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ULTRASTRUCTURE OF THE STIGMA AND STYLE OF SPINACH IN RELATION TO POLLEN GERMINATION AND POLLEN TUBE GROWTH

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SUMMARY

Structural events in the stigma and style of spinach pistils have been investigated in detail with regard to pollination and pollen tube growth. The upper portion of the stigma contains only papillae, papillate cells with a wide central part and a narrow spiral tail part. The lower portion of the stigma also has cylindric parenchymatous cells. In each stigma a central core is formed, initially by the tail parts, later together with the small inner parenchymatous cells. In the style these central cores join and fuse to one central core of transmitting tissue. At the base of the style the central core bends towards the location of the ovular micropyle. In the various portions of the stigma and style the intercellular spaces differ in sizes and also in electron-density of their matrix.

Pollen germinates within 10–20 minutes after pollination. Within 7–10 minutes the pollenkitt fuses with the pellicle of the papillar wall. The pollen tube penetrates the pellicle and the eroded cuticle and grows through the outer cell wall layer downwards. Tube growth in the stigma and style is initially intercellular, but following tubes can follow various pathways. In all cases they come out in the space between the carpel and the outer integument. The interactions between pollen/pollen tube and stigma/stylar transmitting tissue are discussed.

1. INTRODUCTION

The flower biology of spinach was studied earlier with special interest being devoted to sex expression and breeding (ROSA 1925; SNEEP 1957). Less attention has been paid to the fundamental process of sexual reproduction and the ultrastructure of the pistil with regard to pollination and pollen tube growth. The stigmas are long with a papillate structure and belong to the "dry" stigma type (WOITTIEZ & WILLEMSE 1979). Dry stigma surfaces were studied in a number of species of the Cruciferae (KROH 1964; KROH & MUNTING 1967; DICKINSON & LEWIS 1975) and Malvaceae (HESLOP-HARRISON et al. 1975). Such stigmas are dry only in a relative sense, since the surface is known to bear a hydrated proteinaceous pellicle (MATTSON et al. 1974). This pellicle lies over the cuticle of the stigma, the cuticle regulating the passage of water into the pellicle from the underlying protoplasts. The pistil of spinach has a single ovule. The solid style contains no vascular tissue and has specifically structured transmitting tissue (WILMS & VAN AELST 1978). This paper deals with the cellular organisation and ultrastructure of the stigma and style. Mature pistils have been studied before and after pollination. Morphological changes in relation to the penetrating pollen tubes are described and discussed.

2. MATERIALS AND METHODS

Unpollinated and hand-pollinated pistils of the spinach (*Spinacia oleracea* L.) cv. Prévital were used.

Scanning electron microscopy: both fresh and prepared material was studied. The prepared material was fixed in 3.5% glutaraldehyde in 0.2 M phosphate buffer pH 7.2 for 3 hrs at room temperature, rinsed in buffer and dehydrated in a graded ethanol-amylacetate series. The material was critical point dried and sputter-coated with gold.

Transmitting electron microscopy: the pistils were fixed in 3.5% glutaraldehyde in 0.2 M phosphate buffer pH 7.2 and 0.2 M saccharose for 3.5 hrs at room temperature. They were post-fixed in 2% OsO_4 in 0.2 M phosphate buffer pH 7.2 and in a graded ethanol-propylene oxide series and embedded in Epon. Sections were post-stained with uranylacetate and leadcitrate and observed in a Philips E.M. 300.

3. RESULTS

3.1. Stigma and style

The morphology of the pistil can be seen in *fig. 1.* Ontogenetically the pistil results from one carpel and consists of a round ovary with a single ovule and a short style, which usually forks into 4 stigmas. The style has a length of approximately 0.4 mm. The stigmas are 6–7 mm in length and are covered with papillae over their entire surfaces *(fig. 2)*. The extension of the papillae marks the maturation. They then reach a length of 0.02 mm, which can increase a little if pollination is delayed.

The composition of the upper portion of the stigma can be seen in *figures 3* and *4*. In transverse sections of it *(fig. 3)* the cells gradually decrease in diameter from the outside to the centre. In none of the longitudinal sections (*fig. 4*) a complete lengthwise view of a cell can be obtained, since the cells have a spiral form. The upper portion of the stigma consists of only papilla cells with three parts. These are the papillar part extending into the air, a wide central part and a narrow tail part directed towards the centre of the stigma. The cell wall, which is in contact with the air, is relatively thick and covered with a distinct cuticle and pellicle (*figs. 5* and *10*). These walls reveal a layered structure. The other walls are thin and have some plasmodesmata. The cells are highly vacuolated.

The ultrastructure of the cytoplasm is different for the successive parts of the stigmatic cells. These differences concern the quantity rather than the quality of the constituents (*figs. 5*, *6* and *7*). In the papillar part mitochondria, endoplasmatic reticulum (ER), ribosomes and dictyosomes are more frequent, whereas plastids are concentrated in the tail part. Mitochondria are uniformly distributed over the papillar cytoplasm. Their shape is in sections round to oval, their average diameter being 0.7 μm. They contain short cristae, which partly appear to be tubular.

There is no special arrangement of the cristae. At the tip of the papillar part

dictyosomes are concentrated. Each dictyosome consists of 4–5 flat cisternae, of which the middle ones are longest. The average length of the cisternae is 0.9 μm. Associated with the dictyosomes are some small vesicles, about 0.1 μm in diameter, located at the ends of the cisternae. Many ribosomes are present (*figs. 6* and *13*). Some are observed as free ribosomes, some are attached to the ER and others, especially near the plasma membrane, are seen as polyribosomes (*fig. 6*). Near the plasma membrane of the papillar part electron-dense material is accumulated (*fig. 6*). From the top to the base of the stigma the width of the central part of the papillae diminishes gradually from 20 to 10 μm. The intercellular spaces between these parts are initially filled with an electron-dense matrix (*fig. 8A*), which gradually decreases downwards (*fig. 8B*). The nucleus usually located at the base of the papillar part is flattened and has a clear nucleolus (*fig. 5*). The plastids (*fig. 7*), mostly located in the tail part of the cells, are large and have well developed thylakoids with small grana. They contain an extensive electron-transparent peripheral reticulum. The stroma reveal some plastoglobuli. In the tail part of the cell both smooth and rough ER are present, together with free ribosomes and polysomes. The intercellular spaces are filled with an electron-dense substance (*fig. 8C*). The basal part of the stigma consists of two types of cells: the papilla and the parenchymatous cells, the latter being cylindric in shape. The inner parenchymatous cells have few peripheral cytoplasm and large vacuoles. The lobed nucleus (*fig. 14B*) is located at the centre of the cell. The intercellular spaces are filled with an electron-dense substance (*fig. 14A*).

