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Microspore wall organisation and ultrastructure in two species of *Selaginella* (Lycophyta) producing permanent tetrads

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Abstract

Spore morphology and ultrastructural features of the permanent microspore tetrads of *Selaginella convoluta* and *S. lepidophylla* were studied with light microscopy, scanning and transmission electron microscopy. The four members of each tetrad in *S. convoluta* are linked together through a common envelope and form an unbroken complex system of alveoli that connects the equatorial and distal regions of the microspores. In the proximal exospore of microspores, no differentiation referable to an apertural fold was observed but an interruption of the exospore is evident in that area in germinating microspores. The distal exospore is composed of three strata: inner zone, intermediate zone and outer alveolar. In *S. lepidophylla* tetrads, the four microspores are assembled within a common tetrad envelope and are equatorially connected by a prominent ridge. The distal regions of the microspores are free. In the proximal exospore of microspores, no differentiation referable to an apertural fold was observed. The tetrad envelope appears as a wide common coat, which, on the surface and depending on the regions, is granular or alveolate, centrally perforated and, in section, consists of a single layer or forms a two-layered, more or less intricate, network. This envelope has the same contrast as the exospore outer layer and shows, in its median zone, cavities with opaque contents, quite similar to those of the microspore exospore layer. These two different types of tetrads emphasise the outstanding structural diversity of the microspore sporoderm in living Selaginellaceae.

Keywords: *Selaginellaceae, microspore tetrads, exospore, ultrastructure*

While megaspores in *Selaginella* have received wide attention, only a few studies have been performed on microspore tetrads in this genus; consequently, Lugardon (1972) started studying *S. selaginoides* (L.) Schrank & C. F. P. Mart. tetrads and later described the tetrads produced by *S. martensii* Spring based on observations with transmission electron microscopy (TEM) (Tryon & Lugardon, 1978) and microspore tetrads of several species of *Selaginella* with scanning electron microscopy (SEM) (Tryon & Lugardon, 1991), such as *S. selaginoides*, *S. rupestris* (L.) Spring and *S. stenophylla* A. Br.

The microspore morphology in *Selaginella* is very variable (Knox, 1950). Tryon and Tryon (1982) reported that in *Selaginella* species from tropical America, microspores were more diverse than the megaspores, and Morbelli (1977) noticed that microspores were more diverse than megaspores in species growing in Argentina. The sporoderm ultrastructure of *Selaginella* microspores has been studied intensively in the last decades (Robert, 1970, 1971*a*, 1971*b*, 1972; Lugardon, 1972, 1976, 1978, 1990; Tryon & Lugardon, 1978, 1991; Uehara, 1999; Morbelli et al., 2001; Rowley & Morbelli, 2002; Lugardon & Morbelli, 2004). The exospore is generally compact and has two or three layers (Uehara, 1999) in the microspore wall of *S. tamariscina* (Beauv.) Spring. The microspore exospore with all the layers forms a protruding apertural fold, both sides of which are more or less fused together. This exospore includes a system of perforations consisting of either three tangentially cleft small areas arranged between the rays of the trilete aperture, or scattered radial channels. In many species, the elements of the ornamentation are formed by the exospore outer layer and, in most of these species, a

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perispore consisting of material clearly distinct from the exospore constitutes a surface layer that is usually stuck to the exospore, and is much thinner than the exospore.

In a few species, the exospore is covered with a wide, more or less thick and complex, envelope that bears the ornamentation. This envelope is generally connected to the exospore only between the apertural and equatorial regions, and forms two lips in the apertural area that extend beyond or merge with the apertural fold of the exospore. It has similar characteristics to that of the exospore outer layer and develops at the same time as this layer; this has led Lugardon (1972) to name it as 'para-exospore'.

Large numbers of undivided microspore tetrads are present in end-Permian sediments worldwide, megaspore tetrads also occur occasionally (Looy, 2001; Looy et al., 2005; Visscher et al., 2004); both kinds of tetrads were assigned to lycopsids. The botanical affinity of those microspore tetrads was inferred based on the ultrastructure of the preserved sporoderms; Looy (2001), Looy et al. (2005) and Visscher et al. (2004) also made ecological interpretations concerning the survival of the group in harsh environments. Looy et al. (2005) additionally documented well preserved specimens of other microspore tetrads that were assigned to *Densoisporites* and *Uvaesporites imperialis* Utting. These two tetrad types had a continuous outer coating, which is typical of microspore tetrads produced by extant lycopsid species (Looy et al., 2005).

