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Spore morphology and wall ultrastructure of *Actinostachys pennula* (Sw.) Hook. and *A. subtrijuga* (Mart.) C. Presl. (Schizaeaceae)

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ABSTRACT

Actinostachys is a genus of the Schizaeaceae with two species reported from America: *A. pennula* and *A. subtrijuga*. The spores of the two species are studied using light microscopy, scanning electron microscopy and transmission electron microscopy. The spores are monolete, the equatorial diameter is 46–84 μm and the polar diameter is 26–50 μm. In *A. pennula* the ornamentation of the spores is foveolate and in *A. subtrijuga* it is striate, composed by parallel ridges separated by ornamented grooves. In both species, the exospore is two-layered and it is the wall that constitutes the ornamentation of the spores. The perispore is two-layered and thin with granules, and covers the outer surfaces of the exospore including the foveolae. On the spore surface of both species, abundant spheroids are observed. The ultrastructural analysis reveals that some of them are spherules and others globules. The studied species can be easily identified by their spores analysed with light microscopy even without prior treatment. We recommend that the spore ornamentation be included in dichotomous keys and descriptions of *Actinostachys*. The sporoderm ultrastructure of the species is described for the first time. The results contribute to the identification of the species and also may be valuable characters for systematic and phylogenetic purposes.

KEYWORDS

Actinostachys; Schizaeaceae; spores; morphology; ultrastructure

1. Introduction

The Schizaeaceae is a monophyletic family that comprises only two genera: *Actinostachys* Wall and *Schizaea* Sm. The two genera are closely related, and traditionally they were both included in *Schizaea*. They can be distinguished by subdigitate sporangiophores and subterranean, tuberous and nongreen gametophytes in *Actinostachys*, or pinnate sporangiophores and green and filamentous gametophytes in *Schizaea* (Tryon & Tryon 1982; Smith et al. 2006).

Actinostachys is a genus of about 15 species, with a worldwide distribution in tropical regions (Mickel & Smith 2004; PPG I 2016). In America only two species are reported, growing from the USA (the state of Florida) to the south of Brazil: *Actinostachys pennula* (Sw.) Hook. and *A. subtrijuga* (Mart.) C. Presl. The two species are very similar, and according to da Silva et al. (2015) they can be differentiated by the rectangular or triangular cross-section of the leaves.

The species were treated in America as a part of the genus *Actinostachys* or *Schizaea,* such as in the works by Murillo (1986) from Colombia and León et al. (2005) from Peru, or in the context of floristic works and catalogues (Lellinger 1969; Sehnem 1974; Tryon & Stolze 1989; Mickel & Smith 2004; Funk et al 2007; Góes-Neto et al. 2014; da Silva et al. 2015; Santiago & Almeida 2017).

Concerning palynological analysis of the genus, Selling (1944) studied with light microscopy (LM) the spores of several extant and fossil species of *Schizaea* from the Old World and the New World. The author included in this work the spores of *A. pennula*, and mentioned the surface as pitted with the pits irregularly distributed. Van Konijnenburg-van Cittert (1991), in a comparative study of fossil and extant spores of Schizaeaceae, illustrated using a scanning electron microscope (SEM) the spores of other species of *Actinostachys* and *Schizaea*. The author characterised the spores of *Actinostachys* as having a pattern of parallel ridges and furrows of the exospore, giving them a striate appearance. Parkinson (1994, 1995a, 1995b), in studying the spore wall development in *Schizaea pectinata* from South Africa, observed the sporoderm to have a two-layered exospore and a two-layered perispore. Ramos Giacosa et al. (2015) studied with LM, SEM and transmission electron microscopy (TEM) the spores of *Schizaea fistulosa* Labill. from Chile. They described the spores as verrucate-tuberculate with a two-layered exospore and a thin perispore.

Regarding the two species discussed in the present work, very few studies have been carried out. Murillo & Bless (1978) and Solé de Porta & Murillo-Pulido (2005) studied the spores of *Actinostachys pennula* and *A. subtrijuga*, respectively, from Colombia with LM and described them as foveolate and striate. Tryon & Lugardon (1991), illustrated with SEM the spores of *A. subtrijuga* from Colombia and *A. pennula* from Brazil. They defined the spore ornamentation as striate in *A. subtrijuga* and foveolate in *A. pennula*. They also studied with TEM the spores of *A. pennula* and observed a two-layered exospore and a thin granulate perispore.

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The aim of this work is to analyse the spore morphology of *Actinostachys pennula* and *A. subtrijuga* with LM, SEM and TEM in order to contribute to the palynological knowledge of the family Schizaeaceae, and to give useful spore characters for the identification of the species.

2. Materials and methods

Spores were obtained from herbarium specimens from the following institutions: Instituto de Botánica del Nordeste (CTES), División Plantas Vasculares, Facultad de Ciencias Naturales y Museo, Universidad Nacional de La Plata (LP) and Herbario Nacional de Venezuela, Instituto Experimental Jardín Botánico Dr. Tobías Lasser, Universidad Central de Venezuela (VEN).

The spores were studied using LM, SEM and TEM. For LM, the spores were acetolysed according to the method of Erdtman (1960). Additionally, spores without prior treatment were directly placed onto microscope slides with glycerin jelly, to corroborate the usefulness of this method for the identification of the species.

For SEM, the material without treatment was placed onto stubs with adhesive double-faced tape and coated with gold.

For TEM, dry material from herbarium specimens was hydrated following the technique proposed by Rowley & Nilsson (1972), which consists of the use of phosphate buffer and alcian blue (AB); then the material was fixed with glutaraldehyde + 1% AB in phosphate buffer for 12 h and post-fixed with 1% OsO4 in water plus 1% AB. The spores were dehydrated in an acetone series and then embedded in Spurr soft mixture. Sections 3 μm thick were stained with toluidine blue and analysed with LM. Ultra-thin sections were stained with 1% uranyl acetate for 15 min followed by lead citrate for 3 min.