The transition from stigma to style is not sharp. The various cores of small inner parenchymatous cells of the stigmas gather and fuse. The result of this fusion is a central core of transmitting tissue in the style (*fig. 9*). The electron density of the intercellular substance of the central core increases towards the base of the style (*figs. 14B* and *16*). At a number of places in the central core the cell walls are thicker and contain a "cuticle"-like structure. These electron-dense structures strongly resemble the epidermal cuticle (*figs. 9* and *17*). From stigma to style the epidermal cells loose their papillate extension (*fig. 9*). Between the epidermis and the central core of transmitting tissue there are several layers of large parenchymatous cells. They have less peripheral cytoplasm with an irregular lobed nucleus (*fig. 9*). At the base of the style the central core of transmitting tissue bends towards the location of the ovular micropyle. The inner surface of the carpel at the ovary is not covered by a cuticle (*fig. 19*).

3.2. Growth of the pollen tube in the stigma and style

The pollen lands on the surface of the stigma and sticks to the papillar part of a papilla (*fig. 2*). Within 10–20 minutes germination starts at a pore close to the place of contact. From scanning electron microscopic observation one gets the impression that the tube growth is over the surface towards the base of the papillar part and downwards to the style. The tube seems to follow the surface of the epidermal cell and seems to penetrate into the stigma at the place where the epidermal cells border each other (*fig. 2*). From transmission electron microscopic (TEM) observations it is obvious that within 7–10 minutes there has been a

fusion between the pollenkitt and the pellicle of the papillar wall while the cuticle is still intact (*fig. 10*). The pollen tube penetrates the pellicle and cuticle of the papillar wall and grows within and along the outer cell wall layer (*figs. 11* and *12*). The cuticle near the place of penetration has eroded. The ultrastructure of the papillar wall has changed. The outer papillar wall layer becomes more electron-dense near the penetrating pollen tube (*figs. 12* and *13*) and looks strongly swollen at other places (*fig. 12*). As the diameter of the tube increases the papillar part is crushed, the total circumference remaining about the same.

After the pollen tubes have entered the stigma, they continue their growth within the intercellular spaces which are filled with an electron-dense substance. The shape of the tubes changes from round to triangular (*fig. 14*). More to the base and in the centre of the stigma the intercellular spaces get larger and most tubes appear to be roundish. The intercellular growth of the pollen does not alter the ultrastructure of the adjacent parenchymatous cells (*fig. 15*).

Whereas the first arriving pollen tubes grow intercellularly in the style, the next can follow a different pathway (*figs. 16* and *17*). Somewhere in the top of the style some tubes grow through the cell walls and continue their growth between the cell wall and the plasma membrane. Near the "cuticle"-like structures the pollen tubes grow through the outer layer of the cell walls. When the pollen tubes grow inside the cell walls, it often happens that the surrounding intercellular spaces are not affected by pollen tubes at all (*fig. 16*). Sometimes the cytoplasm of the affected cell degenerates (*fig. 18*). The nucleus becomes more lobed and the nucleoplasm stains more intensively. The plastids and mitochondria deform till only the outer membranes are present with a homogeneous electron-dense content. Vacuolar membranes, ER and dictyosomes get disorganized, ribosomes are not distinguished anymore. The total cytoplasm becomes even much more electron-dense than the pollen tube cytoplasm (*fig. 18*).

The loosely organized cells below the base of the style facilitate the pollen tubes to grow further towards the micropyle through the space between the carpel and the outer integument (*fig. 19*).

4. DISCUSSION

4.1. Pollen-stigma interactions

Each female flower of spinach has long stigmas with many unicellular papillae. Such a great receptive surface enables the contact and attachment of the wind transported pollen. Pollen germination on these "dry" stigmas is rapid. Fusion of the pellicle with the pollenkitt, which is released from the exine of the pollen-grain, occurs almost immediately after contact. Soon after this fusion, pollen germination and tube growth start. However, spinach has no incompatibility system; recognition of the pollen by the stigma apparently needs only a very short time. It is likely that the pellicle is involved in recognition, as is proposed for *Populus* (KNOX et al. 1972) and for *Raphanus* (DICKINSON & LEWIS 1973b).

The cuticle and outer layer of the papillar wall are quickly affected, suggesting either that the enzymes involved are already present or that they are rapidly

synthesized. In certain Caryophyllaceae the entry of the tube in incompatible pollinations is prevented or greatly delayed when the proteinaceous pellicle of the stigma is removed enzymatically by pronase (HESLOP-HARRISON & HESLOP-HARRISON 1975). According to these authors the pellicle carries a factor, possibly a protein, which enhances the activity of a pollen-borne sporophytic or gametophytic originating cutinase. HEINEN & LINSKENS (1961) have established the presence of an active cutinase in developing pollen tubes of Cruciferae. In pollen of *Petunia hybrida*, however, no cutinase is present (LINSKENS & HEINEN 1962). This species represents the "wet" type stigma, which lacks a cutin layer at the stigma surface.