In extant species of *Selaginella*, the microspores are usually released individually, while in a number of species, which are most probably few, the microspores may remain grouped in tetrads due to different sorts of connections. In these species, the microspores are linked together due to fusion of spore walls; the nature of this fusion may differ considerably between different species. These structures were illustrated with two examples (Lugardon 1972, 1978): (1) exospore connections in the equatorial area, as in *S. martensii*, are probably the most common type [Tryon and Lugardon (1978) illustrated a part of a tetrad in *S. martensii* from the apertural folds of three microspores in the proximal pole area to the junction between the exospores in the equatorial region]; (2) links in the area of the apertural folds, as in *S. selaginoides*, seem to be less common. Moreover, this species is remarkable for its junction system, consisting of several rather long, in transverse sections nearly round, extensions that are arranged along each ray of the aperture as revealed by Lugardon (1972) illustrating a central area of an immature tetrad with three microspores interconnected by exospores extensions, developed at the apex of the apertural rays. Tryon and Tryon (1982) documented the permanent microspore tetrads produced by *S. estrellensis* Hieron. from Costa Rica and those of *S. delicatissima* Linden from Mexico with SEM. Hellwig (1969, p. 463) noted that 'perhaps the most distinctive microspore specialisation is found in those species characterised by the plants curling into a ball in dry seasons. Apparently, the heavy outer wall is common to the whole tetrad and is ornamented by several ridges.' Two of these tetrad types were selected for this study, i.e. *S. convoluta* (Arn.) Spring and *S. lepidophylla* (Hook. et Grev.) Spring.

The microspore tetrads of *Selaginella lepidophylla* were studied by Tryon (1949) and Hellwig (1969). Initial light microscopy (LM) and SEM studies of *S. convoluta* microspore tetrads by Morbelli (1977) and Morbelli et al. (2001) revealed that the four members of each tetrad are linked together through a common envelope forming an unbroken complex system of alveoli. While the apertural rays were very well defined at the proximal contact face (Tryon & Lugardon, 1978), in *S. martensii* and in *S. selaginoides*, the upper distal part of each ray was connected with the other members of the tetrads. The aim of the present study is to highlight the structure and organisation of the permanent microspore tetrads of *S. convoluta* and *S. lepidophylla*.

Material and methods

For investigations by TEM, the microspore tetrads of *Selaginella convoluta* were processed by John Rowley and Marta Morbelli at the Department of Botany, Stockholm University. Whole microsporangia from dry material from herbarium specimens were imbedded in agar and hydrated with phosphate buffer plus 1% alcian blue (AB), prefixed with 2% glutaraldehyde (GA) 0.1 N in phosphate buffer plus 1% AB and post-fixed with 1% osmium tetroxide (OsO_4) plus 1% AB (1 h, 20 °C). Living material was fixed with 2% GA in phosphate buffer and post-fixed with 1% OsO_4 in distilled water.

After osmium treatment, both were washed with phosphate buffer, dehydrated in an acetone series and embedded in Spurr's hard mixture. A Hitachi HU 11B transmission electron microscope was used for observations and photomicrographs. Sections were stained with 1% uranil acetate (UA) for 15 minutes and subsequent staining by lead citrate (Pb) for three minutes.

Complete and fractured microsporangia were examined with LM and SEM. For the study with LM, semi-thin sections were stained with 1% toluidine blue. For SEM studies, chemically untreated tetrads of both species were suspended in distilled water, fractured with ultrasound, dehydrated, fixed on stubs with double sided adhesive tape and coated

