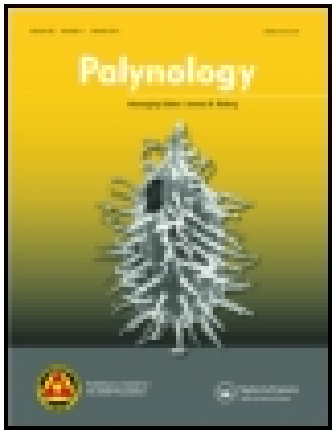

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Substructural components in the sporoderm of the Family Cyatheaceae

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Substructural components in the sporoderm of the Family Cyatheaceae

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The ultrastructure of the sporoderm of three genera of the Family Cyatheaceae was studied in detail, with the aim to elucidate its constituent elements. While the perispore of *Alsophila* and *Sphaeropteris* is thick and complex, that of *Cyathea* is reduced and with a different structure; nevertheless, their basic elements seem to be similar. We found that the substructural components are threads with a diameter of 50–100 nm and that each one is composed of an inner channel, around which there are several smaller coiled units. The channels are interconnected and form a complex system, which is continuous within the exospore and in a similar pattern. This network of threads could be related to the transfer of substances between the outer and inner parts of the spores during their development.

Keywords: substructural components; sporoderm; Cyatheaceae; threads; channels; Argentina

1. Introduction

Cyatheaceae is the major Family among the tree ferns, with about 500 species distributed throughout the tropics, the subtropics and the south-temperate zone. It is characterised by the presence of scales on the bases of the petioles. There are three lineages within the family, corresponding to three genera: *Alsophila*, *Cyathea* and *Sphareopteris* (Korall et al. 2007; Korall & Pryer 2014).

The morphology of spores has been studied in many palynological works (Harris 1955; Nayar & Devi 1966; Erdtman & Sorsa 1971; Gastony 1974; Murillo & Bless 1974; Barth 1975; Gastony & Tryon 1976; Liew & Wang 1976; Gastony 1979; Esteves & Felippe 1985; Braggins & Large 1990; Simabukuro et al. 1998; Lorscheitter et al. 1999) and systematic (Holttum & Sen 1961; Gastony 1973; Tryon 1976; Barrington 1978; Tryon & Tryon 1982; Conant 1983; Lellinger 1987; Conant et al. 1996; Korall et al. 2007; Moran et al. 2008).

However, not many studies have analysed the wall ultrastructure of the Cyatheaceae in depth. Lugardon (1971, 1974) studied spores of two species of the genus *Sphaeropteris* from Oceania with transmission electron microscopy (TEM), and described the exospore as blechnoid and the perispore as two-layered (Lugardon 1971, 1974).

Tryon & Lugardon (1991), in their study of fern spores, carried out a general analysis of the wall structure of Cyatheaceae by means of TEM and scanning electron microscopy (SEM). The exospore had two well-differentiated layers, and the perispore of *Alsophila* and *Sphaeropteris* was shown to be very complex with three strata, while in *Cyathea*, two strata composed of rodlets were found.

In the last few years, the wall morphology and ultrastructure of Cyatheaceae spores from southern South America have been widely studied (Marquez 2009; Marquez et al. 2009, 2010a, 2010b), and it has been observed that the features of the spores are typical of each genus.

The purpose of this study is to carry out a comparative analysis of the sporoderm ultrastructure in the Cyatheaceae from South America and to discuss, on the basis of the information available at present, the wall formation and stratification of the spores. Our aim is also to identify spore ultrastructural components at the generic level. Likewise, we propose a hypothesis on the possible function or functions they might have.

2. Materials and methods

Dry material was obtained from herbarium specimens from Museo Argentino de Ciencias Naturales 'Bernardino Rivadavia' (BA), Instituto de Botánica del Nordeste (CTES), Museo de Ciencias Naturales de La Plata (LP), Instituto Anchietano de Pesquisas (PACA), Instituto de Botánica Darwinion (SI), Universidade de São Paulo (SPF) and the Smithsonian Institution (US). The living samples were obtained in field trips made in Misiones province, Argentina.

For SEM, the material was treated with hot 3% sodium carbonate at 90°C, washed, dehydrated, put in 96% ethanol and then transferred to acetate plates. After drying in air they were coated with gold.

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For TEM, the material was treated as follows:

(1) Mature spores of dry material from herbarium specimens was hydrated with phosphate buffer plus alcian blue (AB), then the sample was fixed with 1% glutaraldehyde (GA) + 1% alcian blue in phosphate buffer for 12 h, rinsed with phosphate buffer + AB and post-fixed with 1% osmium tetroxide (OsO_4) in water plus 1% AB (Rowley & Nilsson 1972).
(2) Mature spores of living material were fixed with 1% GA + 0,0025% ruthenium red (RR) in phosphate buffer washed in phosphate buffer + RR, then post-fixed with 1% OsO4 in water plus 0.0025% RR in phosphate buffer.
(3) Next, the spores were dehydrated in an alcohol series and then embedded in Spurr medium mixture. Ultrathin sections were stained with 1% uranyl acetate for 15 min followed by lead citrate for 5 min.

The observations with SEM were performed with a JEOL JSMT-100. The TEM observations were made with a Zeiss M-10.