In spinach the pollen grain germinates and the tube penetrates the eroded cuticle and wall layer beneath the cuticle. The electron-dense substance, which is observed near the place of penetration, indicates a disturbance of the organisation of the outer wall layer. It might be a product of enzymatic degradation of the cell wall components. KROH (1964) suggested that for Cruciferae it is a product of degradation of pectins. According to Kroh the cellulose microfibrils of the stigma wall are displaced by the growth of the pollen tube, while the pectic substances are degraded by enzymes secreted by the tube. Secretion of pectinases by pollen tubes was also observed by PATON (1921) and DICKINSON & LEWIS (1973a).

In the Cruciferae the pollen tubes grow through the wall of the papilla to enter the style (CHRIST 1959). In spinach the pollen tube starts similarly but has to pass much more stigmatic tissue before entering the style. The pathway of the pollen tube through the stigmatic tissue is shown in *fig. 20.*

4.2. Pollen tube growth in stigma and style

After the growth of the pollen tube within and along the papillar cell wall, it enters the more massive stigmatic tissue by penetrating the intercellular spaces. These intercellular spaces are small and the tubes appear to adapt themselves to the available space. From TEM observations it is clear that the characteristics of the content of the intercellular spaces gradually changes from the stigma to the base of the style. There is initially a decrease in electron-density, followed by a gradual increase. The electron-dense substance in the intercellular spaces closely resembles the intercellular substances as observed in the mature *Lycopersicum* styles (CRESTI et al. 1976). It is likely that in spinach the electron-dense substance also consists of pectins and proteins as is shown for *Lycopersicum.*

During the passage of the pollen tubes no changes in ultrastructure of the stigmatic cells are observed. Spinach pollen is trinucleate and both pollen and pollen tubes are rich in reserve material of all types: starch, lipid and proteins. This suggests the spinach pollen in a stage of development which needs few metabolic substrates to give germination and pollen tube growth. Where the pollen tubes grow through the intercellular spaces the electron-density of the intercellular substance has disappeared. Digestion of parts of this material by pollen tubes is probable. In cotton with empty intercellular spaces in the stigma and pollen tubes also rich of reserve material, the pollen tube growth in the

intercellular spaces causes some crushage but no visible ultrastructural changes in the adjacent cytoplasm (JENSEN & FISHER 1969).

The transition from the stigmatic tissue to the stylar tissue is gradual. The growth of the pollen tube in the stylar tissue is initially the same as in the stigma, which is through the intercellular substance. In all angiosperm plant species studied the pollen tubes always follow one specific pattern of growth in the style. In *Petunia* (VAN DER PLUYM & LINSKENS 1968; SASSEN 1974), *Lycopersicum* (CRESTI et al. 1976), *Nicotiana* (BELL & HICKS 1976), *Capsella*, *Lythrum* (SASSEN 1974) and *Diplotaxis* (KROH & MUNTING 1967) the tubes grow through the intercellular substance. In cotton (JENSEN & FISHER, 1969) the cells of the transmitting tissue have thickened cell walls and the pollen tubes penetrate and grow in layer 3 of these walls.

In spinach the first tubes continue their growth through the intercellular substance. The next tubes can follow various pathways, namely a. intercellular ones, b. through the outer part of the cell wall and c. after having passed the cell wall between the plasma membrane and the cell wall. The pathway of the pollen tubes through the style is determined by the structure of the cell walls and the morphology and distribution of the central core of the transmitting tissue in the style. In what way the tubes penetrate and pass the cell wall is not clear. It does not result from a changing anatomy of the stylar tissue or from a shortage of intercellular spaces. The growth through the outer layer of the cell wall is similar to the growth at the stigma papilla. This indicates that the tube has enzymes which can affect cell walls. The strands of cuticles in the basal parts of the style are not affected by passing pollen tubes. This indicates that pollen tubes at this stage do not produce cutinases.

When the tubes grow within the cell walls, the cytoplasm of the penetrated cells has to give way to expanding tubes, which results in a slow degeneration of the cytoplasm. The impression gained from the present study of the ultrastructure and composition of the tissues of the stigma and style in relation to pollen germination and tube growth in spinach is that these tissues function primarily as a guiding route for the growth of the tube and, possibly, as active agents in the control of tube development.

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REFERENCES

BELL, J. & G. HICKS (1976): Transmitting tissue in the pistil of tobacco; Light and electron microscopic observations. *Planta (Berl.)* **131**: 187–200.

CHRIST, B. (1959): Entwicklungsgeschichtliche und physiologische Untersuchungen über die Selbststerilität von Cardamine pratensis L. *Z. Bot.* **47**: 88–111.

CRESTI, M., J. L. VAN WENT, E. PACINI & M. T. M. WILLEMSE 1976): Ultrastructure of transmitting

tissue of Lycopersicon peruvianum style. Development and histochemistry. *Planta (Berl.)* **132:** 305–312.

DICKINSON, H. G. & D. LEWIS (1973a): Cytochemical and ultrastructural differences between intraspecific compatible and incompatible pollinations in Raphanus. *Proc. Roy. Soc. Lond. B.* **183:** 21–38.

— & —(1973b): The formation of the tryphine, coating the pollen grains of Raphanus, and its properties relating to the self-incompatibility system. *Proc. Roy. Soc. Lond. B.* **184:** 149–165.

— & — (1975): Interaction between the pollen grain coating and the stigmatic surface during compatible and incompatible intraspecific pollinations in Raphanus. *Biol. J. Linn. Soc.* **7:** 165–175.

HEINEN, W. & H. F. LINSKENS (1961): Enzymatic breakdown of stigmatic cuticula of flowers. *Nature (Lond.)* **191:** 1416.

HESLOP-HARRISON, J. & Y. HESLOP-HARRISON (1975): Enzymic removal of the proteinaceous pellicle of the stigma papilla prevents pollen tube entry in the Caryophyllaceae. *Ann. Bot.* **39:** 163–165.

—,— & J. BARBER (1975): The stigma surface in incompatibility responses. *Proc. Roy. Soc. Lond. B.* **188:** 287–297.

