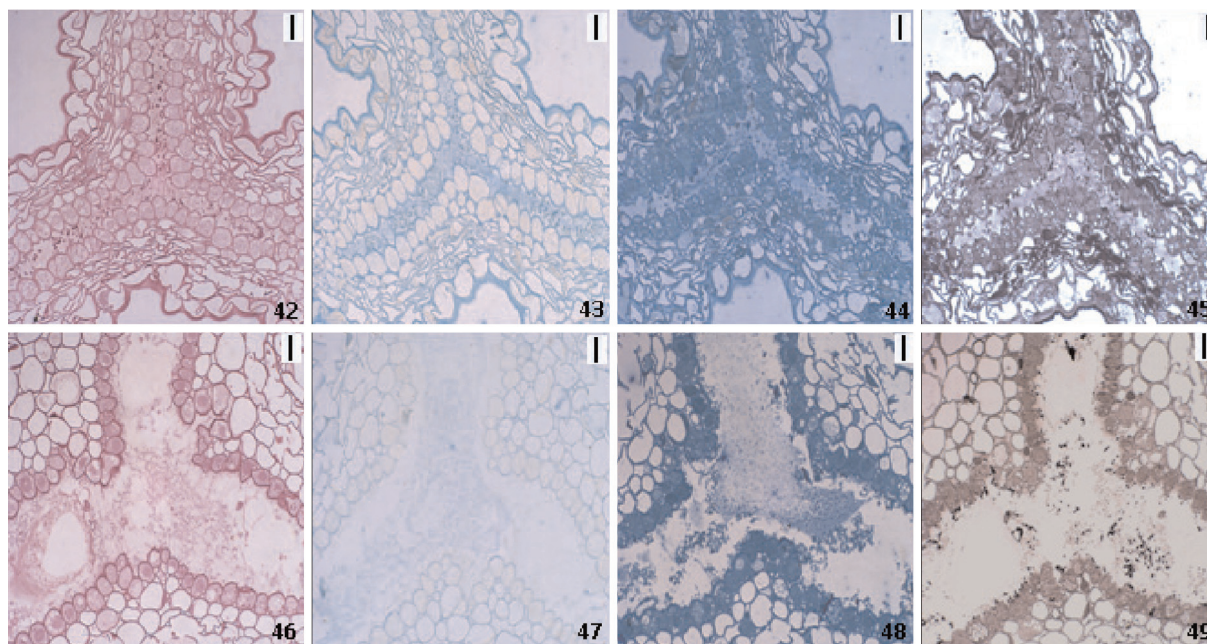
Figs. 28–33. Canal cells in *Ornithogalum sigmoideum* in pollinated pistil. **Fig. 28.** Canal cell with irregularly shaped nucleus, $\times 10,000$. **Fig. 29.** Canal cell with numerous small vacuoles, $\times 10,000$. **Fig. 30.** Plastid (p) without starch in canal cell, $\times 10,000$. **Fig. 31.** Expanded radial cell wall for material transfer (arrow), $\times 30,000$. **Fig. 32.** SEM transverse section through style, showing secretory material filling canal at maturity, $\times 270$. **Fig. 33.** Pollen tubes (arrows) growing in stylar canal, $\times 550$.

canal terminals, meaning that secretion started in the center and proceeded to the terminal side of the canal. At post-anthesis the canal was entirely filled with secretion material (Figs. 34-49; Tab. 2).

SEM and cytochemical staining of the style revealed that the secretion materials in the canal are homogenous at post-anthesis (Fig. 32).



Figs. 34–41. Semithin transverse section of upper region of *Ornithogalum sigmoideum* style. **Figs. 34–37.** From unpollinated pistil. **Figs. 38–41.** From pollinated pistil. **Figs. 34,38.** PAS. **Figs. 35,39.** Alcian blue. **Figs. 36,40.** Coomassie brilliant blue. **Figs. 37,41.** Sudan black B. Bars in Figs. 34–41 = 20 μ m.



Figs. 42–49. Semithin transverse section of lower region of *Ornithogalum sigmoideum* style, stained. **Figs. 42–45.** In unpollinated pistil. **Figs. 46–49.** In pollinated pistil. **Figs. 42,46.** PAS. **Figs. 43,47.** Alcian blue, **Figs. 44,48.** Coomassie brilliant blue. **Figs. 45,49.** Sudan black B. Bars in Figs. 42–49 = 20 μ m

DISCUSSION

The stigma of *Ornithogalum sigmoideum* is of dry and papillate type, and the papillae are covered by cuticle and pellicle. In the pellicle, which shows a positive reaction to Coomassie brilliant blue, non-

specific esterase, acid phosphatase and peroxidase activity were visualized in the form of a continuous layer at the pre-anthesis stage; these reactions were more intense at post-anthesis.

Before anthesis, nonspecific esterase and acid phosphatase activity were identified in the pellicle

TABLE 2. Staining reactions of stylar canal cells and canal in upper and lower regions of unpollinated and pollinated pistils. ++, intense positive stain; +, positive stain

Stain	Specificity	Upper region of canal				Lower region canal			
		Unpollinated		Pollinated		Unpollinated		Pollinated	
		Canal cells	Canal	Canal cells	Canal	Canal cells	Canal	Canal cells	Canal*
PAS	Insoluble polysaccharides	++	+	+	++	+	+	++	+
Alcian blue	Pectins and acidic polysaccharides	++	+	+	++	+	+	++	+
Coomasie brilliant blue	Proteins	++	+	+	++	+	+	++	+
Sudan black B	Lipids	++	+	+	++	+	+	++	+

*The canal enlarges and the secretory material spreads through the canal.

layer of the stigma of *Arachis hypogaea* (Mayer and Pickersgill, 1990). In *Linum grandiflorum*, acid phosphatase, unlike nonspecific esterases, was localized in randomly distributed pockets on the stigma surface, which is not a discrete layer (Ghosh and Shivanna, 1980). Nonspecific esterases occurred as a continuous layer on the stigmas of *Zephyranthes candida* and *Z. citrina*. Acid phosphatase did not occur in a continuous layer as did the nonspecific esterases, but was distributed as granules (Ghosh and Shivanna, 1984). Similarly, in *O. sigmoideum*, acid phosphatase activity was very intense in the cytoplasm of some papillae, unlike other enzymes. Papillae showing a very intense reaction were randomly dispersed on the surface.

