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Structure of wall layers in *Selaginella kraussiana* microspores (Lycophyta)

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Abstract
A similarity was found in both construction and ultrastructure between the two exospore layers in microspores of *Selaginella kraussiana*. The exospore is made up of two different kinds of rods. One of the kinds of rods are large, 100–150 nm in width, while the other are tubular rods 10–15 nm in diameter. The large rods are wider at the base of the spines than in the upper part, possibly due to flattening or compression. Both the outer and the inner exospores have a stranded surface that is very pronounced in the microspores of this species. Fibrous strands persisting the scanning electron microscope and transmission electron microscope (TEM) fixations were observed on the spore surface proximally and through perforations (exospore channel openings). This net of fibres penetrates and fills the space of the cavities within large channels through the outer and inner exospore and within the gap. According to our interpretation, these strands would be produced by the tapetum and are probably related to the nourishment of the developing microspores. Contrast varies in TEM sections after cytochemical stains, but this appears to be due to transitory substances, e.g. carbohydrates, rather than to be a substantial difference in basic composition between inner and outer exospore layers.

Keywords: Selaginella, *microspore, exospore, structure, gap, rods, channels, fibres*

Construction and ultrastructure of the microspore wall in *Selaginella kraussiana* (Kze.) A.Br. have been variously described and interpreted since the earliest studies of microgametophyte development by Millardet (1869), Pfeffer (1871) and Belajeff (1885). The latter two authors recognised that the spore has two separate walls; Pfeffer (1871) called them 'exine' and 'intine'. Heinsein (1894), in contrast, recognised three wall layers: an outer 'exine' and two inner layers termed the 'intine externe' and 'intine interne'. Stainiér (1965) demonstrated the importance of careful preparation of *S. kraussiana* microspores in her study of structure and composition of these unusual microspores at a relatively early time.

Some *Selaginella* species, as for instance *S. kraussiana*, have microspores with two sporopolleninous walls separated by a gap (Lugardon, 1972, 1990). Lugardon (1978, 1990) and Tryon and Lugardon (1991) based their conclusions for *S. kraussiana* on the different structure of the two layers, and the continuity of the outer layer over the laesurae. Morbelli et al. (2001) recognised two layers separated by a gap as a characteristic structure in microspores of *S. peruviana* (Milde) Hieron. and *S. sellowii* Hieron. The authors named these walls an outer and an inner exospore and considered the perispore as a continuous, thin layer, closely attached to the outer sporopolleninous layer.

We referred to these layers as the outer and the inner part of the exospore to express our view that both exospore layers are related structurally. The space between inner and outer exospore was termed 'the gap'. The layer on the cytoplasm is referred to as endospore (Rowley et al., 2002). The aim of our work is to study the surface and structure of the inner layer of *S. kraussiana* microspores using a scanning electron microscope (SEM) and a transmission electron microscope (TEM), in order to

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compare their characteristics with those of the outer layer and to determine if these two distinguishable layers are parts of one single wall or comprise two different walls. We will also consider the reasons for the space (gap) between the two layers.

Materials and methods

The living material of *Selaginella kraussiana* sampled here was collected in eastern Africa and cultivated in a greenhouse of the Botany Department of the University Stockholm, Sweden. Samples were filed as SV-6-00, 365. In this study, intermediate and mature stages in microspore development were examined. Fresh material for the TEM was fixed using 0.003% ruthenium red, 3% glutaraldehyde, and 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7, 48 hours, 4°C) and post-fixed with 1% osmium tretroxide plus 0.003% ruthenium red for one hour. Samples were then washed with phosphate buffer with 0.003% ruthenium red, dehydrated in an acetone series and then included in Spurr hard mixture (Spurr, 1969). Section staining was processed according to the PA-PTA-C procedure: samples were treated with 1% periodic acid (PA) for 60 minutes followed by a treatment with 0.1% phosphotungstic acid (PTA) in 10% chromic acid (C) for 35 minutes according to the procedures outlined in Roland et al. (1972). This is a simple and clean contrasting method, giving results similar to the periodic acid-Schiff (PAS) reaction of the Thiéry procedure for carbohydrates (Thiéry, 1967). The material was examined with a Zeiss EM109 Turbo TEM at the School of Medicine, Buenos Aires National University, Argentina.

For SEM study, living material from the greenhouse was used without any chemical treatment. Ten strobili of different sizes were dissected on moist butting paper under a stereomicroscope. Sixteen microsporangia in different degrees of maturation were selected from base to top of the same strobilus and dissected in order to expose their microspore contents. The microsporangia open in halves were placed on six stubs for analysis with scanning electron microscopy. The material was sputter coated with gold/palladium and studied with a JEOL JSM6300 SEM in the physiology section of the Department of Botany, Stockholm University, Sweden. Four samples were processed by a freeze-fracture and cytoplasmic maceration technique (Blackmore & Barnes, 1986). This technique gives the possibility of analysing 192 microspores per sample, resulting in a total of 3072 microspores studied. In several of these, the two walls were pulled apart due to maceration techniques; consequently the inner and outer surfaces of each wall were scanned separately.

Results

Outer exospore structure and surface

A primary finding is that the rod structure, which is such a prominent part of the surface of these microspores, not only occurs throughout the exospore but is also composed of rods of two very different sizes (Figures 1A–C, 2C). The large rods are 100–150 nm in width, while the small rods are ten times smaller, being only 10–15 nm in diameter. In cross-section, the large rods appear to be elliptic (Figure 1B); small tubular rods are more roundish in transverse section (Figures 1A–C, 2C). At the fracture site, large rods have been pulled apart (separated), while the small tubular rods extend across the fractured separation of the exospore (Figure 1A). Rods are present inside the outer exospore layer, on its inner surface as well as continuously occurring on the distal and lateral surfaces. Rods may be seen on the spines on the outer surface (Figure 3A, B) and on the inner surface (Figure 3A) on the fracture of the outer exospore that was removed from the inner exospore.