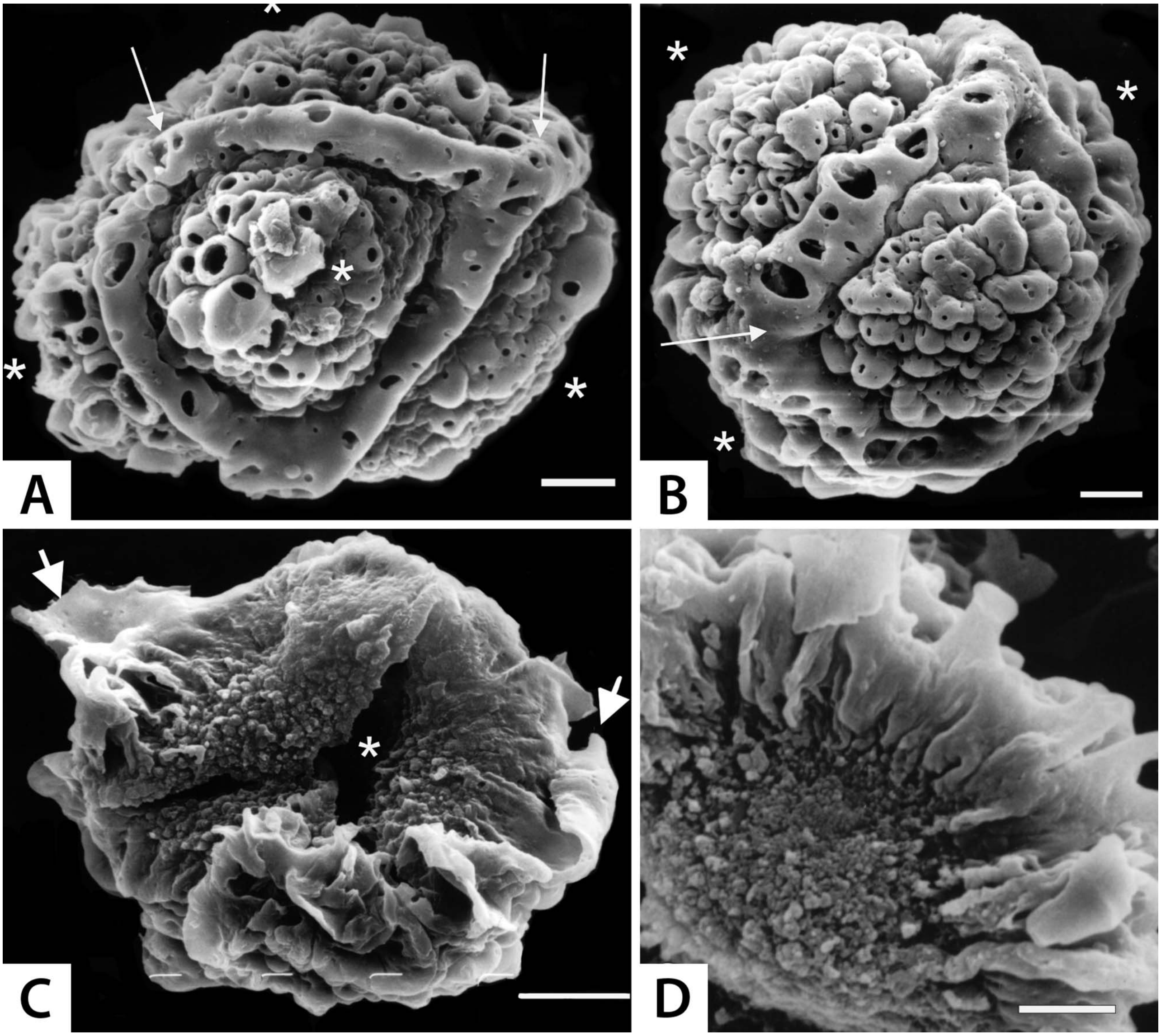


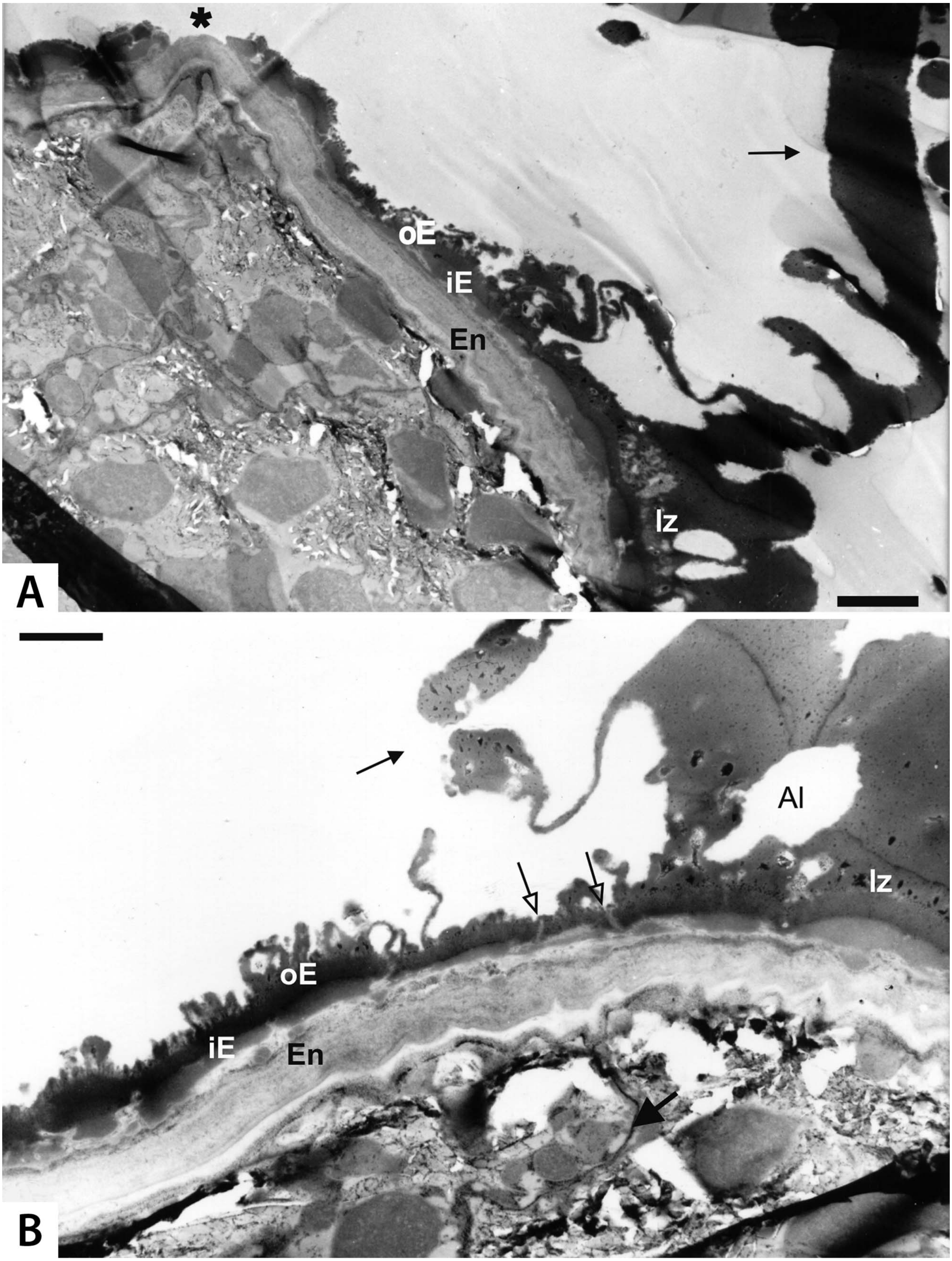
Figure 1. *Selaginella convoluta* microspore tetrads in SEM. **A, B.** Rounded projections (*arrows*) mark the contact between the microspores distal faces (*asterisks*), which bear packed small domes of irregular size; the rounded projections and the distal domes have rather large and abundant openings in these tetrads, but may be smaller and rarer in other tetrads produced by the same specimen. **C.** Proximal face of three tetrad members from the proximal polar zone (*asterisk*) up to the equator lined with alveoli and torn pieces of the bridges (*arrows*) that joined the four members of the tetrad. **D.** Polar view of the proximal face of a microspore surrounded by alveoli marking the equator; the proximal face has a uniformly irregular surface that is formed of packed small projections; it does not show any morphological indication of an apertural differentiation comparable to the three radiated protrusion that usually characterises the proximal face of the microspores in *Selaginella*. Scale bars – 10 μm (A–C), 2.5 μm (D).

with gold. The study was performed with a JEOL JSM-6360 LV scanning electron microscope.

Results

Selaginella convoluta *(Figures 1–3)*

The four members of each tetrad are linked together through a system of bridges and alveoli, forming a common envelope that connects the equatorial and distal regions of the microspores (Figure 1A, B). The proximal face has a uniformly irregular surface formed of low heterogeneous projections (Figure 1C, D). In TEM sections, the proximal exospore (Figure 2A, B) is composed of a light inner layer with irregular thinning or even interruptions, and a dark outer layer forming dense, multiform and small surface projections. Radial channels cross the whole exospore at the level of the thinnest parts of the inner layer. There is also a separation (Figure 2A) through which endosporal structures tend to protrude. The separation appears to have the same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three-radiated layout as the normal trilete apertures. However, every examined separation break appeared in section as composed of both exosporal layers. Thus, the inner exospore is thick and larger than the outer exospore. However, no trace of ultrastructural differentiation resembling the usual apertural fold was observed. In equatorial (Figure 2A, B) and distal areas (Figure 3), the exospore is alveolate. An intermediate zone (Figure 2A, B), including small, often radially elongated cavities, more or less filled with opaque material, is evident. The exospore outer layer, the intermediate zone and the alveolate envelope seem to consist of the same substance and have the same contrast, and there is no trace of splitting between them. In germinating microspores (Figures 2, 3), the endospore is heterogeneous and thick. It comprises two distinct structures, a thick outer layer and a thinner inner layer that is connected to intercellular walls (Figure 3).