JENSEN, W. A. & D. B. FISHER (1969): Cotton embryogenesis: the tissue of the stigma and style and their relation to the pollen tube. *Planta (Berl.)* **84:** 97–121.

KNOX, R. B., R. R. WILLING & A. E. ASHFORD (1972): Role of pollen wall proteins as recognition substances in interspecific incompatibility in poplars. *Nature (Lond.)* **237:** 381–383.

KROH, M. (1964): An electron microscopic study of the behaviour of Cruciferae pollen after pollination. In: *Pollen physiology and fertilization* (H. F. LINSKENS, ed.) p. 221–224, Amsterdam, New York: North-Holland American Elsevier.

— & A. J. MUNTING (1967): Pollen germination and pollen tube growth in Diplotaxis tenuifolia after cross-pollination. *Acta Bot. Neerl.* **16:** 182–187.

LINSKENS, H. F. & W. HEINEN (1962): Cutinase-Nachweis in Pollen. *Z. Bot.* **50:** 338–347.

MATTSON, O., R. B. KNOX, J. HESLOP-HARRISON & Y. HESLOP-HARRISON (1974): Protein pellicle of stigmatic papillae as a probable recognition site in incompatibility reactions. *Nature (Lond.)* **247:** 298–300.

PATON, J. B. (1921): Pollen and pollen enzymes. *Amer. J. Bot.* **8:** 471–501.

PLUYM, J. VAN DER & H. F. LINSKENS (1968): Feinstruktur der Pollenschläuche im Griffel von Petunia. *Züchter* **36:** 220–224.

ROSA, J. T. (1925): Sex expression in Spinach. *Hilgardia* **1:** 259–274.

SASSEN, M. M. A. (1974): The stylar transmitting tissue. *Acta Bot. Neerl.* **23:** 99–100.

SNEEP, J. (1957): *De stand van de veredeling bij spinazie.* Thesis, Wageningen; Van Putten & Oortmeijer, Alkmaar.

WILMS, H. J. & A. C. VAN AELST (1978): Fertilization in spinach: The pathway of the pollen tubes in the style. *Bull. Soc. Bot. Fr.* **126:** 243–247.

WOITTIEZ, R. D. & M. T. M. WILLEMSE (1979): Sticking of pollen on stigmas: The factors and a model. *Phytomorphology* (in press).

ABBREVIATIONS

CP = central part of the papilla cell
CU = cuticle
D = dictyosome
EP = epidermis
II = inner integument
IS = intercellular space
OI = outer integument
M = mitochondrium
PD = plasmodesmata
PE = pellicle
PG = pollen grain
PK = pollenkitt
PP = papillar part of the papilla cell
PT = pollen tube
RER = rough endoplasmic reticulum
S = starch
TP = tail part of the papilla cell
TW = wall of the pollen tube
W = cell wall

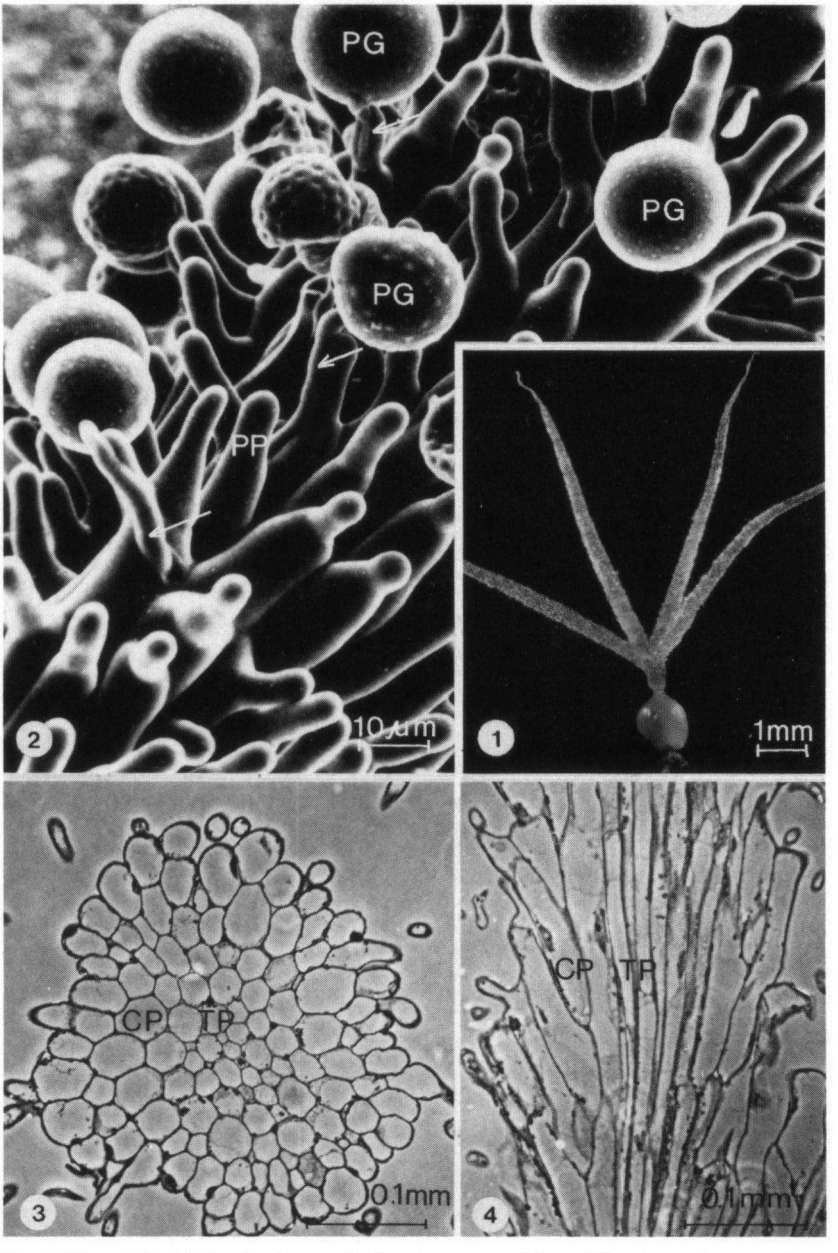

Fig. 1. The pistil of *Spinacia oleracea L.* showing ovary, style and four long stigmas. × 6.
Fig. 2. Stigma papillae and germinating attached pollen, 4 hrs after pollination. Note the pollen tubes (arrow). × 900.
Fig. 3. Cross section of the upper part of the stigma. Only papillae are present. GA-OsO_4 fixation. × 160.
Fig. 4. Longitudinal section of the stigma shows the three parts of the papillae, the papillar parts, the wide central parts and the narrow tail parts. GA-OsO_4 fixation. × 160.