TEM thin sections reveal that the outer exospore layer shows a variety of circular sites. The most common are small tubules with a dark margin and a low density central spot. These tubules are 10–15 nm wide and run parallel with the large 100–150 nm wide rods (Figures 1A, 2C). These large rods are, to some extent, shaped or arranged like honeycombs (Figure 1C), but transverse sections of 100–150 nm diameter are very rare. The outer exospore shows also many large channels (Figures 1A–C, 2C), striations and perforations, openings of large channels on the outer surface (Figure 2B) and on the inner surface (Figure 3A).

The rods on the proximal surface are short and separated in intermediate stages of development (Figure 4C, E, F). In mature microspores, rods on the proximal surface are long, arranged close together and sheet-like in some microspores (Figures 2A, B, 3B). The laesurae in intermediate stages have many closely spaced short spines (Figure 4A–F). In intermediate stages, the rods that characterise the structure of the spines are seen to be wider than at the upper surface of the spines (Figure 4C, E, F). The rods in the outer exospore run parallel with the long direction of the outer exospore in the thin portions of the exospore and in the lower portions of spine bases. In the middle parts of the spines, the rods become canted towards a radial orientation. In the upper part of the spines, the rods are radially orientated.

In favourable views, the laesurae seem to be composed of several rods (Figure 4A, D). At the base of each laesura, there are four or five supports that are 100–150 nm wide, thus similar in width to the rods seen in the TEM fracture (Figure 1A).

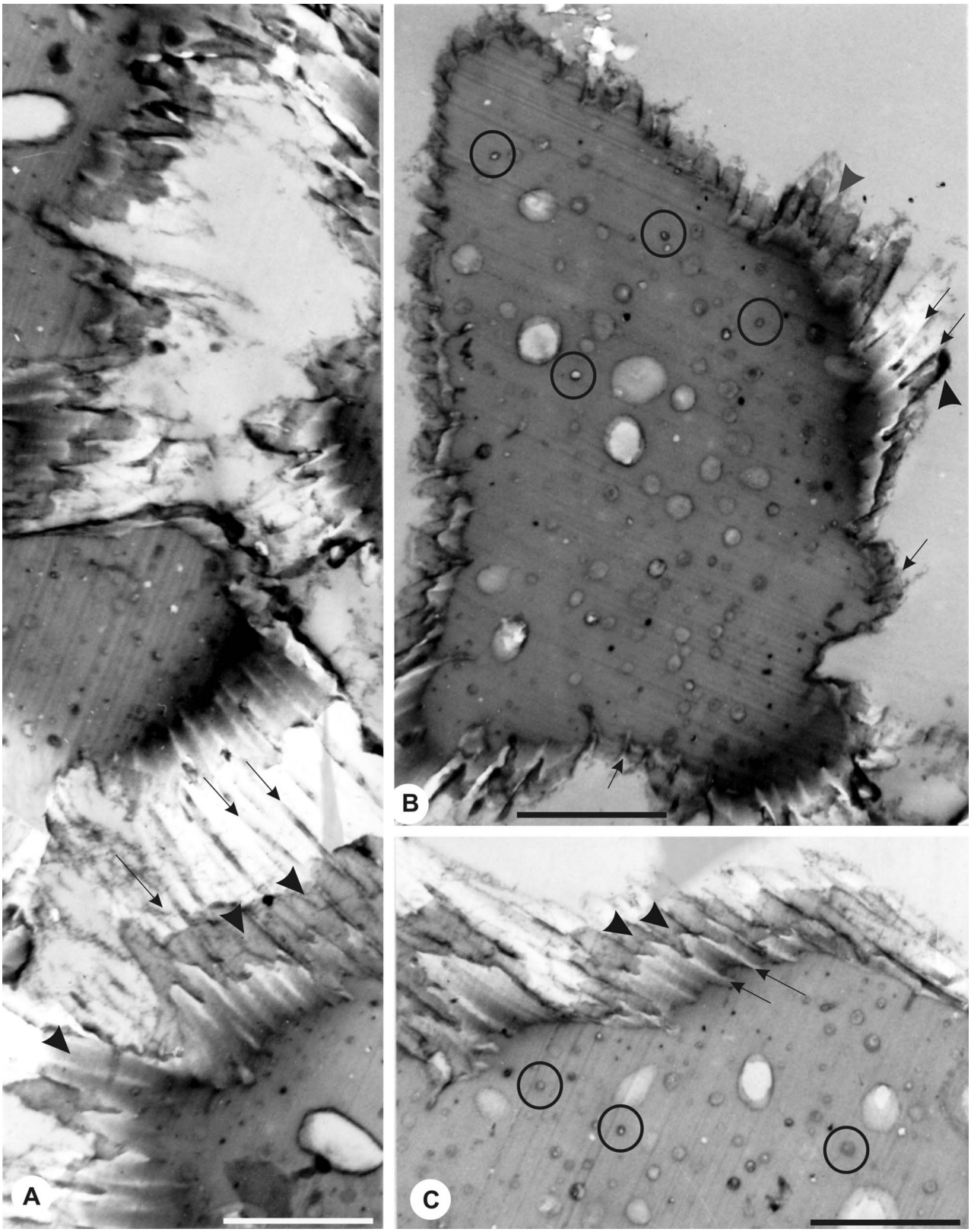

Figure 1. TEM micrographs of fractures in a microspore that separated portions of the outer exospore ultrastructure. **A.** The large rods (arrowheads) in this fracture are 100–150 nm in width, while the small rods (arrows) are tubular and 10–15 nm in diameter and can be seen in the transverse sections. The large rods have been pulled apart during the fracture but the small tubular rods extend to some extent across the fracture, suggesting that the small tubular rods are strong or elastic. **B.** Unlike (**A**), where the fractured parts remain

