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ORIGINAL ARTICLE

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Stages in development of *Selaginella diffusa* megaspores

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Abstract In mature megaspores of *Selaginella diffusa* (C. Presl) Spring the units of the exospore are ordered and become unordered toward the outer and inner surfaces. The exospore surface is coated with silica at maturity. The insertion of the future gap begins in early stages with formation of many minigaps within the inner part of the exospore distally. The mesospore, like the exospore, is resistant to the acetolysis reaction and can, thus, provisionally be considered to consist of sporopollenin. Unit structures within the outer part of the mesospore are unordered, but become ordered in the middle and inner parts. The inner surface of the mesospore appears verrucate. In maturing megaspores, the mesospore is mostly disintegrated and the inner exospore, which encapsulated the mesospore, remains as a somewhat isolated structure, and is again near the outer exospore. There are connecting strands across the gap between the inner surface of the outer exospore and the surface of the inner exospore. There are also spheres on the outer surface of the inner exospore.

Key words Development · Exospore · Megaspores · Mesospore · *Selaginella* · Ultrastructure

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G. El-Ghazaly, our dear friend and collaborator, died on 13 January 2001. We will miss him very much.

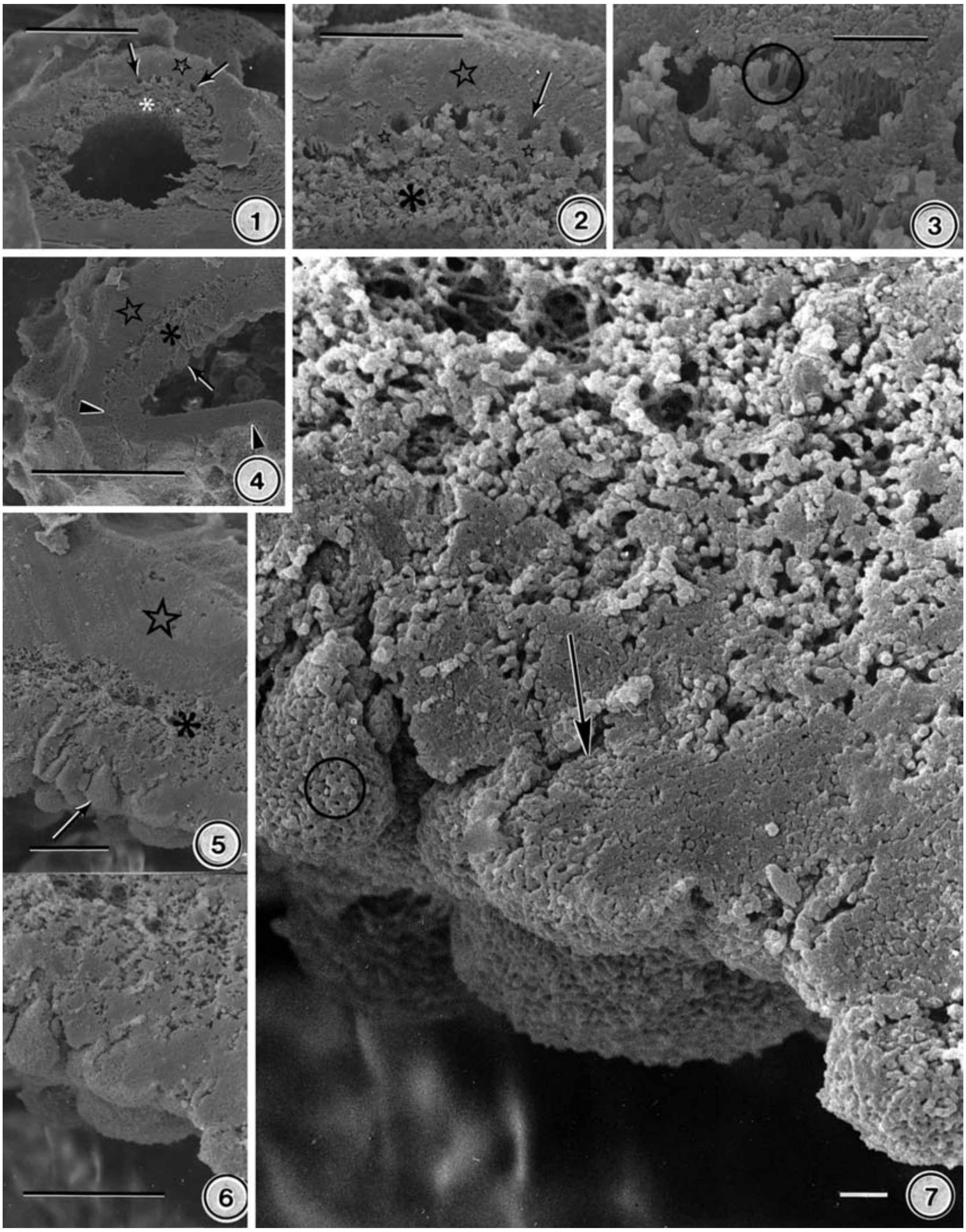
Introduction

Megaspore development in *Selaginella diffusa* was previously studied by Morbelli and Rowley (1999). They reported the characteristics for the middle stages for this species. The megaspores are large with reticulated surfaces, very thick walls and apertures with high laesurae. The exospore has an ordered pattern.

Campbell (1902), Denke (1902), Fitting (1900) and Heinsen (1894) cited extension of elements of the structure across the gap. Lyon (1901, 1905) considered the gap as a functional part in megaspore development and this was supported by the findings of Rowley and Morbelli (1993, 1995) with respect to the presence of lipoidal material and polysaccharides within the gap. Rowley and Morbelli (1993, 1995) showed rod-connections between the outer and inner exospore across the gap in middle stages of development in *Selaginella argentea*.

