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Plant anatomy: history and future directions

Spore wall cytochemistry and structure in five species of the liverwort *Riccia* (Ricciaceae)

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Abstract

The spore wall of *Riccia plano-biconvexa*, *R. fruchartii*, *R. cavernosa*, *R. stenophylla*, and *R. curtisii* was analyzed using light microscopy. The study involved cytochemical analysis to distinguish the different layers of the sporoderm and assess their chemical composition. In all the species studied, the sporoderm was found to have a tri-layered exine, consisting of a lamellar exine 1 that forms ridges and depressions, an intermediate exine 2, and a thick and generally discontinuous exine 3. Cytochemical studies indicated that exine 1 and 2 are composed of sporopollenin, while exine 3 is a mixture of sporopollenin and polysaccharides. The intine, on the other hand, is the only layer that is purely cellulosic and contains pectins. Based on the chemical composition and structural organization, exine 1 and 2 can be categorized as ectexine while exine 3 corresponds to an endexine. We emphasize the importance of using cytochemical tests to study the spore wall in liverworts and other bryophytes.

Key words: exine, intine, liverwort sporoderm, *Riccia*, sporopollenin.

Resumo

Os esporos de *Riccia plano-biconvexa*, *R.fruchartii*, *R. cavernosa*, *R. stenophylla* and *R. curtisii* foram analisados sob microscopia de luz. O estudo citoquímico foi realizado para distinguir as diferentes camadas do esporoderma e para determinar a sua natureza química. O esporoderma de todas as espécies estudadas apresenta uma exina com três camadas, composta por uma exina 1, lamelar, que forma cristas e depressões, uma exina 2, intermediária, e uma exina 3, espessa e geralmente descontínua. Os testes citoquímicos indicam que as exinas 1 e 2 são compostas por esporopolenina, enquanto a exina 3 é uma mistura de esporopolenina e polissacarídeos. A intina é a única camada só celulósica, além de conter pectinas. Com base na composição química e na organização estrutural, as exinas 1 e 2 podem ser agrupadas numa ectexina, enquanto a exina 3 corresponde a uma endexina. Nós ressaltamos a importância do uso de testes citoquímicos para o estudo da parede de esporos em hepáticas e outras briófitas.

Palavras-chave: exina, intina, esporoderma de hepática, *Riccia*, esporopolenina.

Introduction

Riccia L. (Ricciaceae, Marchantiales) represents a monophyletic lineage (Wheeler 2000; Villarreal *et al.* 2016) characterized by a remarkable diversity in vegetative and reproductive morphological traits, including significant variations in spore morphology (Schuster 1992). These spore features are central to taxonomic concepts, aiding species diagnosis.

Riccia spores are among the largest in liverworts, with diameters ranging from 60 to 200 µm. The shape and ornamentation of the spores are crucial criteria for species identification, making the sporoderm (or spore wall) ornamentation an essential feature in identification keys and descriptions within the family Ricciaceae (Jones 1957; Thaithong 1982; Perold 1989; Jovet-Ast 2005; Singh *et al.* 2010). Various traits, such as

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spore shape (ranging from spherical to angular), the presence of a trilete scar on the proximal face, and the ornamentation of the exine (comprising ridges and depressions), are commonly used for identification purposes. The ridges may form areolae or reticules with papillae or tubercles at the angles, and the spores may have complete or incomplete, smooth or crenulated wings, sometimes with papillae. Furthermore, spores can occur as monads or tetrads (Jovet-Ast 1966, 1975, 2005; Vianna 1985; Perold 1989; Schuster 1992; Gradstein & Costa 2003).

The early 20th century witnessed researchers exploring sporogenesis and sporoderm stratification in the genus *Riccia*. Beer (1906) was the first to examine spore development in *Riccia* and described the sporoderm as consisting of three layers in mature spores: (1) a laminated primary wall, (2) a secondary wall with two sublayers, and (3) a homogeneous inner layer. Black (1913) further investigated sporoderm development in *R. frostii* Aust. and observed the primary wall being deposited by the "mother cell membrane". At maturity, this spore wall gave rise to the outer coating of the spore, which exhibited a system of irregular ridges, together with a secondary layer, forming the exospore (exine), while the endospore (intine) formed inward. The sporoderm of *R. cavernosa* Hoffm. had an ornamented outer layer and an inner layer with two subdivisions - one granular and the other homogeneous - without a clear definition of the limit between them. In contrast, the inner layer of *R. crystallina* L. was not subdivided, according to Jovet-Ast (1966). Additionally, Jovet-Ast (1975) described three well-defined layers (sexine, nexine, and intine) in *R. personii* S.A. Kalm and *R. curtisii* (James *ex* Austin) Austin.

Riccia californica Austin, *R. campbelliana* M. Howe, *R. sorocarpa* Bisch., and *R. trichocarpa* M. Howe were reported to have a tri-layered exine (regions I, II, and III) and a unistratified intine (Steinkamp & Doyle 1979). The thickness of the exine layers and spore size were the primary distinguishing features among these species.

Thaithong (1982) classified the sporoderm of the genus *Riccia* into two types: those with three layers (sexine, nexine, and intine) and those with two layers (sexine and intine). Although these features have taxonomic significance, they were not entirely conclusive in grouping the species in their study.

Prior studies using light microscopy (Beer 1906; Black 1913; Jovet-Ast 1966, 1975) laid the groundwork for understanding the stratification and organization of spore walls in *Riccia*. Subsequent analyses by Steinkamp & Doyle (1979) and Thaithong (1982) with transmission electron microscopy provided further insights.

Detailed investigations into the chemical composition of sporoderm layers in the genus *Riccia* are lacking, and the sporoderm layers have been assigned different names (Tab. 1). In this study, we aim to elucidate the structure and chemistry of the sporoderm in five *Riccia* species using light microscopy. We seek to provide a more precise interpretation of the stratifications and variations among species.

Material and Methods

Gametophytes of five species of *Riccia* from three subgenera (*Riccia*, *Thallocarpus*, and *Ricciela*) were collected in the state of Rio Grande do Sul, Brazil (Tab. 2): *R. plano-biconvexa* and *R. fruchartii* (subg. *Riccia*), *R. cavernosa* and *R. stenophylla* (subg. *Ricciela*), and *R. curtisii* (subg. *Thallocarpus*). Voucher material is deposited at the ICN Herbarium at the Instituto de Biociências of the Universidade Federal do Rio Grande do Sul (UFRGS), with duplicates in the RB Herbarium of the Instituto de Pesquisas Jardim Botânico do Rio de Janeiro (JBRJ).

The gametophytes were dissected under a stereomicroscope (Leica M165C stereomicroscope) to select the sporangia containing mature spores, identified by the dark color of the capsule for chemical fixation. Mature sporangia were then macerated directly in a drop of pure glycerin on a histological slide and covered with a coverslip to analyze and record unmodified spores before they were submitted to fixation, dehydration, and inclusion.