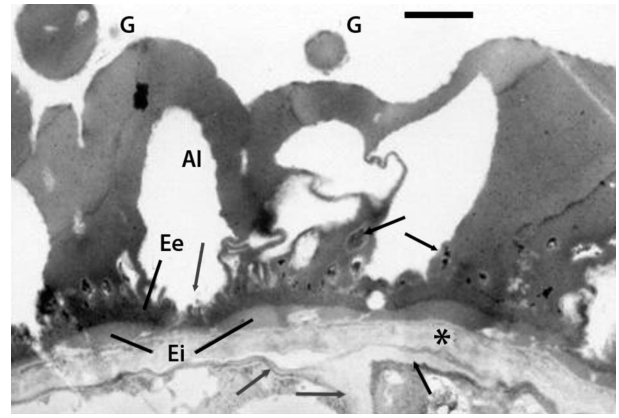

Figure 3. Distal wall structure of a microspore tetrad of *Selaginella convoluta* in TEM; the exospore has two layers (Ee, Ei) and perforations (*arrows*) similar to those of the proximal face, which is closely joined with the alveolar layer (Al); the junction zone includes small, often radially elongated cavities partly filled with opaque material (*arrows*); note the outer exospore layer, junction zone and whole alveolar layer including micro-cavities with dark contents; globules (G) are rather abundant near these tetrads investigated before release from sporangium; note the germinating microspores comprising two distinct structures at the site of the endospore: a thick outer layer (*asterisk*) and a thinner inner layer connected to intercellular walls (*arrows*). Scale bar – 1 μm.

Selaginella lepidophylla *(Figures 4, 5)*

The four microspores are assembled within a wide common tetrad envelope (Figure 4A, B) of variable appearance (Figure 4A, C, F). The sculptured outer surface of the tetrad envelope can be seen as verrucate with a granular surface and with domes or alveoli with distal large opening in others (Figure 4A, upper right corner; 4B, upper left corner, 4C). The microspores are seen separated from each other (Figures 4F, 5A). In section and depending on the regions, the tetrad envelope consists of a single layer or forms an intricate two-layered network. The outer layer has irregular thickness and cavities in the middle zone, while the inner layer is composed of rods of different thickness with expanded ends (Figures 4D, 5A), which, appear circular according to the plane of section (Figure 5A, B). This envelope has the same contrast as the microspore exospore outer layer and shows, in its median zone, cavities with opaque contents, quite similar to those of the exospore outer layer (Figure 5B).

The microspore exospore shows the usual two-layered structure (Figure 5A, B). It consists of a thin inner layer and a thick, usually darker, outer layer in TEM sections. It forms the equatorial thickening, typical of these microspores, and has radial channels that completely traverse both exospore layers. Some particularities concerning the microspore outer exospore layer are numerous cavities with opaque contents in its inner part and a usually nearly uniform thickness, but shows strongly reduced or expanded areas that seem to result from small ontogenetic disruptions.

A layer is commonly found following the microspore exospore surfaces (Figure 5A). It is constituted of a network of dark fibres with small globules (see Lugardon, 1981) like elements of different sizes, irregularly arranged. Sometimes, this layer is found closer to the tetrad envelope's inner surface (Figure 5B). A thin, discontinuous, dense layer, possibly consisting of silica, is loosely applied to the outer surface of the tetrad envelope (Figure 5B).

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Figure 2. Sections of *Selaginella convoluta* microspore tetrads through the proximal face and equatorial area of contact in TEM. **A.** Large part of the proximal face and, to the right, a portion of the alveolar layer comprising few alveoli and intermediate zone (Iz) and exospore (Ex); a part of the bridge (*arrow*) between two microspores; the exospore comprises two layers (oE) and (iE); it shows a prominent outline with an apical break (*asterisk*) through which the endosporal structures (En) tend to protrude; it can be assumed that this break corresponds to the apertural opening. **B.** The proximal exospore (*left side*) has the usual two-layered structure with some special features among which the inner layer (iE) has irregular reductions and even interruptions, the outer layer (oE) is more uniform in thickness, forms small, multiform, densely packed surface projections, and perforations (*arrows with white arrowheads*) usually arranged at the level of the thinnest parts of the inner layer; in the equatorial region (*right side*), this exospore is surmounted by irregular arches of the alveolar layer (Al) and intermediate zone (Iz) and exospore (Ex) with the basal part of the bridge (*dark arrow*) linking the microspores together; note the thick, heterogeneous endospore (En) and the thin intercellular walls (*thick dark arrow*) proving that the microspores have reached a multicellular gametophytic stage. Scale bars – 2 μm.

