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Sporoderm development in *Nymphaea mexicana* (*Nymphaeaceae*)

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Key words: *Nymphaeaceae, Nymphaea.* – Microsporogenesis, sporoderm development, tapetum, exine substructure, morphogenesis.

Abstract: Mature pollen grains of *Nymphaea mexicana* have a verrucate proximal surface, a psilate distal surface and an anazonasulculus (encircling-sulcate aperture). The developmental events of microspores and tapetal cells were observed with TEM and SEM. Radially oriented substructural elements are seen in the microspore surface coating of *Nymphaea mexicana* from the early tetrad stage through the whole exine development. These elements, being the structural units of the microspore surface matrix (glycocalyx), are associated with sporopollenin precursor accumulation. In young free microspores, radially oriented elements are observed at both proximal and distal poles as a "palisade" between the endexine and plasmalemma. – Several points are discussed: (1) the initial and mature forms of exine substructure elements; (2) the significance of exine substructure for realisation of morphogenetic processes; (3) the ways by which verrucate and psilate sculpture patterns are developed.

Several pollen morphological studies have dealt with members of the *Nymphaeaceae* which combine both primitive and derived characters (see Gabarayeva & Rowley 1994 and references cited therein). Only a few species were subjects of ontogenetic investigations of the sporoderm: *Nuphar variegatum* Engelm., *Nuphar luteum* (L.) Sm. (Flynn & Rowley 1971), *Nuphar japonicum* De Candolle (Takahashi 1992), *Nymphaea coerulea* Andr. (Gabarayeva 1991), *Nymphaea colorata* Peter (Gabarayeva & Rowley 1994) and *Nymphaea mexicana* A. Gray in the present study. Our aims are (1) to explain the appearance of multiple verrucae at the proximal pole of pollen grain surface and their absence at the distal pole; (2) to study the developmental processes of exine substructure and its significance for morphogenetic processes; and (3) to investigate the development of tapetal cells.

Material and methods

Flower buds of *Nymphaea mexicana* were collected from the Victoria House of the Bergianska Botanical Garden, Stockholm. Stamens were fixed in 3% glutaraldehyde (GA) and 2.5% sucrose in 0.1 M phosphate buffer (pH 7.3, 20 °C, 24 h). After post-fixation with 1% osmium tetroxide (pH 7.4, 20 °C, 1 h) and dehydration with acetone, the samples were embedded in Spurr's resin. Ultrathin sections were contrasted with a saturated solu-

tion of uranyl acetate in ethanol and 0.2% lead citrate. Sections were examined with a TEM Hitachi H-600 and with a Zeiss EM-10A.

Fresh material was also prepared for SEM (JEOL JSM-6300) study. Anthers were fixed, dehydrated in acetone, frozen in liquid nitrogen, freeze fractured and then critical point dried (BARNES & BLACKMORE 1984). The fractures were mounted on double stick tape attached to the SEM stub, coated for 2 min with gold.

Results

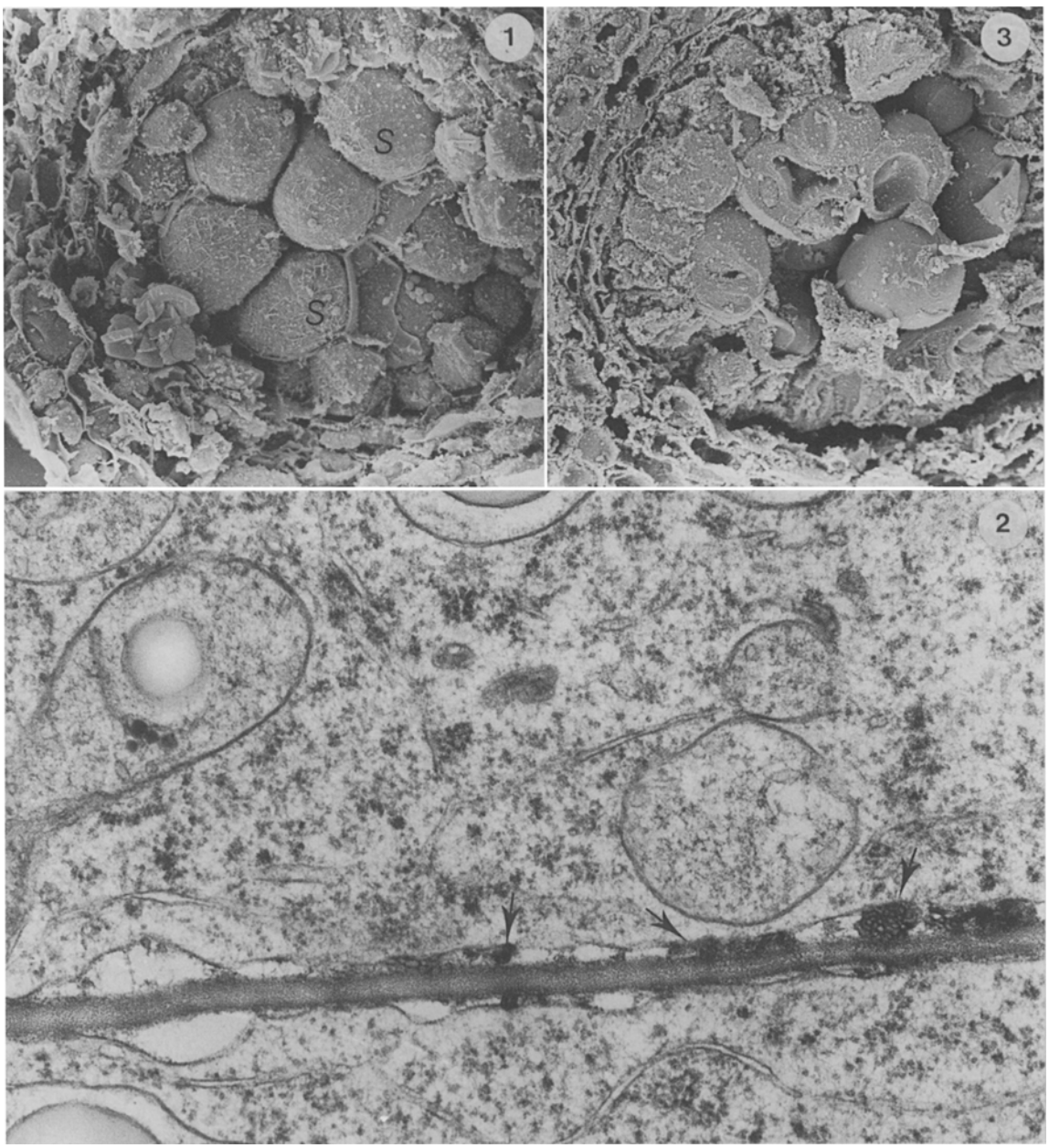
Sporogenous cells. The cells are tightly packed in the loculus (Fig. 1) and appear multiangular. The nucleus is slightly irregular; the nucleolus is rather compact and has a granular structure. There are many ribosomes and polysomes in the cytoplasm (Fig. 2). The cisternae of rough endoplasmic reticulum (RER) are long and disposed separately from each other, but locally form stacks of 3–4 cisternae.

Mitochondria are in non-differentiated state and have few cristae. Plastids with large starch grains occupy a considerable part of the cell volume. The most numerous organelles in the cytoplasm are the dictyosomes. They are active and pinch off small vesicles with fibrillar contents. The plasma membrane is wavy, and many convoluted parts are seen in the periplasmic space (Fig. 2) between the cell wall and plasmalemma.