The fine structure of gap formation early in development has been studied in megaspores of *Selaginella argentea* by Morbelli and Rowley (1999). Using living megaspores in the middle stages of development they found the periodic acid-Schiff reaction for carbohydrates positive for the gap region in *S. bigelowii* and *S. argentea*. Morbelli and Rowley (1999) stained living megaspores of *S. diffusa* with Sudan black-B resulting in a strong sudanophilic reaction in the region of the gap indicating the presence of lipids. After loss of the mesospore (the stages of this loss are illustrated by Morbelli and Rowley 1999), the central lumen of megaspores of *S. bigelowii* and *S. kraussiana* was found to be filled by material that presumably had been stored in the gap and transported from it to the central lumen (Rowley and Morbelli 1995; Morbelli and Rowley 1999). The so-called minigaps, referred to in the text of this paper, are considered to be the result of exospore expansion.

Our aims were to analyse stages in megaspore development of *Selaginella diffusa* from an early stage to maturation to see if rod connections across the gap could be stablized in TEM and SEM preparations, as they were in *S. argentea and S. kraussiana* (Rowley and Morbelli 1995;



Figs. 1–7. SEM illustrations of stages in development of the gap

Figs. 1,2. There are many minigaps (*arrows*) in the inner part of the exospore. The enlargement of this region in Fig. 2 shows that the minigaps are within the exospore. *Stars* indicate the distal part of the exospore. Both figures show that the mesospore (*asterisk*) is morphologically distinct from the exospore. *Bars* 20 μm

Fig. 3. In this enlargement of the minigap region in Figs. 1 and 2 the exospore unit structures can be seen to be rods (*circled*) that extend generally perpendicular to the surface of the transverse section plane. *Bar* 5 μm

Fig. 4. A section that includes equatorial/distal (*left and top portion*) and proximal areas (between arrowheads); there are minigaps forming in the former but few in the proximal region. On the surface of the exospore (*star*) the framework for silica deposition is evident and has a foam-like aspect. The inner surface of the mesospore (*asterisk*) in this early stage of development, prepared in the living state, has many rounded (verrucate) structures (*arrow*). *Bar* 10 μm

Fig. 5. Enlargement of equatorial/distal portion of Fig. 4. The sectioned surface extends from the foam-like coating on the exospore (*star*) and mesospore (*asterisk*) to the inner verrucate-appearing surface of the mesospore (*arrow*). *Bar* 20 μm

Fig. 6,7. Enlargement of the mesospore and its verrucate-appearing inner surface. Fig. 7 illustrates the complex arrangement of structural units (several are in the *circle*) on these verrucae. Some internal structure is exposed in a fractured verruca (*arrow*). The internal structure appears to have the ordered arrangement of units typical of the outer portion of the exospore in *Selaginella diffusa*. *Bars*: 20 μm in Fig. 6; 1 μm in Fig. 7