Chemical fixation in microwaves (MW)

Mature sporangia were immersed and macerated (with the help of a histological needle to release the spores) in 600 µl of a fixative solution containing 2.5% glutaraldehyde and 2% formaldehyde in a sodium phosphate buffer (0.05 M, pH 6.8). The samples were kept at a temperature of 5 ºC (using ice) in a microcentrifuge under a negative pressure of 600 mmHg for 5 minutes (Roland & Vian 1991). The samples were then transferred to a microwave

Table 1 – Terminology used to describe the sublayers of sporoderm in previous studies on the genus *Riccia*. The columns represent different authors' nomenclature for the corresponding layers, while the rows represent the different sublayers of sporoderm identified in those studies.

Beer (1906)	Black (1913)	Jovet-Ast (1966)	Steinkamp & Doyle (1979)	Jovet-Ast (1975) and Thaithong (1982)	Thaithong (1982)
Primary spore wall	Primary spore wall	*Couche externe a*	Exine region III	Sexine	Sexine
Secondary spore wall	Secondary spore wall	*Couche interne b1*	Exine region II	Nexine	
		Couche interne b2	Exine region I		
Endospore	Endospore	–	Intine	Intine	Intine

processor (MW; PELCO BioWave® Pro), where three fixation cycles were performed, each lasting two minutes (150 W, O W, 150 W), followed by a wash in a sodium phosphate buffer (0.05 M, pH 6.8; three changes, two minutes each, under 150 W).

Sections at ambient temperature in acrylic resin

The fixed spores were dehydrated via a series of washes in solutions of increasing ethanol concentration (10, 30, 50, 70, 90, and 100%) in a MW (two minutes for each stage at a potency of 150 W). Subsequently, they were embedded in hydroxyethyl methacrylate-based acrylic resin (Technovit® 7100 Kulzer; Gerrits & Smid 1983). Alternatively, fixed spores of *R. plano-biconvexa* were transferred to an acetolysis solution (Erdtman 1960) at 70 ºC for 2 minutes, dehydrated, and included in acrylic resin.

Sections of 1 and 2 µm thickness were obtained using a Leica RM2265 motorized rotary microtome equipped with a glass knife. The sections were adhered to glass slides on a heating plate at 60 °C.

Staining and cytochemistry

The acrylic resin sections were stained with 0.05% aqueous Toluidine Blue O (C.I. 52040), pH 4.4 (O'Brien & McCully 1981). The following cytochemical reactions were performed: 1) Periodic acid-Schiff reaction (PAS) to identify total polysaccharides (Lillie 1965); 2) 0.01% aqueous Calcofluor White M2R (C.I. 40622), followed by excitation under ultraviolet light (340–380 filter) to detect cellulose (O'Brien & McCully 1981); 3) 0,01%, Auramine O (C.I. 41000) in 0.05 M Tris buffer, followed by excitation in ultraviolet light (340–380 nm filter), to localize sporopollenin (Heslop-Harrison 1977; Nepi & Franchi 2000); 4) 0.05% aqueous Basic Fuchsin (C.I. 42510), for staining and differential identification of the exine sublayers (Faegri & Iversen 1964; Punt *et al*. 2007); 5) saturated Sudan Black B (C.I. 26150) in ethanol 70%, for lipid identification (Jensen 1962); 6) 0.25% Coomassie Brilliant Blue R-250 (C.I. 42660) in 7%

Table 2 – Data from the *Riccia* species studied. The coordinates are given in degrees, minutes, and seconds format for latitude and longitude.

Herbarium voucher number	Specie	Sample locality (city, state, country)	Coordinates
ICN 175632	*R. plano-biconvexa*	Caçapava do Sul, Rio Grande do Sul, Brazil	30º45'2.57"S, 53º31'42.08"W
ICN 192153	*R. fruchartii*	Caçapava do Sul, Rio Grande do Sul, Brazil	30º45'2.57"S, 53º31'42.08"W
ICN 175634	*R. cavernosa*	Barra do Ribeiro, Horto Florestal Barba Negra, Rio Grande do Sul, Brazil	30º21'40.02"S, 51º16'18.93"W
ICN 175635	*R. stenophylla*	Barra do Ribeiro, Horto Florestal Barba Negra, Rio Grande do Sul, Brasil	30º21'40.02"S, 51º16'18.93"W
ICN 175633	*R. curtisii*	Caçapava do Sul, Rio Grande do Sul, Brazil	30º45'2.57"S, 53º31'42.08"W

aqueous acetic acid, for total protein localization (Southworth 1973); 7) 1% Alcian Blue 8GX (C.I. 74240), in 3% aqueous acetic acid, to locate acid polysaccharides and pectic acid (Jensen 1962).

All the histological preparations were photomicrographed under a Leica DMR light microscope equipped with a Leica DFC 500 digital camera and Leica LAS v. 4.2 imaging software.

Results

The spores of the studied *Riccia* species (Fig. 1-5) exhibit different shapes: *R. plano-biconvexa* spores are spherical (Fig. 1a-b), while *R. fruchartii* (Fig. 2a-b), *R. cavernosa* (Fig. 3a-b), and *R. stenophylla* (Fig. 4a-b) have angular spores with a trilete scar on the proximal face (Figs. 2a; 4a). On the other hand, *R. curtisii* spores occur in tetrads (Fig. 5a-b). The spores have ornamentation in the form of ridges and depressions. In *R. plano-biconvexa*, the ridges form areolae on the entire spore surface (Fig. 1a-b), while in *R. fruchartii* and *R. stenophylla*, they form reticules on the proximal face and are present on the distal face as well (Figs. 2a-b; 4a,b). *R. cavernosa* also shows reticules on the proximal face (Fig. 3a). Tubercles are present on both faces of *R. plano-biconvexa* (Fig. 1a-b) and *R. curtisii* (Fig. 5a-b). *R. fruchartii* and *R. stenophylla* have smooth wings (Figs. 2a-b; 4a-b).

Exine

All species of *Riccia* studied in this work have a tri-layered exine (exine 1, exine 2, and exine 3).

Exine 1 is the outermost layer of the sporoderm and forms ridges and tubercles on both faces of the spores (Figs. 1c; 2c; 3c; 4c; 5c). It has a lamellar structure that is clearly visible in all species (Fig. 1d-g,j-k for *R. plano-biconvexa*; Fig. 2d-g,j-k for *R. fruchartii*; Fig. 3d-g,j-k for *R. cavernosa*; Fig. 4d-g,j-k for *R. stenophylla*; and Fig. 5d,j for *R. curtisii*). Exine 1 appears with an orange-yellow to brown color, reducing the intensity of some staining reactions. It partially stains with Toluidine Blue O in *R. plano-biconvexa*, *R. fruchartii*, *R. cavernosa*, *R. stenophylla*, and *R. curtisii* and is weakly PAS-positive. In immature spores of *R. cavernosa*, it reacts more intensely to the PAS test and stains with Basic Fuchsin. The lamellae of exine 1 are also stained with Sudan Black B in immature spores of *R. cavernosa*, partially in *R. fruchartii* and *R. curtisii*, and weakly with Auramine O in *R. plano-biconvexa*, *R. fruchartii*, and *R. curtisii*. After acetolysis treatment, the spores of *R. plano-biconvexa* show incomplete bleaching of exine 1 and a greater intensity in the reaction for Auramine O (Fig. 6a-b) and Toluidine Blue O (Fig. 6c-d). The tetrads of *R. curtisii* show a fine PAS-positive line at the point of contact between the spores, which is also stained by Basic Fuchsin, Auramine O, and Sudan Black B (Fig. 5f-h,j).