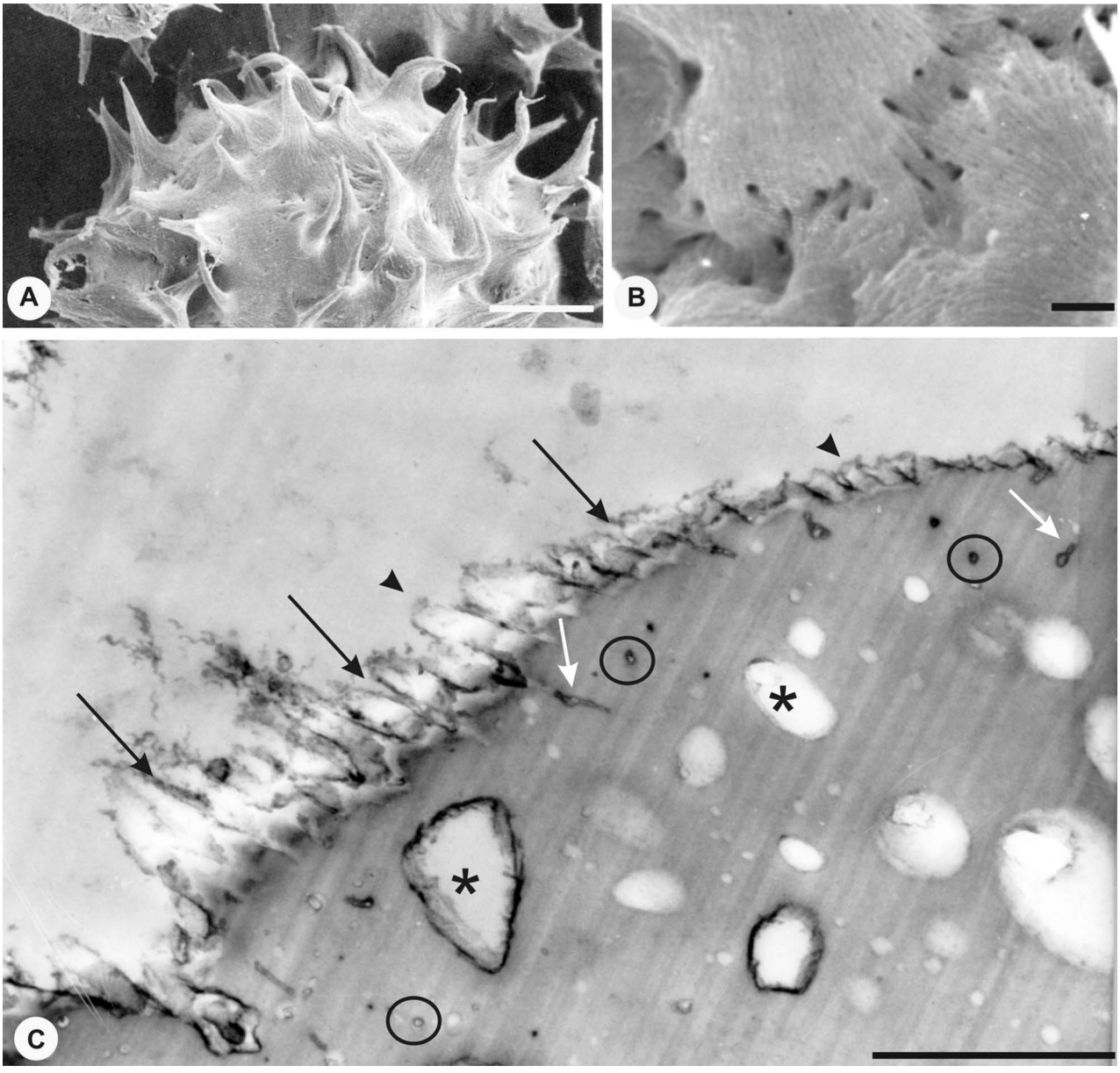

Figure 2. **A.** SEM micrograph giving an example of the rods on the spines that are typical of *Selaginella kraussiana* microspores. **B.** SEM micrograph of the basal portion of spines showing many perforations that are the openings of the large channels to the surface. **C.** TEM micrograph of the outer exospore cut very obliquely, showing the rods (arrowheads) that are prominent. Small tubules (black arrows) are seen with the rods. These tubules are also seen in profile (white arrows) and in section (circled). The asterisks indicate large channels. Scale bars – 10 μm (A), 1 μm (B, C).

Inner exospore surface and structure

The inner exospore layer may be better appreciated when it is seen without the dramatic spines of the outer exospore that dominate the character of this microspore. The microspore in Figure 3B was fractured, with the result that part of the outer exospore with spines was left intact, while the surface of the inner exospore including the laesurae is exposed at

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associated, the rods here have been pulled apart from other wall components. The large rods were 'broken off' near the exospore surface (arrowheads). Several small rods are circled; others at the surface are marked by arrows. **C.** Broken large rods are marked by arrowheads and the small tubular rods by arrows. The angles of the broken rods give a three-dimensional view of the exit of rods from the cut exospore surface. There are many cut off tubular rods in evidence (several are circled). Scale bars – 1 μm.