The observations were made with a Nikon E200 light microscope, a JEOL JSMT-100 SEM and Zeiss T-109 TEM. For spore descriptions, the terms proposed by Tryon & Lugardon (1991) and Punt et al. (2007) were used.

2.1. Specimens investigated

Actinostachys pennula: Bolivia, Departamento Beni., Provincia Vaca Diez, 17-IV-1979, Krapovickas & Schinini 35.063 (LP); Departamento La Paz, Provincia Iturralde, Siete Cielos, Río Manupare, 8 June 1987, Solomon 17.007 (LP). Brazil, Estado Amazonas, Manaus, near Ponta Negra, 16 June 1966, Andrade Lima 15.732 (LP); Estado do Pará, Alto Tapajós, Rio Cururú, 7°35′S, 57°31′W, 6 February 1964, Anderson 10.545 (LP); Serra de Cachimbo, BR 163 Cuibá-Santarém, km 823, 7 November 1977, Prance et al. 24.979 (LP); Estado Paraná, Mun. Paranagua, Rio Pereque, 31 May 1962, Hatschbach 9.152 (LP); Mun. Pontal do Paraná, Ponta do Poco, 9 November 2003, J. M. Silva et al. 3.895 (CTES); Estado Pernambuco, Goiana, 28 December 1965, Andrade Lima 13.721 (LP). Venezuela, Estado de Amazonas, Dpto. Casiquiare, caño Pimichin, 3 July 1979, Bunting 4.083 (VEN), 4 km NE of San Carlos de Río Negro, 1°56′N, 67°03′W, 7 May 1979, Liesner 7.280 (VEN); Estado de Apure, Distrito Pedro Camejo, calcetas de las Galeras, 3 April 1989, Gómez et al. 629 (VEN); Estado de Bolívar, cumbre de Cerro Guaiquinima a lo largo del Río Szczerbanari, 5°44′N, 63°41′W, 20 January 1977, Steyermark & Dunsterville 113.268 (VEN).

Actinostachys subtrijuga: Brasil, Estado Amazonas, Mun. de Humaitá, Río Madeira, road Humaitá to Labrea, 25 November 1966, Prance et al. 3.291 (LP); Basin of Rio Negro, Río Uneiuxi, 8 November 1971, Prance et al. 16.189 (LP). Venezuela, Estado de Amazonas, Dpto. Atabapo, E del caño Perro de Agua, 3°47′N, 67°00′W, 30 November 1978, Huber & Tillet 2.746 (VEN), Dpto. Río Negro, Cuenca del brazo de Casiquiare, 3 February 1992, Stergios et al. 15.549 (VEN); Mucuruapi, 1°20′N, 66°26′W, 28 June 1984, Davidse & Miller 26.768 (VEN); granitic outcrop on south side of lower Rio Guasacavi, 3°12′N, 67°25′W, 2 March 1996, Berry et al. 5.850 (VEN); Estado Apure, Distrito Pedro Camejo, Río Juriepe, 25 February 1979, Davidse & González 15.770 (VEN).

3. Results

3.1. Actinostachys pennula

The spores are monolete and elliptic in polar view. In equatorial view, the proximal face is plane to convex and the distal face is convex. The equatorial diameter is 46–84 μm, and the polar diameter is 26–50 μm. The laesurae are 29–54 μm long.

The spores observed with LM (Plate 1, figures 1–2) are foveolate with some spheroids distributed on the spore surface. The laesurae are prominent and somewhat translucent.

Observed with SEM, the spores are foveolate (Plate 2, figures 1–3). The foveolae are circular or elliptical, irregular in size (Plate 2, figure 5) and distributed uniformly in the spore. The laesura is thick and protruding (Plate 2, figure 3).

Single or fused spheroids are observed on the spore surface, and sometimes their location coincides with that of some foveolae (Plate 2, figures 2, 5). The abundance of spheroids is variable according to the specimens analysed (Plate 2, figures 3, 4) and can be located even on the laesura.

Small granules are found between the foveolae and constituted the ornamentation of the perispore (Plate 2, figure 5). In some areas where the perispore was detached a smooth exospore is evident (Plate 2, figure 6).

The foveolate exospore is clearly observed in the fractured spores. The perispore is very thin and covers the exospore surface (Plate 2, figure 7).

When analysed with TEM (Plate 4), the exospore is 0.7–2.2 μm thick and two-layered: having an inner layer 0.2–0.3 μm thick and an outer layer 0.4–1.9 μm thick. The outer exospore layer forms the ornamentation of the spores (Plate 4, figures 1–3).

The perispore is 0.03–0.13 μm thick and two-layered. The inner layer (P1) is 21–29 nm thick and more contrasted, and is attached to the outer exospore. The outer layer (P2) is 20–50 nm thick with an irregular surface (Plate 4, figure 5). The perispore covers the outer surfaces of the exospore including the foveolae (Plate 4, figure 4). Some membranes (scales) are observed immersed in the perispore (Plate 4, figure 5).

Spheroids are found on the spore surface. The most abundant are strongly contrasted and have a similar contrast to the perispore. They have a granular structure and may be single or joined together by threads of perisporal material (Plate 5, figures 1–3). Other spheroids are also evident on the spore surface. They have an inner portion which has similar

structure to that of the exospore, and an outer layer with the same structure as the perispore. They may be fused with the perispore (Plate 5, figure 4) or with the exospore (Plate 4, figure 1; Plate 5, figure 5).

3.2. *Actinostachys subtrijuga*

The spores are monolete and elliptic in polar view. In equatorial view, the proximal face is plane and the distal face is convex. The equatorial diameter is 54–77 μm, and the polar diameter is 29–43 μm. The laesurae are 28–49 μm long.

The spores observed with LM (Plate 1, figures 3–4) are striate with low ridges somewhat parallel to the equator. Some spheroids are evident on the spore surface and may be single or fused to one another.