Exine 2 occurs under exine 1 as a fine layer, more visible in *R. cavernosa* and *R. stenophylla*. Its natural color is similar to exine 1 in all species. In *R. stenophylla* and *R. cavernosa*, particularly in the immature spores of *R. cavernosa*, exine 2 stains with Toluidine Blue and is PAS positive. Exine 2 also exhibits staining with basic fuchsin and Sudan black B, which stain the aforementioned species, including *R. curtisii*. However, identifying exine 2 in *R. curtisii* is more difficult due to its tetrad arrangement.

Exine 3 is the last layer of the exine and is colorless and thick. It often presents discontinuities or less thick zones aligned below the ridges of exine 1. Exine 3 is stained by Toluidine Blue O, PAS-positive in all species, and reacts positively to Basic Fuchsin, Auramine O, and Sudan Black B. In *R. plano-biconvexa* and *R. fruchartii*, exine 3 has a fine "interlaced lamellae" structure.

Intine

The intine is the innermost layer of the sporoderm and has a continuous and homogeneous appearance. Its thickness varies among the species, with *R. cavernosa* having the thickest intine. The intine is PAS-positive and is stained with Calcofluor White and Alcian Blue in all species. Toluidine Blue O also stained light purple in *R. fruchartii* and *R. plano-biconvexa*.

A summary of the cytochemical results is presented in Table 3, and the profile of the sporoderm of each species, showing the position and thickness of each layer, is displayed in Figure 7.

Discussion

Terminological clarity

Early reviews of sporoderm structures aimed to differentiate between the spore and pollen grain walls. Wallace *et al.* (2011) employed the terms "exospore" for the outermost layer and "intine" for the innermost layer of the sporoderm in bryophyte and pteridophyte spores; however, the justification for this terminology mix remains unclear. In contrast, the terms "exine" and "intine" are conventionally used for pollen grains. Earlier

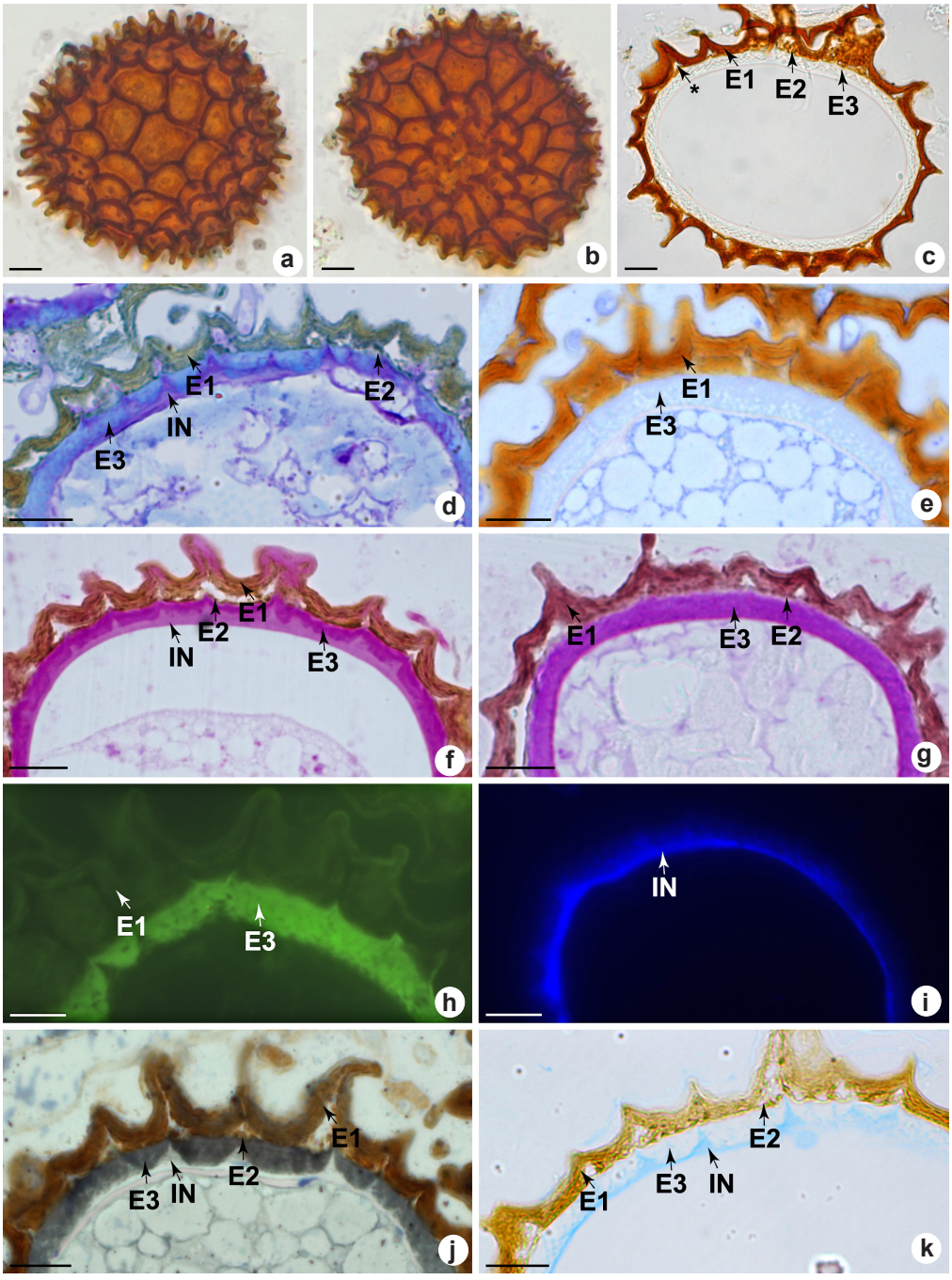

Figure 1 – a-k. *Riccia plano-biconvexa* – a. proximal face of the spore; b. distal face of the spore; c. spore under section, showing exine 1, with the typical orange natural color, a cavity (*) between exine 1 and exine 2, and exine 3, transparent; d. detail of the sporoderm after staining with Toluidine Blue O, showing exine 1 and exine 2 partially stained in greenish-blue, exine 3 stained blue, and the intine stained purple and filling the exine 3 discontinuities; e. detail of the sporoderm, stained with Coomassie Blue, showing exine 1 and exine 3; f. details of the sporoderm show that exine 3 and the intine are PAS-positive; g. detail of the sporoderm after staining with Basic Fuchsin, showing the difference in staining between exine 1, exine 2, and exine 3 (stained); h. detail of the sporoderm, with a positive reaction to Auramine O in exine 3; weak reaction in exine 1; i. detail of sporoderm, showing the intine with a positive reaction for Calcofluor White; j. detail of the sporoderm showing exine 3 stained with Sudan Black B; k. detail of the sporoderm, with intine stained by Alcian Blue. (E1 = exine 1; E2 = exine 2; E3 = exine 3; IN = intine). Bars = 10 µm.