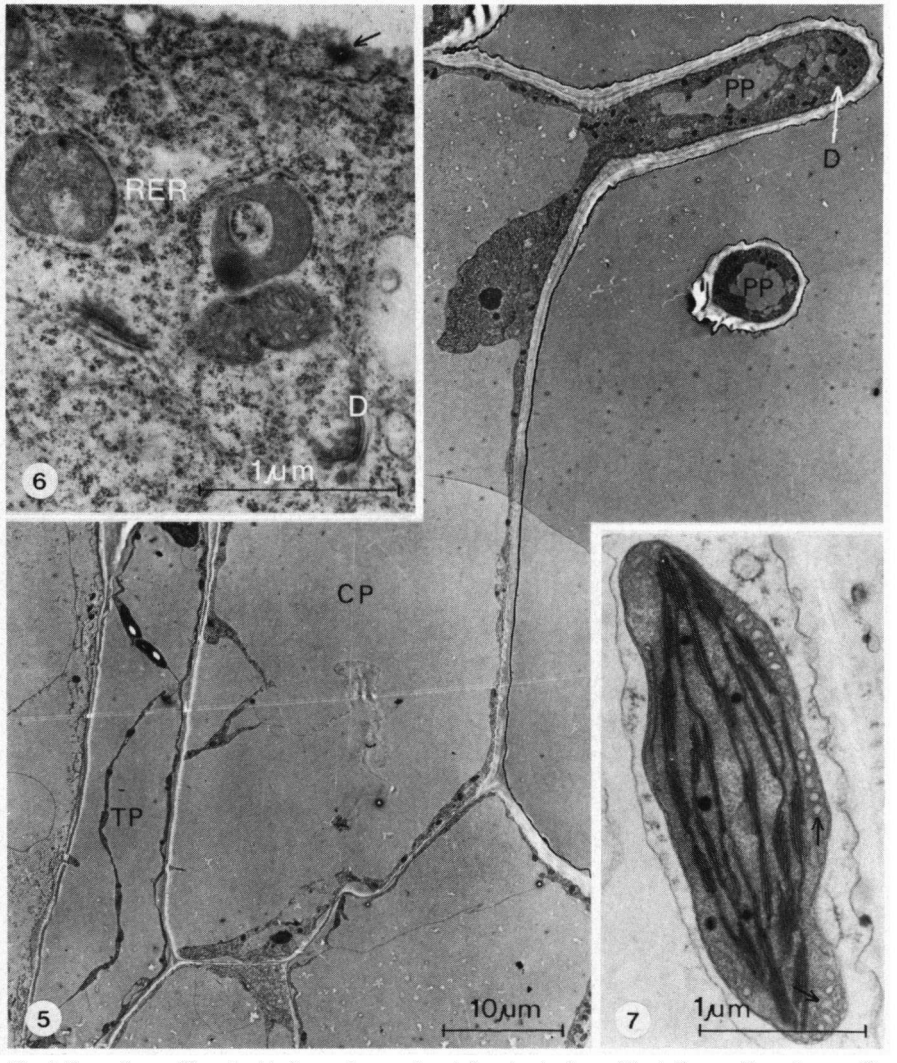

Fig. 5. Part of a papilla cell with the nucleus and peripheral cytoplasm. The tail part of another papilla cell shows some plastids. The cross section is of the upper papillar part. Note the presence of several cell wall layers at the papillar part. GA-OsO_4 fixation. × 1600.

Fig. 6. Enlarged portion of the papillar cytoplasm in cross section showing RER cisterns and numerous free ribosomes. Most free ribosomes are present as polyribosomes. Note the presence (arrow) of electron-dense material near the plasma membrane. GA-OsO_4 fixation. × 27.000.

Fig. 7. Plastid of the tail part of the papilla cell showing well developed thylakoids, plastoglobuli and abundant peripheral reticulum (arrow). Ga-OsO_4 fixation. × 22.000.