In previous papers, some photos were published (Marquez et al. 2009, 2010a, 2010b) in which the wall ultrastructure and stratification were described. This contribution focuses on other topics since the wall substructural components are already defined.

Specimens studied:

Alsophila capensis (L.F.) J. Sm. subsp. *polypodioides* (Sw:) D.S. Conant

BRAZIL: MINAS GERAIS, Bocaina, Rio Vérmelo, 19/7/1937, *Castellanos* (BA 20555), MP 4101.

Alsophila odonelliana (Alston) M. Lehnert

ARGENTINA: JUJUY, Parque Nacional Calillegua, Río de las Piedras, 2/10/1986, *Iudica and Ramadori 343* (SI), MP 4110; SALTA, Sta. Victoria, San Andrés, 13/7/1945, *Capurro 279* (BA), MP 920.

Alsophila setosa Kaulf.

ARGENTINA: MISIONES, Frontera, 28/8/1950, *Diem 1503* (SI); *Idem*, Frontera, San Antonio, 21/7/1945, *Krapovickas 2501* (LP). BRAZIL: PARANA, Villa Velha, 30/4/1914, *Dusén 14839* (SI). PARAGUAY: CAAGUAZU, Pastoreo, Col. Sommerfeld, 28/1/1951, *Saprre and Vervoorst* 2269 (LP).

Sphaeropteris gardneri (Hook.) Tryon

BRAZIL: SANTA CATARINA, Mun. Papanduva, Serra do Espigao, 20/04/1962, *Reitz & Klein 12656* (PACA); Biguaçu, Antinha, 04/03/1943, *Reitz 232* (PACA); Ilhota, Morro de Baú, 21/01/1953, *Reitz 5170* (PACA).

Cyathea atrovirens (Langsd. & Fisch.) Domin

ARGENTINA, CORRIENTES, Ituzaingó, *Tressens et al. 372* (LP, CTES); ÍDEM, MISIONES, Gral. Manuel Belgrano, *Partridge s/n* (BA 70619 a-b-c), MP 4099; ÍDEM, Iguazú, *Rodriguez 430* (SI, BA); ÍDEM, Guaraní, 28/4/1997, *Morrone et al. 2181* (SI), MP 4080; ÍDEM, San Pedro, P.P. Piñalito, *Marquez & Carrión 181* (LP); ÍDEM, San Ignacio, P.P. Teyu Cuare, *Marquez et al. 230* (CTES, LP); ÍDEM, San Antonio, *Capurro 935* (BA). BRAZIL, RIO GRANDE DO SUL, Rio Pardo, *Jürgens s/n* (Rosenstock 257) (SI). PARAGUAY, SAN PEDRO: Col. Guayaibí, *Krapovickas et al. 14282* (SI).

Cyathea corcovadensis (Raddi) Domin

BRAZIL, PARANA, *Pereira 8224* (LP); ÍDEM, Curitiba, *Krapovickas et al. 23143* (LP); ÍDEM, Guaratuba, *Dusén 13729* (SI), MP 4102; SANTA CATARINA, Lages, *Spanagel s/n* (Rosenstock 240) (LP), MP 4103.

Cyathea delgadii Sternb.

ARGENTINA, CORRIENTES, Ituzaingó, *Meyer 6278* (US 2361678); BRAZIL, SANTA CATARINA, Sao Jose, *Fernandes 1132* (SPF); PARANA, Paranagua, *Fernandes et al. 1117* (SPF), MP 4127; *Ídem*, Piraquara, *Fernandes 1115* (SPF).

3. Results

3.1. Substructural units of the perispore of Alsophila and Sphaeropteris

Alsophila and *Sphaeropteris* have a very complex perispore, with two well-differentiated layers (Plate 1, figures 1, 2): the outer layer (oP) is 0.8—1.5 μm thick and the inner layer (iP) is 0.3—0.6 μm thick.

The oP is composed of threads of 60−80 nm diameter and greater than 4 μm in length in both genera (Plate 2, figures 3−5). These threads tend to be arranged tangentially to the inner perispore surface and they are circular in section (Plate 2, figures 1, 2, 4, 5; arrowheads). Inside this layer, the threads are intertwined randomly, although in some areas they seem to be parallel to each other (Plate 2, figure 1; arrows). While these subunits can be clearly observed in *Alsophila*, they are difficult to identify in *Sphaeropteris*; in the latter case, the threads are tightly packed and can be differentiated in few places (Plate 2, figure 2; arrows).

The inner perispore layer (iP) consists of three strata in both genera, named outer (o), middle (m) and inner (i). At first appearance, the external stratum (o) looks homogeneous, but at higher magnifications it shows small dots inside circular units. Those images were