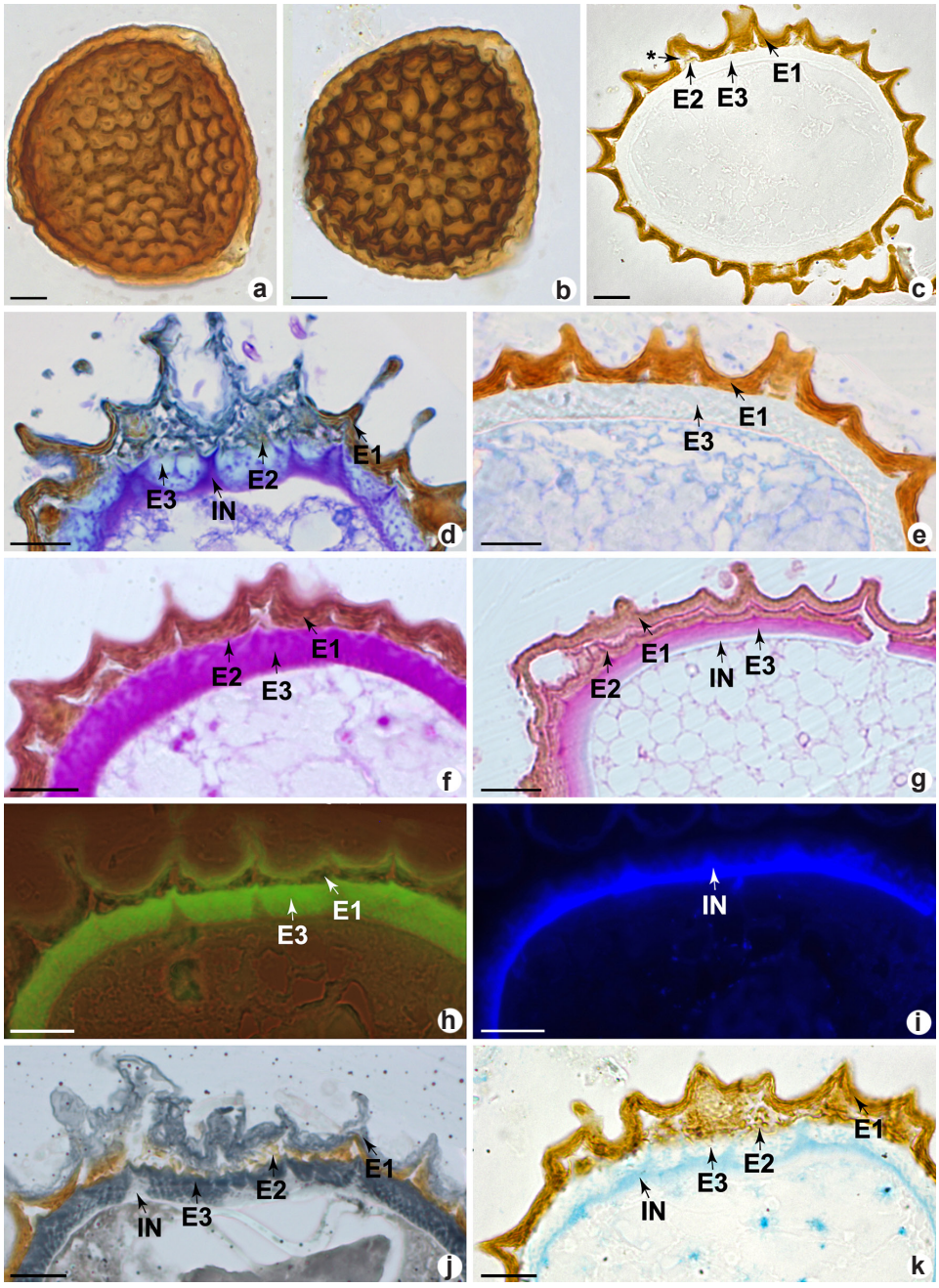

Figure 2 – a-k. *Riccia fruchartii* – a. proximal face of the spore; b. distal face of the spore; c. spore under section, showing exine 1 with the typical orange color, a cavity (*), between exine 1 and exine 2, and exine 3, transparent; d. detail of the sporoderm after staining with Toluidine Blue O, showing exine 1 and exine 2, irregularly stained with greenish blue, exine 3 stained blue, and the intine stained purple, filling in the exine 3 discontinuities; e. detail of the sporoderm, stained with Coomassie Blue, showing weak exine 3 staining; f. detail of sporoderm showing exine 3 strongly stained by the PAS reaction. It is not possible to distinguish the limits between exine 3 and intine, both stained; g. detail of sporoderm, stained with Basic Fuchsin, showing differential staining between exine 1, exine 2 and exine 3; h. detail of sporoderm stained with Auramine O, showing exine 3 and a weak color on the surface of exine 1; i. detail of sporoderm, showing intine, after staining with Calcofluor White; j. detail of sporoderm, showing exine 3, stained with Sudan Black B and partial staining of exine 1; k. detail of sporoderm, with intine stained by Alcian Blue. (E1 = exine 1; E2 = exine 2; E3 = exine 3; IN = intine). Bars = 10 µm.