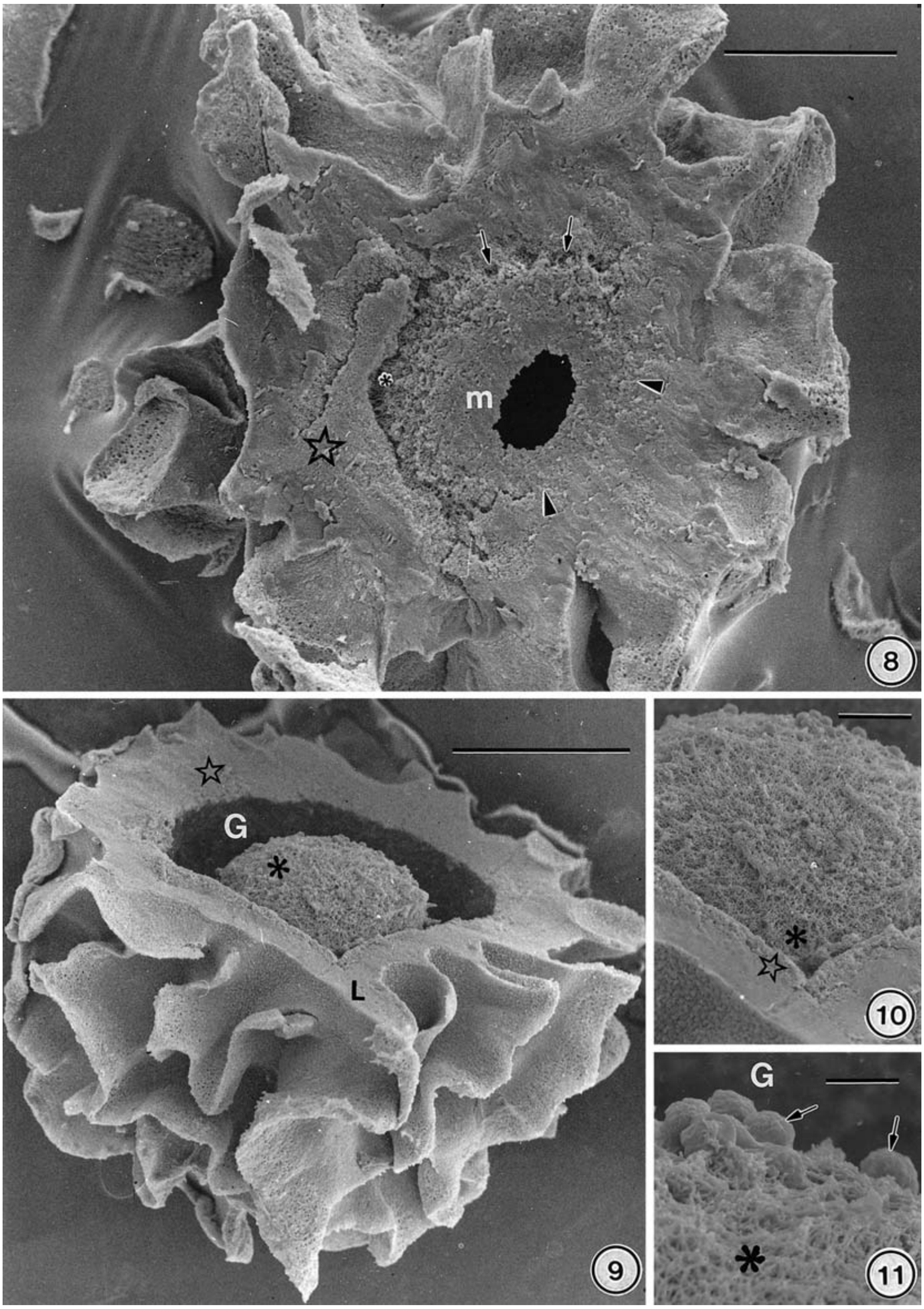


Fig. 8–11. SEMs of more advanced stages than in figures 1 to 7

Fig. 8. There are some minigaps (*arrows*) in the equatorial region, but in the distal region much of the inner exospore and mesospore (*m*) is separated by the gap (*asterisk*) from the bulk of the exospore (*star*). There is no gap across the proximal area (between *arrowheads*). The massive configuration of the exospore and its foam-like coating is emphasized in this oblique exposure. *Bar* 100 μm

Fig. 9–11. SEMs of middle stages

Fig. 9. Nonmedial section passing through one arm (*L*) of the triradiate aperture. The outer exospore (*star*) is separated by the gap (*G*) from the inner exospore (*asterisk*) that envelopes the mesospore. *Bar* 200 μm