Early tetrad stage. At this stage the callose envelope is thick (Fig. 3), and the form of a tetrad is more or less spherical. The profile of the plasmalemma is rather smooth (Fig. 4: distal surface), and is coated with a thin layer of glycocalyx. This glycocalyx appears like fine fibrils (Fig. 4), and partly as roundish dark particles. At the proximal surface of the microspores the plasma membrane has many irregularities (Fig. 5), and a thicker layer of glycocalyx. Small radially oriented units are occasionally detected on the proximal surface of the plasmamembrane (Fig. 5: arrows). The cytoplasm shows active dictyosomes and smooth endoplasmic reticulum cisternae (Fig. 6). The plastids contain starch grains. The endoplasmic reticulum cisternae are mainly smooth (without ribosomes) and some of them are in contact with the plasmamembrane.

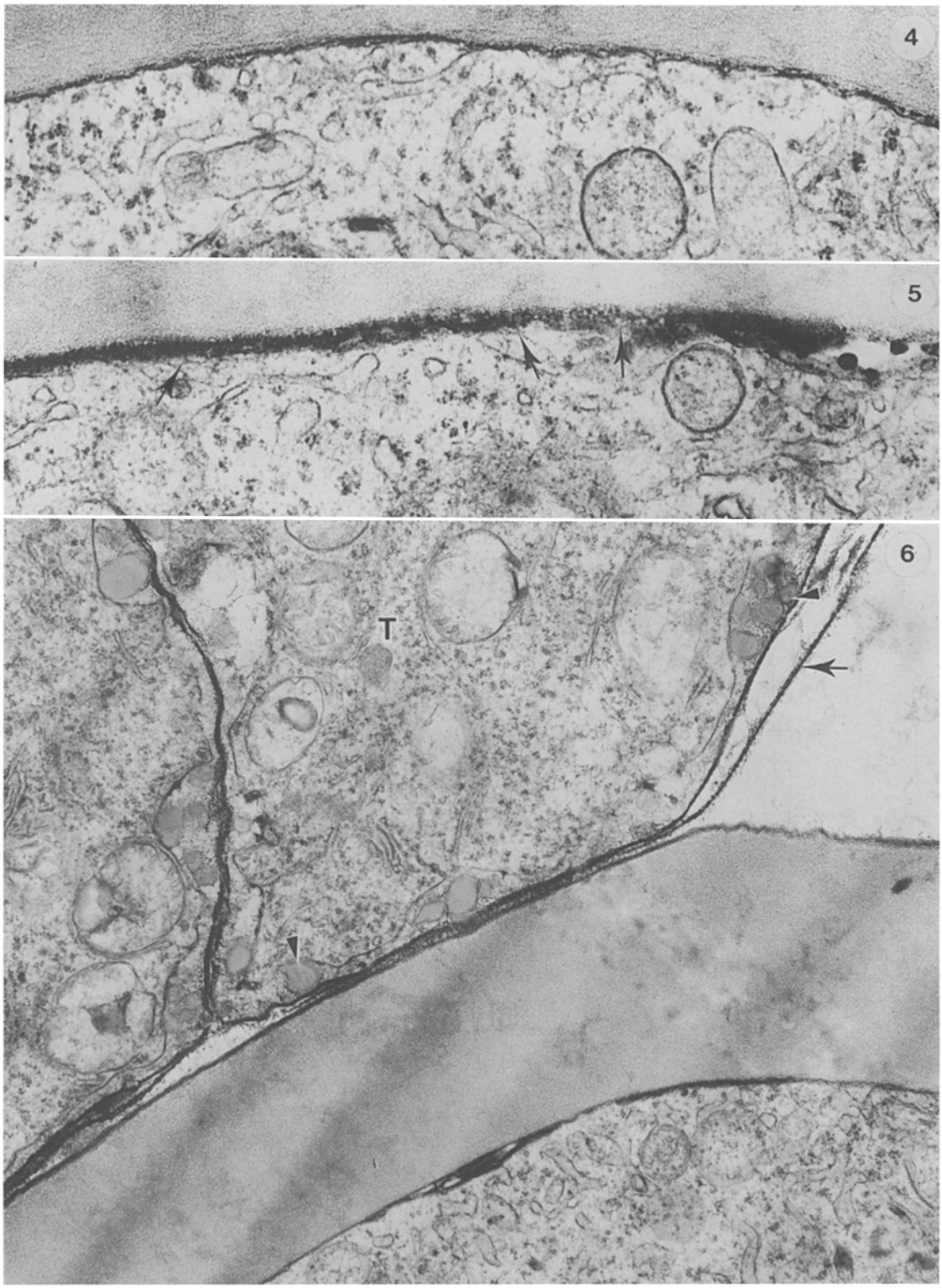
Many lipoidal globules are observed in the invaginations of the tapetal plasmalemma. They are either single or in clusters (Fig. 6).

Middle tetrad stage. Our TEM observations show that the tetrads are still pressed together (Fig. 8). In SEM, flat areas are observed on the surface of the callosic envelope (Fig. 9). The tetrads are loosely arranged when observed with SEM, probably because some of them have fallen out during preparation. Several tapetal cells penetrate between the tetrads (Fig. 7). Many lipid globules are seen in the plasmalemma invaginations of the tapetal cells (Fig. 10: arrowheads). The surface coating on the microspore plasma membrane becomes rather thick on the proximal pole but remains thin at the distal pole (Fig. 10). In the region of the future aperture (anazonasulculus type) the surface coating of the plasmalemma is very thin and poorly discernible (Fig. 10). At the border of the aperture region there is a part of the surface coat which is essentially thicker than the rest of the coating (Figs. 10, 14). The distal and the proximal microspore surface coatings are considerably different. The distal surface (Fig. 11) consists of two layers: the outer is more or less homogenous, and the inner is mainly fibrillar. Short radially oriented rod-like elements extend through both layers (Fig. 11). At the proximal