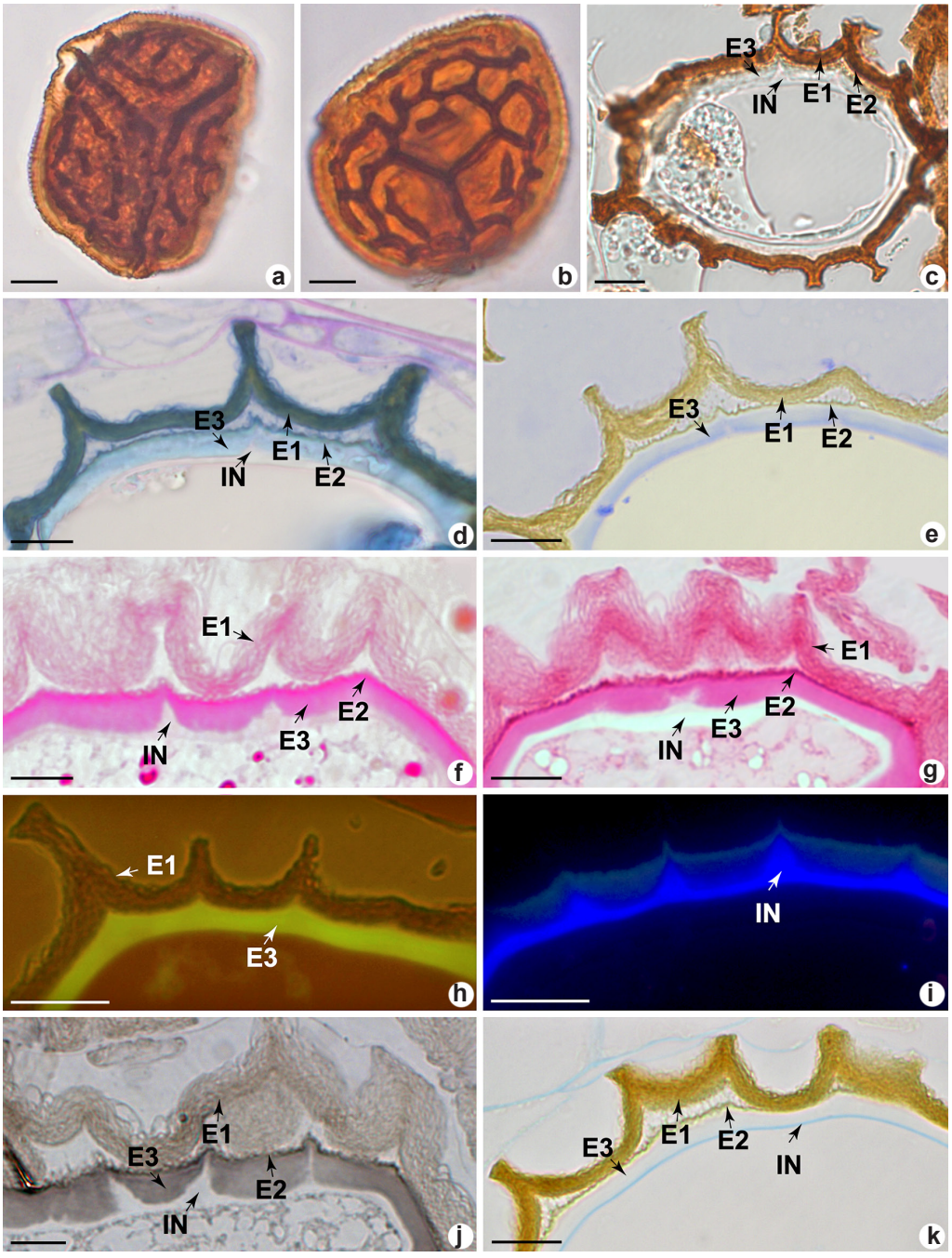

Figure 3 – a-k. *Riccia cavernosa* – a. proximal face of the spore; b. distal face of the spore; c. spore under section, showing exine 1 and exine 2, with typical orange color and exine 3, transparent; d. detail of sporoderm, stained with Toluidine Blue O, showing exine 1 and exine 2, greenish blue, exine 3 stained blue, and the intine, colorless and filling the discontinuities present in exine 3; e. detail of the sporoderm, stained with Coomassie Blue, showing exine 3; f. detail of the sporoderm of immature spore, showing all exine sublayers PAS-positive and the lamellation of exine 1; g. detail of the sporoderm of immature spore, showing differential staining of exine sublayers (1, 2 and 3) with Basic Fuchsin; h. detail of sporoderm, stained with Auramine O, showing exine 3; i. detail of sporoderm, showing intine after staining with Calcofluor White; j. detail of sporoderm, showing exine 2 and exine 3, stained with Sudan Black B and weak staining of exine 1 lamellae; k. detail of sporoderm with intine stained with Alcian Blue. (E1 = exine 1; E2 = exine 2; E3 = exine 3; IN = intine). Bars = 10 µm.