When stigmas attain maturity they are characterized by high levels of peroxidase activity (Dupuis and Dumas, 1990; Dafni and Motte Maues, 1998), but the biological function of stigma peroxidases is not known (McInnis et al., 2006). Esterase activity shows no appreciable change during stigma development. Peroxidase activity increases dramatically as pistils mature, peaking when the stigma is most receptive to pollen (Dupuis and Dumas, 1990; Seymour and Blaylock, 2000; Stpiczynska, 2003). Our study confirms that peroxidase enzyme activity increases from early stages to maturity.

Ornithogalum sigmoideum has a hollow-type style. The stylar canal usually is bordered by a single layer of specialized glandular canal cells (Tilton and Horner, 1980; Ciampolini et al., 1981; Dickinson et al., 1982). The inner tangential wall of canal cells in *Lilium* shows numerous ingrowths of the type characteristic of transfer cells (Rosen and Thomas, 1970; Vasilev, 1970). Inner tangential walls of *Gladiolus* (Clarke et al., 1977), *Citrus* (Ciampolini et al., 1981), and *Sternbergia* (Ciampolini et al., 1990) have been shown to be thicker than other walls, and the radial walls of

O. caudatum (Tilton and Horner, 1980) showed expansion. In *O. sigmoideum* we observed wall expansion on the surface facing the canal, which increased at post-anthesis. The walls of *O. sigmoideum* canal cells also exhibited ingrowths between two cells and on the side of the cell facing the canal, increasing the surface area of the wall. Tilton and Horner (1980) argued that the increase of surface area facilitates intracellular transport and transfer of the secretion products in the canal cells into the canal.

Rosen and Thomas examined (1970) the fine structure of canal cells in *Lilium* before and after anthesis and concluded that there is no relationship between anthesis and fine structure; however, the thickness and complexity of the secretion zone increased with age of the pistil. Our findings indicate significant differences in the fine structure of canal cells at pre- and post-anthesis. Canal cells of *O. sigmoideum* are very rich in plastids, dictyosomes, mitochondria, ribosomes and endoplasmic reticulum at pre-anthesis; at post-anthesis the amount of cytoplasm and the number of organelles decrease. These data suggest that metabolic activity is very high at pre-anthesis; secretory synthesis and secretory transfer are intense. The low activity during post-anthesis means that synthesis of secretion products in the cell has slowed down and almost stopped. These changes are similar to those that occur in flowers such as *Lilium regale* and *L. davidii* just before anthesis (Vasilev, 1970).

The presence of ER, active dictyosomes and abundant starch-containing plastids in canal cells is correlated with the synthesis and secretion of proteins, polysaccharides and lipids required for pollen tube growth (Tilton and Horner, 1980; Kandasamy and Kristen, 1987; Ciampolini et al., 1990; Ciampolini and Cresti, 1997). *O. sigmoideum* also contains many active dictyosomes

and plastids, and the abundance of polysaccharides and lipids in the canal is related to their synthetic activity.

Before secretion from the canal cells begins there are many lipid droplets in the canal cells of *O. caudatum*, and after the cuticle is disrupted and secretion starts the lipid droplets rapidly disappear in the canal cells, leading to an increased number of lipid droplets inside the canal (Tilton and Horner, 1980). This is also the case in *O. sigmoideum*, in which the lipid droplets in canal cells at pre-anthesis are reduced at post-anthesis. The number of lipid droplets inside the canal is also higher in the post-anthesis stage. The lipid droplets are surrounded by a light-colored region which shows that catabolism is proceeding from the outer to the inner areas.

In *Hypericum* the content of the canal is rich in lipids but poor in proteins and polysaccharides. A close relationship has been shown between lipid accumulation and the presence and activity of well-developed smooth ER with abundant plastids. The presence of abundant and active dictyosomes has been closely linked with synthesis and secretion of polysaccharides (Ciampolini et al., 1988).

Our findings on fine structure in *O. sigmoideum* were supported by cytochemical staining. Secretions in canal cells pass through the canal when the cuticle on the cells begins to disrupt. Disruption of the cuticle at post-anthesis was visualized with Auramine O by fluorescence microscopy. Secretion starts in the cells in the center of the canal and continues at the terminal part of the canal, as in *O. caudatum* (Tilton and Horner, 1980).

Stylar secretions are usually similar across genera and in particular contain polysaccharides and proteins. Secretion products found in the canal of *O. sigmoideum* are rich in insoluble polysaccharides, proteins, and lipids. Secretion is intense in the part of the canal that is close to the stigma at pre-anthesis, and in the lower regions at post-anthesis. At the post-anthesis stage, secretion products are dispersed throughout the enlarged canal.

Similarly, in *Citrus* (Ciampolini et al., 1981) and *Zephyranthes* (Ghost and Shivanna, 1984) the secretion products inside the canal are rich in lipids, carbohydrates and proteins. In *Lilium* (Loewus and Labarca, 1973), *Annona* (Vithanage, 1984) and *Sternbergia* (Ciampolini et al., 1990) the canal secretions are rich in polysaccharides but show a weak reaction for proteins. Light and EM observations of these canals revealed the almost complete absence of lipids.

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