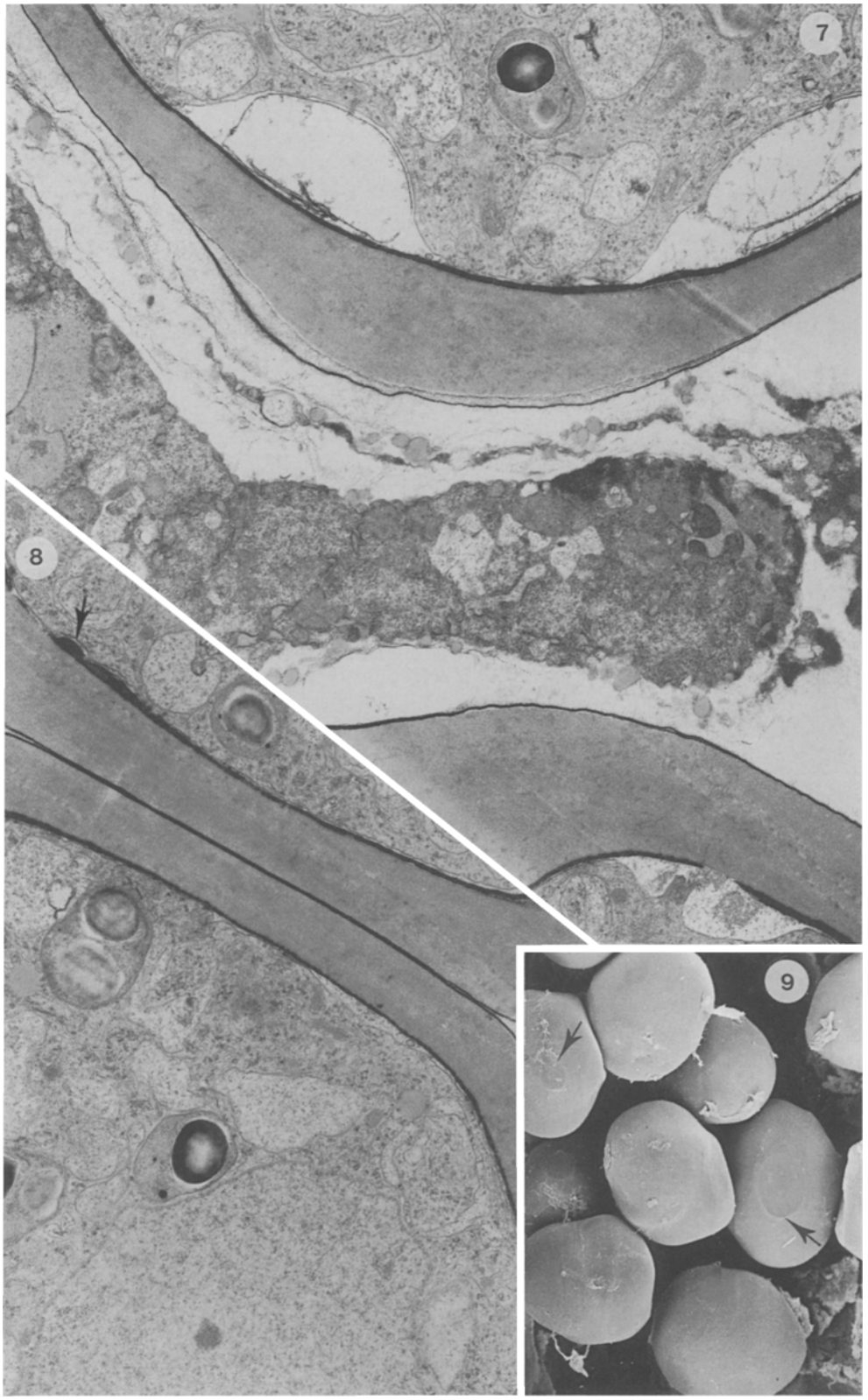


Figs. 1–3. *Nymphaea mexicana*. Sporogenous cells, early tetrad stage. – Fig. 1. Freeze fracture of an anther showing sporogenous cells (S) that are packed in a loculus. SEM, × 990. – Fig. 2. Sporogenous cells with globular aggregates and convoluted parts of plasmalemma (arrows), × 30500. – Fig. 3. Freeze fracture showing tetrads with thick callosic envelope. SEM, × 990

surface (Fig. 12), distinct radial rod-like structures, located rather close to each other, are well recognizable. Some of them look like helicals and protrude out into the callose (Fig. 12: arrows). It is important to note that at this stage there is a narrow space between the plasma membrane and the developing sporoderm which



Figs. 4–6. *Nymphaea mexicana*. Early tetrad stage of microspores. – Fig. 4. Distal surface. The plasmalemma is covered with a thin fibrillar electron dense layer. × 35000. – Fig. 5. Proximal surface. The plasmalemma is coated with a thick electron dense layer containing radially oriented units (arrows). × 44000. – Fig. 6. Part of a microspore, thick callosic envelope and tapetal cells (T). A membrane-like structure (arrow) is observable between the callosic envelope and the tapetum. Single or clustered lipoidal globules are seen in invaginations of the tapetal plasmalemma (arrowheads). × 22000



Figs. 7–9. *Nymphaea mexicana.* Middle tetrad stage. – Fig. 7. Tapetal cell between tetrads. × 4900. – Fig. 8. Two adjacent microspore tetrads with comparatively thick plasmalemma surface coating, particularly close to the proximal apeture region (arrow). × 11800. – Fig. 9. Tetrads with rather thin callosic envelopes which shows outline of the microspores (arrows) within each tetrad. SEM, × 820