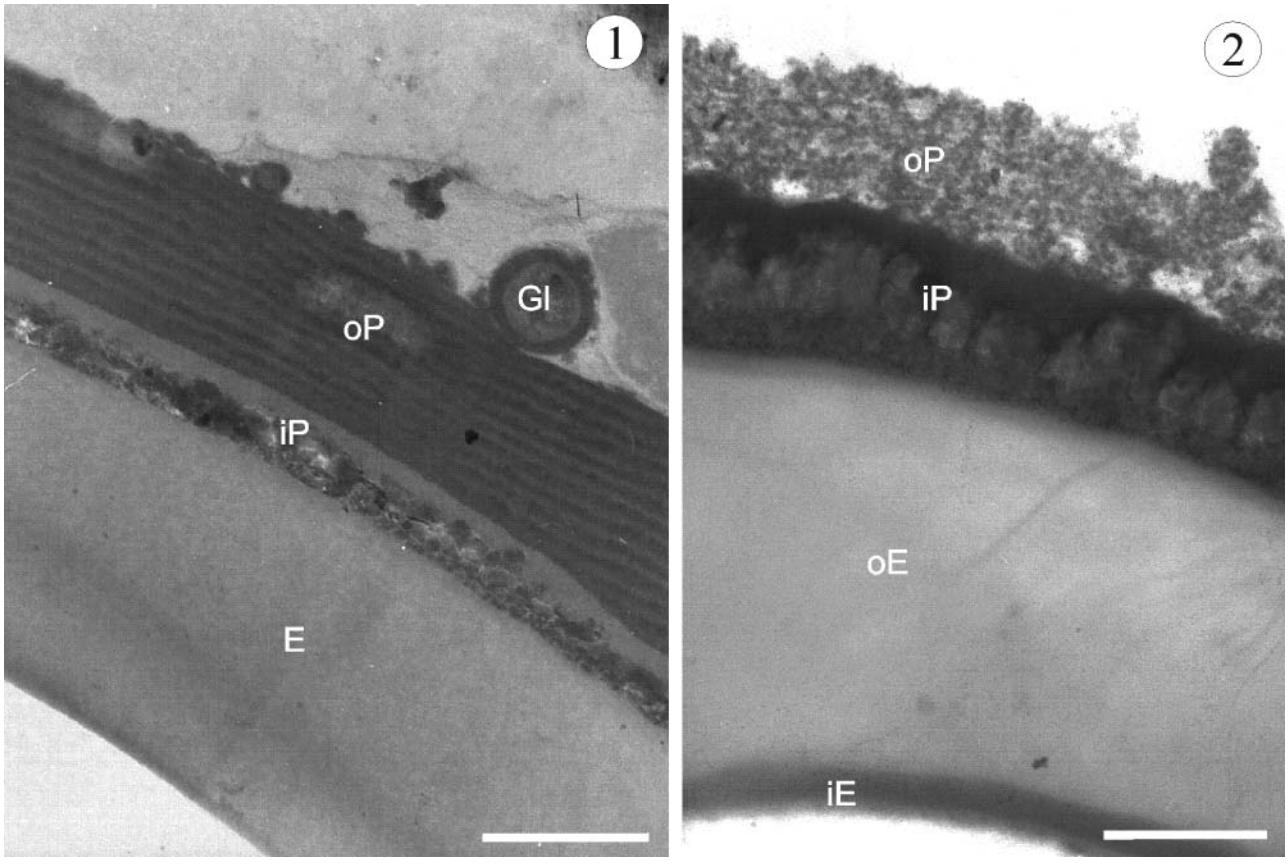

Plate 1. **1**. Sporoderm section of *Sphaeropteris gardneri* with transmission electron microscopy (TEM). The exospore (E) is less osmiophilic than the perispore. The perispore is composed of two layers, the complex inner perispore (iP) and the outer homogeneous perispore (oP). A large globule is seen (Gl) on the right on the outer perispore surface, and a small one is on the left. Several small ones are located between them. Bar: 1 μm. **2**. Sporoderm section of *Alsophila setosa* with TEM. The exospore is composed of the inner exospore (iE), which is thinner and electron denser than the outer exospore (oE), and the oE. The perispore is formed of the inner perispore (iP), with three strata, and the outer perispore (oP), formed of intermixed threads that constitute a lax structure. Bar: 1 μm.

interpreted as short threads in cross section (Plate 3, figures 1, 2; white arrowheads). The middle stratum (m) consists of threads of 40–60 nm in diameter that fuse to the adjacent strata (o) and (i), and are perpendicular to the spore surface and immersed in a homogeneous, less contrasted substance (Plate 3, figures 1, 2; arrows). In the inner stratum (i), there are densely packed threads (Plate 3, figures 1, 2; black arrowheads).

The threads that form a part of this iP layer are 50–80 nm in diameter, and have channels filled with an electron dense material.

3.2. *Structural units of the perispore in Cyathea*

The perispore in *Cyathea* consists of two layers. The outer one (P2) is thin and electron dense, and it covers the threads of the inner layer (Plate 4, figure 2, arrowhead). The inner layer (P1) consists of a three-dimensional network of threads arranged in a lax way, that are differentiated into two strata: the inner (iP1) and the outer (oP1; Plate 4, figures 1–3). The diameter of these threads is about 100 nm, with one or two channels (10–20 nm diameter) inside each one (Plate 4, figures 4–5).

3.3. *Structural units of the exospore*

The structure of the exospore in all the species studied is similar: it consists of two layers, a thick outer exospore (oE) and a thin inner exospore (iE; Plate 5, figure 1). Channels have been observed along both exospore layers (Plate 5, figures 1–4); these were continuous with the channels present in the inner perispore (Plate 5, figure 2). Towards the inner exospore, the channels are ramified and connected to cavities, especially near the laesurae (Plate 5, figure 1). At higher manifications, these channels show a complex system of compound interwoven substructures (Plate 5, figure 4).