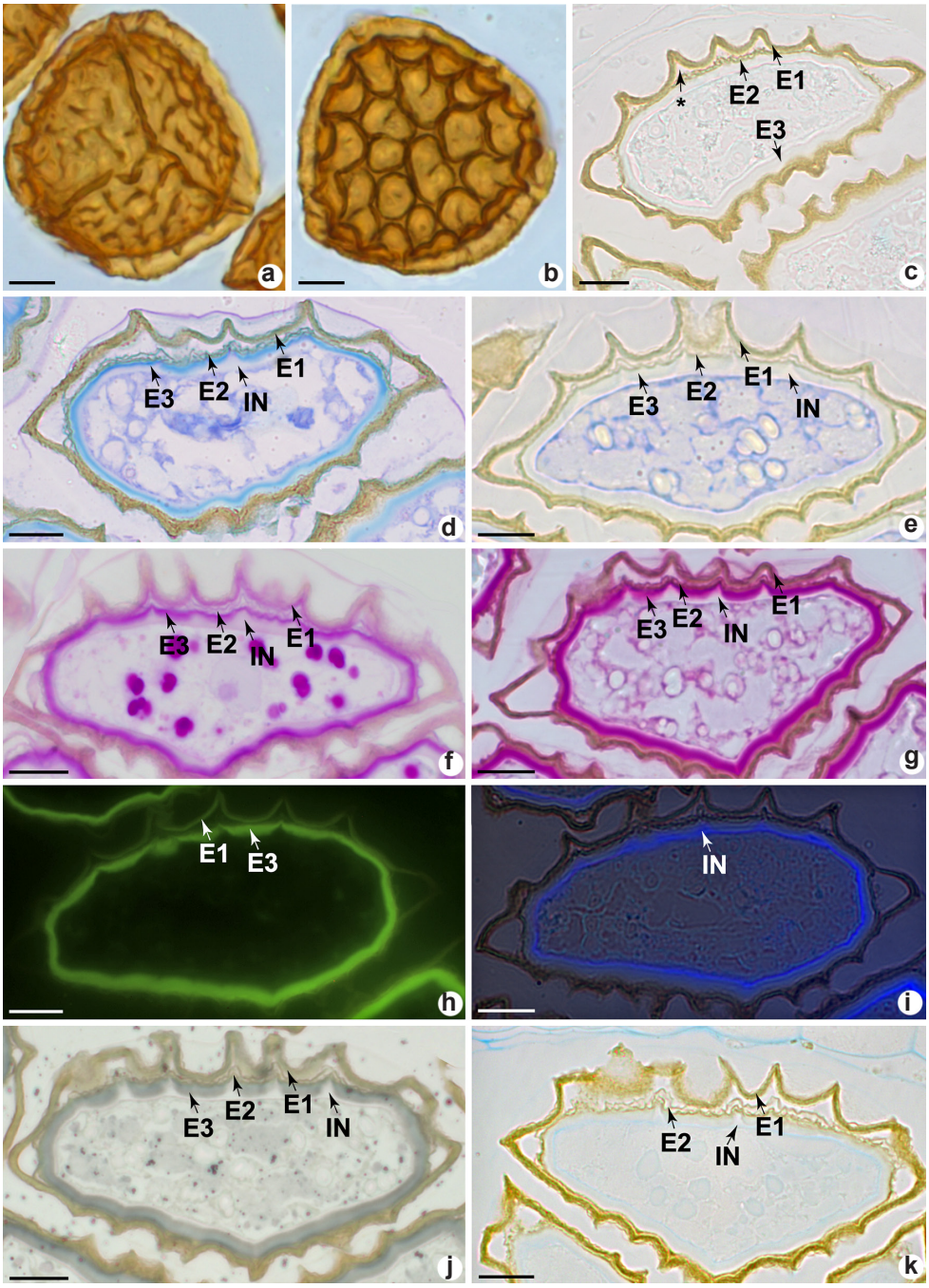

Figure 4 – a-k. *Riccia stenophylla* – a. proximal face of spore, showing the trilete scar; b. distal face of the spore; c. section of spore, showing exine 1 and exine 2, with typical yellow color, a cavity (*) between exine 1 and 2, and exine 3, transparent; d. detail of sporoderm, stained with Toluidine Blue O, showing exine 3, stained blue and the intine, colorless and filling the discontinuities present in exine 3; e. detail of sporoderm, stained with Coomassie Blue, showing exine 3, weakly stained; f. detail of sporoderm, showing the PAS-positive exine 3 and a weak reaction in exine 1 and the intine; g. detail of sporoderm, showing differential staining of exine sublayers (1, 2 and 3) with Basic Fuchsin; h. detail of the sporoderm, stained with Auramine O, showing exine 3 and a weak reaction on the surface of exine 1; i. detail of sporoderm, showing the intine, after staining with Calcofluor White; j. detail of sporoderm showing exine 3, stained with Sudan Black B and weak color of exine 1 lamellae; k. detail of sporoderm, with intine stained by Alcian Blue. (E1 = exine 1; E2 = exine 2; E3 = exine 3; IN = intine). Bars = 10 µm.

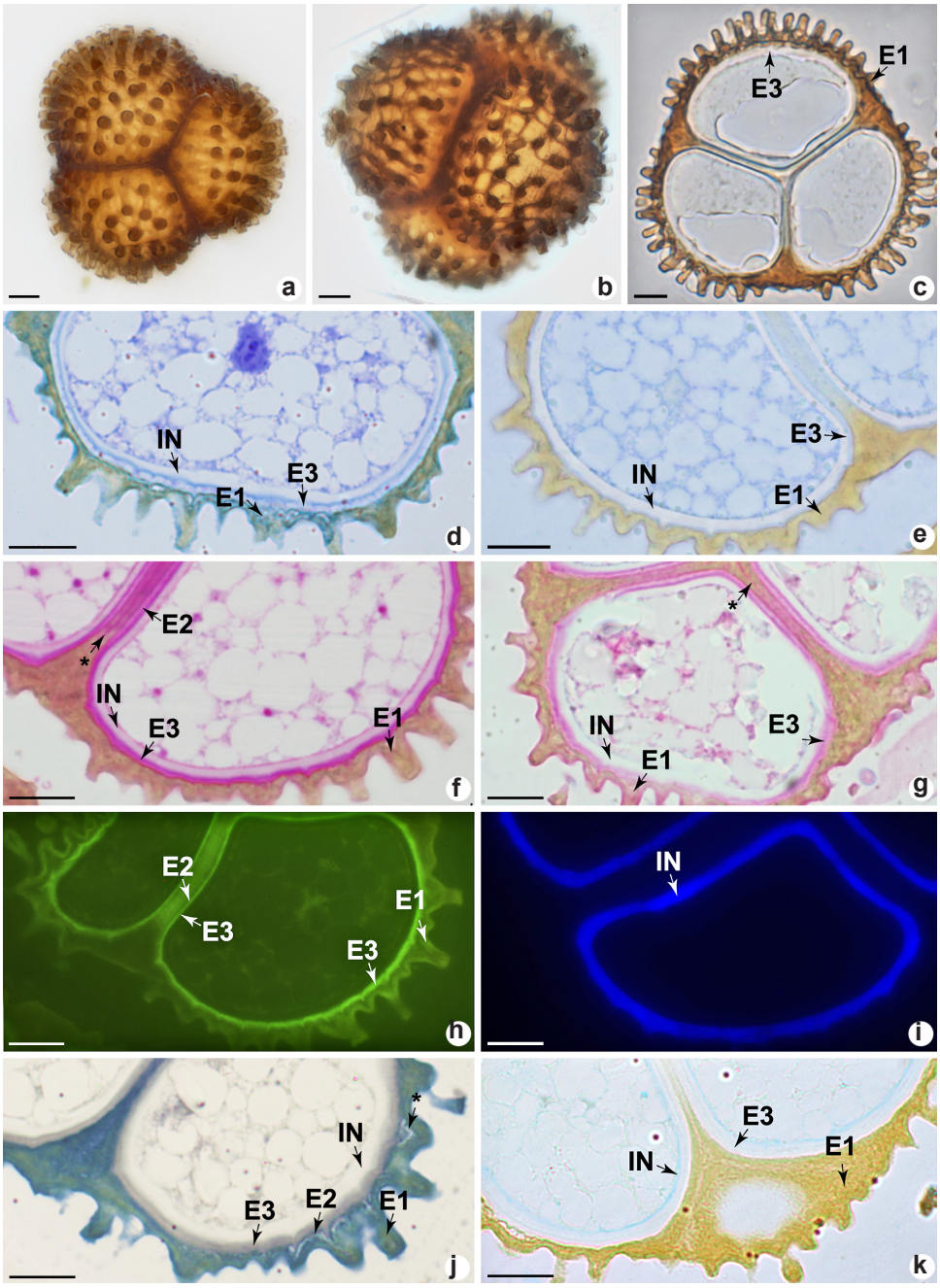

Figure 5 – a-k. *Riccia curtisii* – a-b. tetrad of spores; c. section of spores in the tetrad, showing the outer layers of exine 1, orange colored, and exine 3, transparent; d. detail of sporoderm, stained with Toluidine Blue O, showing exine 1 and 2, partially stained greenish-blue, exine 3, stained blue, and the intine, colorless; e. detail of sporoderm, stained with Coomassie Blue, showing exine 3, weakly stained; f. detail of sporoderm, showing the PAS-positive exine 3 and a weak reaction in the intine. Note a thin PAS-positive layer between the spores (*); g. detail of the sporoderm, showing differential staining of the exine sublayers (1, 2 and 3) with Basic Fuchsin. Note a thin layer stained with Basic Fuchsin between the spores (*); h. detail of sporoderm stained with Auramine O, showing exine 3 and a weak reaction on the surface of exine 1. A positive reaction occurred in the exine between the spores; i. detail of sporoderm, showing intine, after staining with Calcofluor White; j. detail of sporoderm, showing exine 3, stained with Sudan Black B, reaction in exine 1 and 2 and cavity (*) between exine 1 and 2; k. detail of sporoderm, with intine stained with Alcian Blue. (E1 = exine 1; E2 = exine 2; E3 = exine 3; IN = intine). Bars = 10 µm.

works by Black (1913) and Beer (1906) referred to the outer layer of the sporoderm in the genus *Riccia* as an "exospore". Subsequently, Jovet-Ast (1975) and Thaithong (1982) described the term "exine", which they subdivided into "sexine" and "nexine" (*sensu* Erdtman 1952), while Steinkamp & Doyle (1979) adopted the term "exine" and divided it into regions I (more internal), II (intermediate), and III (the outermost).