Observed with SEM, the spores are striate (Plate 3, figure 1–3). The exospore is composed by parallel ridges separated by ornamented grooves. The ridges are frequently interrupted by depressions of variable size which have an irregular circular shape with a defined edge. In some cases they are located between two ridges connecting them (Plate 3, figure 4). A few ridges are anastomosed (Plate 3, figure 2) or bifurcated (Plate 3, figure 3). The grooves are ornamented by verrucae of the exospore (Plate 3, figure 4).

The perispore is thin and covers the surface of the exospore, including the depressions, and is ornamented by small granules (Plate 3, figure 6).

Single or fused spheroids can be observed on the spore surface, distributed over the whole spore, even on the laesura (Plate 3, figures 1–3, 5, 6).

When analysed with TEM (Plate 6), the exospore is 1–2.3 μm thick and two-layered, having an inner layer 0.2–1.3 μm thick and an outer layer 0.7–1.6 μm thick and less contrasted than the inner layer. The inner exospore layer is thicker in the area of the laesura and clearly distinguished around the entire outline of the spore. The outer exospore layer forms the ornamentation of the spores which consists of ridges (Plate 6, figure 1–3) and some verrucae located on the grooves (Plate 6, figure 6).

The perispore is 0.09–0.5 μm thick and two-layered. The inner layer (P1) is 7–14 nm thick, highly contrasted and strongly attached to the outer exospore, following its contours. The outer layer (P2) is 70–500 nm thick, with an irregular surface (Plate 6, figure 5). Sectioned small granules that form the perispore ornamentation are observed (Plate 6, figure 5).

Spheroids are located on the spore surface. Some of them are strongly contrasted, with similar structure to the perispore (Plate 6, figure 4). Others are grouped together and have an inner portion with similar structure to that of the exospore, and an outer layer with the same structure as the perispore (Plate 6, figure 6). The spheroids are surrounded by abundant perisporal material and some membranes (scales) which are oriented in several directions (Plate 6, figure 7).

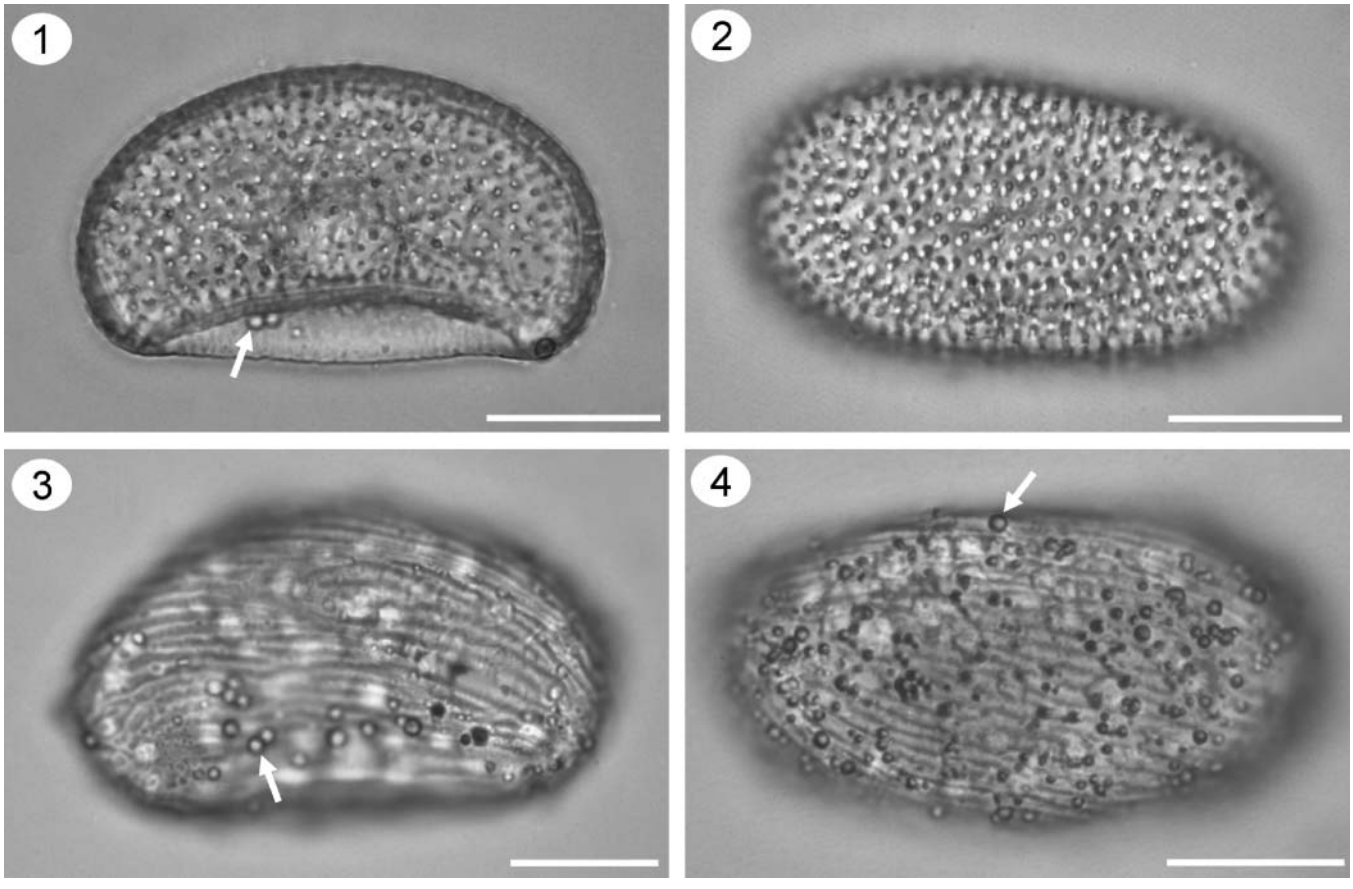

Plate 1. Spores of *Actinostachys pennula* and *A. subtrijuga* with LM.
1–2. *A. pennula*. **1.** Equatorial view of a foveolate spore with a translucent laesura. Some spheroids are evident (arrow). **2.** Distal view of an elliptic spore. **3–4.** *A. subtrijuga*. **3.** Equatorial view of a striate spore. Some spheroids are observed on the spore surface (arrow). **4.** Distal view of a spore. Numerous spheroids are also evident in this view (arrow). Scale bars = 20 μm.