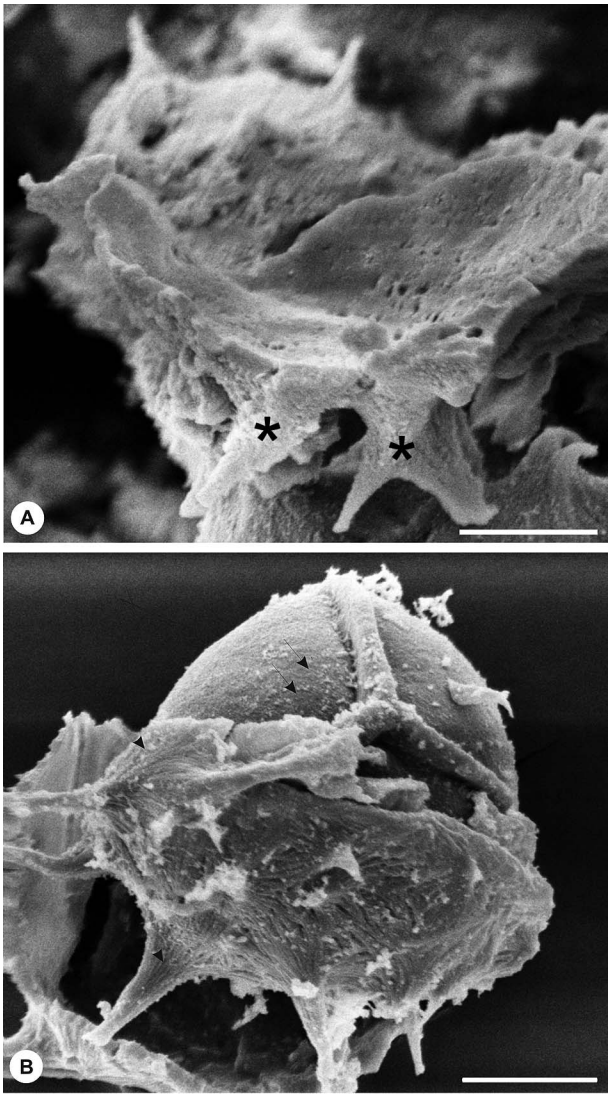

Figure 3. SEM micrographs of fractures that expose inner and outer surfaces of the exospore. **A.** The fracture exposes the inner surface of the outer exospore. There are many striations and large channels, as on the outer surface. Two outer surface spines are in evidence (asterisks) at the lower part of the fracture. **B.** The proximal fracture has part of the outer exospore removed (stripped off), thus exposing the outer surface of the inner exospore including the laesurae. The surface of the inner exospore is covered with striations (arrows) like those (arrowheads) on the outer surface of the microspore. Scale bars – 10 μm.

the upper part. The surface of the inner exospore is striate (Figure 3B), similar to that of the outer surface of the microspore.

The space between inner and outer exospore

In TEM and SEM images, a gap between the inner and outer exospore was visible in samples of both fresh and fixed microspores (see Discussion for initiation and development of the gap in *Selaginella kraussiana*) (Figures 5A, 6A–C, 7). TEM observations of fixed sections revealed fibrous, strand-like material within large channels in the outer exospore and inside the gap (Figures 5A, 6A–D). SEM observations of freeze-fractured and cytoplasm macerated microspores showed that fibrous strands were also present over channels of the outer exospore (Figure 5B). Similar strands were also observed in TEM sections of both the outer and the inner exospore layers (Figures 5A, 6A–C) and on the surfaces of both the outer and the inner exospore facing the gap (Figure 6A–D). As seen in TEM sections, these strands are composed of dark interwoven fibres. This net of dark fibres penetrates and fills the space of the cavities (Figure 6A–C).

Stain reactions of the inner and outer exospore layers and the endospore

TEM analysis of adjacent sections of the outer and inner exospore (Figure 7) from fresh microspores fixed with ruthenium red in glutaraldehyde and paraformaldehyde and exposed to the staining sequence PA-PTA-C (a stain procedure that contrasts carbohydrates) indicated that the inner and outer exospore give different levels of contrast. The contrast is much higher for the inner exospore than for the outer exospore and still higher for the endospore. The dark contrast indicates the presence of carbohydrates in the inner exospore, in the endospore and in the small tubules in the outer exospore.

Discussion

With surface features as distinct as the rods in the microspore of *Selaginella kraussiana* it is always interesting to know or wonder if such ornamentation occurs throughout the wall and on the inner surface. Our study that combines sections and fractures shows that rod-like structures like those observed on the surface of the outer exospore also occur on the inner surface of the inner exospore and that these consist of the same kind of substance as that of the outer exospore. The study also shows that there are similar striations at the outer surface and in the structure of the inner exospore. The rods constituting the prominent ribbing of the outer exospore run throughout, as also shown by Rowley et al. (2002, figures 9–15). The microspores of *S. muscosa* Spring show evidence of a rod substructure that is similar to that of *S. kraussiana*, as in Morbelli et al. (2001, figures 17, 18).

Two layered exospore in Selaginella *microspores besides* S. kraussiana

It has been shown that a two layered exospore separated by a gap characterises the microspores of