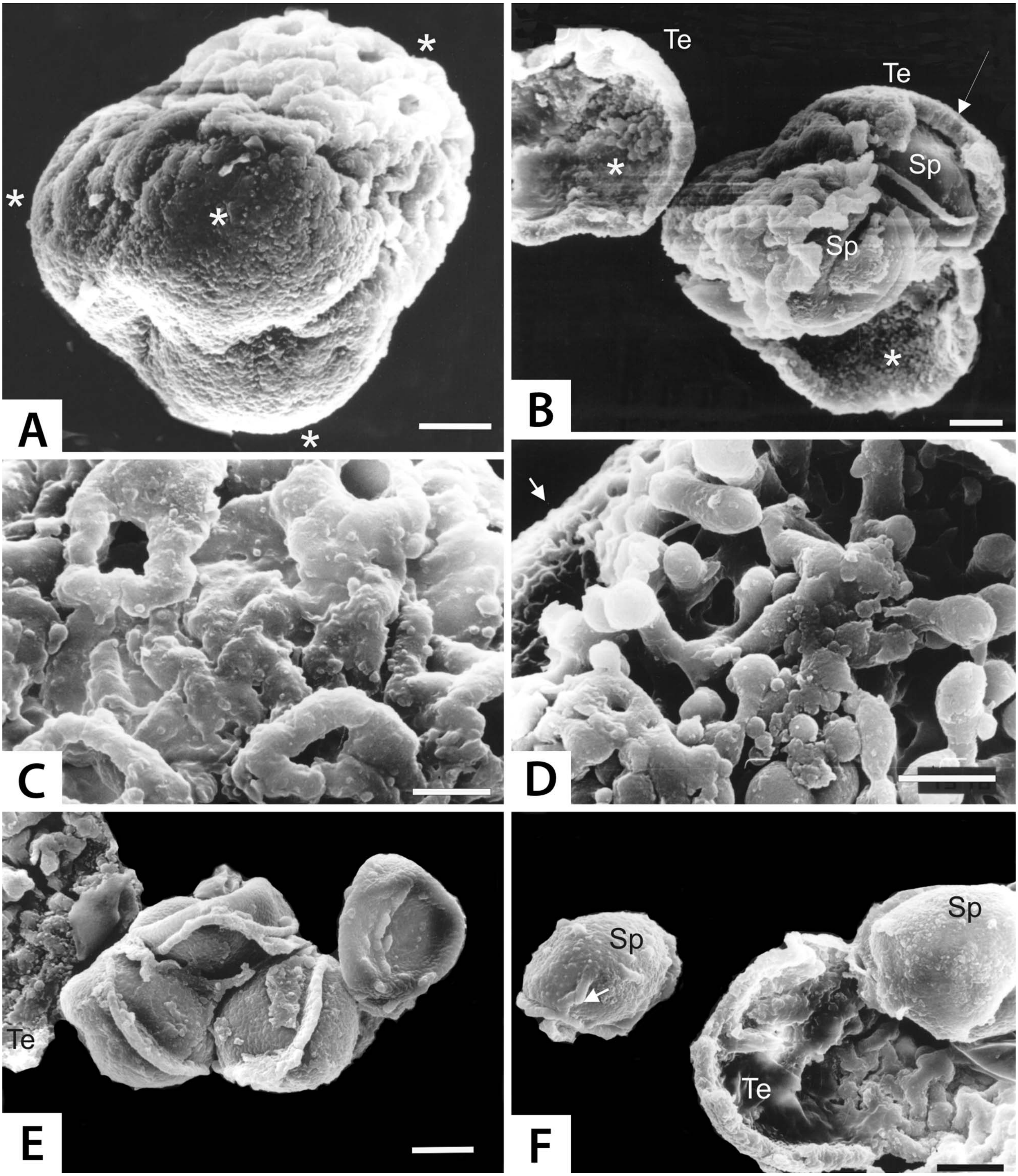

Figure 4. *Selaginella lepidophylla* microspore tetrads in SEM. **A.** The four microspores wrapped by a common continuous envelope; the size of each microspore is visible below the tetrad envelope (*asterisks*); note the changing surface of the tetrad envelope, which is almost smooth above two spores (at the top) and strongly sculptured above the others. **B.** Fractured tetrad, where the tetrad envelope is split into several fragments (Te), allowing seeing two of the microspores (Sp), its thickness (*arrow*) and varied appearances of its inner surface (*asterisks*). **C.** Magnification of the sculptured outer surface of the upper right part of the tetrad envelope of the tetrad in (**A**); each alveolus has a distal opening. **D.** Cross fracture of the tetrad envelope with double-layered complex structure; the *white arrow* marks the outer surface of the envelope; the inner layer consists of rods with expanded ends, arranged in all directions and irregularly fused. **E.** After the loss of the tetrad envelope (Te), three microspores remain close together showing a lateral ridge. **F.** The fractured tetrad envelope (Te) has released the microspores (Sp); Note the changing inner surface of the tetrad envelope and the irregular surface of the microspore, and a sinuous protrusion (*arrow*). Scale bars – 10 μm (A, B, E, F), 5μm (C, D).

It penetrates the tetrad coat and fills the spaces between rods of its inner layer.