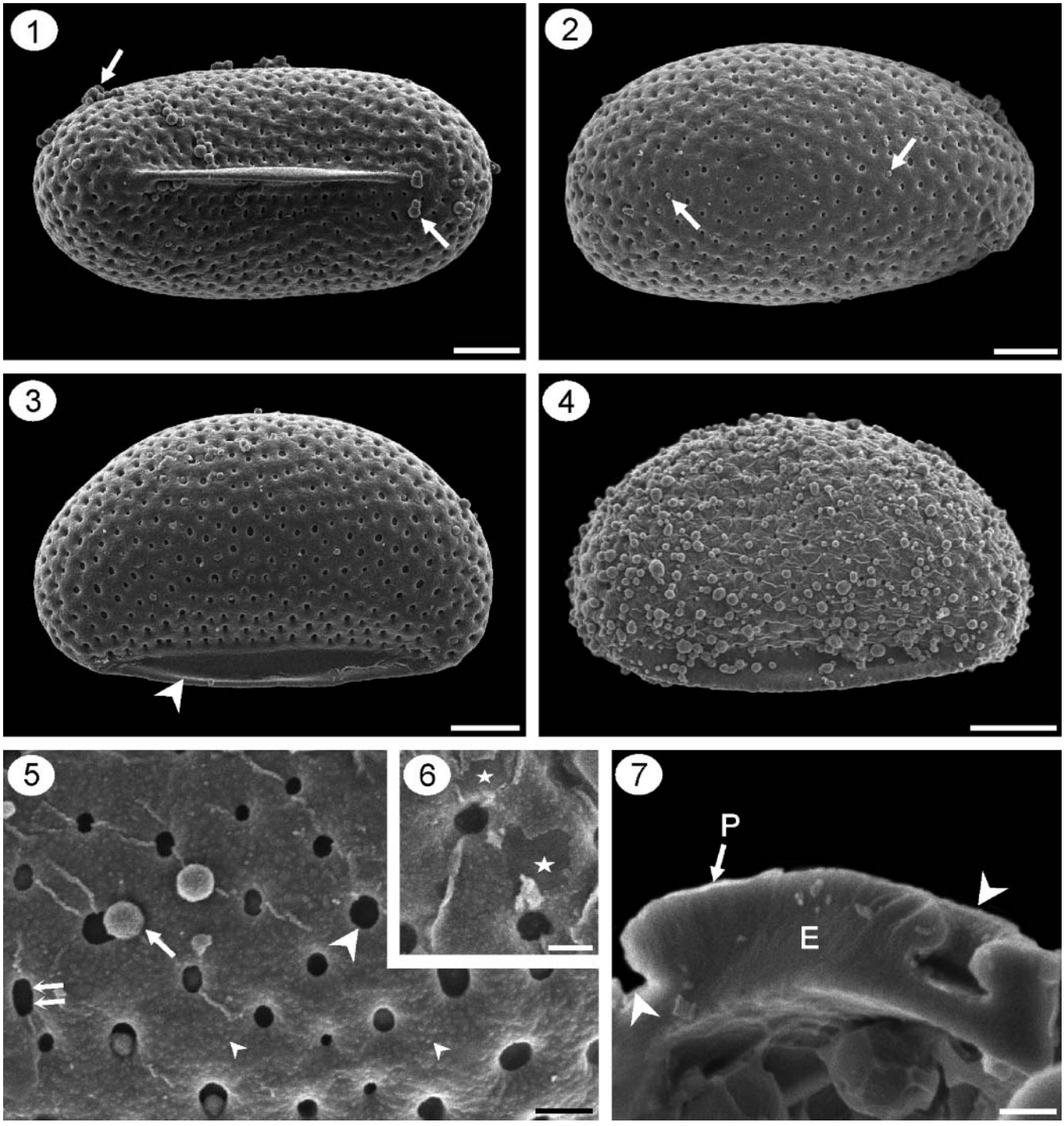

Plate 2. Spores of *Actinostachys pennula* with SEM.
1. Proximal view of a foveolate spore. Single or fused spheroids are observed on the spore surface (arrows). **2.** Distal view of a spore. The location of some spheroids coincides with some foveolae (arrows). **3.** Equatorial view of a spore with a prominent laesura (arrowhead). **4.** Equatorial view of a spore with numerous spheroids on the spore surface. **5.** Detail of the spore surface. The foveolae may be circular (arrowhead) or elliptical (double arrow). Small granules (small arrowheads) are observed between the foveolae and constitute the ornamentation of the perispore. Some spheroids are also observed (arrow). **6.** In some areas where the perispore was detached a smooth exospore is evident (stars). **7.** Fractured spore. The exospore (E) forms the foveolated ornamentation (arrowheads). The thin perispore (P) covers the outer surface of the exospore. Scale bars: 1–4 = 10 μm; 5 = 2 μm; 6–7 = 1 μm.

4. Discussion and conclusions

In the present work we defined the spores of *Actinostachys pennula* as foveolate and those of *A. subtrijuga* as striate with low ridges. This observation is similar to the ornamentation mentioned by other authors (Tryon & Lugardon 1991; Van Konijnenburg-van Cittert 1991). Nevertheless, we also noticed some details in the spore morphology of *A. subtrijuga* that were not previously mentioned. Some verrucae are found on the grooves and this ornamentation corresponds to the exospore. Also, the ridges are frequently interrupted by depressions of variable size which have an irregular circular shape with a defined edge.

