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FORMATION OF POLLEN IN THE ANTHER OF LILIUM
1. DEVELOPMENT OF THE POLLEN WALL

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SUMMARY

The ultrastructural changes during the microsporogenesis and gametogenesis in *Lilium* are studied in relation to the surrounding tissues. In this article the development of the microspore and pollen wall is presented. In relation to the increase in pollen volume the nexine is increased but this process is discontinuous. It is concluded that four periods of sporopollenin deposition can be distinguished. The mechanism of exine formation during the young microspore stage is the embedding of sporopollenin on a sexine matrix of polysaccharides, some proteins and osmophilic material. During this period the sporopollenin deposition on membrane-like lamellae takes place on both sides of the exine. During the vacuolated microspore stage globules, containing sporopollenin are deposited on the exine too. After mitosis the "Pollenkitt" also contributes in sporopollenin addition to the nexine and sexine.

Folds of the plasma-membrane of the microspore are the origin of the membrane-like lamellae. The pollen storage consists mainly of starch, lipid granules and lipoprotein.

1. INTRODUCTION

Sporopollenin deposition on the exine of pollen has been described for some plant species, including *Lilium* (HESLOP-HARRISON 1971; DICKINSON 1976). The developing microspore and tapetal cells are involved in the formation of the exine. Membrane-like lamellae are present in structures containing sporopollenin (ROWLEY & DUNBAR 1967; ROWLEY & SOUTHWORTH 1967; GODWIN et al. 1967; DICKINSON & HESLOP-HARRISON 1968, 1971). In relation to the deposition of sporopollenin the receptor surfaces are described as ordered polymerization surfaces (DICKINSON 1976) and are related to the glycocalyx of the plasma-membrane (ROWLEY & SKVARLA 1975; ROWLEY & PRIJANTO 1977). Ultrastructural studies as well as chemical experiments during the development of the pollen have shown that the exine has its own pattern and chemical composition (FAEGRI 1956; DICKINSON 1973). This may indicate the existence of more than one precursor and probably a discontinuous development of the exine and sporopollenin. Therefore attention is paid to the sources of the sporopollenin, its deposition on membranes and the moment of production in relation to the ultrastructure of the exine (HESLOP-HARRISON 1968a, b). In this study the formation of the "Pollenkitt" (pollen glue) is studied in relation to the formation of the exine. The pollen development and wall formation is related to all the surrounding tissues. The ultrastructural changes in the cells of the anther are

studied from the tetrad stage till the mature pollen. Attention is focussed on the structures containing sporopollenin.

Based on physiological data a survey and interaction model during the microspore development can be made. In this article the changes in the cell volume in relation to the different dimensions of the exine are studied. The changes in the pollen wall and the cell of the developing microspore are related to the formation of the structures containing sporopollenin.

2. MATERIALS AND METHODS

The length of the flower buds of *Lilium*, hybrid "Enchantment", cultivated in a greenhouse, was measured and correlated to the different stages of pollen development. In pollen wall terminology the exine is divided into a sexine (tectum and bacula) and nexine (the foot layer).

During the different stages of development the maximal length and width of fresh microspores and pollen were measured from photographs. The volume of the developing microspore was determined by the formula $V = \frac{3}{4}\pi LK\frac{L+K}{2}$. In this formula L and K are the semiminor and semimajor axis (KHESIN 1967). Exine elements were measured on photographs of thin sections made from frozen material. The height of the sexine was determined as the difference between the height of the whole exine and the nexine. For the determination of the cell volume and the exine and nexine, 30 measurements were made for each stage. The volume of the nexine was calculated as the difference between the mean cell volume including the nexine and the same without the nexine.

Transverse sections of about 1.5 mm were obtained with a razor blade from an anther held in the fixation medium. To prevent the loss of microspores the sections were enveloped by a collodion film. From each stage material was fixed in 2.5% vol/vol glutaraldehyde in 0.03 M phosphate buffer at pH 6.6 with 0.01 M sucrose at 4°C for 6 h. After washing three times in the same medium for 15 min the specimens were postfixed in 2% OsO_4 in buffer at 4°C for 1 h. The material was stained in 1% vol/vol uranyl acetate in 70% vol/vol alcohol. After dehydration and embedding in Epon, sections were poststained with Reynolds lead citrate and studied with a Philips E.M. 301 at 60 kV.

For detection of carbohydrates and proteins in the exine the sections were treated according to the method of Thiery for carbohydrates and with phosphotungstic acid in 10% acetone or in 10% chromic acid as described by ROWLEY & PRIJANTO 1977.