4. Discussion

According to the results presented here, the substructural components of the Cyatheaceae sporoderm are

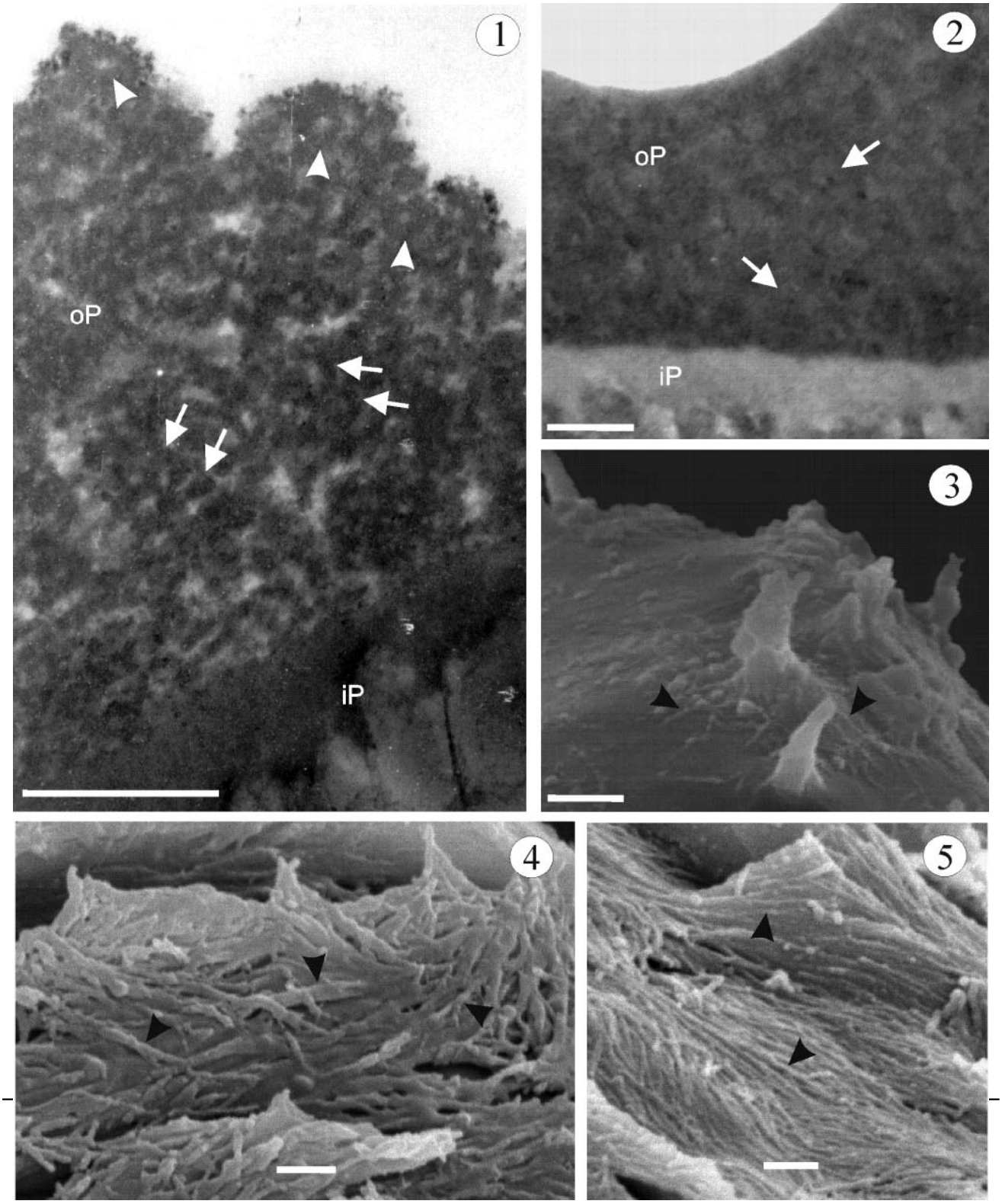

Plate 2. **1**. Perispore section of *Alsophila setosa* (transmission electron microscopy, TEM). Within the bulk of the outer perispore (oP), the ultrastructure is composed of threads (arrows), while on the surface these are seen on ends and appear as circular units (arrowheads). In this section, only two of the three strata of the inner perispore (iP) are shown; it has an outer stratum which is apparently homogeneous and tangentially oriented with respect to the middle stratum. Bar: 0.5 μm. **2**. Perispore section of *Sphaeropteris gardneri* (TEM). The ultrastructure of the outer perispore (oP). Threads in transverse sections show the electron-dense center (arrows). In the inner perispore (iP), the outer stratum is evident, which seems to be homogeneous. Bar: 100 nm. **3**. Spore surface of *Sphaeropteris gardneri* in detail with scanning electron microscopy (SEM). Threads are seen on the surface (arrowheads). Bar: 1 μm. **4**. Surface detail of *Alsophila capensis* (SEM). Bunches of threads are seen forming the outer spore surface (arrowheads). Bar: 1 μm. **5**. Spore surface of *Alsophila odonelliana* with SEM. Slim threads that form the outer perispore are distinguished on the spore surface (arrowheads), and they constitute the ornamentation. Bar: 1 μm.