The spores of the two species studied were observed with abundant single or fused spheroids distributed on the surface

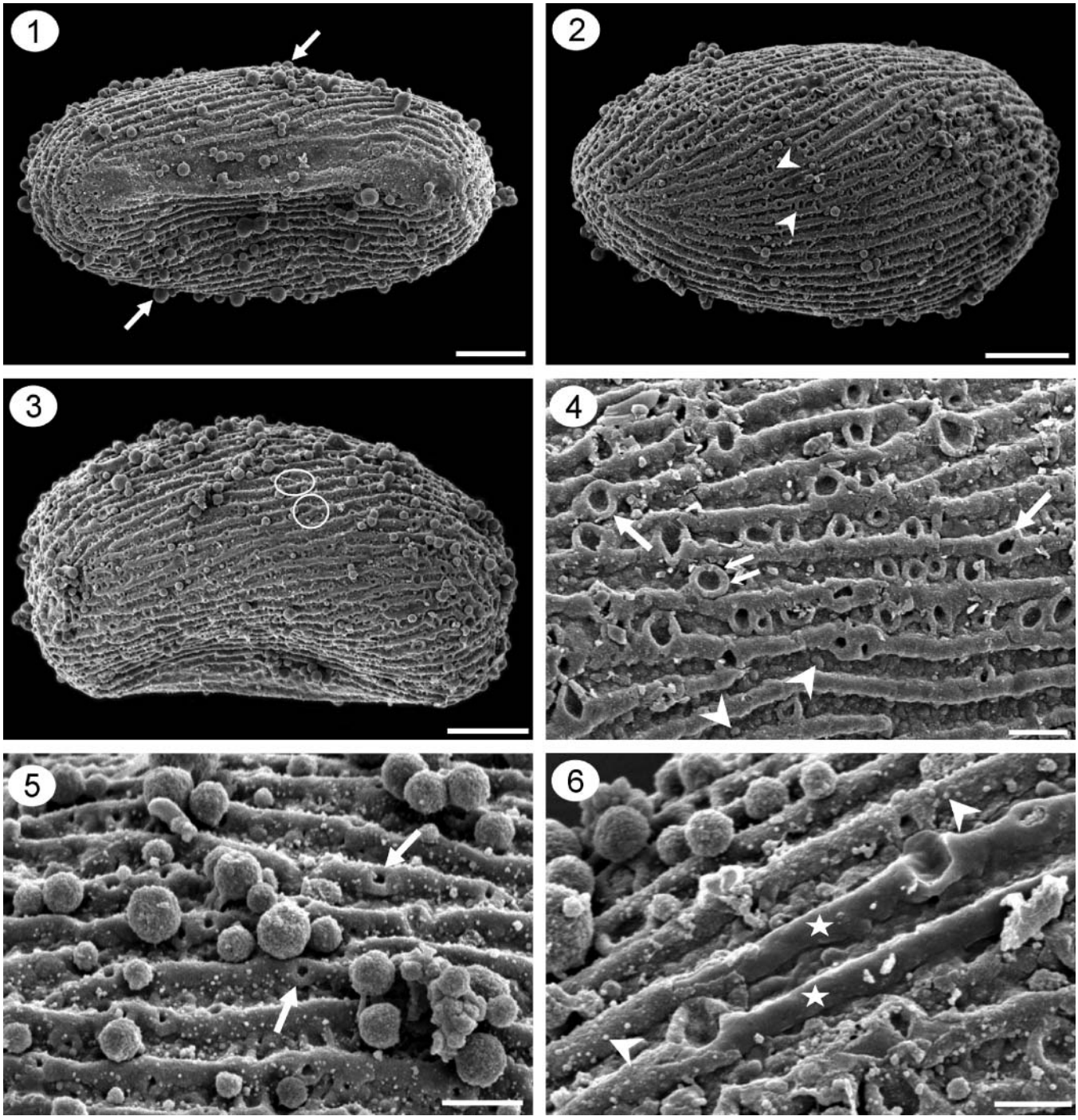

Plate 3. Spores of *Actinostachys subtrijuga* with SEM. **1.** Proximal view of a striate spore. Single or fused spheroids are observed on the spore surface (arrows). **2.** Distal view of a spore. The ridges are parallel to the equator and some of them are anastomosed to others (arrowheads). **3.** Equatorial view of a spore. Some ridges are bifurcated (circles). **4.** Detail of the spore surface. The ridges are interrupted by depressions which have an irregular circular shape with a defined edge (arrows). In some cases they are located between two ridges (double arrow). The grooves are ornamented by verrucae (arrowheads). **5.** Detail of the single and fused spheroids located on the spore surface. Ridges with small circular depressions are also evident (arrows). **6.** The perispore is composed by small granules (arrowheads). In two ridges and depressions the perispore was detached and a smooth exospore is observed (stars). Scale bars: 1–3 = 10 μm; 4–5 = 2.5 μm; 6 = 2 μm.

of the spores, even on the laesurae. During the course of the study, the abundance of spheroids observed was variable in different studied specimens of the same species. This condition can be explained by several stages of development of the analysed spores. The presence of these spheroids on the spore surface was cited in *Actinostachys pennula* and *A. subtrijuga* by Tryon & Lugardon (1991), and they were also found on the spores of several species of the related genus *Schizaea* (Large & Braggins 1991; Tryon & Lugardon 1991; Ramos Giacosa et al. 2015).

Another difference between the two taxa was the morphology of the laesurae. Those of *A. pennula* were plane to convex, prominent and translucent, as observed by LM. In the case of *A. subtrijuga* the laesurae were plane and less prominent.