Recent reviews mention the sporoderm using the term exine, referring to the inner and outer layers as exine 2 and exine 1, respectively, when discussing spores of *Anthoceros* and other liverworts (Brown *et al.* 2015; Renzaglia *et al.* 2020).

In this study, we adopted a similar approach to Steinkamp & Doyle (1979) for *Riccia* and Renzaglia *et al.* (2020) for other liverworts by

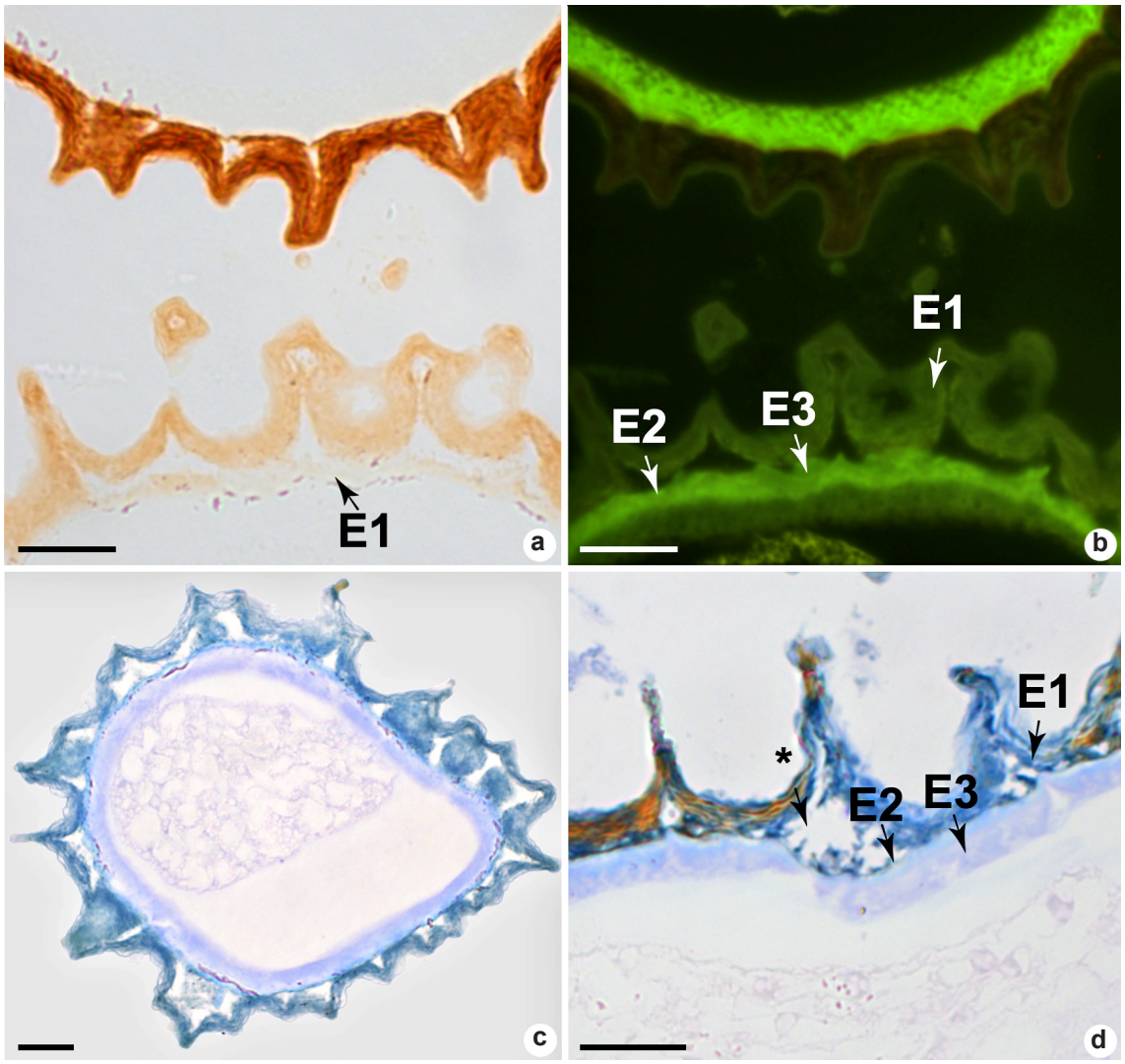

Figure 6 – a-d. Spores of *Riccia plano-biconvexa* submitted to acetolysis – a. detail of sporoderm of two adjacent spores, showing a differential action of acetolysis. In the lower spore, greater extraction of components of exine 1 occurred, and there was a clear reduction of the natural color of this sublayer; b. the same section as the previous figure (a), stained with Auramine O, showing a more intense reaction in exine 1, after extraction due to acetolysis; c. spore stained with Toluidine Blue O, with a greater exine 1 and 2 staining after acetolysis (compared to Fig. 1d); d. detail of sporoderm, after acetolysis and stained with Toluidine Blue O, showing differential lamellae staining in exine 1. (E1 = exine 1; E2 = exine 2; E3 = exine 3). Bars = 10 µm.

using the term "exine", but with different layer designations. Based on our results, we used the terms "exine 1" and "exine 2" to represent the outer and inner layers of the ectexine, respectively, and "exine 3" to describe the endexine. This terminology aligns with Brown & Lemmon (1993), who used "E1" (exine 1) and "E2" (exine 2) to describe the outer and inner layers of the sporoderm in *Fossombronia wondraczekii* (Corda) Dumort. *ex* Lindb., and Renzaglia *et al.* (2020) in *Sphaerocarpos michelii* Bellardi, and with the concept of ectexine and endexine found in pollen grains of seed plants, providing a systematic approach to describe the stratification of the sporoderm in liverworts. Current studies, such as those by Blackmore & Barnes (1987), Brown & Lemmon (1988), Brown *et al.* (2015), Renzaglia *et al.* (2020), and Renzaglia *et al.* (2023), which studied other bryophyte species, have already consolidated this nomenclature, solidifying the homology between these layers in different species.

Structural characteristics

The lamellar structure observed in the *Riccia* exine is considered characteristic of the Ricciaceae (Steinkamp & Doyle 1979) and is also found in the spores of other liverworts, such as *Aneura pinguis* (L.) Dumort. [=*Riccardia pinguis* (L.) Gray] (Horner Jr. *et al*. 1966), *Clevea hyalina* (Sommerf.) Lindb. [=*Athalamia hyalina* (Sommerf.) S.Hatt.] (Steinkamp & Doyle 1981), and *F. wondraczekii* (Brown & Lemmon 1993). Transmission electron microscopy studies (Steinkamp & Doyle 1979; Renzaglia *et al.* 2015; Brown *et al.* 2015) have revealed that the lamellar structure comprises branched and interconnected lamellae with electron-dense material and an electron-transparent core. Our results validate the presence of this lamellar character in the *Riccia* exine. These layers resemble those in *Sphaerocarpos* spores illustrated in the transmission electron microscopy images (Renzaglia *et al.* 2015).