3. RESULTS

3.1. Increase in cell volume and cell wall

The changes in the volume of the cell and the height of exine, sexine and nexine during the different stages of development represented in *fig. 1*. After the tetrad stage MS1 the volume of the microspore increases intensively from the moment

that the cell starts to vacuolize (MS4). Till the moment before the late vacuolated microspore stage (MS5) the cell volume increases strongly. From this moment (MS5) to the phase that the generative cell is near the cell wall (P3) the volume increases most strongly. Thereafter during the phase that a lens-like generative cell is formed (P4) the volume is still increasing till three to four days before dehiscence (P6). Thereafter it decreases slightly. The height of the sexine is nearly

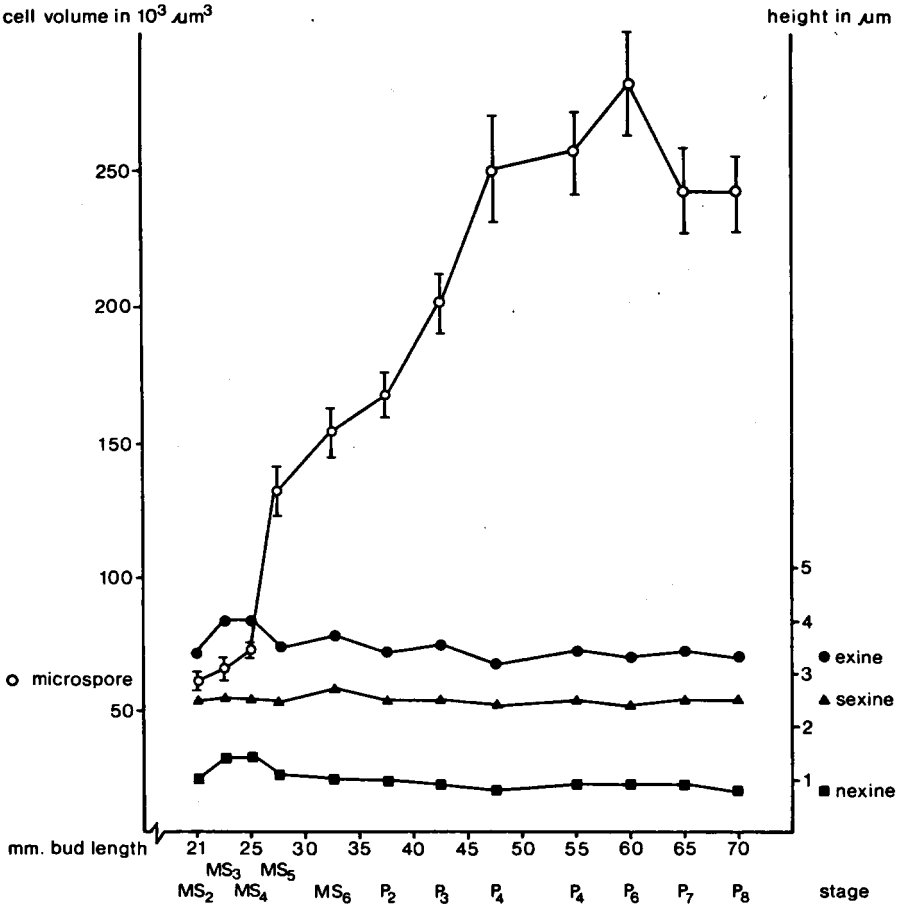

Fig. 1. Changes in cell volume and height of total exine, sexine, and nexine during microsporogenesis and gametogenesis in *Lilium*. The variation in height of the exine is between 0.08–0.12, sexine 0.06–0.12, and nexine 0.02–0.08 µm.

Abscissa: developmental stages, MS2, microspore with noticeable wall, MS3, microspore acquires vacuoles, MS4, vacuolated microspore MS5, late vacuolated microspore, MS6, late interphase microspore, P, pollen just after mitosis, P2, young pollen grain, P3, pollen grain with the generative cell near its wall, P4, pollen grain with lens-like generative cell, P6, pollen 4 days before dehiscence, P7, 2 days before dehiscence, P8, 1 day before dehiscence.

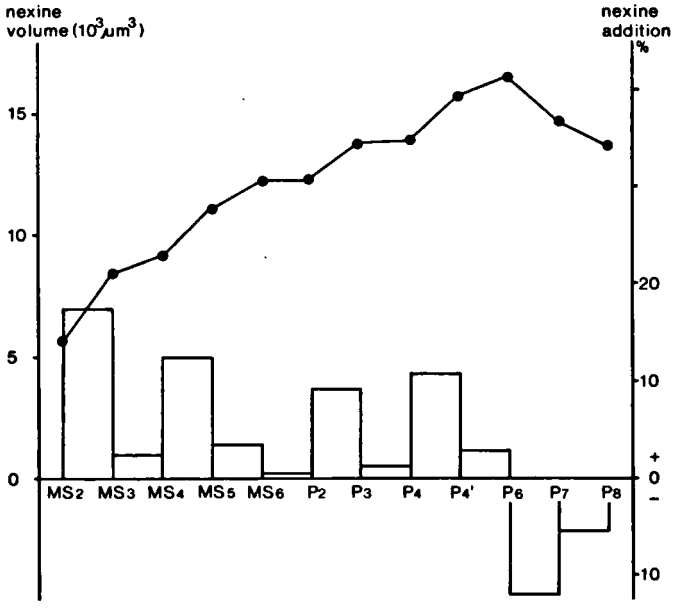

Fig. 2. Changes in nexine volume during the development of the pollen grain.