Fig. 10. In this enlargement of the section in Fig. 9 the structural of units at the inner surface of the outer exospore (*star*) correspond with the structure of the inner exospore (*asterisk*). *Bar* 20 μm

Fig. 11. The surface of the inner exospore (*asterisk*) facing the gap (*G*) has several spheroidal structures (*arrows*). *Bar* 5 μm

Morbelli and Rowley 1999). Further, we wanted to test the resistance of the mesospore during young and middle stages to the acetolysis reaction. We wanted also to see if the mesospore, which is exceptionally thick in *S. diffusa*, was absent in mature, pre-germination stages, as it is in other species.

Materials and methods

Fresh material was collected from greenhouses at the Botany Department, Stockholm University. These were sputter coated with gold/paladium.

In preparation for exposure to the Erdtman (1960) acetolysis method, living megaspores were cut in half along selected planes. One half was sputter coated as above and the other half was dried in acetone and acetolysed for 4 min while another megaspore, sectioned with a razor blade, was acetolysed for 12 min at 100°C before washing and sputter coating.

Examination and micrographing was by a Cambridge Stereoscan 600 or JEOL-6300 scanning electron microscopes (the latter at 5 kV).

Results and discussion

The initiation of the future gap in *Selaginella diffusa* begins with the formation of many minigaps within the inner part of the distal exospore. This is comparable with our finding for *S. argentea*, based upon TEM micrographs (Morbelli and Rowley 1999). Thus, the mesospore becomes encapsulated by a separated part of the exospore in all but the proximal portion of the megaspore (Taylor 1994; Morbelli and Rowley 1999). Proximally there are few minigaps and no separation within the exospore. In this way, the future gap is limited to the equatorial and distal zones of the megaspore.

An early stage in initiation of the gap is illustrated in Figs. 1–4.

In our earliest stage of *Selaginella diffusa*, the mesospore is unordered (Fig. 2). Later in development, but before there is a definite or continuous gap, the units of the inner part of the mesospore become ordered (Figs. 4–7). The inner surface of this region of ordered units is verrucated (Figs. 5–7).

When the gap between the outer and inner exospore opens, the surface of the inner exospore can be seen to

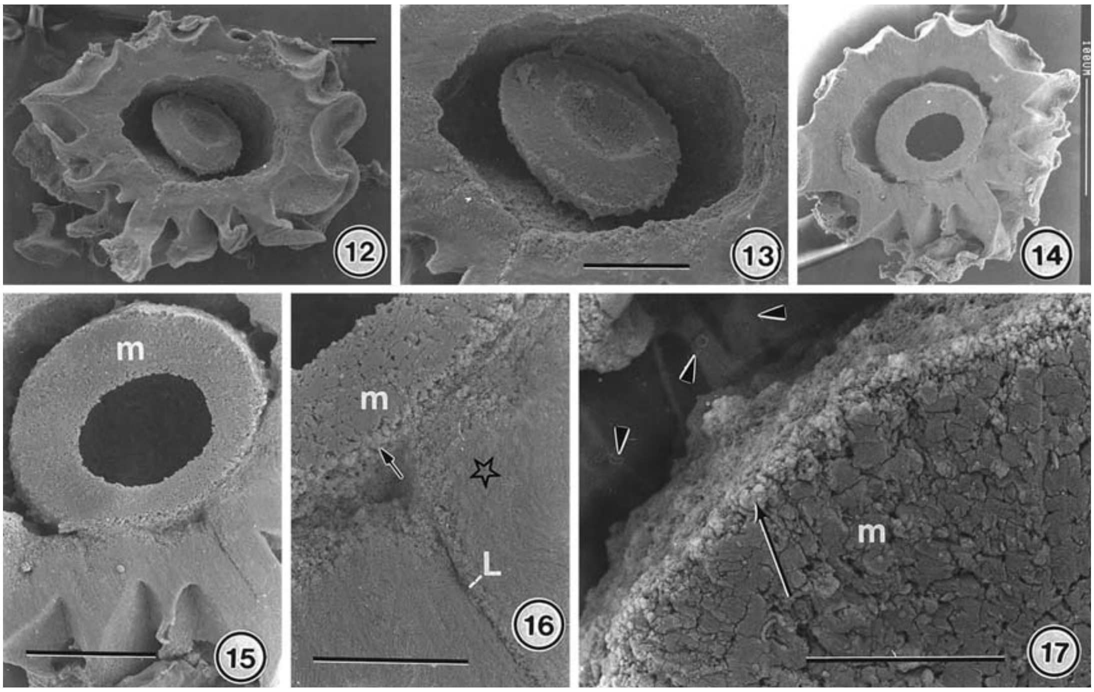


Fig. 12–17. SEMs of middle stages. The megaspore in these figures was cut in two while alive. The "half" in Figs. 12 and 13 was sputter coated soon after being cut. The part of that megaspore in Figs. 14–17 were dried in acetone then acetolyzed for 12 minutes at 100°C