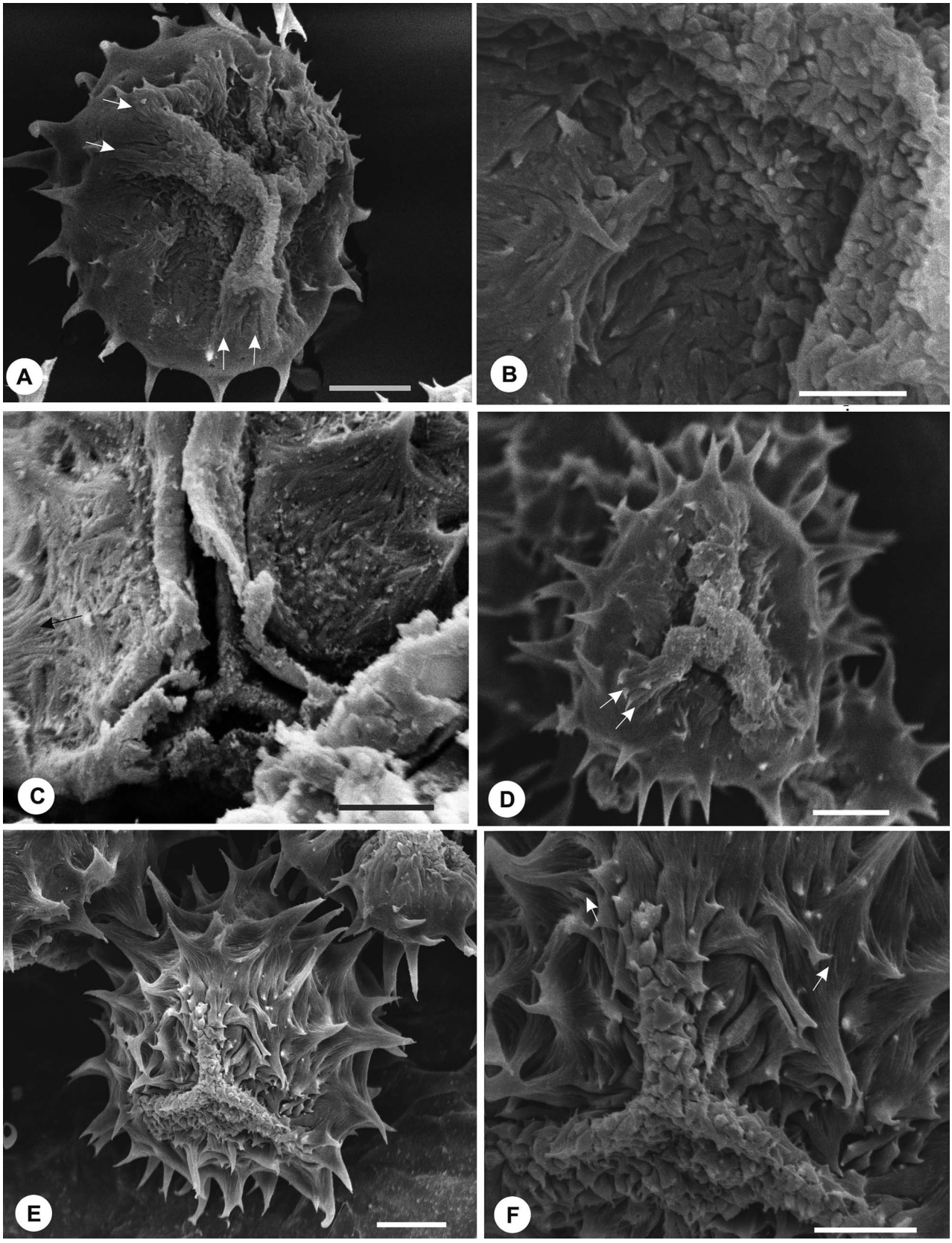

Figure 4. SEM micrographs of fresh prepared microspores from a living plant. **A.** Proximal face of a young microspore showing laesurae with many small spines and wide rods between laesurae and others leading up to each laesura (arrows). See differences in laesurae of

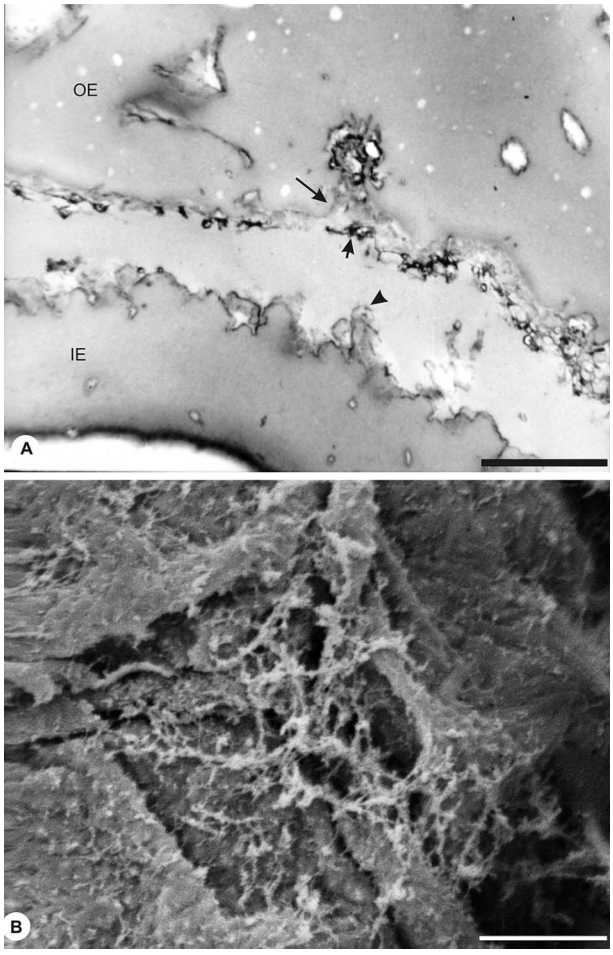

Figure 5. **A.** TEM micrograph of outer and inner exospore. The outer exospore (OE) has a large channel (large arrow) that opens to the inner surface where there are filamentous strands (short arrow). Several of the raised parts of the inner exospore (IE) show the ends of the small rods. One of these is at the top of a large rod (*c.* 100 nm, arrowhead) like those on the right side of the fracture in Figure 1B. At the central of this *c.* 100 nm wide rod there is a *c.* 10 nm wide small tubule. **B.** SEM micrograph taken on a freeze-fractured microspore. Filamentous strands like these are abundant on the surfaces in, e.g. Figure 4E. Scale bars – 5 μm (B), 1 μm (A).