constant. The height of the exine is maximal at the stage of the early vacuolated microspore, MS3. After mitosis of the microspore it decreases slowly. The height of the nexine shows a comparable sequence with the exine. It increases from the young microspore (MS2) to the stage of the vacuolate microspore (MS4). It decreases slowly after microspore mitosis. *Fig. 2* represents the increase in volume of the nexine and shows how this layer increases during the different stages as a percentage, fixed at 100% in the stage four days before dehiscence (P6).

The volume of the nexine reaches its maximum four days before dehiscence of the anther. From the stage of the young microspore the volume increases in a way comparable to the cell volume. But the addition during the different stages shows great differences. There are four periods with a high increase before the nexine reaches its maximum volume.

3.2. Formation of pollen wall after tetrad stage

In *fig. 3* a scheme of the different stages of pollen wall formation is given with the changes in cell organelles in the microspore and the pollen.

After the release of the callose wall (MS1), the sexine is formed as an electron-dense, rod-like tectum in which electron-transparent small round pores are present in the fine fibrillar material. This material rests on the original disc-like tectum formed in the tetrad and reacts weakly positively to a test for polysaccharides and is nearly positive to the test for proteins. The very thin nexine

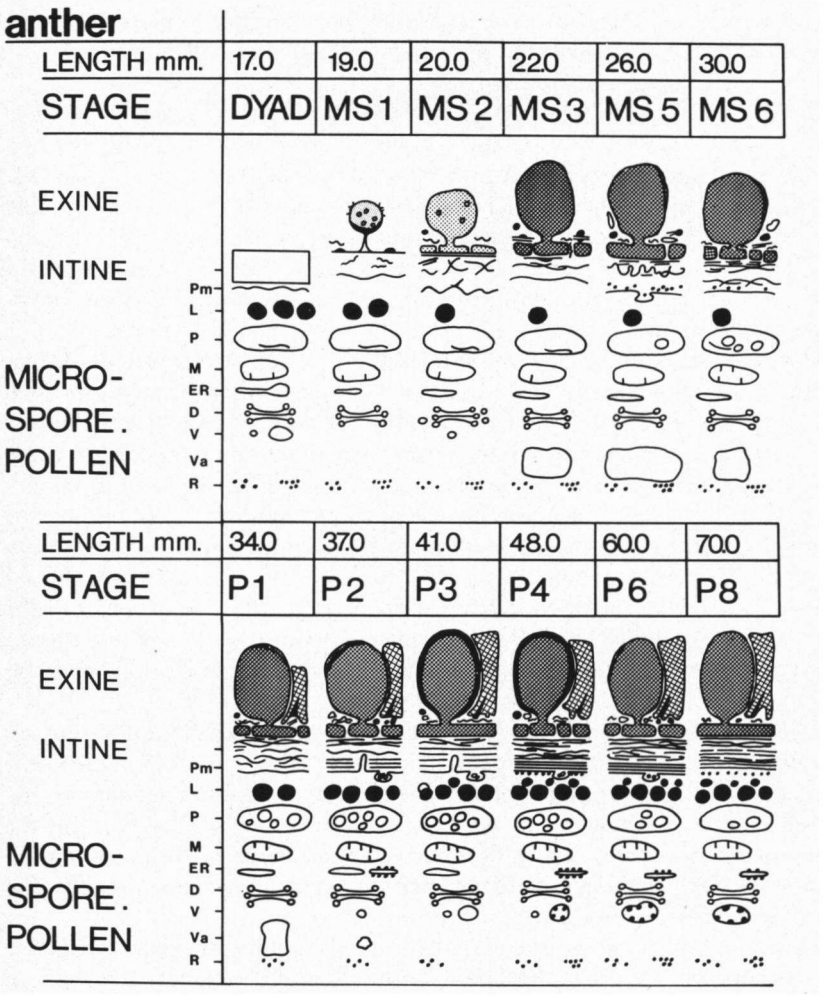

Fig. 3. Scheme of pollen wall formation. Developmental stages as in Figure 1. MS1 = microspore just after the release of the callose wall. Used abbreviations: PM = plasma-membrane, L. = lipid granule, P = plastid, M = mitochondria, ER = endoplasmic reticulum, D = dictyosome, V = vesicle, VA = vacuole, R = ribosome and polysome.

appears as an irregular electron-dense layer and contains proteins too. The fine fibrillar material which is positive for polysaccharide tests covers the nexine Between the nexine and the plasma-membrane, this fine fibrillar material also appears. The plasma-membrane is positive for the presence of polysaccharides (*figs. 4, 5*).