Discussion

Morphological features, as described here for the exospore in *Selaginella lepidophylla* microspores, such as many thin radial fissures in the exospore and the junction areas between microspores, are similar to those observed in the equatorial zone of *S. convoluta*, and were also shown by Tryon and Lugardon (1978) for microspores in tetrads produced by *S. martensii*. An external pattern similar to that of *S. convoluta* was also found in *S. stenophylla* from Mexico by SEM studies (Tryon & Lugardon, 1991).

Other kinds of tetrads referred to Selaginella *microspores*

For immature tetrads of *Selaginella selaginoides*, which in mature state are shed as single units, Lugardon (1978) described exospore extensions at the apex of each apertural fold that linked the four members of the tetrad. This type of extension is not frequently found in either single *Selaginella* or tetrahedrally joint microspores. Moreover, in mature *S. selaginoides* tetrads, the apertures were proximally well defined and separated from those of the other tetrad members.

We have found a similar morphology between tetrads produced by extant taxa and fossils studied by Looy et al. (2005) who assigned the tetrads to *Densoisporites* and *Uvaesporites*. In SEM records of well-preserved material of *Densoisporites*, the microspore members in the tetrads are interlocking by the outer layer or para-exospore (Looy et al., 2005). In *Selaginella lepidophylla* and *S. convoluta*, the microspore members are also separated and only interlocked at the distal and equatorial areas.

Uvaesporites imperialis unseparated microspore tetrads recognised as having a selaginellaceous affinity by Looy (2001) and Looy et al. (2005), have two similarities, one referring to interlocking system and the other concerning the stratification and ultrastructure of the outer coat. The *Uvaesporites* tetrads illustrated with TEM in Looy et al. (2005), show similarity with the extant mature tetrads of *Selaginella convoluta*. In *S. lepidophylla*, each microspore in the undivided tetrads is fused by a prominent ridge of the distal exospore to the continuous distal two-layered envelope, into such a unique and efficient structure that keeps the four members of the tetrad tightly fixed and next to each other when mature.

The two species selected for the present study both thrive in extreme dry environments and demonstrate that the undivided microspore tetrads produced by extant *Selaginella* maintain the same system of retention of their male members. More studies, both on fossil and extant material, are needed in order to improve our understanding of how these types of complex covers consisting of several layers and how the fusion of microspores evolved.

Conclusion

The microspores of *Selaginella convoluta* and *S. lepidophylla* are joined in undivided tetrads and share significant features such as exospores without laminated zones and radial channels that cross both exospore layers. Nevertheless, both species appear to be different in many aspects. The apertural features are very different from those described in previous studies, both for extant and fossil material. As typically encountered in Selaginellaceae, where microspores disperse as single units, the exospore comprises two layers, which form a proximal protruding well-defined apertural fold. However, in the microspores of *S. convoluta* and *S. lepidophylla*, the trilete aperture on the proximal face seems to be appreciably altered. In *S. convoluta*, it can be assumed that the proximal break is related to the development of the gametophyte and corresponds to the apertural opening, while in *S. lepidophylla*, the proximal break probably is the result of ontogenetic phenomena associated with the special tetrad condition. It could also be probably due, in part, to free rotations of the microspores within the tetrad envelope.

In *Selaginella lepidophylla*, the tetrad members are included within a shared, thick, complex envelope. Each microspore is detached from the other three, but each of them is fused to the common outer envelope. To date, such a type of microspore tetrad is not