Fig. 12,13. In the nonmedial sections of the megaspore the inner exospore and mesospore became detached from the outer exospore at one arm of the triradiate aperture whereas they remain attached in the "half" in Figs. 14–17. *Bars*: 100 μm in Fig. 12; 50 μm in Fig. 13

Fig. 14,15. The outer and inner exospore (see Figs. 16 and 17) and mesospore (*m*) remain intact through acetolysis. *Bars*: 100 μm in Fig. 14; 50 μm in Fig. 15

Fig. 16. Detail of a laesura (*L*) at a junction between outer exospore (*star*) and inner exospore (*arrow*). The inner exospore covers the mesospore (*m*). *Bar* 20 μm

Fig. 17. The mesospore (*m*) within the thin enveloping inner exospore (*arrow*) is low in contrast compared with the mesospore. A small edge of the outer exospore is at the *top left*. There are strands (*arrowheads*) across the gap between the outer and inner exospore. *Bar* 10 μm

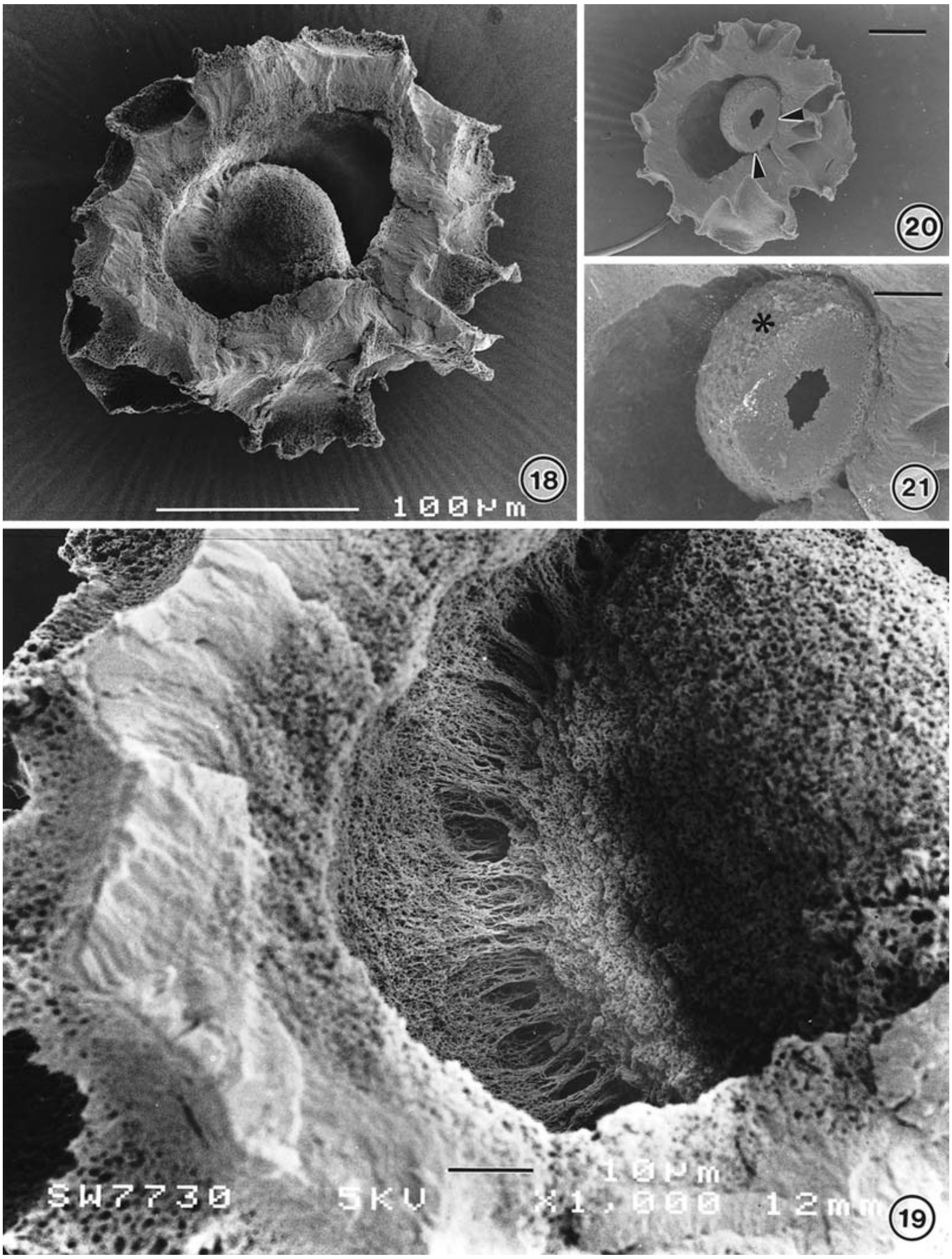


Fig. 18–21. SEMs of sections of early to middle stage megaspores which had been acetolyzed for 12 min at 100°C. Figs. 18 and 19 belong to an early stage whereas 20 and 21 are middle stages

Fig. 18. The SEM shows the mesospore encapsulated by the inner exospore. The unit-structures connect the outer exospore, inner exospore and mesospore. In an intact megaspore these units crossing the gap would number many thousands. *Bar* 100 µm