By the time the microspore has a noticeable wall (MS2), the fibrillar structure is nearly compact. The sexine still shows some electron-transparent holes. The nexine is thickening and shows open channels. Against the nexine fine globules

about 0.15 μm are visible. Between the nexine and the now undulating plasma-membrane, fine fibrillar material and membrane-like lamellae covered with electron-dense material are present (*figs. 6, 16*).

When the microspore acquires vacuoles (MS3) electron-dense globules of about 0.25 μm can be observed. These globules are in connection with membrane-like lamellae and fibrils. This exine is compact and totally electron-dense and strongly increases in volume. The thickened nexine has small open channels, some filled with electron-dense material. Some electron-dense material on lamellae and more fine fibrillar material fill up the smaller space between the nexine and the less undulating plasma-membrane. In the nexine two layers can be distinguished (*fig. 7*).

Against the wall of the late vacuolated microspore (MS5) dense fibrillar material is present, the globules are about 0.30 μm and electron-transparent vesicles are added. Around the sexine and the globules a thin electron-transparent layer is observed. The channels in the nexine are sometimes closed. Under and against the nexine the electron-dense membrane-like lamellae are more numerous. Fibrillar material increases between the plasma-membrane and nexine and the intine appears. Some electron-dense material appears against the convulated plasma-membrane (*fig. 8*).

The wall of the late interphase microspore (MS6) has, in contrast with the earlier stage (MS5) open channels in the nexine. Between the plasma-membrane and the nexine, more fibrillar material of the intine is visible; also dotted material appears.

Just after mitosis (P1) between the sexine the "Pollenkitt", containing carotene (REZNICKOVA 1978), is present. The "Pollenkitt" shows fine sheet-like structures which are connected with the sexine. Numerous enlarged membrane-like lamellae connected with the globules and the nexine can be seen on the surface of the nexine. Electron-transparent vesicles appear in this place (*figs. 9, 10*). In the wall fewer changes than in the previous stage MS6 are noticeable. The channels in the nexine are open.

In the young pollen grain stage (P2) the same situation still exists. The intine against the nexine has fine fibrillar material. Near the plasma-membrane a distinct layer of parallel fibrillar material is present. The plasma-membrane shows long extensions and lomasome-like structures become visible (*fig. 11*).

When the generative cell is near the wall (P3), the sexine shows an electron-dense border and the nexine has no channels. Next to the nexine a narrow layer of the intine (Layer 1) is visible, a compact electron-transparent layer borders the plasma-membrane (*fig. 12*).

During the stage that the pollen grains acquire a lens-like generative cell (P4), the "Pollenkitt" now makes a clear contact with the exine by fibrillar material. Between the nexine, with open channels, and the plasma-membrane three layers of the intine are visible, layer 1 a layer of fine fibrillar material. Layer 2 has a more homogeneous structure. Layer 3, against the plasma-membrane, consists of parallel fibrils (*figs. 13, 14*).

Four days before dehiscence or anthesis, P6, the exine and intine are almost

completed. The "Pollenkitt" is attached to the exine and the structure of the "Pollenkitt" is not changed. One day before dehiscence (P8) the channels in the foot layer are closed again. The intine shows three distinct layers (*fig. 14*).

3.3. The changes of cell organelles in the microspore and pollen

In the dyad ribosomes are abundant, polysomes are present too. The endoplasmatic reticulum (ER) forms multi-membraned structures. Dictyosomes produce large and small vesicles. Mitochondria are small and have distinct cristae. Plastids have some thylakoids. Numerous lipid granules are present (*fig. 15*). At the stage of the young microspore, MS1 and MS2, the dictyosomes produce numerous vesicles and small vacuoles appear. The lipid granules decrease in number. The plasma-membrane shows undulations.

At the stage of vacuolization, MS3, the plasma.membrane shows undulations and is in contact with membrane-like lamellae. The vacuoles appear and the mitochondria have few cristae (*fig. 16*).

At the stage of the late vacuolated microspore, MS5, the cytoplasm is rich in ribosomes and polysomes. The dictyosomes produce vesicles. Some starch is formed in the plastids.

Just before mitosis, MS6, near the plasma-membrane cisternes of the ER are present. Mitochondria are large and have distinct cristae. Starch accumulation proceeds. The lipid granules still decrease in number.

At the stage of the young pollen grain, P2, the plasma-membrane shows undulations and lomasome-like structures. Some vesicles can be observed outside the plasma-membrane. The dictyosomes produce vesicles. The amyloplasts contain some electron-dense granules. The lipid granules start to increase in number (*figs. 11 and 17*).