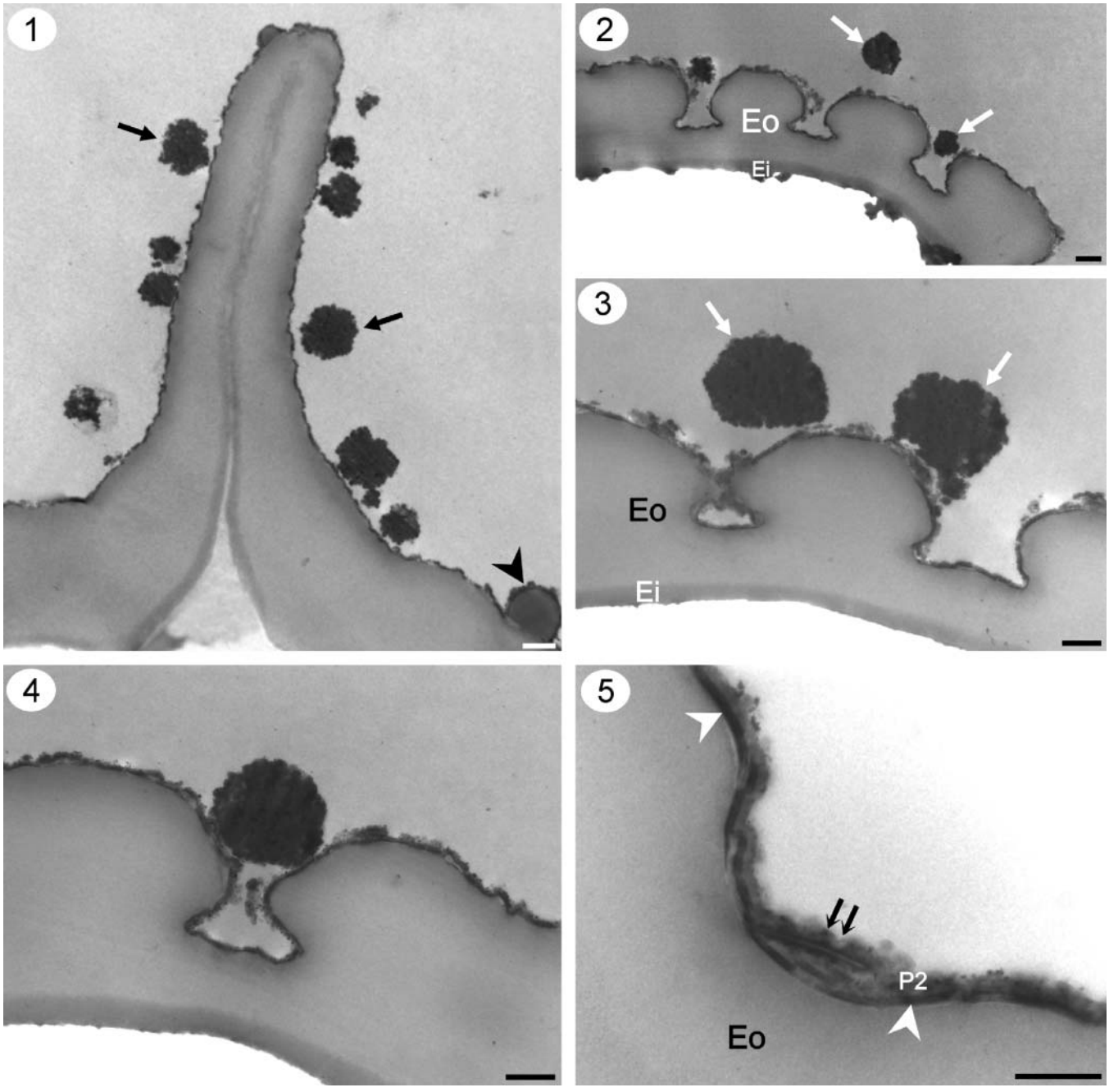

Plate 4. Spores of *Actinostachys pennula* with TEM. **1.** Section through the laesura. Spheroids are seen on both sides of the laesura, free (arrows) or attached to the exospore (arrowhead). **2, 3.** Section through the sporoderm. The exospore consists of two layers. The inner exospore layer (Ei) is more contrasted and thinner than the outer exospore layer (Eo). The outer exospore forms the ornamentation of the spore. Some spheroids are also seen (arrows). **4.** A spheroid is located on a foveola as can be seen in the SEM image of Plate 2, figure 2. **5.** Detail of the perispore. It is two-layered: The inner layer (P1, arrowheads) is more contrasted, thin and is attached to the outer exospore (Eo). The outer layer (P2) has an irregular surface. A scale is observed immersed in the perispore (double arrow). Scale bars: 1, 2 = 0.5 μm; 3, 4 = 0.4 μm; 5 = 0.2 μm.

The spore morphology of Schizaeales (Anemiaceae, Lygodiaceae and Schizaeaceae) is a useful character to identify related species, as in the case of *Lygodium* (Ramos Giacosa et al. 2013; Arana et al. 2015). According to our results, the different ornamentation observed in the spores of the two species of *Actinostachys* can be easily identified by placing the spores, without prior treatment, onto microscope slides with glycerin jelly or even water and analysing them with LM. This technique constitutes an efficient and quick method to check the identity of fertile plant specimens. For the reason that the two species are very similar plants and sometimes confused in the herbarium material, we recommend that the spore ornamentation should be included in dichotomous keys and species descriptions as an additional useful character to consider.

Regarding the sporoderm analysis, the exospore is the thickest wall and the one that bears the spore ornamentation constituted by foveolae in *A. pennula* and ridges in *A. subtrijuga*. This wall is visible with LM, SEM and TEM. The perispore is thin and covers the surface of the exospore and is also ornamented in