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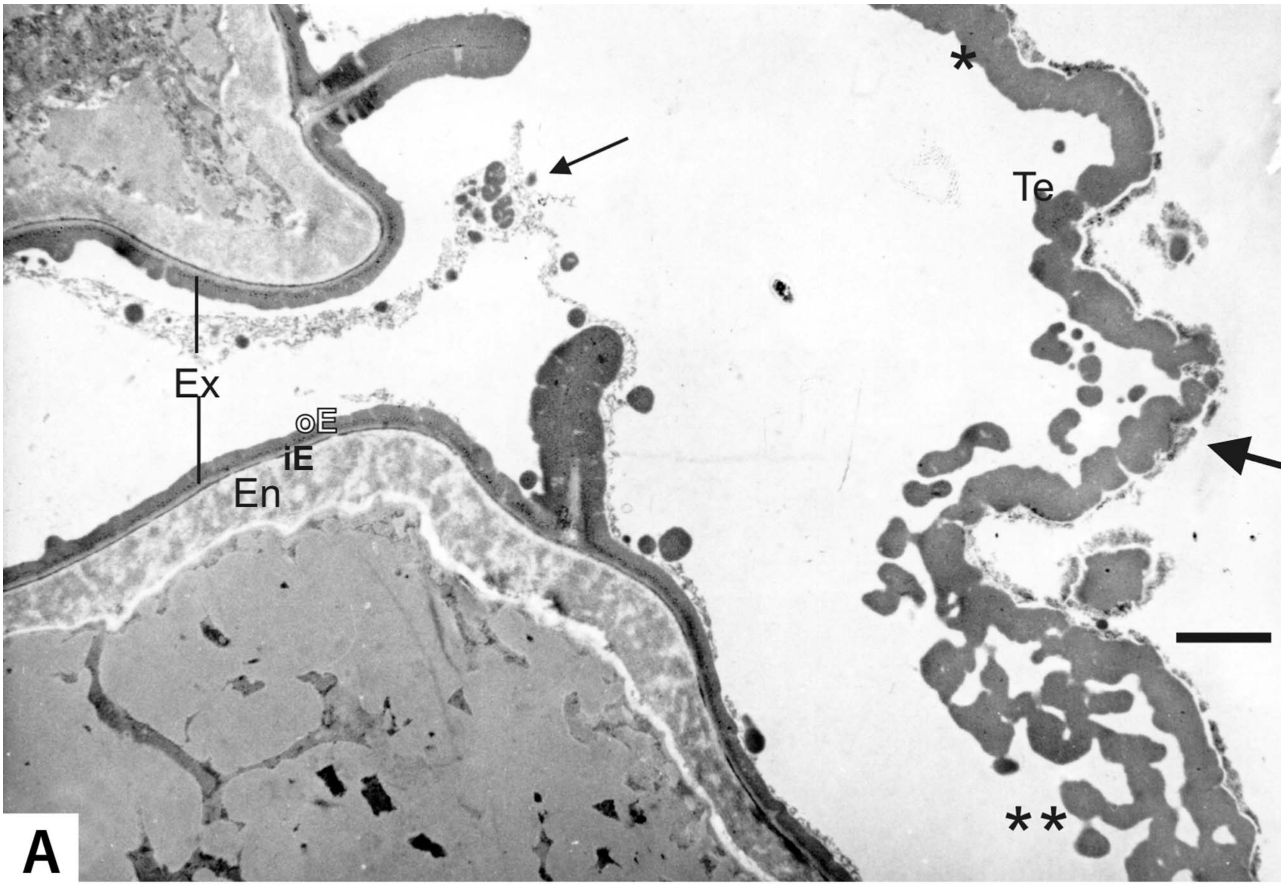
Figure 5. *Selaginella lepidophylla* microspore tetrads in TEM. **A.** Lateral area of two microspores and tetrad envelope; the microspores are totally separated from one another and from the single-layered (*) or complex two-layered (**) tetrad envelope (Te); radial conduits traverse completely the outer layer of the tetrad envelope; microspore sections show the two-layered exospore (Ex) forming a protruding fold; the thick heterogeneous endospore (En) is surrounding the spore cell. **B.** The tetrad envelope (Te) is sectioned and radial conduits traverse completely the outer layer of the tetrad envelope; a particulate network with small globule-like elements (*thin arrow*), irregularly arranged around the microspores, a thin, discontinuous dense layer, possibly consisting of silica, loosely applied to the outer surface of the tetrad envelope (*thick arrow*), are visible; the microspore exospore consists of a thin inner layer and a thicker, usually darker, outer layer, which more or less fuse together and form a fold; radial channels completely traverse both exospore layers. Scale bars – 4 μm (A), 1 μm (B).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known in any other living pteridophyte 'nor would in living seed-plant – but it has perhaps existed in some very ancient land-plants' (B. Lugardon). Another odd feature of the tetrads of *S. lepidophylla* lies in the morphological and structural differences within the tetrad envelope.

In *Selaginella convoluta*, the four tetrad members are linked by bridges between their thick distal alveolar layers. Thus, these microspores are not free within the tetrad. Such tetrad organisation is not known in ferns, but it might be compared with that of some kind of tetrads in some angiosperms.

The present data strongly suggest that the apertural system in *Selaginella convoluta* and *S. lepidophylla* is vastly altered. In both species, the proximal exospore consists of two thin layers as in microspores of other *Selaginella* species. However, it does not show any further morphological or structural differentiation comparable to the usual apertural fold, while in germinating microspores, a separation appears to have the three-radiated arrangement of the normal trilete aperture.

These two different types of tetrads demonstrate the outstanding structural diversity of the microspore sporoderm in living Selaginellaceae. They also emphasise the considerable importance of further ultrastructural and ontogenetic studies on the spores of this complex group of lycopsids.

Acknowledgements

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Specimens investigated

Selaginella convoluta (Walk. et Arnoff) Spreng. Paraguay, Cerro Pelado, near Paraguari, Balansa, 3293 (BAF, SI).

Selaginella lepidophylla (Hook. et Grev.) Spring. Mexico 77/259a, Incl. 862; 77/257b, B. Lugardon, Lab. Bio. Vég. Toulouse, France.

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