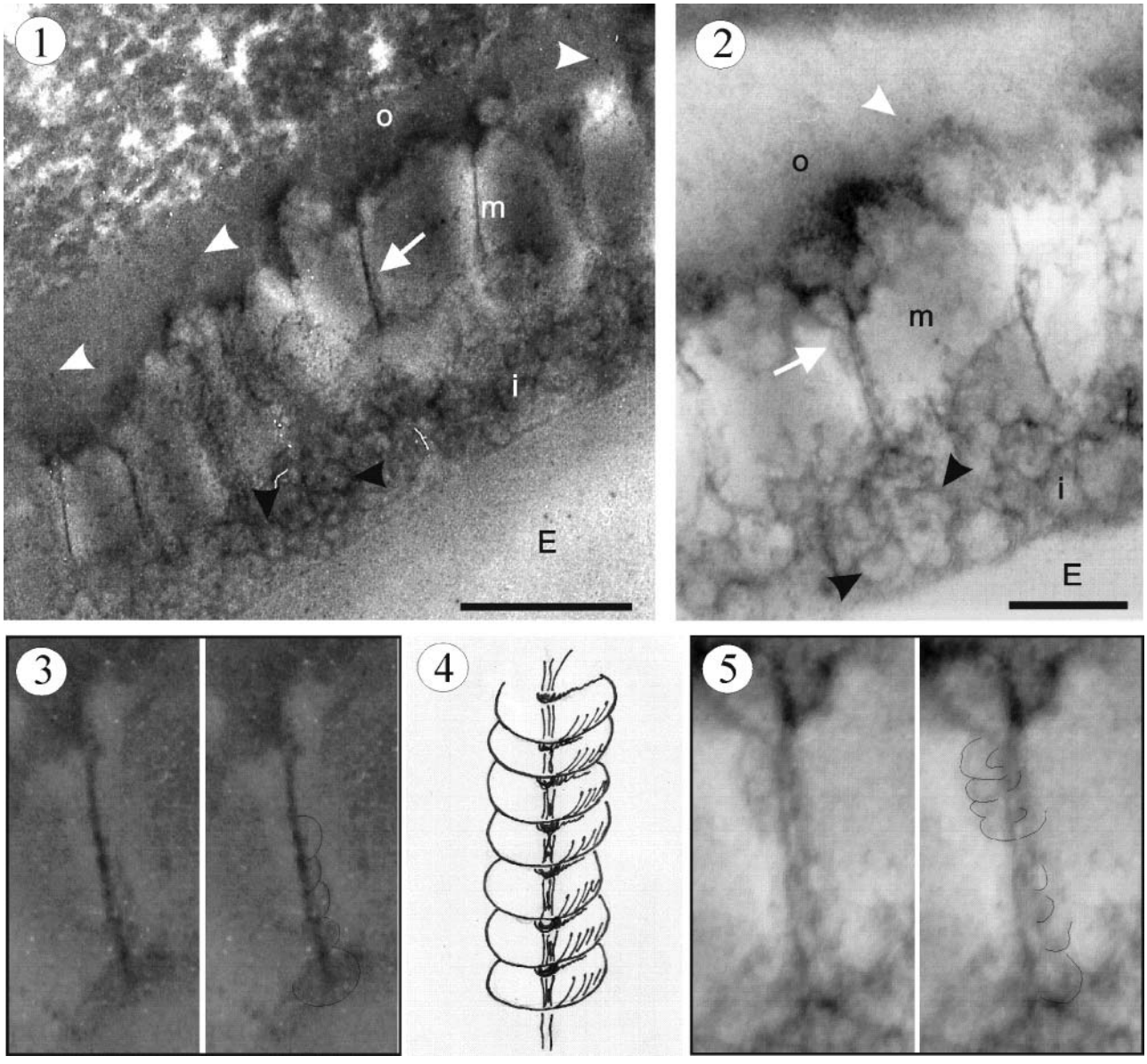

Plate 3. **1**. Perispore section of *Alsophila setosa* (transmission electron microscopy, TEM). Inner perispore (iP), in detail; three strata are distinguished: outer (**o**), medium (**m**) and inner (**i**). The arrowheads show transverse sections of the inner channels, passing along the threads. The arrows point to the threads in longitudinal view. E: exospore. Bar: 0.5 μm. **2**. Perispore section in *Sphaeropteris gardneri* with TEM. The inner perispore has three strata. In the outer stratum (**o**), the white arrowhead shows a section of a thread with an inner channel. In the middle stratum (**m**), the arrow shows a longitudinal section of a thread, and in the inner stratum (**i**), the black arrowheads shows threads transversally sectioned. Bar: 250 nm. **3**. The untouched image to the left shows a detail of a thread in the middle strata of iP. Its channel and subunits that roll up each thread are pointed out with an arrow in Plate 3, figure 1. To the right, traces are added to enhance the edges of loops. **4**. A scheme of a substructural coiled element. This scheme explains the disposition of the coiled elements and the core channel. **5**. The magnified portion of Plate 3, figure 2, that shows a thread in the middle stratum (iP, arrow). To the left, the inner channel and the coiled elements are discernible, according to the authors' interpretation. To the right, the edges of loops were enhanced by the authors.

threads, which constitute a complex system. These threads are large and circular in cross-section, as shown in SEM photographs. They have inner channels, around which smaller units are coiled, forming striations (see model, Plate 3, figure 4). These results, however, differ from those interpreted by Lugardon (1971, 1974), who described a granular substructure associated with a lumpy substance.