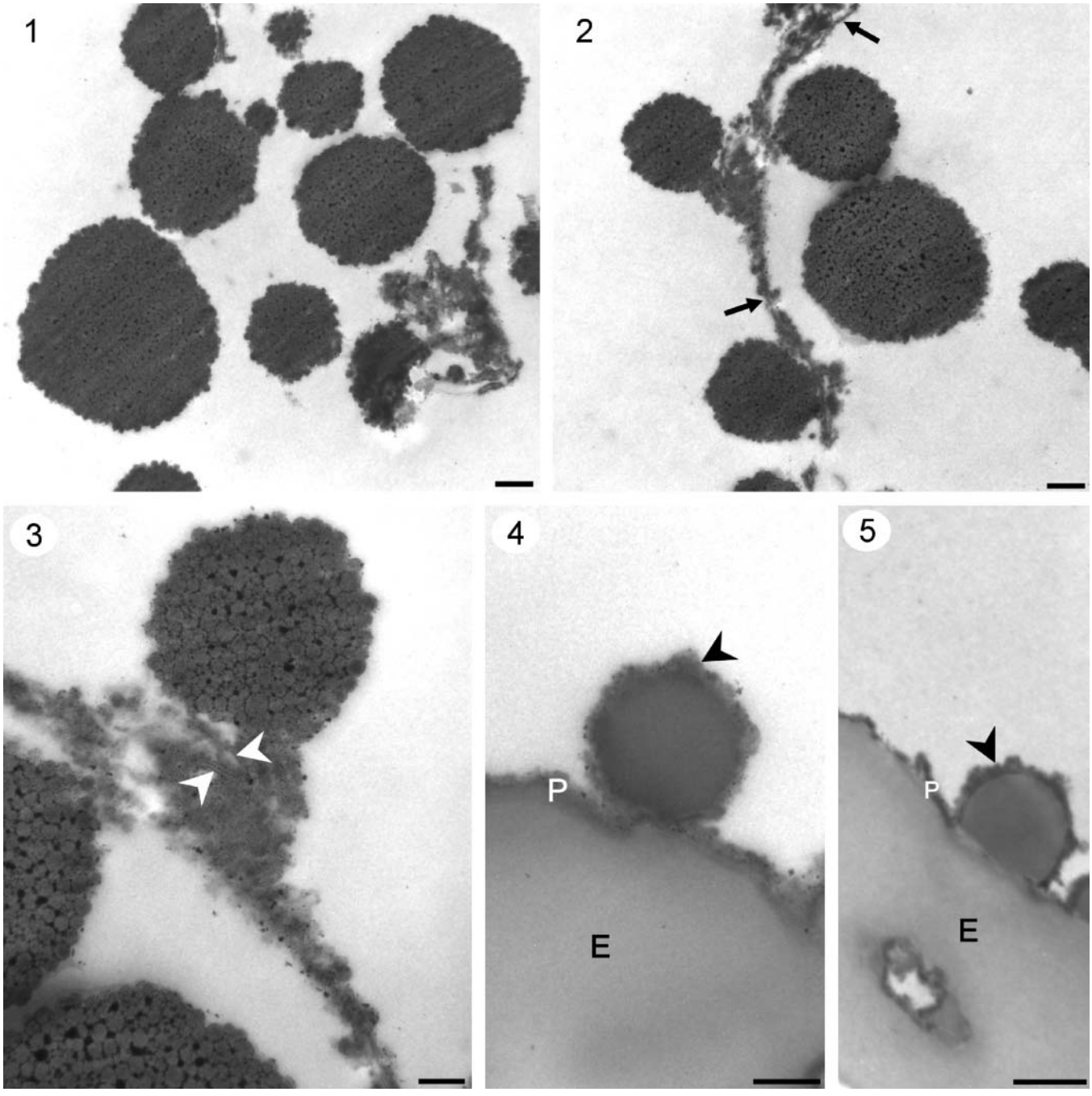

Plate 5. Spheroids of *Actinostachys pennula* with TEM. **1–3.** Spheroids consisting only of perisporal material. **1.** Spheroids of variable size are frequently observed in groups. They are strongly contrasted. **2, 3.** Some of them are joined together by threads of perisporal material (arrows). They have a granular structure. Some scales (arrowheads) are also found. **4, 5.** Single spheroids consisting of an inner portion with similar structure to the exospore (E), and an outer layer (arrowhead) with the same structure as the perispore (P), are observed. They may be fused with the perispore (figure 4) or with the exospore (figure 5). Scale bars: 1, 2 = 0.4 μm; 3, 4 = 0.2 μm; 5 = 0.5 μm.

both species. The ornamentation consists of small granules that can be observed with SEM and TEM.

Until now, the sporoderm ultrastucture of the species studied here was almost unknown. The results of the present work reveal that the spores of *Actinostachys pennula* and *A. subtrijuga* have the same sporoderm stratification: a two-layered exospore and a two-layered perispore with the inner layer strongly adhered to the outer exospore.

Spheroids are frequent on the spore surface of the two analysed species. Based on the information provided by the TEM analysis, we conclude that two types of spheroids can be observed. The first type has an inner portion with similar structure to the exospore, and is surrounded by material with the same structure as the perispore. According to their size, location, resistance to acetolysis and ultrastructure, they appeared to be 'globules' as defined by Lugardon (1981), and they may be homologous to Ubisch bodies of the spermatophytes. These globules were observed fused with the perispore or with the exospore. The presence of globules was also reported for *Schizaea fistulosa* (Ramos Giacosa et al. 2015).

The second type of spheroid is strongly contrasted and has a similar contrast to the perispore. They have a granular structure and may be single or joined together by threads of perisporal material. These superficial bodies, wholly consisting of