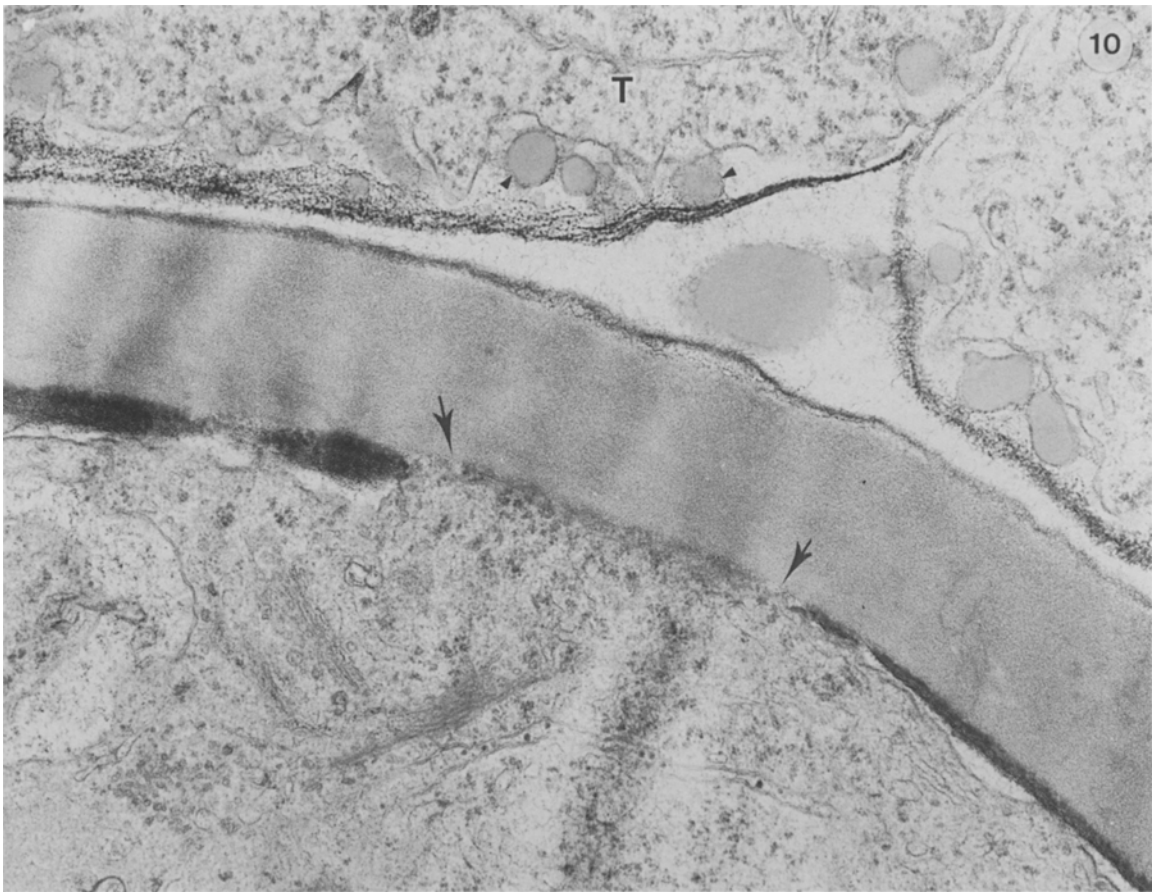
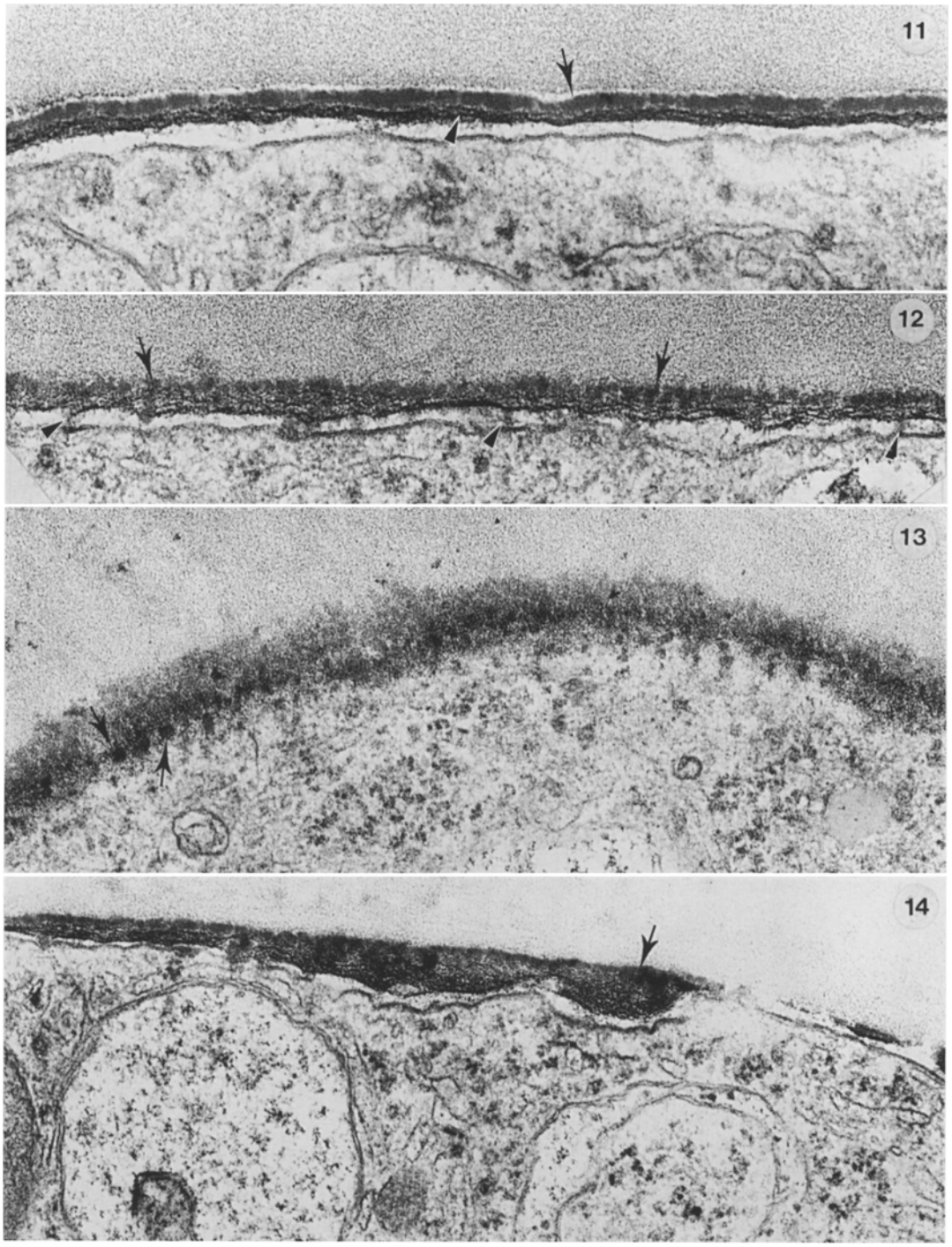
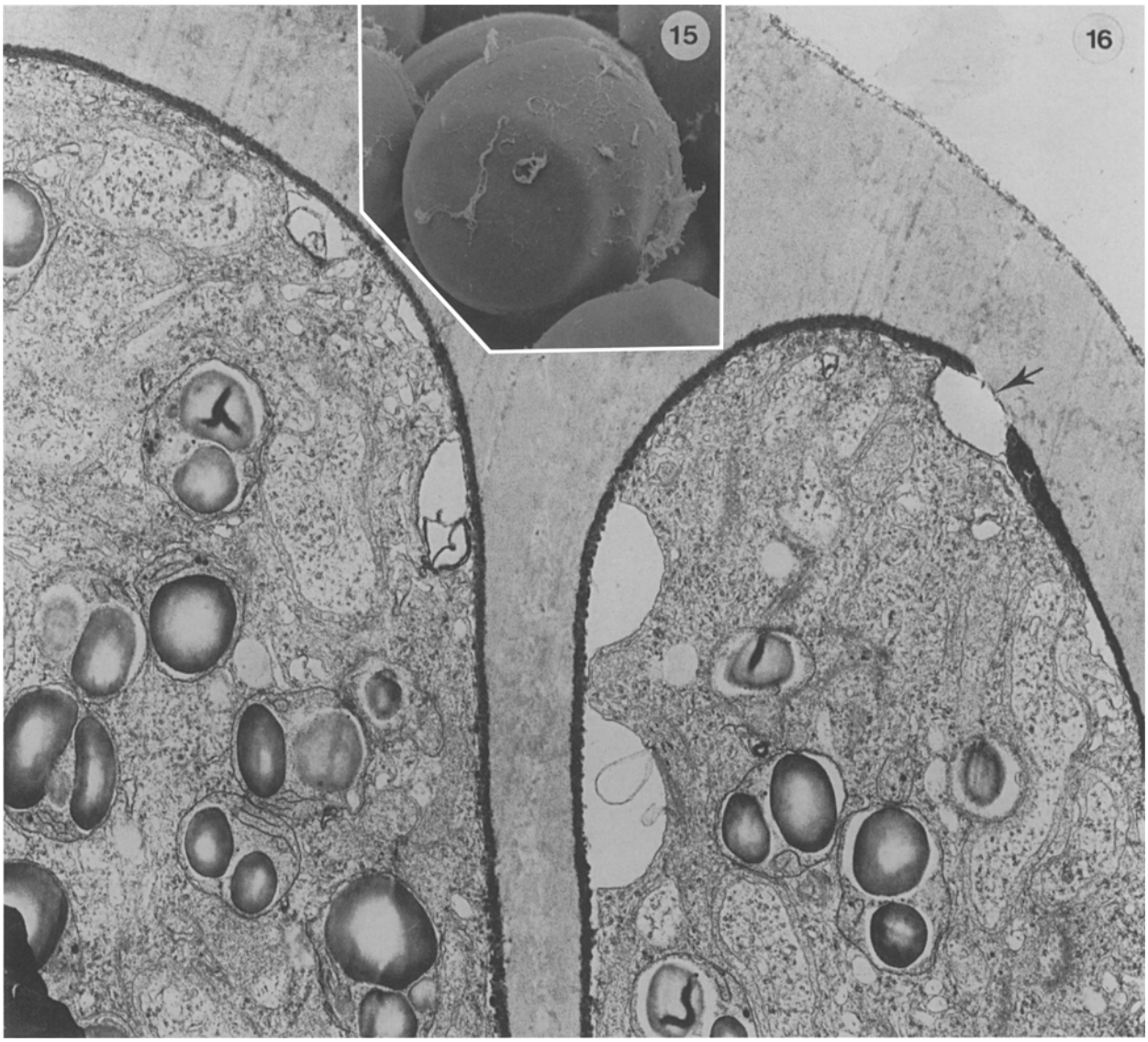


Fig. 10. *Nymphaea mexicana*. Middle tetrad stage. Tapetal cells (T) and part of a microspore. Note lipid globules in tapetal cells (arrowheads). The region of the future aperture (between arrows) is apparently without surface coating. At the proximal margin of the aperture site the surface coating is rather thick. × 28600

persists during later stages. The radial rod-like structures are extending through the periplasmic space, and their new parts appear in this narrow periplasmic space (Fig. 12: arrowheads). The "knobs" of a homogenous substance (probably first accumulation of sporopollenin) are seen mainly around the tops of radial elements. These elements appear in tangential section as small discs very close to each other (Fig. 13: arrows). When the tetrads are examined with SEM, the callosic envelope appears pressed against the tetrad units. Therefore it was possible to observe the general configuration of the tetrad units inside the callosic envelope (Figs. 15, 16). Radially oriented elements which are evidently procolumellae, are now dumb-bell-shaped (Fig. 17). The distance between them has increased probably because of stretching of the surface coating during microspore growth (Fig. 18). There are electron-dense accumulations, often looking like spherical concretions at the basal parts of columellae (Figs. 19–21). The basal parts of the procolumellae are attached to the slightly wavy plasma membrane. Where small