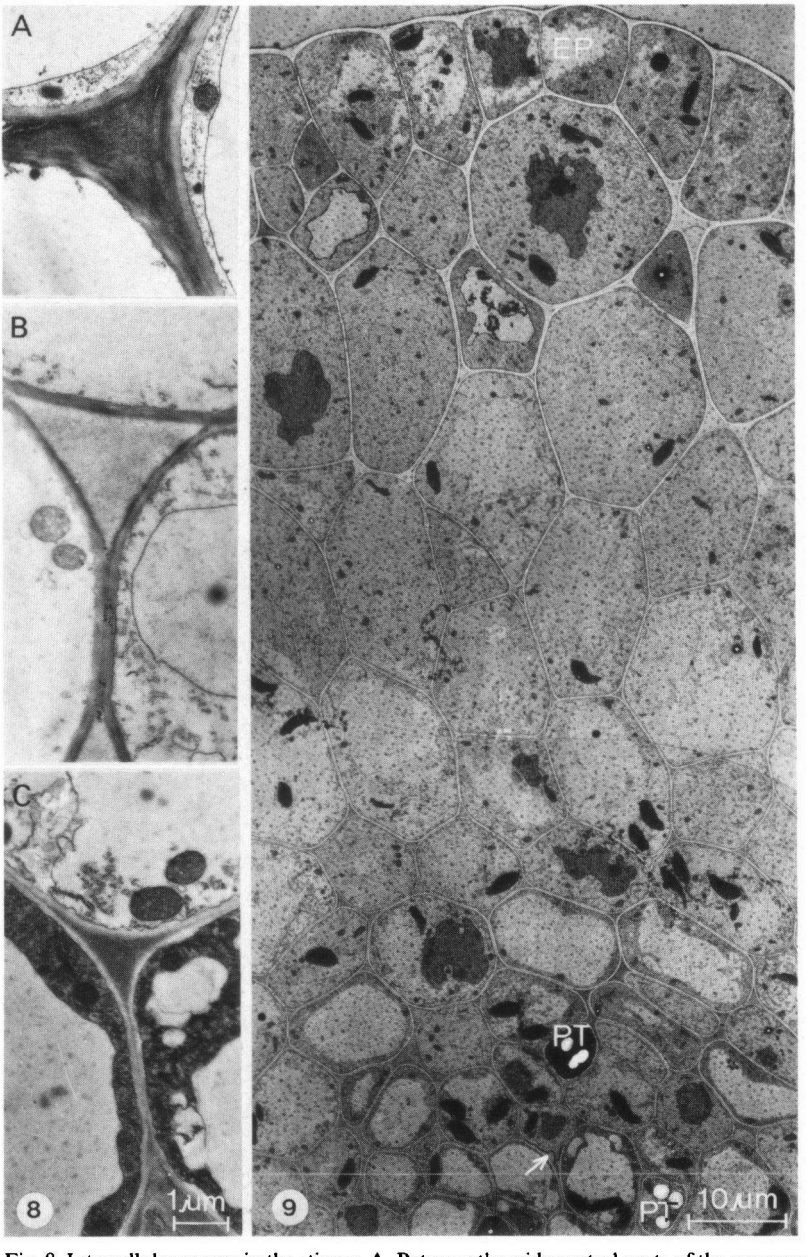

Fig. 8. Intercellular spaces in the stigma. A. Between the wide central parts of the upper papillae. B. Between the wide central part of the lower papillae. C. Between the narrow tail part of the papillae. GA-OsO_4 fixation. × 7000.

Fig. 9. Cross section of the style with epidermis, large parenchymatous cells and the cells of the central core through which pollen tubes grow. Note the cuticle strands in the central core (arrow). GA-OsO_4 fixation. × 1300.

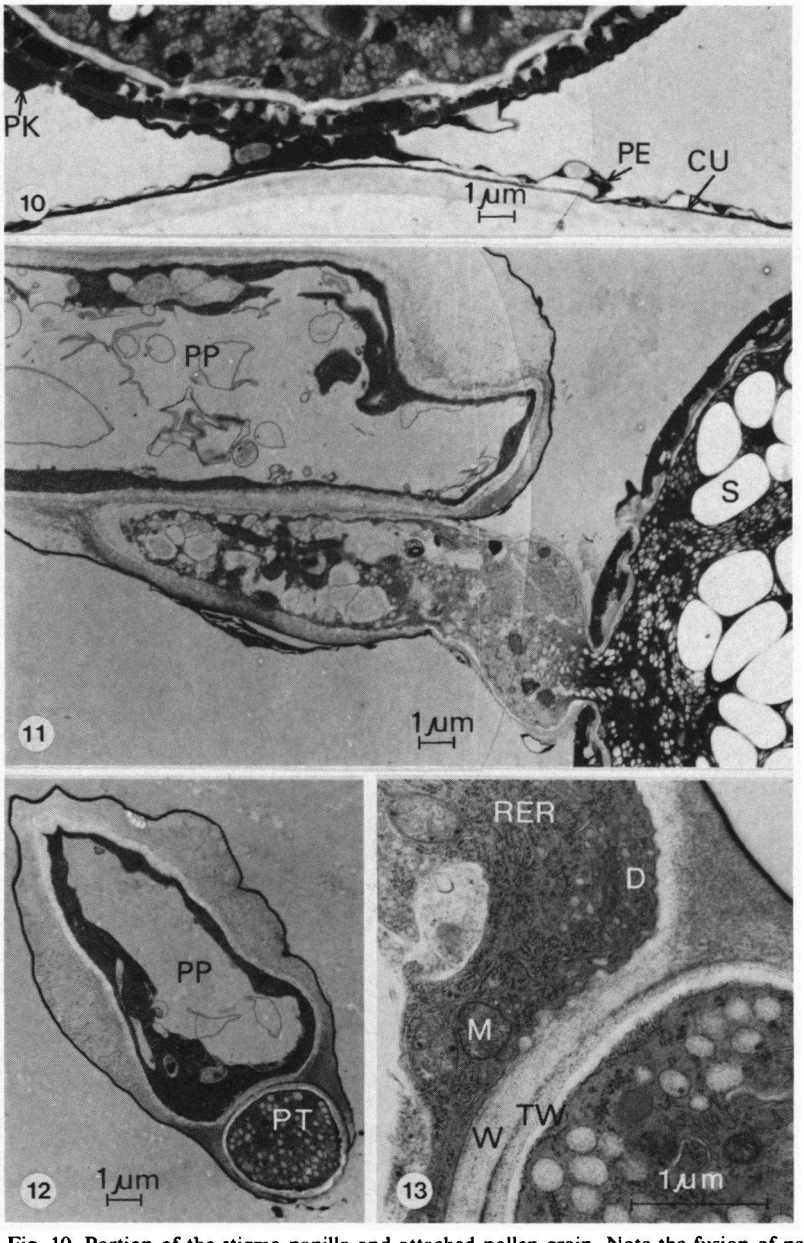

Fig. 10. Portion of the stigma papilla and attached pollen grain. Note the fusion of pellicle and pollenkitt. GA-OsO_4 fixation. × 4500.