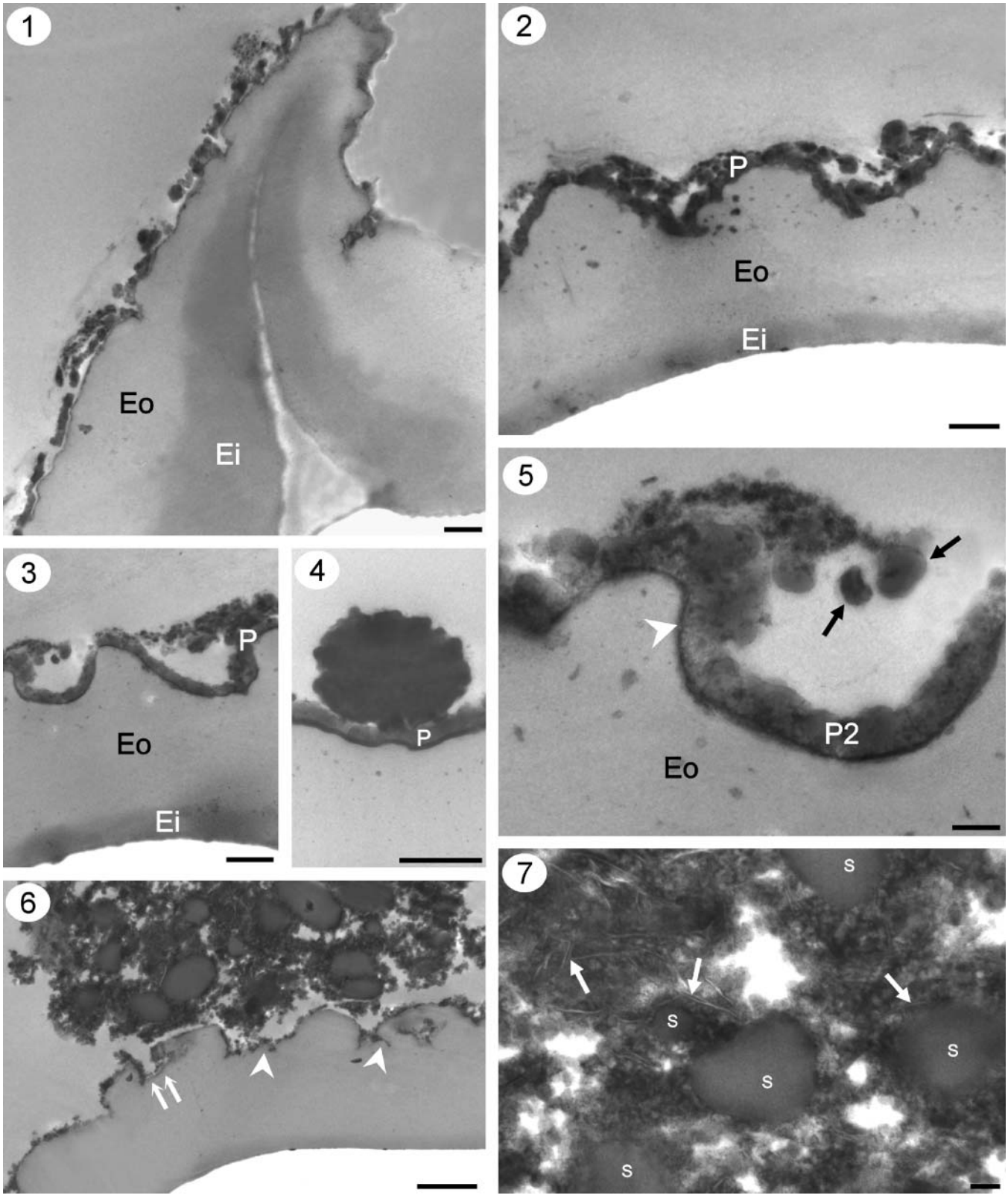

Plate 6. Spores of *Actinostachys subtrijuga* with TEM. **1.** Section through the laesura. The exospore consists of two layers. The inner exospore layer (Ei) is more contrasted than the outer exospore layer (Eo). **2, 3.** Section through the sporoderm. The inner exospore layer (Ei) is thinner than in the laesura area. The outer exospore (Eo) forms the ornamentation which consist of ridges. On the exospore, a more contrasted perispore (P) is evident. **4.** A single spheroid consisting of perisporal material. Below, a two-layered perispore is observed (P). **5.** Detail of the two-layered perispore. The inner layer (P1, arrowhead) is more contrasted, thin and is strongly attached to the outer exospore (Eo). The outer layer (P2) is thicker, with an irregular surface. The small granules observed in the SEM images can be seen sectioned in the perispore surface (arrows). **6.** Group of spheroids located on the spore surface. They have a core with similar structure to the exospore, and an outer layer with similar structure and contrast to the perispore. Verrucae of the exospore are observed on the grooves (arrowheads). Depressions observed in Plate 3, figure 4, are seen sectioned (double arrow). **7.** Detail of the spheroids (s). They are irregular in size and are surrounded by abundant perisporal material interwoven with scales (arrows). Scale bars: 1–4 = 0.4 μm; 5–7 = 0.1 μm.

perisporal material and developed within the sporangium cavity at the same time as the perispore, are recognised as spherules (Tryon & Lugardon 1991).

Thus, the importance of TEM studies is evident because globules and spherules are impossible to differentiate under LM or SEM observation.

In other species of *Actinostachys* and *Schizaea* the presence of spherules were reported, and also in other Schizaeales such as the genus *Lygodium* Sw. (Lygodiaceae) (Ramos Giacosa et al. 2013). The presence of scales surrounding globules is also found in other fern families such as Anemiaceae (Ramos Giacosa 2014) and Blechnaceae (Tryon & Lugardon 1991; Ramos Giacosa et al. 2009).

The sporoderm stratification described in the present work coincides with that described by Parkinson (1995a), who studied the spore wall development of *Schizaea pectinata* from South Africa. This author refers to a clear distinction between the inner and outer perispore layers, a feature that is also evident in the perispore of *A. pennula* and *A. subtrijuga* described in our contribution.

Moreover, in *S. pectinata* are mentioned different types of bodies that consist exclusively of perisporal material and are associated with the formation of the perispore. In addition, a combination tapetum consisting of a cellular, parietal component and a plasmodial component occur in the species (Parkinson 1995a, 1995b). Although the type of tapetum is unknown for *Actinostachys pennula* and *A. subtrijuga*, we suggest that the spherules with granular structure found in the spores of these species are spherical bodies that contribute to the perispore formation and remain in the spore surface as a remnant of the tapetum. More ultrastructure research is needed to corroborate whether the sporoderm stratification and the presence of spherules are stable characters in the Schizaeaceae family.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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