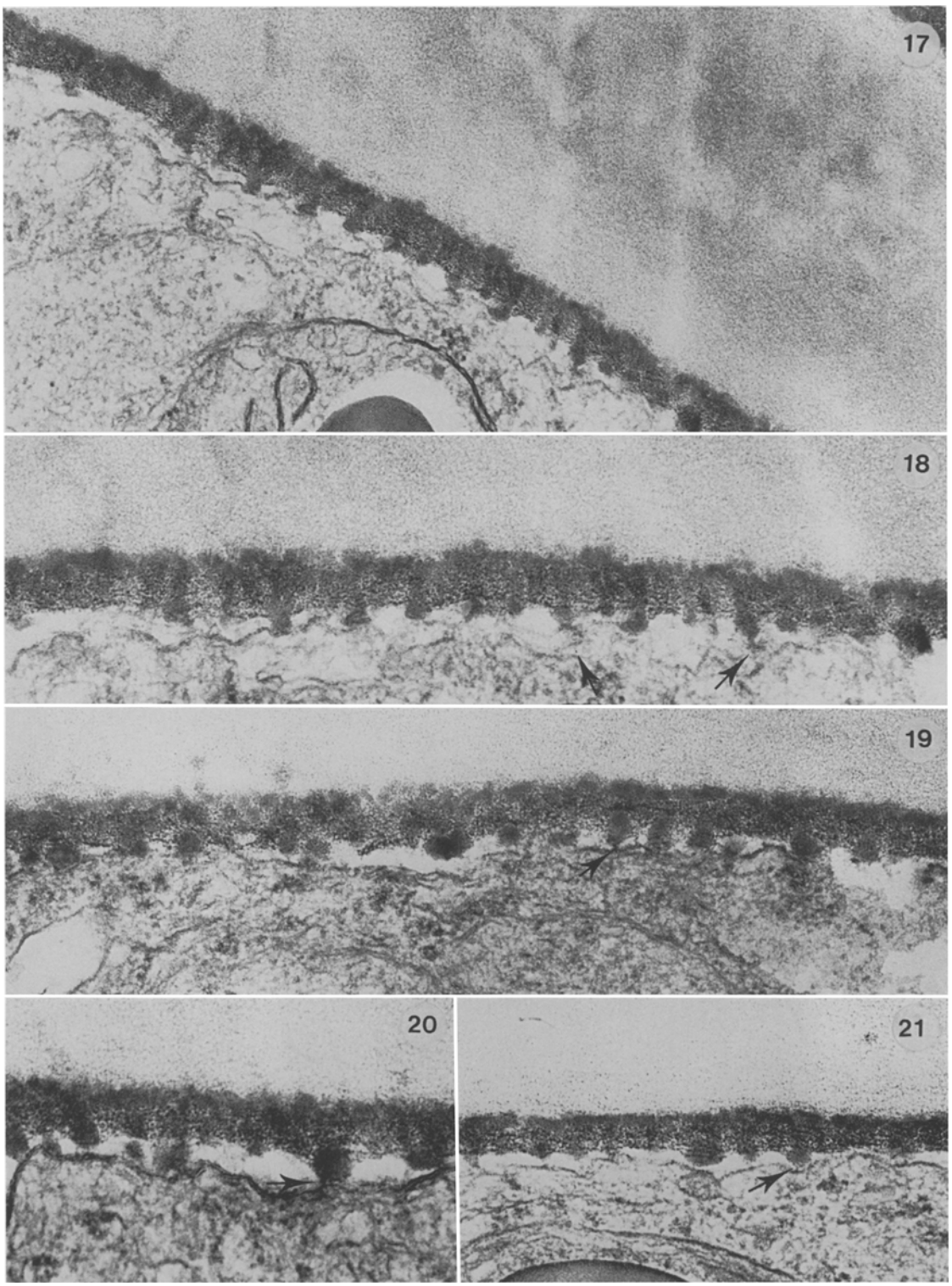


Figs. 11–14. *Nymphaea mexicana*. Microspores in middle tetrad stage. – Fig. 11. Distal surface with two layers of surface coating: a homogenous layer (arrow) and a fibrillar inner layer (arrowhead). × 42000. – Fig. 12. Proximal surface with numerous radial rod-like elements (arrows). Arrowheads show rod-like structures in the periplasmic space. × 50000. – Fig. 13. Slightly tangential section with rod-like elements appearing as small discs (arrows). × 35500. – Fig. 14. Section close to the pro-aperture. Note thick surface coating at the proximal border of aperture site (arrow). × 42000



Figs. 15, 16. *Nymphaea mexicana*. Late tetrad stage. – Fig. 15. General configuration of the tetrad inside the callosic envelope. SEM, × 1160. – Fig. 16. Two adjacent microspores. The cytoplasm contains dictyosomes, numerous vesicles, plastids with starch grains, and endoplasmic reticulum. Plasmamembrane surface coat densely stained. Arrow shows a pre-aperture site. × 10700