In the pollen grain with the generative cell near the wall, P3, the plasma-membrane still has lomasome-like structures. These structures are present near mitochondria and lipid granules too. The vacuole becomes smaller. Lipid granules are numerous.

In the stage with a lens-like generative cell, P4, small electron-dense granules accumulate near the plasma-membrane. The vesicles are partly filled with electron-dense material, reacting positively to proteins and osmium. In the amyloplasts, the starch disappears (*figs. 18* and *19*).

Before anthesis, P6 and P8, the electron-dense granules near the plasma-membrane increase to a diameter of about 50 nm. The proteins accumulate in the vacuoles (*fig. 13*).

4. DISCUSSION AND CONCLUSION

4.1. The changes in exine dimensions in relation to cell volume

The dimensions of the exine depend on growth of the cell, deposition of sporopollenin and elasticity of the exine. In some cases reduction in the exine dimensions was demonstrated (BANERJEE et al. 1965). The volume of the cell

increases by uptake of water. After mitosis cell organelles and storage products increase.

The different stages and their duration related to the increase in volume imply an irregularity in the development of the microspore. Based on the irregular increase in cell volume and of the nexine, the conclusion can be made that the production of the sporopollenin is discontinuous. The height of the sexine, which shows a slow decrease, is less dependent on the changes in cell volume, because the sexine forms only muri and not a continuous layer.

The measurements reveal that the cell volume increases till about 4 days before anthesis so that sporopollenin is produced until about 4 days before dehiscence. The irregular increase per stage in the volume of the nexine indicates a discontinuous production of sporopollenin, subdivided in 4 periods with higher production. During the periods that the cell volume increases, the discontinuity in the production of the sporopollenin can be observed in the structure of the nexine. In the nexine of the wall of the young microspore open channels are visible. When the volume of the cell increases and the wall formation is not yet completed, the stress on the thin nexine opens its channels. When the sporopollenin accumulates, the nexine is built up and the channels are closed. The channels will be closed during and after periods of a high accumulation of the nexine.

After the young microspore stage, during vacuolization, MS3, -4, -5, the channels are closed. Before and after mitosis, MS6 – P2, they are open. When the generative cell is near the wall, P3, they are closed again. After this stage they are open, P4–P6, but from about 2 days before anthesis they are closed. Also, from the stage of the young microspore 3 periods of sporopollenin accumulation can be determined. These structural changes coincide with the moments of a high accumulation found during the changes in the volume of the nexine. In the period from MS4–MS5 and P2–P3, the whole period of P4 is followed by an increase in cell volume till P5. After this period the channels are closed. During the first period of accumulation (MS2) mainly the basal layer of the nexine is completed.

4.2. Formation of pollen wall

During the formation of the exine different periods as well as mechanisms can be postulated. In the beginning of the stage of the young microspore the structure of the sexine is finely fibrillar and the osmophilic substance contains also polysaccharides and some proteins. The nexine with probably the same constitution is very thin. This composition and structure can be compared with the finding and ideas of ROWLEY and coworkers (1975, 1977, 1978). The matrix of the sexine consists of osmophilic polysaccharides and some proteins: the sporopollenin penetrates in this matrix. The matrix of the nexine is thin at that moment.

Nexine forms from two sides. The membrane of the microspore undulates and forms thin folds on which sporopollenin deposition starts. This plasmamembrane reacts positively to polysaccharides during this period. These membrane-like lamellae are connected to the nexine and will form the nexine 2. This process is most active at the stages of young and vacuolated microspores

(DICKINSON 1971), but after these periods it continues. On the outer surface of the nexine and sexine the fibrillar material takes on a more compact form. The sporopollenin deposition starts on membrane-like lamellae. These structures are described as a universal mode of sporopollenin deposition and their origin is related to elements of ER or to vesicles (ROWLEY & SOUTHWORTH 1967; DICKINSON & HESLOP-HARRISON 1968, 1971; DICKINSON 1971). The origin of the membrane-like lamellae outside the microspore is not clear but they may be elements of the plasma-membrane of the tapetal cells.

Apart from the lamellae an electron-transparent layer on the surface of the exine is present when it becomes more compact. This means that the lamellae are dependent on the presence of sporopollenin.

When in the young microspore stage the matrix is formed and sporopollenin embedding starts, membrane-like lamellae are present. During vacuolization of the microspore, the sexine has a compact structure. Close to the exine, globules are present which enlarge and are related to membrane-like lamellae. These globules add to the exine. The membrane-like lamellae which remain on the exine are numerous. After mitosis the "Pollenkitt", consisting partly of carotenes, adhere to the exine. Membrane-like lamellae and subsequently the electron-transparent vesicles are strongly related to forming this contact which occurs mainly near the nexine. There is also direct contact with the sexine. Thus during this stage, the addition to the nexine originates probably from the "Pollenkitt". Nothing can be said about the nature and contents of the vesicles.