Fig. 11. Longitudinal section of a germinated pollen grain on a papilla showing the ingrowth of the tube in the outer cell wall layer. The molarity of the fixative has been adapted to the pollen tube cytoplasm and not to the papillar cytoplasm. GA-OsO_4 fixation. × 4500.

Fig. 12. Cross section of the papillar tip and the tube tip. Note the electron-dense material near the tube tip in the outer cell wall layer of the papilla cell. GA-OsO_4 fixation. × 4500.

Fig. 13. Enlarged portion of the papillar tip and the tube tip. GA-OsO_4 fixation. × 18.000.

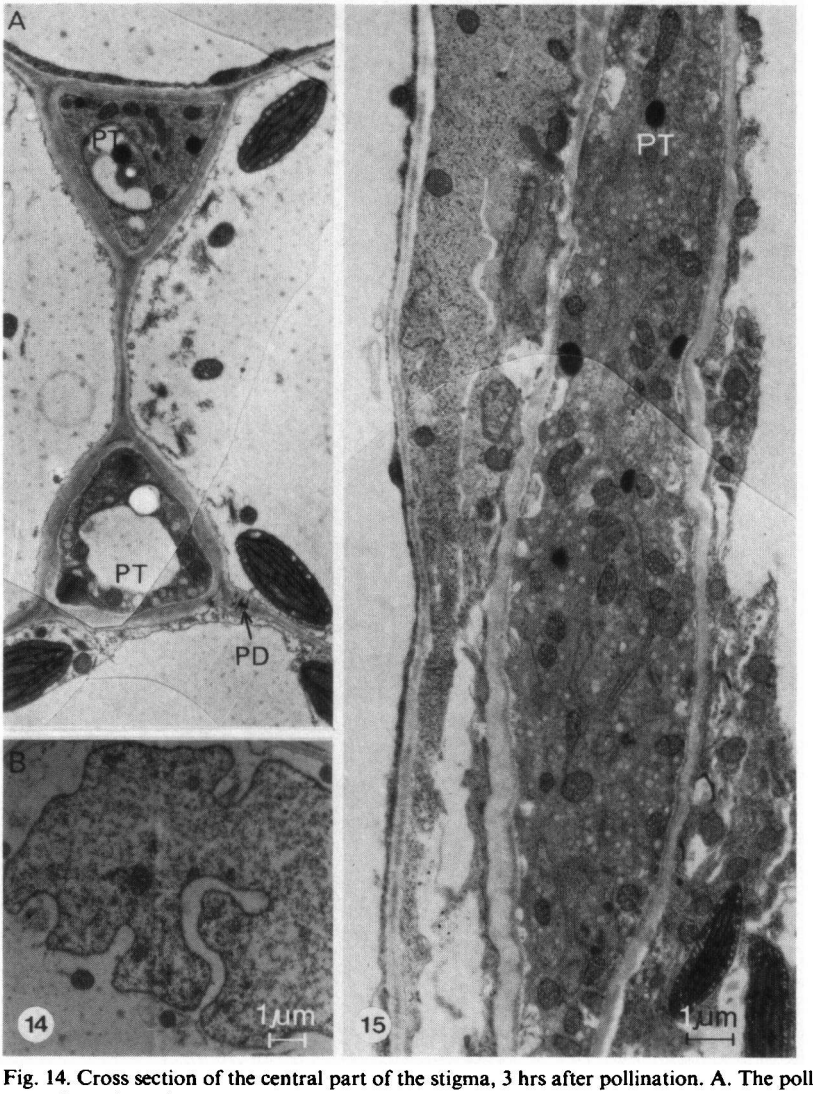

Fig. 14. Cross section of the central part of the stigma, 3 hrs after pollination. A. The pollen tubes grow through the intercellular spaces, which are filled with an electron-dense substance. B. Lobed nucleus of the inner parenchymatous cells. GA-OsO_4 fixation. × 4500.

Fig. 15. Longitudinal section of the central part of the stigma with a pollen tube. Stigmatic cells near the tube are not changed by the immediate presence of the pollen tube. GA-OsO_4 fixation. × 6000.