Several authors who have studied the ultrastructure of the sporoderm in *Lycophyta* and pollen grains of Gymnosperms and Angiosperms, also observed the presence of thread-like elements. Rowley (1995)

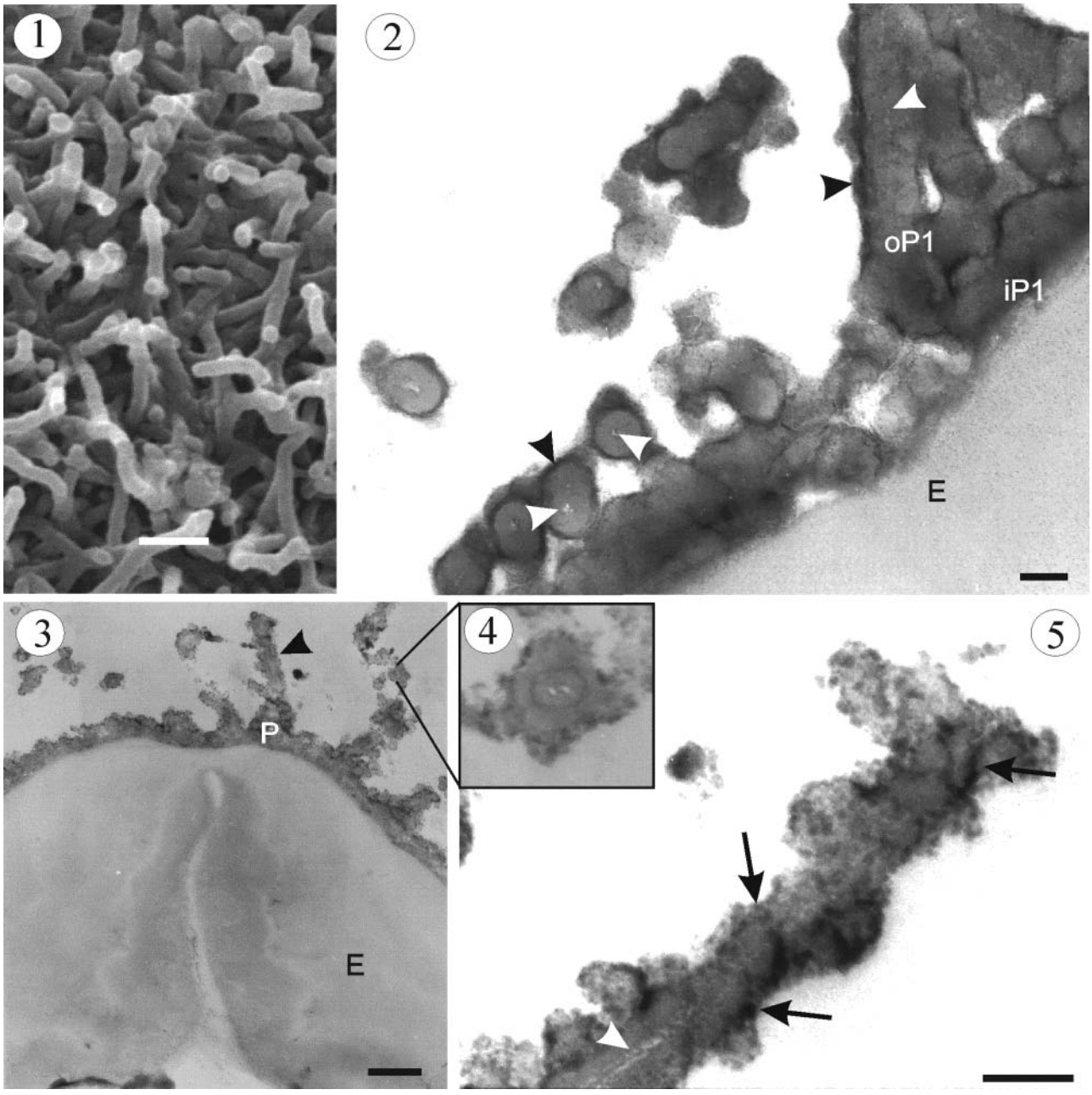

Plate 4. **1**. A magnified portion of the spore surface of *Cyathea corcovadensis* with scanning electron microscopy (SEM), that shows a three-dimensional network of threads. The ends of threads can be free or fused, forming spines. Bar: 1 μm. **2**. Section of *Cyathea atrovirens* with transmission electron microscopy (TEM). Transverse and longitudinal sections of threads in the outer stratum (oP1) and the inner stratum (iP1). The section shows the disposition of the inner channels (white arrowheads). The black arrowheads point to the outer perispore layer (P2), which has a high electron density. E: (exospore). Bar: 100 nm. **3**. Sporoderm section in the laesurae zone of *Cyathea delgadii*, with TEM. The exospore (E) is thicker than the perispore (P), which is formed of threads (arrowhead), with their main axes oriented tangentially to the exospore surface. Bar: 0.5 μm. **4**. Detail of the transverse section of a thread in Plate 4. 3. In a thread, two central channels are distinguished. Hence, the threads are bifurcate, and binders are seen around them. **5**. Longitudinal section of a thread of *Cyathea atrovirens*. The central channel (arrowhead) and binder subunits are evident (arrows). Bar: 100 nm.