Fig. 19. An SEM of the section in Fig. 18 taken at higher magnification. It is apparent that a myriad of unit-structures cross the gap. *Bar* 10 µm

Fig. 20,21. A sectioned megaspore. The gap is large. The mesospore is thick and is encapsulated by the inner exospore (*asterisk*). The surface of the inner exospore at this middle stage is similar to that in Figs. 9, 10 and 11. The mesospore and inner exospore is only attached to the outer exospore in the region of the proximal pole (between *arrowheads*)

include units stretched between the inner and outer exospore (Fig. 8). These units are better presented in the later stages in Figs. 17–19. The great frequency of the units crossing the gap can be appreciated in Fig. 19.

The gap is confined to equatorial and distal zones of the megaspore (Figs. 8, 9, 14, 15, 18, 20, 21).

The inner exospore surface is evident in Figs. 9–11 and 16. Its surface is similar to that of the inner part of the outer

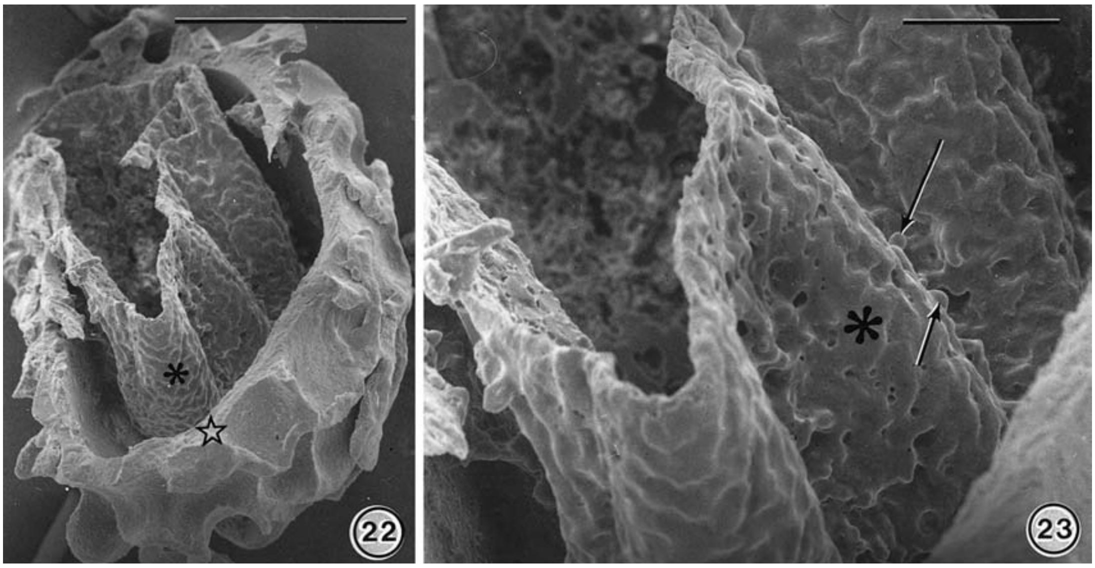


Fig. 22–23. SEMs of an almost mature megaspore. There was no acetolysis treatment
Fig. 22. The inner exospore (*asterisk*) is separated from the outer exospore (*star*) except near the laesura, at the top of the figure. A well circumscribed mesospore is not in evidence, it is degraded. *Bar* 200 μm
Fig. 23. Detail of the inner exospore surface (*asterisk*). It has perforations and spheres (*arrows*) on its surface. *Bar* 100 μm