Selaginella peruviana and *S. sellowii* (Morbelli et al., 2001). Morbelli et al. (2001) named these layers an outer and an inner exospore and considered the perispore as a thin and continuous wall that is closely attached to the outer layer of sporopollenin. In *S. peruviana* and *S. sellowii*, the structure of both layers was similar, but the stratification of the outer layer was different to that described by us for *S. kraussiana* microspores. Radial channels completely traversing the exospore (our inner exospore), in a similar way to the material described here, were also mentioned by Lugardon (1986, 1990) as being present in *Selaginella* microspores and Lugardon (1986, p. 256, figure 11) could prove that similar radial channels are distinct in immature microspores of *S. kraussiana*.

The differentiation between the outer and inner exospore

The gap between the inner and outer exospore is clearly evident in the material illustrated by Stainiér (1965, figure 18). The author received results that are very similar to those presented here, using the same staining method (i.e. ruthenium red) as we have done. In both studies, the inner exospore, small sites in the outer exospore and the endospore are darkly contrasted.

The differences in stain contrast within the inner exospore and endospore layers appear to be due to transitory substances such as pectins and/or carbohydrates, rather than to differences in the basic composition of the wall (Figures 6A–D, 7). This is especially the case in the endospore, which has a cytoplasmic component similar to that of the intine of pollen grains.

Material within the gap and its probable function

In a developmental study of the microgametophytes of *Selaginella kraussiana* by Slagg (1932), it was shown that a new layer had developed within the exospore. This inner exospore was not in close opposition to the 'outer exospore' from the time of its initiation. The outer exospore was at first smooth, but after it had enlarged to a diameter of about 20 μm, papillate projections formed on the outer surface. These projections grew into long spines. As the spines enlarged, the outer exospore outstripped the inner exospore in growth. As a result, the gap between the two layers widened.

Our studies reveal that the gap between these layers contains filament-like strands that persist through the preparations used for SEM (Figure 5B) and TEM (Figures 5A, 6A–D). These have been

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young (**A**, **D**) and mature (**E**, **F**) microspores. **B.** Detail of the proximal surface. These short rods have about the same width as the rods in Figure 1A–C. **C.** On the proximal surface of the outer exospore (to either side of the laesurae), the rods are seen to be wider than on the upper surfaces of the spines. In favourable views they are seen to be composed of several rods (arrow). **D.** Small spore in proximal view. The base of each laesura has four or five supporting rods (white arrows) that are 100–150 nm in width, similar in width to the rods seen in Figures 1A–C, 2C. **E.** Proximal face of a mature microspore showing laesurae with many small spines and wide rods between laesurae. **F.** Magnification at the junction of the laesurae in **E**. Note differences in these laesurae and those in the young microspores in (**A, B** and **D**). Scale bars – 10 μm (A, D, E), 6 μm (F), 5 μm (C), 4 μm (B).