noticed the existence of subunits defined as 'like wire-wound springs' (p. 13) found in the exospore of *Lycopodium* as well as in the exine of pollen grains of *Poa*, *Betula*, *Fagus* and *Artemisia.* Morbelli (1995) found in *Selaginella* megaspores walls units like rod-shaped coiled elements, circular in section. Wittborn et al. (1998) also observed similar cylindrical elements at a substructural level in *Fagus sylvatica* L. and

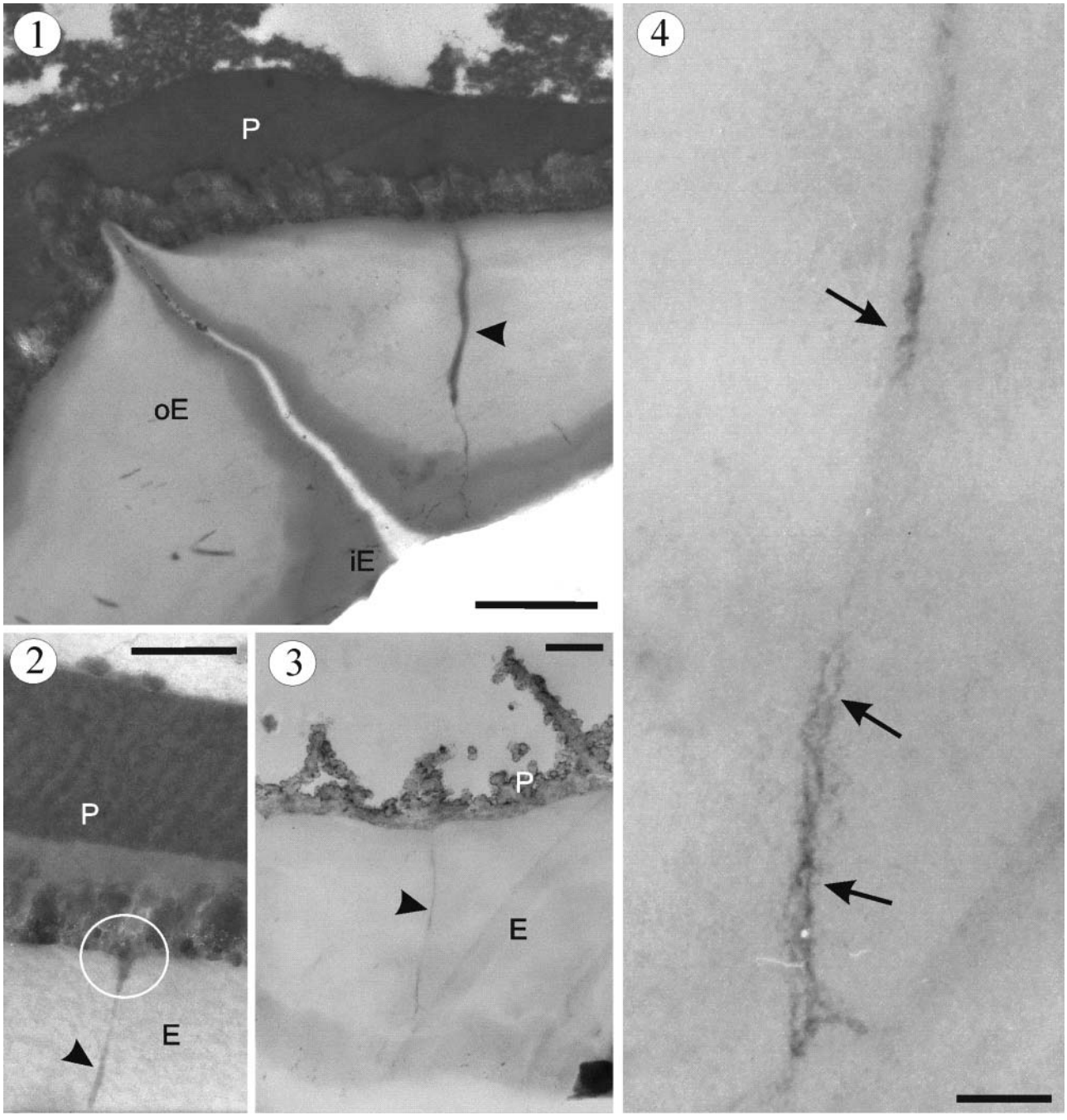

Plate 5. **1**. Sporoderm of *A. setosa* in transverse section in the lesura area with transmission electron microscopy (TEM). The exospore has two well-defined layers: the outer exospore (oE) and the inner exospore (iE). Within the bulk of the exospore channels that cross it through are evident (arrowhead). Bar: 1 μm. **2**. Sporoderm of *S. gardneri* in section with TEM. The arrowhead points to a channel within the exospore (E), which is in contact with the innermost layer of the perispore (P) (circle). Bar: 0.5 μm. **3**. Sporoderm of *C. atrovirens* in transverse section with TEM. The arrowhead points to a channel that runs throughout the exospore (E) and has a higher electron density. P: perispore. Bar: 0.5 μm. **4**. The channel pointed out in Plate 4, figure 3, with higher magnification. The arrows point to the elements that roll up around the central channel. Bar: 100 nm.