Figs. 17–21. *Nymphaea mexicana*. Late tetrad stage. – Fig. 17. Dumb-bell-shaped procolumellae in the proximal surface coating. × 36000. – Figs. 18–21. Sections showing basal parts of procolumellae attached to the plasmamembrane. Tiny elements connect the plasmamembrane with the procolumellae (arrows). Figs. 18–20 × 54000, Fig. 21 × 58000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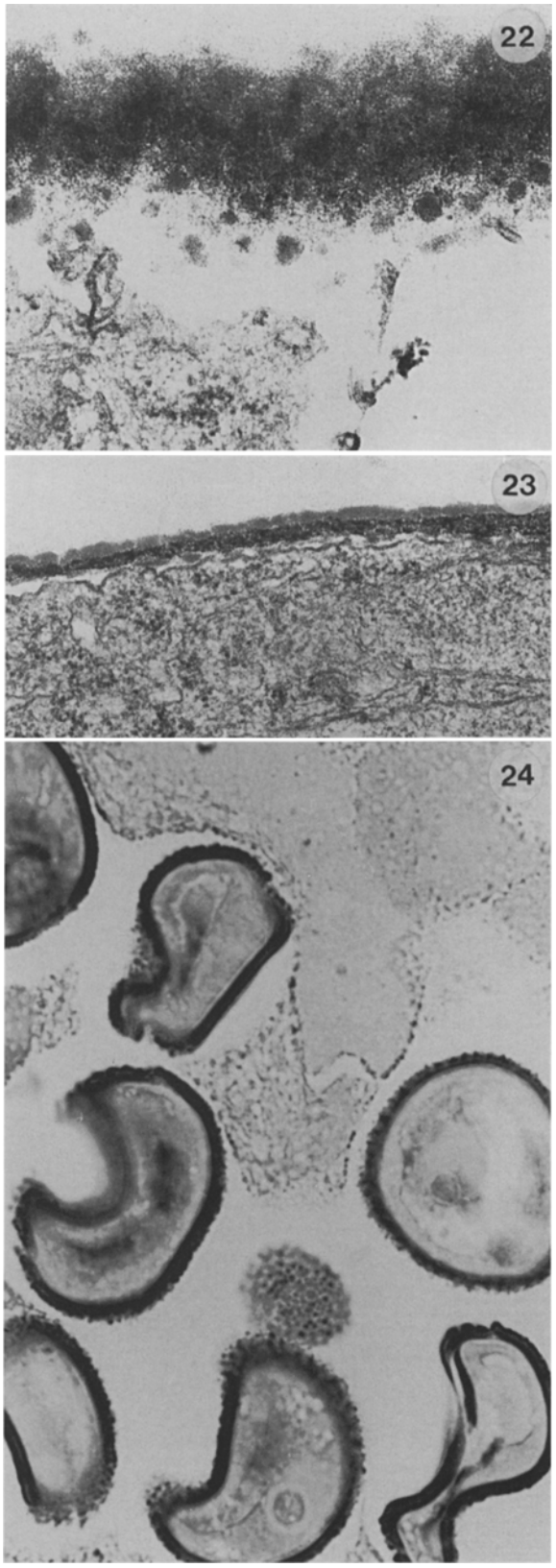
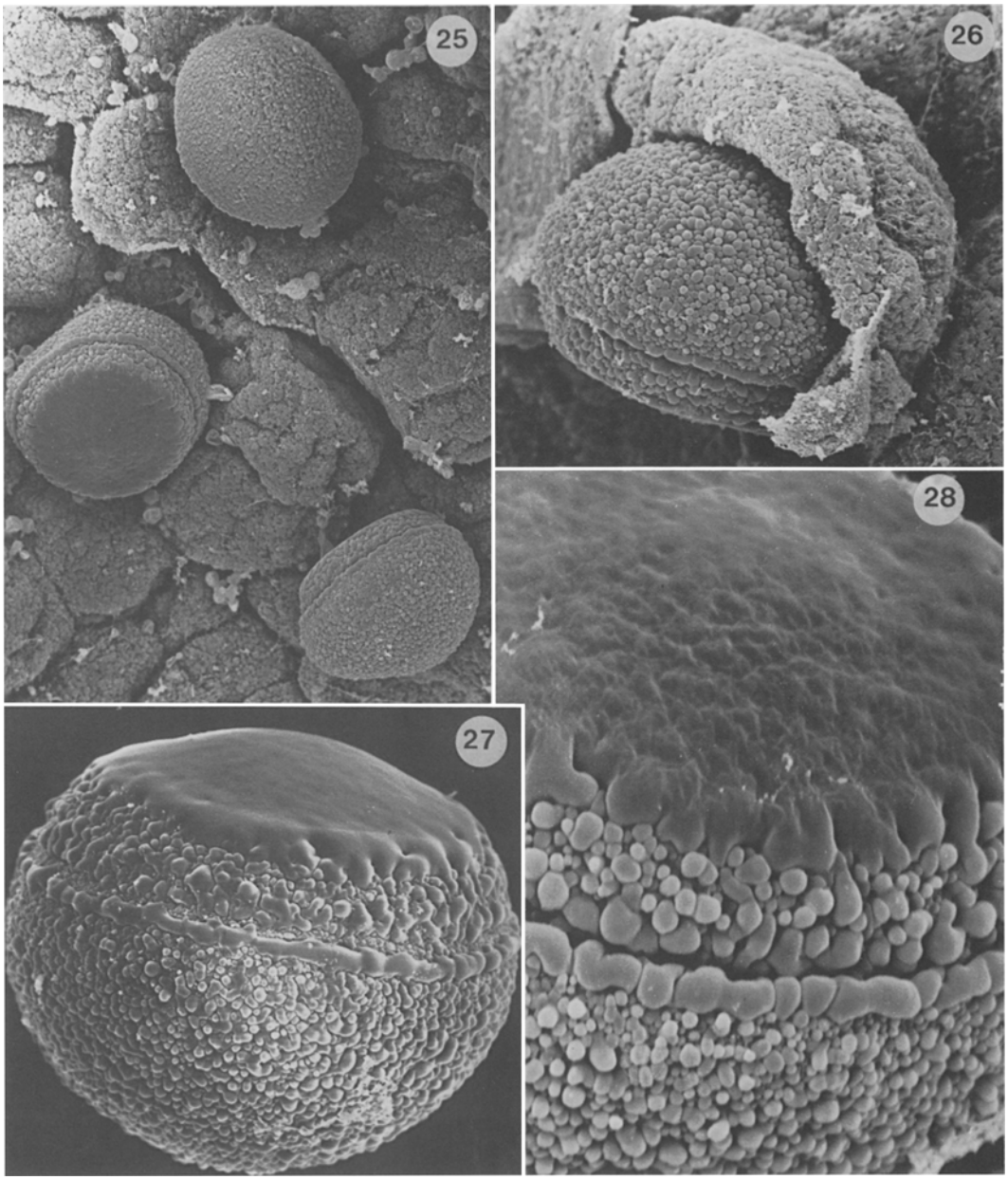


Figs. 22–24. *Nymphaea mexicana*. – Fig. 22. The procolumellae have increased in diameter (cf. Fig. 12) and appear ± circular; tangential, oblique section. × 38500. – Fig. 23. Distal region. Note different electron density of the two layers outside of plasmalemma. × 33700. – Fig. 24. Tapetal cells protruding between the free microspores. LM, × 960



Figs. 25–28. *Nymphaea mexicana*. Free microspore stages. – Fig. 25. Free microspores and tapetal cells. SEM, × 1170. – Fig. 26. A microspore in close contact with tapetal cells. SEM. × 2150. – Fig. 27. A microspore with smooth distal surface and verrucate proximal surface. SEM. × 2240. – Fig. 28. Details of distal surface and aperture region. SEM. × 5400

plasmalemma invaginations take place, procolumellae look to be stretched and connected with plasmalemma by fine thread-like elements (Fig. 18) and with the cytoplasm by microfilaments (Fig. 19). These fine elements are seen either as the axes of procolumellae (Fig. 18), or as the axes of spiral-like units not embedded into sporopollenin. Oblique section shows small discs of cross-sectioned procolumellae (Fig. 22). The distal part of the developing primexine preserves its two-layered character (Fig. 23) with sporopollenin accumulated only on the surface of the glycocalyx.

Free microspore stage. Figures 24–27 show the general configuration of free microspores and tapetal cells. The tapetal cells do not remain in a parietal arrangement as they were in late tetrads, but they protrude and expand between the free microspores (Fig. 24). Figure 26 shows a microspore "nested" into tapetal cells. The distal pole of the pollen grain is flattened and almost smooth, whereas the proximal pole is covered with verrucae (Figs. 27, 28). The anazonasulculus is not equatorially located but is closer to the distal pole (Fig. 27). The proximal margin of the aperture is beset with large verrucae which vary in shape; the verrucae at the distal margin are tooth-like (Fig. 28). The proximal pole of the microspore is convex and the distal one concave. The exine ultrastructure of the two pole regions is obviously different.

In the distal region, the endexine lamellae are loosely arranged (Fig. 32), in the proximal one they are tightly pressed to each other (Fig. 29) and appear as white lines. The infratectal region (which looks granular because of the wavy arrangement of the short columellae) and the thick foot layer of the proximal region (Fig. 29) are absent in the distal one (Fig. 32). The tectum is thick, verrucate in the proximal region (Fig. 29) and non-verrrucate in the distal region (Figs. 32–34).

The periplasmic space is persistently present in all regions of the developing sporoderm (Figs. 29, 32, 33, and 35), as it was earlier in development. Well-discernible radially oriented structures are observed under the endexine lamellae. In places where the plasmalemma is invaginated (Fig. 29: proximal pole) and in the distal pole (Figs. 32, 33) these radial structures are seen especially well. Figures 32, 33, and 35 show a row of "helicals" arranged like a fringe. Thin radial structures which are probably the same helical units, but considerably stretched, go through the whole layer of endexine lamellae, being slightly wavy and arranged perpendicularly to the lamellae (Fig. 34). Moreover, in the periplasmic space low-contrasted parts of these spring-like units are discernible (Figs. 32, 35).