After mitosis the intine begins to show a more compact and layered structure. The pollen cell produces more material for the intine than for the nexine. The pollen wall continues to form by the addition of lamellae, globules and "pollenkitt", until about 4 days before anthesis.

4.3. The microspore

The plasma membrane functions in the production of membrane-like lamellae as well as in the formation of lomasome-like structures. Production and excretion of dictyosome vesicles is probably related to earlier stages of intine formation. Ribosomes are always present, polysomes are mainly present before and after mitosis and about 4 days before anthesis.

Vacuolization originates from dictyosome vesicles too. In the last periods of the formation of the pollen grain the vacuoles contain lipoproteins, probably as a storage product. The mitochondria have fewer cristae during the period of vacuolization. This phenomenon can be observed in megasporogenesis (DE BOER-DE JEU 1978) as well as in microsporogenesis (HOEFERT 1969; WILLEMSE 1971) and can be used as marking point in the development of macrospores or microspores.

In the plastids the starch accumulates during the period before and after mitosis. The pollen cell builds up its storage from elements derived from the surrounding tissues and uses this product partly again for building the intine and lipid granules. The lipid granules disappear during the young and vacuolated microspore stage. During this period these are probably used for nexine for-

mation. Lipid granules increase in number after mitosis and form the main storage product of the pollen. The products for the formation of these granules originate from the surrounding tissues. At the end of the development storage protein is formed in vesicles. The onset of the different sources of sporopollenin as well as the formation of the orbicules are the subject of the next article (part 2) in which the changes in the cells of the surrounding tissues are described (REZNICKOWA & WILLEMSE 1980).

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LEGENDS TO THE FIGURES

Unless mentioned otherwise, the line on the figures represents a length of 0.5 μm.

Plate I.

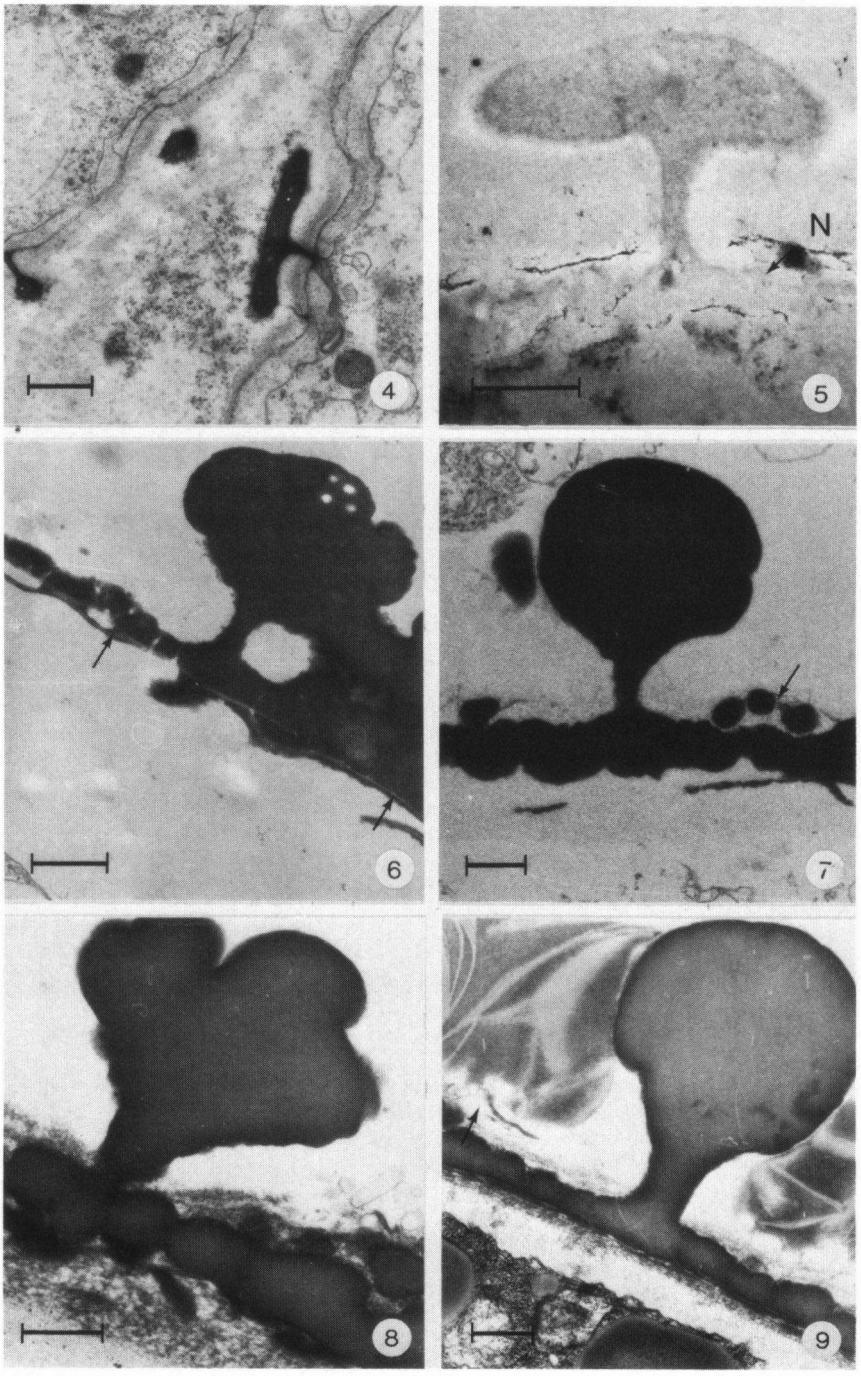
Fig. 4. Pollen wall structure of a very young microspore, MSI, × 16,800.
Fig. 5. Pollen wall of young microspore (MS1, MS2) after treatment with a reaction on polysaccharides according to Thiery. N = nexine, × 30,000.
Fig. 6. Pollen wall of a young microspore (MS2), with channels in the nexine and holes in the sexine. Near the nexine membrane-like lamellae (arrow), × 21,600.
Fig. 7. Pollen wall of the vacuolated microspore, MS3. Note the membrane-like lamellae (arrow). One layer of the intine is formed, × 16,120.
Fig. 8. Pollen wall of the late vacuolated microscope. Against the nexine membrane-like lamellae and vesicles are visible. Two layers of intine are present, × 21,600.
Fig. 9. Pollen wall just after mitosis, Pl. "Pollenkitt" is present and connected with the sexine. Note the direct connection with the sexine and the relation to the membrane-like lamellae (arrow), × 18,000.