Lycopodium clavatum L., that were formed of helical subunits. The elements described in all cases (Rowley 1995, figure 14; Morbelli 1995, figure 4D; Wittborn et al. 1998), are quite similar to the threads described herein. Therefore, we can infer that thread-like elements are the most frequent substructural components in pollen and spore walls, in many different lineages of plants.

Recently, Gabarayeva and Hemsley (Hemsley et al. 1992; Gabarayeva 1993; Gabarayeva & Hemsley 2006; Gabarayeva et al. 2009a, 2009b; Gabarayeva & Grigorjeva 2010) proposed that the wall subunits of pollen and spores are formed by self-assembly. These authors state that although the species-specific nature of the exine structure suggests a genomic key, it appears that self-assembly processes interfere and distort the regular work of the genome, making the results unpredictable because of the non-linear character of self-assembly (Gabarayeva & Grigorjeva 2013). Gabarayeva & Hemsley (2006, 2009a) and Gabarayeva & Grigorjeva (2013) also suggested that, though the genome determines the exact chemical composition of all the substances and their concentrations necessary for exine development in the microspore periplasmic space, the rest of the constructive process is picked up by physico-chemical self-assembly.

These authors suggested a hypothesis to interpret the different stages of spore/microspore wall development on the basis of physical-chemical self-assembly processes unfolding in the colloidal micelle system in the periplasmatic space (see Gabarayeva et al. 2009a, their figure 1), where cylindrical or hexagonally packed cylindrical micelles stages were observed. This hypothesis might explain the mold that gives rise to threads, which we have observed in Cyatheacae spores.

In an article about the experimental destruction of the pollen wall in Gymnosperms and Angiosperms, Gabarayeva et al. (2003) observed cylindrical units in the exine, which were more evident when oxidative reagents were used. When the pollen grains were exposed to oxidative treatment for a long time, the rod-like elements were decreased in width and the diameter of the inner hollow core was increased, which could be explained by the elimination of secondarily accumulated sporopollenin. In this sense, we suppose that the variation in diameter of the threads in spores of Cyatheaceae could be related to the level of deposition of secondarily accumulated sporopollenin.

The diameter of the threads in this study is 50–100 nm, while the diameter of the central channels is about 10–25 nm. These sizes are similar to those pointed out by Rowley & Morbelli (2009) regarding the elements of the pollen walls. In our opinion, the variation in the diameters might be related to the number of minor subunits that constitute the threads.

The model of the substructural units suggested in our study coincides with the model presented by Rowley (Rowley 1981; Rowley & Dahl 1982, 1988, 1990; Rowley et al. 2003) regarding not only the spatial distribution of the constitutive elements, but also the thread diameter. From Rowley's point of view, the structural units of pollen walls are formed of a super-coiled binder around one to many core subunits – a tuft – originating from the plasma membrane-glycocalix system of the young microspores.

Although the presence of channels in the exospore, similar to those found in the tree ferns under our study, has been widely proven (Lugardon 1971; 1974; Van Konijnenburg-van Cittert & Kurman 1994; Tryon & Lugardon 1991; Giudice et al. 2000, 2006; Morbelli & Giudice 2001, 2010; Piñeiro et al. 2006; Ramos Giacosa et al. 2009, 2011, 2012), the presence of channels in the fern perispore has been documented by us for the first time. In fact, these kinds of structures have been found in all three Cyatheaceae genera studied, either inside the threads or in the different strata of the wall. In the genus *Cyathea*, they were found inside the threads, while in the case of *Sphaeropteris* and *Alsophila*, they can be observed all over the strata, although they tend to be more evident in the middle stratum of the internal perispore. As for the latter genera, the channels in the outer layer are seen inside well-differentiated threads, while in the inner layer they run through the different strata among the other elements.

The perispore channels in *Cyathea* were only described by Tryon & Lugardon (1991) but, in their study, the channels were described as having 'a clear central core' (p. 264), and the authors did not develop further explanations.

It has been observed that the threads' inner channels have a sort of communication with each other within the same stratum and even with the ones in different strata, as is shown in Plate 3, figures 1, 2 and Plate 5, figure 2. Thus, the perispore and exospore could be interconnected by a three-dimensional network of channels, which would connect the spore surface with their inner part. If we consider following Rowley (Rowley et al. 1999) in that the 'tufts' of the Angiosperms (Rowley 1986; El-Ghazaly & Rowley 1998; Rowley et al. 2003) as well as the 'wicks' of the *Lycophyta* (Morbelli & Rowley 1993; Rowley & Morbelli 1995) are equivalent to plasmodesmata, it is possible to suggest that the network of channels present in the Cyatheaceae sporoderm may be related to the exchange of substances between the outer and inner parts of a spore during its development. However, more studies are necessary to undertake to affirm this hypothesis.

To summarise, the results presented here make evident that the sub-structural components forming the exospore and perispore of the Cyatheaceae are threads of different size, and they have inner channels. These channels might constitute an interconnected network, which could allow an exchange of substances between the locular space and the spore cytoplasm during their development.

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