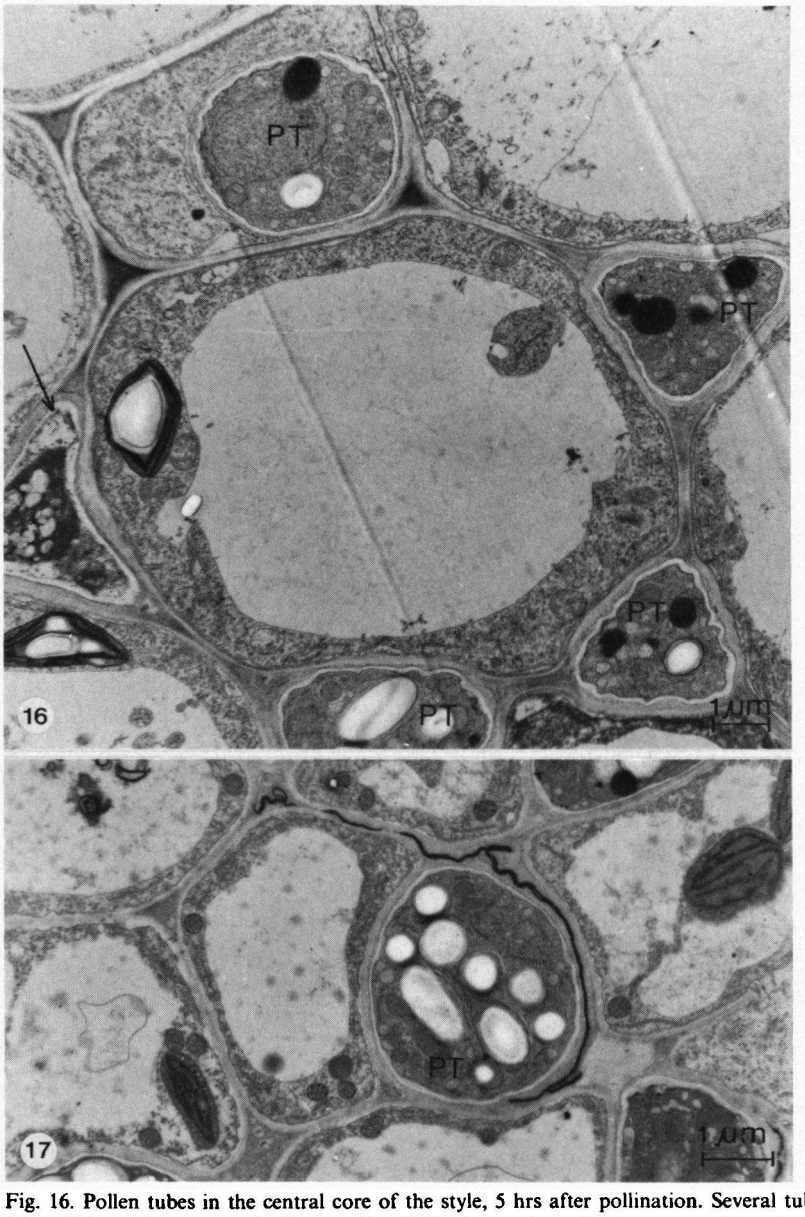

Fig. 16. Pollen tubes in the central core of the style, 5 hrs after pollination. Several tubes grow intercellular and some grow inside the cell wall. The triangular pollen tube (arrow) is cut near the tip of the tube. GA-OsO_4 fixation. × 7000.

Fig. 17. Cross section of a pollen tube in the style, $4\frac{1}{2}$ hrs after pollination. This tube grows in the cell wall layer beneath the cuticle strands. GA.OsO_4. × 9000.

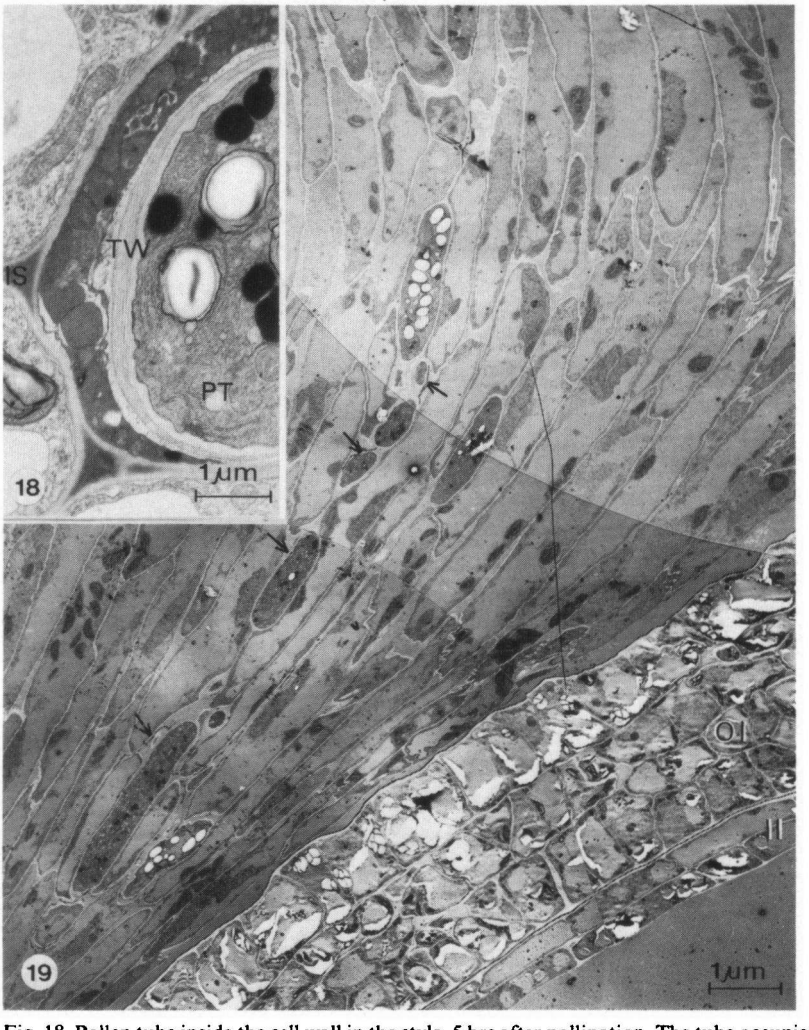

Fig. 18. Pollen tube inside the cell wall in the style, 5 hrs after pollination. The tube occupies most of the volume of the affected cell, resulting in a strong degeneration of the cytoplasm of that cell. GA-OsO_4 fixation. × 10.000.

Fig. 19. Basal part of the stylar tissue, 3 hrs after pollination. The cells of the central core bend to one side. The pollen tubes follow this direction. Note the cuticle strands near the pollen tubes (arrow). GA-OsO_4 fixation. × 900.

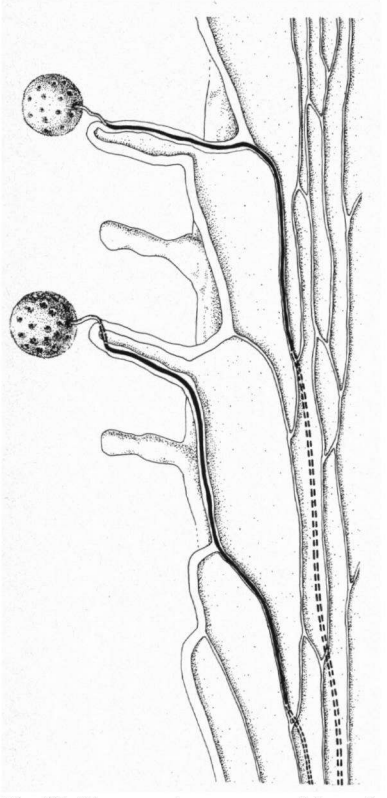

Fig. 20. Diagrammic summary of the pollen tube growth at and in the stigma.