Plate II.

Fig. 10. Detail of "Pollenkitt" and nexine. Note the connection between "Pollenkitt" and nexine by the vesicles, × 19,000.
Fig. 11. Nexine and intine of the young pollen grain P2. Note the lomasome (Lo) on the plasmamembrane, × 17,600.
Fig. 12. Pollen wall around pollen with the generative cell near the pollen wall (P3), × 13.000.
Fig. 13. Pollen with lens-like generative cell, P4. Three layers of intine are visible, × 10,400.
Fig. 14. Pollen wall one day before dehiscence. Channels closed, × 15,600.
Fig. 15. Cytoplasm of dyad with organelles, × 18,000.

Plate III.

Fig. 16. Cytoplasm of young vacuolated microspore. Note the membrane-like lamellae on the plasma-membrane. G = globule, × 24,500.
Fig. 17. Detail of young pollen cytoplasm. D = Dictyosome, × 18,000.
Fig. 18. Detail of cytoplasm of pollen with a lens-like generative cell with electron-dense granules near the cytoplasm. The vesicles contain electron-dense material, × 5,800.
Fig. 19. Same stage, proteins reacting positively on the material in the vesicles, × 11,800.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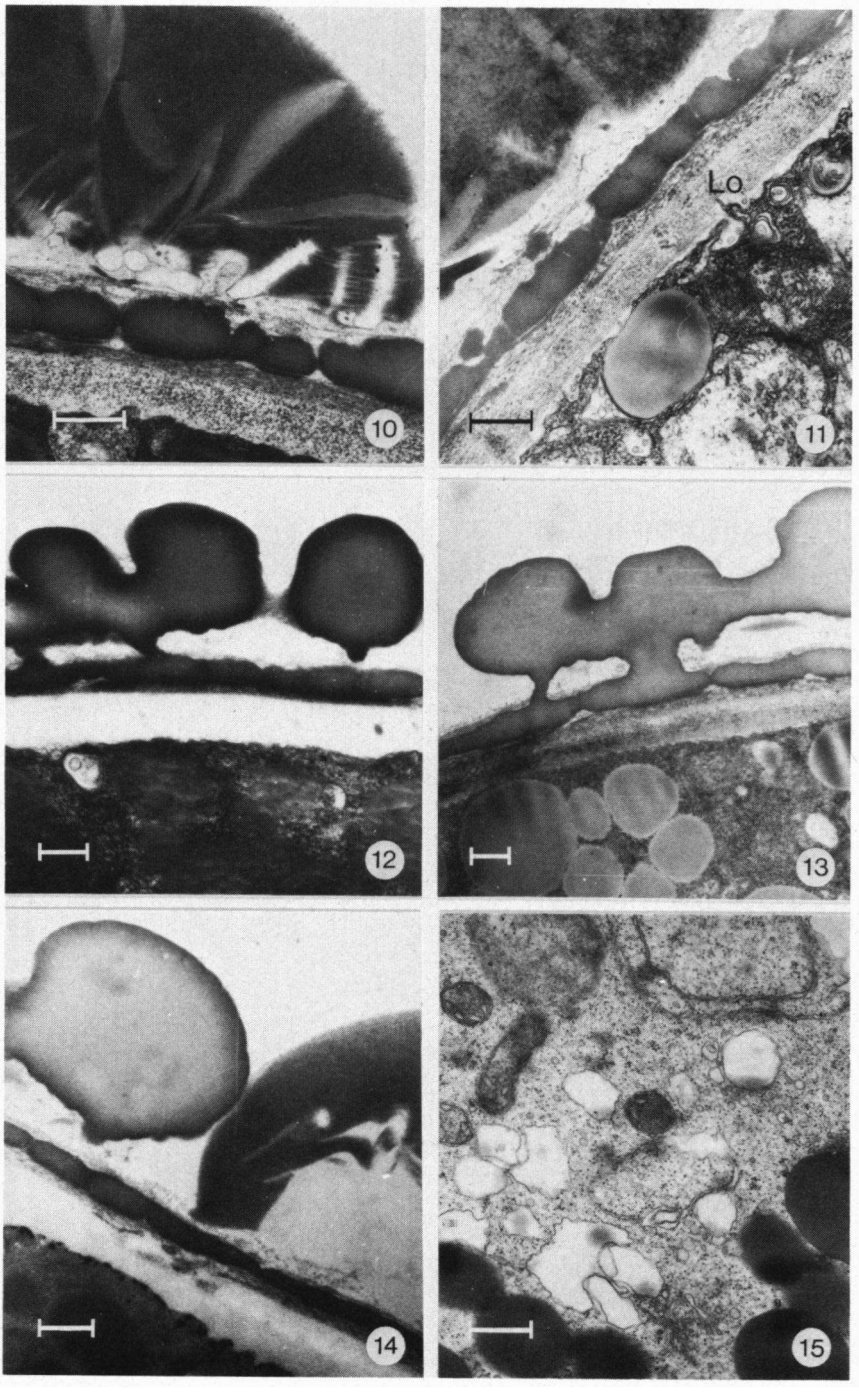
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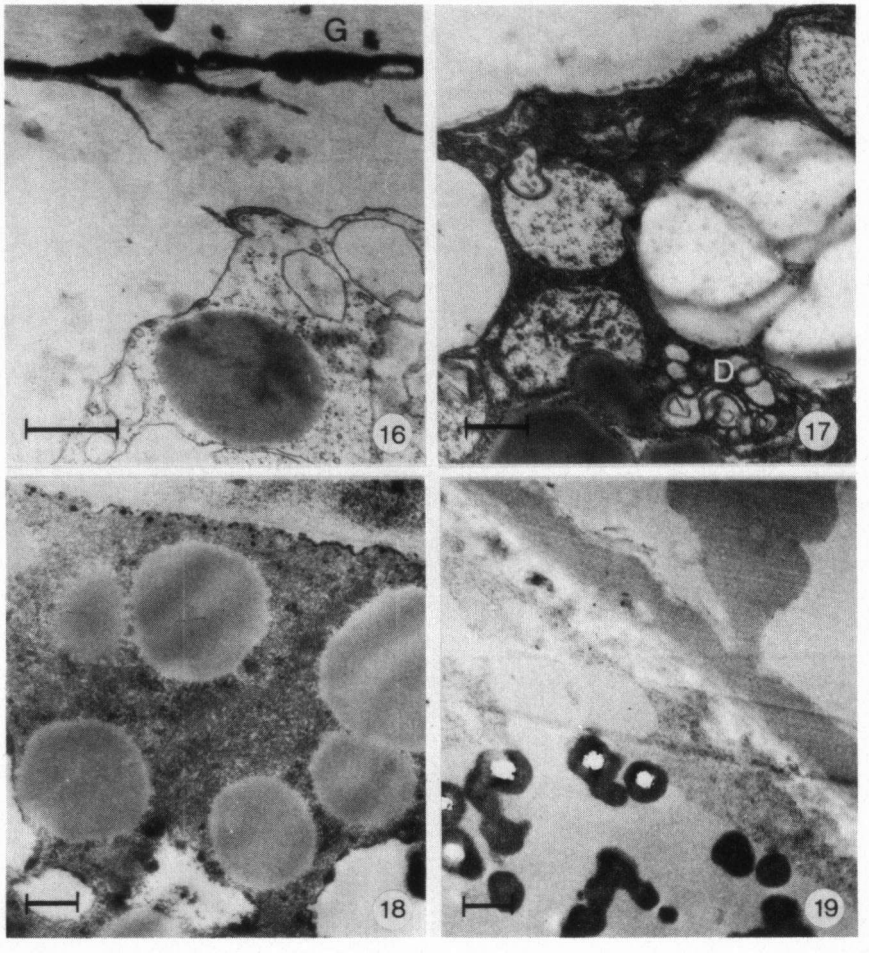
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