Bi-cellular pollen grain stage. At both the proximal (Fig. 30) and the distal pole (Fig. 31) the layer with the procolumellae becomes more distinct. It appears

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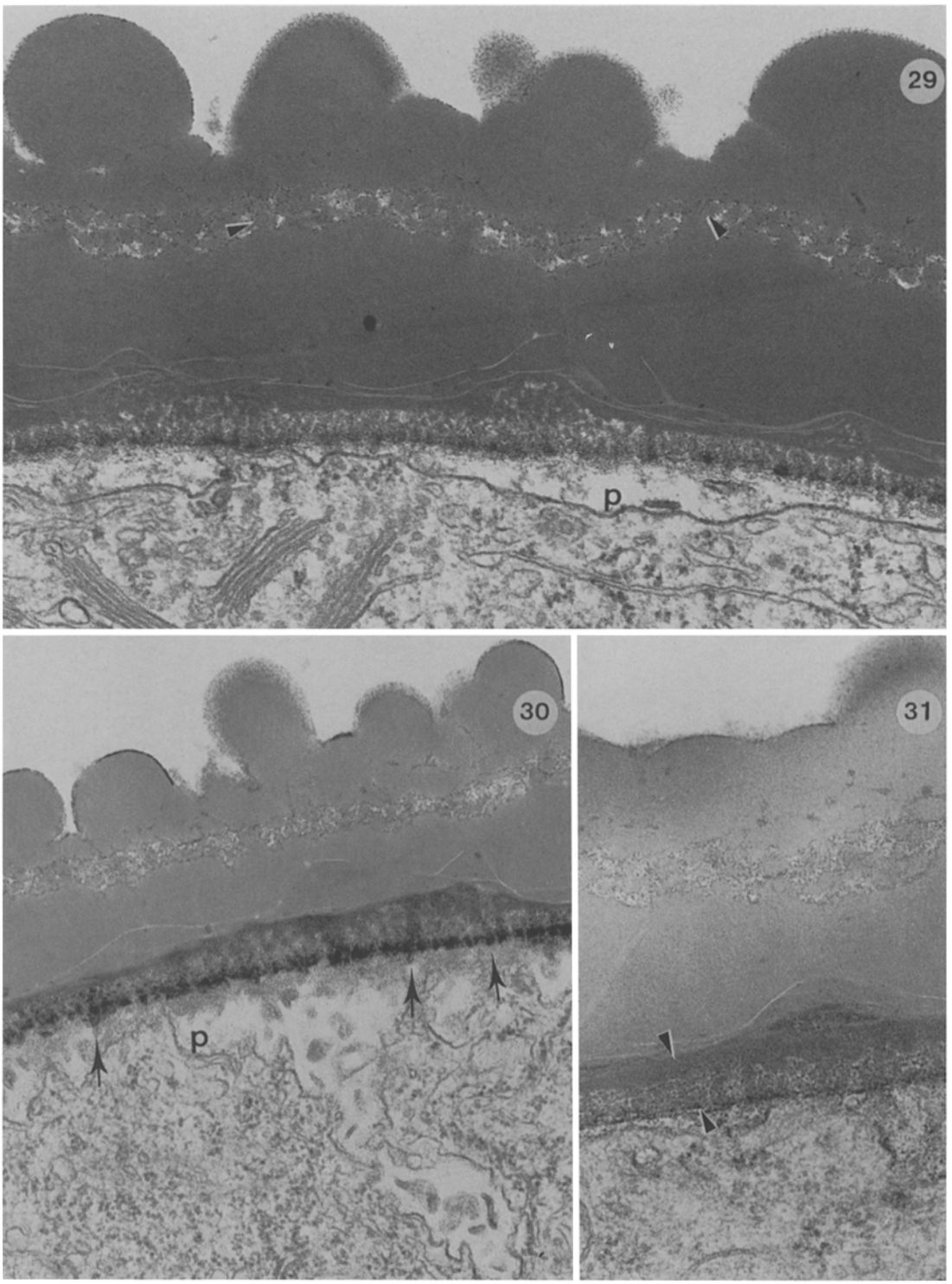
Figs. 29–31. *Nymphaea mexicana.* – Fig. 29. Free microspore stage. Proximal region of a microspore with verrucate tectum, irregular shaped columellae (arrowheads), thick foot layer, and compact lamellae of the endexine with white line centres. Note numerous radial spirals at the inner surface of the endexine and invaginated plasmalemma (p). × 43100. – Figs. 30–31. Pollen grain stages. – Fig. 30. Section in bi-cellular pollen grain stage showing proximal site of the grain. The radially oriented elements at the inner surface of endexine become more evident and they have electron dense inner ends (arrows). × 34500. – Fig. 31. Detail of the proximal site to illustrate the compound layer developed at the inner surface of the endexine (arrowheads). × 51800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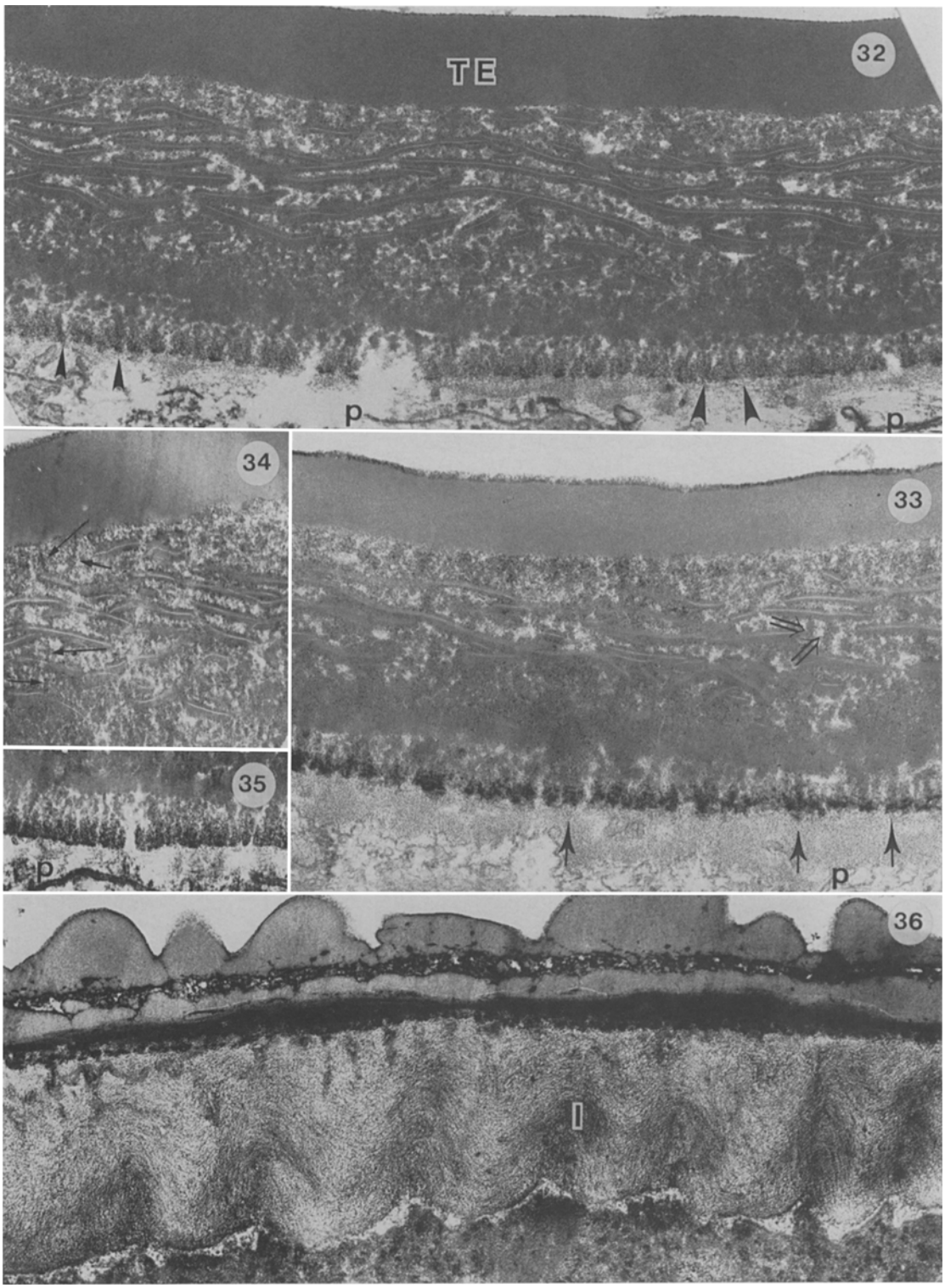
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as a tectate-columellate pattern (Fig. 30) and develops on the spring-like units seen in the periplasmic space during the previous stage. The distal portion of this layer appears homogeneous and irregular in thickness and consists of a tectum-like portion and columella-like radial elements intermingled with fine fibrillar material. The proximal surface of this layer is thin, fibrillar and electron-dense. The highly undulating plasmalemma probably indicates the beginning of the intine formation (Fig. 30). Later the intine becomes very thick. It consists of wavy microfibrils and is crossed by numerous membranous channels (Fig. 36).