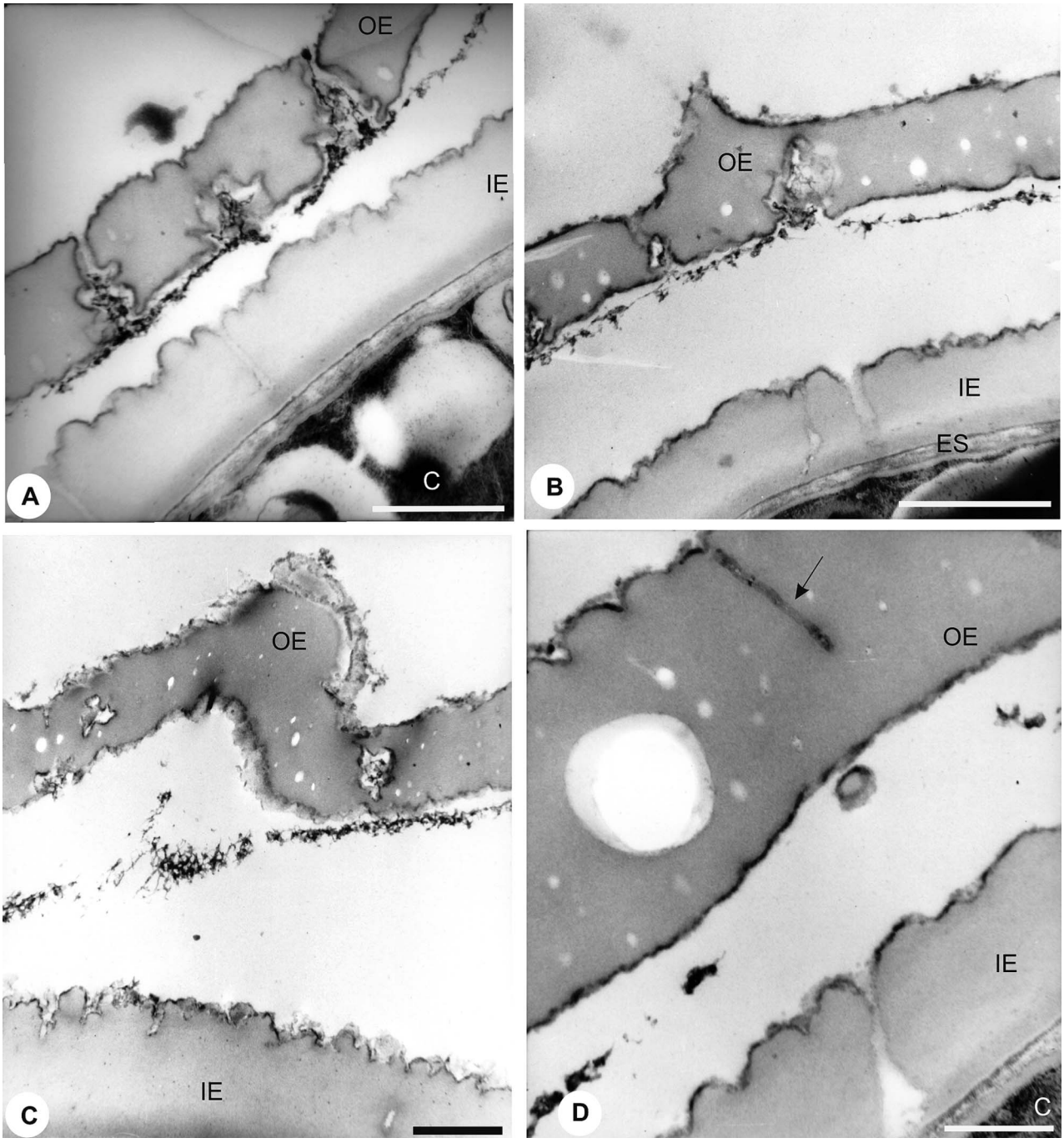

Figure 6. TEM micrographs of outer and inner exospore. **A, B.** Large and small channels are shown in both parts of the exospore and filamentous strands within channels and adjacent to the outer exospore (OE). The thin endospore (ES) is apparent between the inner exospore (IE) and the cytoplasm (C). **C.** There is filamentous material at the outer and inner surfaces. The outer surface of the inner exospore is very irregular, like that seen in the surface in Figure 7. **D.** There are large channels in both the inner exospore and outer exospore. There is a long section of the small (10–15 nm) tubule (arrow) that goes more than half way across the outer exospore. Scale bars – 5 μm.

seen to be associated with the large channels through the outer and inner exospore layers (Figure 6A–D). We consider that these strands and the material with them are related to the nourishment of the developing microspores.

Substructure of the inner and outer exospore layers and source of wall building material

According to our results, there are rod-shaped components in the outer exospore as well as in the inner

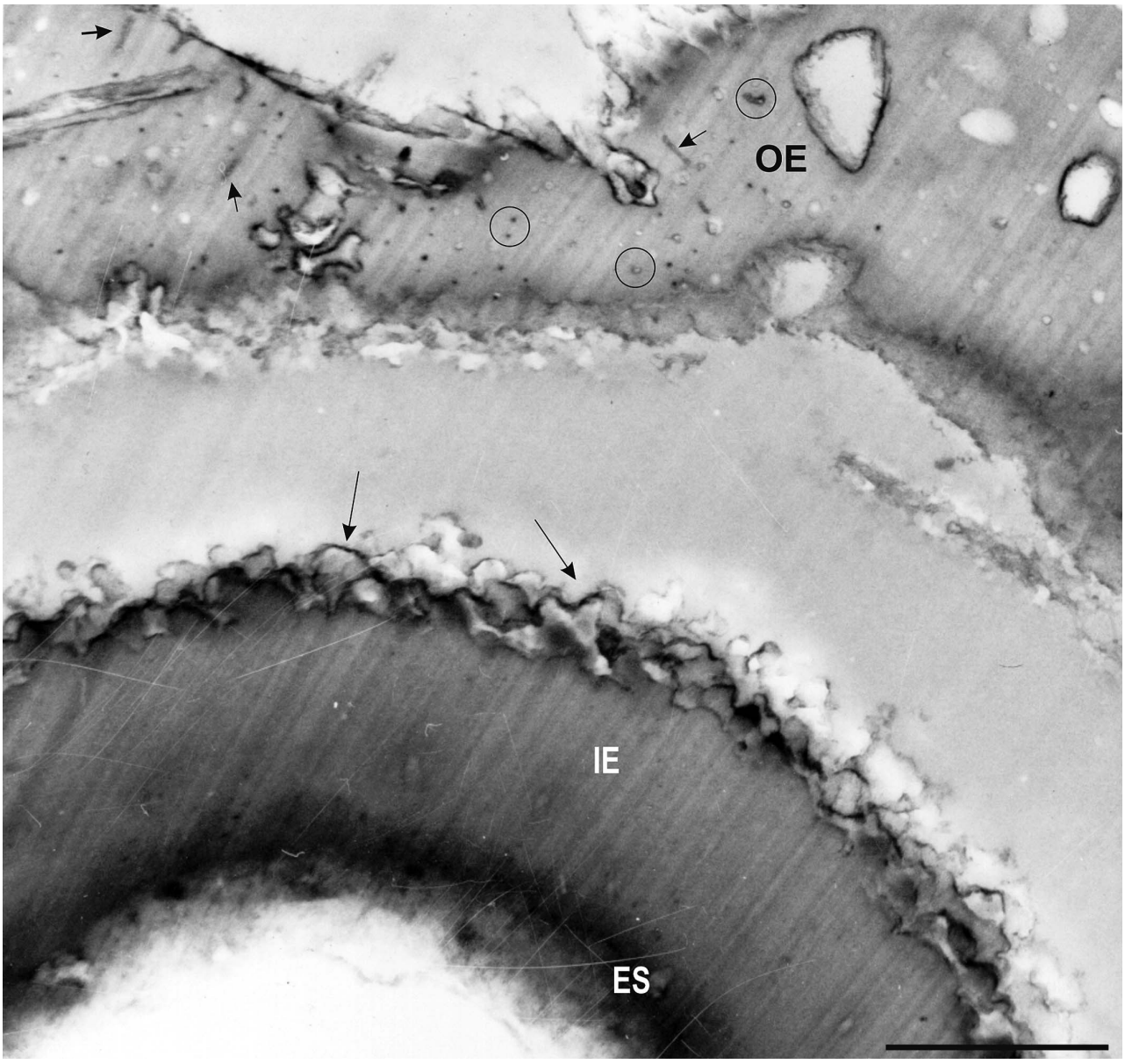

Figure 7. TEM micrograph of a fresh microspore fixed with ruthenium red in glutaraldehyde and paraformaldehyde. The section was exposed to PA and then contrasted with PTA-chromic acid, a stain procedure that contrasts carbohydrates. The dark contrast indicates the presence of carbohydrates in the inner exospore (IE), in the endospore (ES) and in the small tubules (several are circled or marked by arrows) in the outer exospore (OE). The surface within the large channel (arrowheads) at the inner surface of the outer exospore are profiles of large rods. Scale bar – 1μm.