exospore (Figs. 9, 10). The surface configuration of the outer exospore is evident on the mesospore in Fig. 21. Spheres are a common feature of the outer surface of the inner exospore (Figs. 11, 17, 20).

At maturity, the mesospore is degraded and the inner exospore occurs as a thin structure (layer) that is again adjacent to the outer exospore (Figs. 22–25). Its outer surface is rugulated with numerous perforations (Figs. 22, 23). Spheres are evident on the outer surface (Fig. 23). An enlargement of the section in Fig. 25 illustrates the ordered arrangement of units in the exospore (Fig. 26).

Surface ornamentation of mature megaspores is shown in distal (Fig. 27) and proximal views (Fig. 29). Holes in the surface of the exospore are most prominent in the muri (Fig. 28).

Middle stages (Figs. 14–21) were tested for mesospore resistance to acetolysis (early stages in Figs. 1–7 were not tested for acetolysis resistance). Some middle-stage material was untreated (Figs. 9–13) and some was treated (Figs. 14–21).

The acetolysis experiment, illustrated in Figs. 12–17, shows that the acetolysed half of the megaspore and its contained mesospore were resistant to acetolysis. Mesospore resistance to acetolysis (and also to fossilization) was also reported by Höeg et al. (1955) and Pettitt (1966).

The loss of the mesospore late in development indicates that the mesospore was lysed or digested (Figs. 22–25). This interpretation is not much in accordance with the "legendary" resistance of sporopollenin, but this loss of the mesospore occurs presumably under physiological conditions. The legendary resistance of sporopollenin is apparent after oxidation resulting from sporangium dehiscence and exposure to the atmosphere. We consider that the mesospore structures may not be resistant to acetolysis until being cut open and exposed to the atmosphere and hot acid. The basis for such an interpretation arose from a Heslop–Harrison (1968) experiment with the early microspore tetrad stages of *Lilium*. He found that the primexine had resisted acetolysis, showing that sporopollenin is present early in exine formation at a stage of development when stabilization of these early structures (often termed "pre-exine" structures) is extremely difficult in preparations for electron microscopy. Preparations in recent decades have begun with non-oxidative solvents and fixatives. We found that early exine stages were poorly represented or entirely missing without the addition of cations (e.g. ruthenium red, alcian blue) to the first or all solvents and fixatives (Rowley and Walles 1987; Morbelli and Rowley 1993; Morbelli and Rowley 1995).

Concluding remarks

- The mesospore is only present during the early and middle stages of megaspore development.
- During the early and middle stages of megaspore development, the mesospore is resistant to acetolysis.
- Rod structures between outer exospore and inner exospore are resistant to acetolysis. It is likely these rod structures are sporopollenous.
- Presumably, these rod structures are involved in nutrient transport during the early and middle stages. In other species, the entire central space within the mature megaspores is filled by nutrient material (for example *S. argentea*, *S. bigelowii* and *S. kraussiana* in Rowley and Morbelli 1995, Figs. 10–14).