Discussion

Cell surface activities. Sporogenous cells ready to transform into microspore mother cells with subsequent meiosis and acquiring a callosic wall undergo some conspicuous changes, e.g., the formation of multiple convoluted parts of the plasma membrane. These folded parts are seen as multivesicular structures within plasmalemma pockets, known earlier as plasmalemmasomes. They could be reserves of the plasma membrane necessary for the enlargement of the sporogenous cells. They are also known to have a functional role in solute transport (Harris & Chaffey 1986). In any case, these cell surface irregularities indicate that active processes are taking place between sporogenous cells and cells of the anther wall. After meiosis and development of the callosic envelope such activities probably continue, because callosic wall is not an obstacle to substance movements (e.g., Rowley & Dunbar 1970, Dunbar 1973, Mascarenhas 1975, Gabarayeva 1992, Rodríguez-García & Majewska-Sawka 1992, Gabarayeva & Rowley 1994).

We observed that cell surface activities continue after meiosis when the tetrads are enveloped in callose. Many irregularities and also plasma membrane invaginations of developing microspores suggest that transfer of substances through the plasmalemma. We can not point out precisely the direction of substance traffic without tracer experiment, but general knowledge (see above) allows to conclude that bi-directional flow exists.

Radially oriented elements. Tangentially oriented thin fibrils appear first at the microspore surface, and then radially oriented elements are discernible on the microspore plasma membrane very early in the development. At the initial middle tetrad stage they are seen well: they have become longer and begin to accumulate a lipoidal substance, probably sporopollenin. These two components of the glycocalyx – tangential fibrils (primexine matrix sensu Heslop-Harrison 1968) and radially oriented rod-like elements (tufts sensu Rowley & Dahl 1977) are probably constant constituent elements of microspore surface coating. At the advanced

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Figs. 32–36. *Nymphaea mexicana*. Pollen grain stages. – Fig. 32. Part of distal surface of pollen grain wall with smooth tectum (TE) and endexine with numerous loose lamellae. The foot layer is not obvious. Note radially oriented elements beneath the endexine (arrowheads). × 38900. – Figs. 33–35. Distal part of pollen grain wall. The inner radially oriented elements are clearly seen (arrows). They extend through the endexine between the lamellae (double arrows in Fig. 33, and tiny arrows in Fig. 34). Periplasmic space (p). × 43200. – Fig. 36. Thick intine (I) fully developed. Note numerous electron dense membranous units ("channels") embedded in the intine. × 30300

middle tetrad stage, radially oriented elements are dumb-bell-shaped due to accumulation of lipoidal globules on their distal and basal ends. Lipoidal globules or similarly shaped concretions were observed during sporoderm development in many taxa (DICKINSON 1976 a, b, 1982; HESSE 1985; EL-GHAZALY & JENSEN 1990; EL-GHAZALY 1990; DAHL & ROWLEY 1991; ZAVADA & GABARAYEVA 1991; ROWLEY 1992 b; TAKAHASHI 1992, 1993; GABARAYEVA 1995) and were interpreted as nutritive lipids or sporopollenin precursor. We consider the latter more likely, since recent data indicate the lipid nature of sporopollenin precursors (WILMESMEIER & WIERMANN 1994). The lipoidal precursor are first seen on the ends of rod-shaped exine units where they form a continuum of hemispheroids (HESSE 1985, DÍEZ & FERNÁNDEZ 1989, DAHL & ROWLEY 1991, HARLEY 1991, SAHASHI & al. 1991). These lipoidal globules were also observed in *Nymphaea colorata* in connection with radial spiral elements of the glycocalyx (GABARAYEVA & ROWLEY 1994).

The distal surface of the *Nymphaea mexicana* pollen grain is smooth, and similar to the grain surface of *Nymphaea colorata* which is psilate. In early developmental stages the plasmalemma at the distal face of *Nymphaea mexicana* microspores is coated with a thin glycocalyx layer. In the aperture region the glycocalyx layer is absent unlike in *Nymphaea colorata*, where an evenly thick glycocalyx layer was observed over the whole microspore surface (GABARAYEVA & ROWLEY 1994).

We assume that the observed periplasmic space, which is probably increased by fixation, exists nevertheless as a real and necessary space for the concentration of new formative processes.

The ends of the radially oriented elements that appear during the tetrad stage are probably responsible for selective accumulation of sporopollenin and for the formation of the hemispherical verrucae. A similar formation of a verruca-like tectum surface was observed in *Calluna* (DAHL & ROWLEY 1991) where glycocalyx in supratectal sites was considered to accumulate sporopollenin and resulted in a thicker tectum.

An interesting point is how many radial elements participate in the formation of one verruca. Simple calculation of the number of radially oriented elements on a section at middle tetrad stage, and the comparison of it with the number of verrucae on a section at free microspore stage made at the same plane, show the relation between radial elements and verrucae approximately as 6 : 1. It means that three-dimensionally one verruca is based on ca. 36 radially oriented elements.

Three constructive elements are constantly observed in developmental ultrastructural studies of exine substructure: fibrillar network (HESLOP-HARRISON 1968, DICKINSON 1976 a), radially oriented elements (ROWLEY & FLYNN 1968; HIDEUX 1981; ROWLEY 1981, 1990; EL-GHAZALY 1982; HIDEUX & ABADIE 1985; GABARAYEVA 1994, 1995), and globular structures (DICKINSON 1967 b, 1982; HESSE 1985; SOUTHWORTH 1986; EL-GHAZALY & JENSEN 1987; ROWLEY & al. 1992 a). We consider that fibrillar network and radially oriented elements coexist and are not antagonistic. We may suggest that even such profound differences in exine structures could be a result of different distribution of spherical sporopollenin precursors. The diversity of sculptures in pollen grains has, in our view, the same roots, but the developmental process is still obscure.

Tapetum. Tapetal cells do not retain their initial parietal location and penetrate

between tetrads during the middle tetrad stage. In a later tetrad stage they retreat to the initial position, but at free microspore stage they invade again the centre of the loculus as bridges and ingrowths. In mature pollen grains the final position of the tapetum incrusted with orbicules is parietal again. Such extraordinary behaviour of the tapetum was also observed in *Nymphaea colorata* (GABARAYEVA & ROWLEY 1994). The penetration of the tapetal cells inside the loculus at early vacuolate stage and their retreation to parietal position was observed also in *Catharanthus roseus* (L.) G. DON (EL-GHAZALY & NILSSON 1991).

Conclusions

1. The basis for future ect- and endexine structure is formed by radially oriented units which apparently serve as a canvas or as a framework for the appearance of the exine pattern.
2. The diversity of exine patterns appears not on the level of its substructure itself, but on the level of the selective capacity of units of this substructure to accumulate sporopollenin.
3. The behavior of the tapetal cells during microspore development is unusual. The tapetum invades at least twice the loculus and then retreats to the initial parietal position.

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