exospore. Following the rod orientation on the outer surface of the outer exospore, we conclude that the rods are orientated within spines and spine bases in the same direction as the rods on the surface of the components of the outer exospore. If the surface material of the large rods differed from the surface of sections, the cut surface should show a close-packed pattern of rods. Since there is no such pattern, this suggests that these rods have a uniform composition except for the small tubules. The large rods and the cut surfaces (Figures 1A–C, 2C, 7) probably consist of sporopollenin. Morbelli et al. (2003) found that the exospore of *Selaginella* megaspores resisted the hot acetolysis treatment (four minutes at 100°C) of Erdtman (1960).

Slagg (1932, p. 113) revealed that the four microspores remained as a tetrad during the development of *Selaginella kraussiana* until the walls became thickened 'apparently largely by deposits from the tapetal fluid, into a common exospore about the tetrad'. The microspores were finally separated by infolding of

the exospore. After development of spines on the outer exospore, Slagg (1932) detected a new wall layer between the exospore and the protoplast in a sectioned microspore. This new layer was from the outset separated by a gap from the outer spiny exospore. The gap increased because 'the exospore outstrips it in growth' (Slagg, 1932, p. 113).

The broader relevance of this study is that through the study of the fine structure of double-walled microspores in *Selaginella*, selecting *S. kraussiana* as typical and a very controversial case, the authors were able to demonstrate that the two major walls of the microspores are built in the same pattern and they are composed of the same chemical and ultrastructural components. In this special case it means that both layers should be considered as exospore (outer and inner exospore).

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References

Belajeff, W. (1885). Antheridien und Spermatozoiden der heterosporen Lycopodiaceen. *Botanische Zeitschrift*, *43*, 793–802 & 809–819.

Blackmore, S. & Barnes, S. H. (1986). Freeze fracture and cytoplasmic maceration of pollen grains. *Grana*, *25*, 41–45.

Erdtman, G. (1960). The acetolysis technique – a revised description. *Svensk Botanisk Tidskrift*, *54*, 561–564.

Heinsein, E. (1894). Die Makrosporen und das weibliche Prothallium von *Selaginella*. *Flora*, *78*, 466–496.

Lugardon, B. (1972). Sur la structure fine et la nomenclature des parois microsporales chez *Selaginella denticulada* (L.) Link et *S. selaginoides* (L.) Link. *Comptes Rendues de l'Académie des Sciences Paris*, *274*, 1656–1659.

Lugardon, B. (1978). Comparison between pollen and pteridophyte spore walls. In D. C. Bharadwaj, K. M. Lele & R. K. Kar (Eds), *4th International Palynology Conference Lucknow 1976–77. Proceedings Volume 1* (pp. 199–206). Lucknow: Birbal Sahni Institute.

Lugardon, B. (1986). Ultrastructural data of the exospore function in pteridophyte spores. In S. Blackmore & S. H. Barnes (Eds), *Pollen and spores: form and function* (pp. 251–264). London: Linnean Society of London.

Lugardon, B. (1990). Pteridophyte sporogenesis: a survey of spore wall ontogeny and fine structure in a polyphyletic plant group. In S. Blackmore & R. B. Knox (Eds), *Microspores: evolution and ontogeny* (pp. 95–120). London: Academic Press.

Millardet, G. (1869). *Le prothallium mâle des cryptogames vasculaires*. Strasbourg, PhD Diss.

Morbelli, M. A., Rowley, J. R. & Claugher, D. (2001). Spore wall structure in *Selaginella* (Lycophyta) species growing in Argentina. *Boletín de la Sociedad Argentina de Botánica*, *36*, 315–368.

Morbelli, M. A., Rowley, J. R. & El-Ghazaly, G. (2003). Wall structure during stages in development of *Selaginella pulcherrima* and *S. haematodes* megaspores. *Taiwania*, *48*, 77–86.

Pfeffer, W. (1871). Die Entwicklung des Keimes der Gattung *Selaginella*. *Hanstein's Botanische Abhandlungen aus dem Gebiete der Morphologie und Physiologie*, *1*, 1–80.

Roland, J.-C., Lembi, C. L. & Morre, J. (1972). Phosphotungstic acid-chromic acid selective electron-dense stain for plasma membranes of plant cells. *Stain Technology*, *47*, 195–200.

Rowley, J. R., Morbelli, M. A. & El-Ghazaly, G. (2002). Microspore wall structure in *Selaginella kraussiana* (Lycophyta). *Taiwania*, *47*, 115–128.

Slagg, R. A. (1932). The gametophytes of *Selaginella kraussiana*. I. The microgametophyte. *American Journal of Botany*, *19*, 106–126.

Spurr, A. R. (1969). A low-viscosity epoxy resin embedding medium for electron microscopy. *Journal of Ultrastructure Research*, *26*, 31–43.

Stainiér, F. (1965). Structure et infrastructure des parois sporales chez deux Sélaginelles (*Selaginella myosurus* et *S. kraussiana*). *Cellule*, *65*, 219–244.

Thiéry, J. P. (1967). Mise en évidence des polysaccharides sur coupes fines en microscopie. *Journal of Microscopy*, *6*, 987–1018.

Tryon, A. F. & Lugardon, B. (1991). *Spores of the pteridophyta: surface, wall structure and diversity based on electron microscope studies*. New York: Springer.