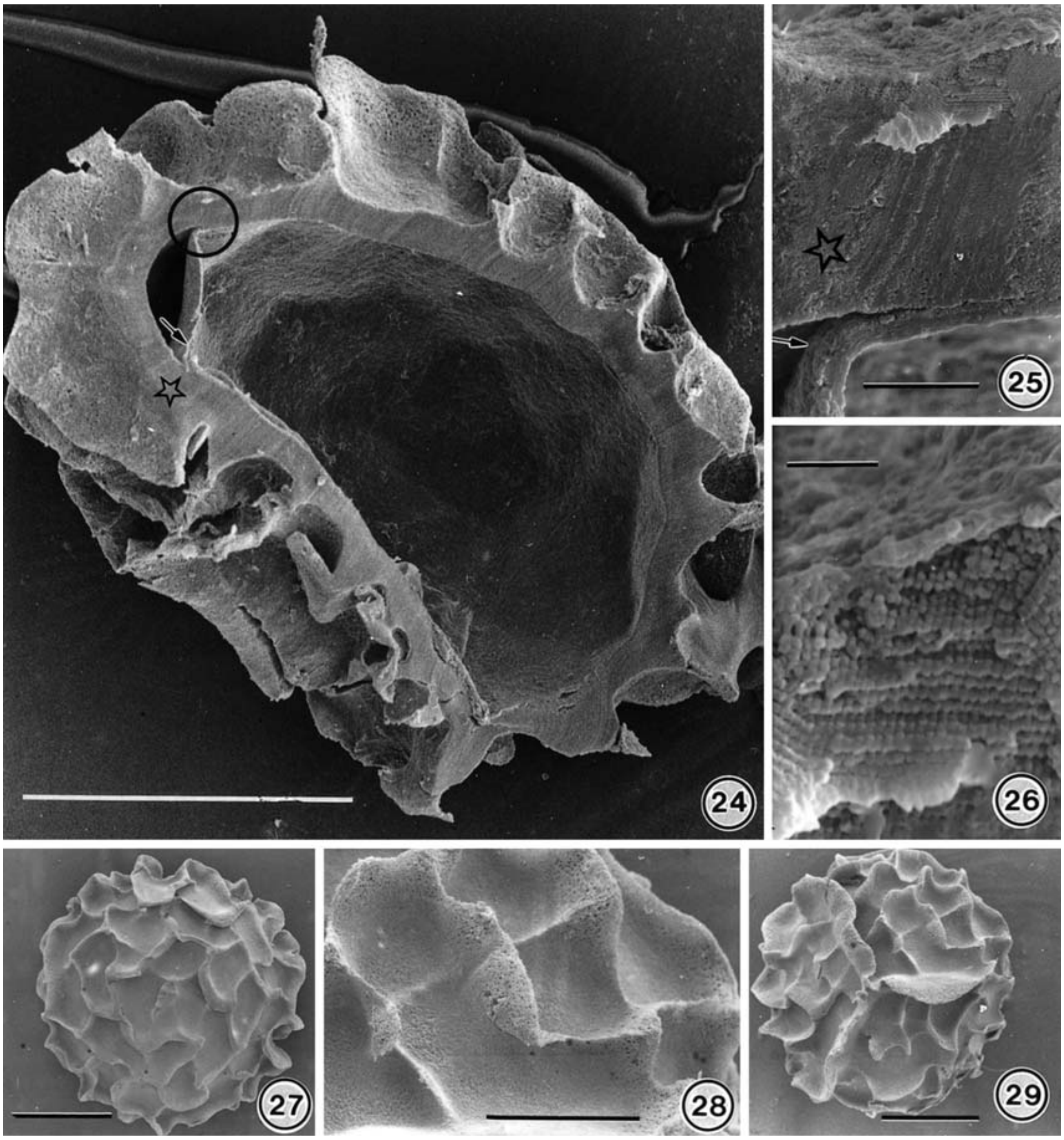


Fig. 24–29. SEMs of mature megaspores

Fig. 24. At maturity the inner exospore (*arrow*) comes again to be close to the outer exospore (*star*). In this micrograph, the only distinct separation is at the top left of the figure (*circle*). *Bar* 200 µm

Fig. 25. This figure shows the separation of the outer (*star*) and inner (*arrow*) exospore in the region circled in Fig. 24. The outer surface at the top is the coating of silica. *Bar* 10 µm

Fig. 26. The ordered exospore units of *S. diffusa* megaspores are evident in this enlargement of part of Fig. 25. There is a coating of silica at the top of the figure. *Bar* 2 µm

Fig. 27–29. SEM micrographs of intact and untreated megaspores of *S. diffusa*. The megaspores have a reticulated surface with irregular polygonal luminae. The entire surface is perforated, but perforations are larger on the sides of the high muri

Fig. 27. Distal view showing the reticulate arrangement of the high muri. *Bar* 200 µm

Fig. 28. Detail of the distal surface showing the large perforations on the sides of the muri. *Bar* 100 µm

Fig. 29. Proximal view showing a distinctive aperture of three high laesurae. The proximal faces are reticulate. The luminae are irregular and some muri are not fused. *Bar* 200 µm

- Rod structures that connect outer and inner exospore in the early and middle stages were not stabilized by our preparative methods.
- The connecting rods and the mesospore are degraded at maturity since neither rod-connections nor the mesospore are seen at maturity.

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