Exine layers, staining properties and composition: Exine 1 in *Riccia* exhibits staining patterns similar to the ectexine of Angiospermae pollen grains (Nepi & Franchi 2000), both in immature and mature spores, staining with Toluidine Blue O, Basic Fuchsin, Sudan Black B, and Auramine O, either partially or wholly after acetolysis. This indicates a composition that is based on sporopollenin, a highly resistant biopolymer that played a crucial role in the colonization of land by plants (Wallace *et al*. 2011; Grienenberger & Quilichini 2021; Suh & Ashton 2022). In the *Riccia* spore analyzed in this study, exine 1 displays a characteristic color for the genus, which appears to "mask" the staining reaction. This natural color has also been observed in spores of *R. glauca* (Beer 1906) and *A. pinguis* (Horner Jr. *et al.* 1966) without a clear explanation of their chemical nature. While the loss of staining reaction in *A. pinguis* has been attributed to the presence of a "characteristic brown pigmentation", we could not definitively identify this substance's chemical composition. Additionally, in spores of *R. plano-biconvexa* subjected to acetolysis, the component responsible for the typical pigmentation or color of the spores was partially removed, suggesting that it is likely unrelated to sporopollenin. This component is absent in the immature spores of *R. cavernosa*, indicating that it might be derived from the transformation of other pre-existing components in exine 1 itself, possibly occurring between the lamellae.

As liverwort sporangia lack tapetal cells (Renzaglia *et al*. 2000; Wallace *et al*. 2011), the components leading to pigmentation or color in exine 1 and 2 are likely synthesized by the spores themselves, not derived from sporangium cells. The electrodense material observed on and between the lamellae in the exine of some *Riccia* species (regions II and III) by Steinkamp & Doyle (1979) cannot be directly related to the components removed during acetolysis, as this study was the first to test the resistance of the exine layer to acetolysis in *Riccia* spores. The fact that this material can be removed by acetolysis suggests that it exposes some radicals or lamellae to the action of stains.

Exine 2 was a thin layer, challenging to detect, but its presence was revealed through cytochemical tests and facilitated by the spaces between this layer and exine 1, as previously described by Beer (1906) and Thaithong (1982). According to Steinkamp & Doyle (1979), these spaces would be "filled by air". These spaces occur in the region of the ridges and wings in *R. plano-biconvexa*, *R. fruchartii*, *R. stenophylla*, and *R. cavernosa*, similar to other species of *Riccia* described by Steinkamp & Doyle (1979) and Thaithong (1982), as well as in the domes between exine 1 and exine 2 in *C. hyalina* (Steinkamp & Doyle 1981). However, its specific function is likely to allow it to float (Glime 2017).

Previous studies based on light microscopy did not detect exine 2 in *Riccia* spores (Beer

Table 3 – Sporoderm sublayers of *Riccia* species under different stains and cytochemistry tests. (+ = weak reaction; ++ = medium reaction; +++ = strong reaction; – = not reacted; 1. After the acetolysis treatment).

Species	Stain / cytochemical test	Sporoderm layers			
		Exine			Intine
		Exine 1	Exine 2	Exine 3	
R. plano-biconvexa	Toluidine Blue O	+ (green), ++, (green)[1]	+ (green), + (green)[1]	++ (blue), + (blue)[1]	+ (purple), –[1]
	Comassie Blue	–	–	+	–
	PAS	–	–	+++	++
	Basic Fuchsin	–	–	+++	++
	Auramin O	–, +[1]	–, –[1]	+++, +++[1]	–, –[1]
	Calcofluor White	–	–	–	++
	Sudan Black B	–	–	+++	–
	Alcian blue 8GX	–	–	–	+
R. fruchartii	Toluidine Blue O	+ (green)	+ (green)	++ (blue)	+ (purple)
	Comassie Blue	–	–	+	–
	PAS	–	–	+++	+++
	Basic Fuchsin	–	–	++	–
	Auramin O	+	–	+++	–
	Calcofluor White	–	–	–	++
	Sudan Black B	+	–	+++	–
	Alcian blue 8GX	–	–	–	+
R. cavernosa	Toluidine Blue O	+ (green)	+ (green)	+ (blue)	–
	Comassie Blue	–	–	+	–
	PAS	+	+++	++	–
	Basic Fuchsin	++	+++	++	–
	Auramin O	–	–	++	–
	Calcofluor White	–	–	–	++
	Sudan Black B	+	++	+	–
	Alcian blue 8GX	–	–	–	+
R. stenophylla	Toluidine Blue O	–	–	+ (blue)	–
	Comassie Blue	–	–	+	–
	PAS	+	–	+++	+
	Basic Fuchsin	–	–	+++	
	Auramin O	–	–	++	–
	Calcofluor White	–	–	–	++
	Sudan Black B	–	–	+	–
	Alcian blue 8GX	–	–	–	+

Species	Stain / cytochemical test	Sporoderm layers			
		Exine			Intine
		Exine 1	Exine 2	Exine 3	
R. curtisii	Toluidine Blue O	+ (green)	+ (green)	++ (blue)	–
	Comassie Blue	–	–	+	–
	PAS	–	–	++	+
	Basic Fuchsin	–	–	++	–
	Auramin O	+	–	++	–
	Calcofluor White	–	–	–	++
	Sudan Black B	+	+	++	–
	Alcian blue 8GX	–	–	–	+

1906; Black 1913; Jovet-Ast 1966, 1975). The identification of exine 2 was possible through ultrastructural studies using transmission electron microscopy, as Steinkamp & Doyle (1979) demonstrated for region II and Thaithong (1982) for nexine. Our analyses using light microscopy and various stains enabled the structural identification of exine 2 in this study, highlighting the importance of employing cytochemical tests to study the spore wall.

Contrary to the descriptions by Jovet-Ast (1966), who referred to just one non-ornamented layer (the nexine) in *R. cavernosa*, we identified two non-ornamented layers, exine 2 and exine 3, as well as in *R. plano-biconvexa*, *R. fruchartii*, and *R. stenophylla*. Although exine 2 is thin and challenging to detect in all species studied, its presence was revealed in the immature spores of *R. cavernosa* through differential staining with the PAS reaction, Basic Fuchsin, Toluidine Blue O, and Sudan Black B. This staining revealed a mixture of polysaccharides and sporopollenin, contrasting with Jovet-Ast's (1966) description of only two layers in this species.

Based on the staining properties, exine 3 emerges as the most complex layer of the *Riccia* sporoderm. Our results indicate a diverse chemical composition, consisting of a combination of sporopollenin (resistant to acetolysis, stained with Toluidine Blue O, Sudan Black B, and Basic Fuchsin, and fluorescent after Auramine O) and polysaccharides (stained purple with Toluidine Blue O and positive for PAS). The fibrillar structure of this exine layer corresponds to the descriptions provided by Beer (1906) and Steinkamp & Doyle (1979). However, in *R. personii and R. curtisii*, Jovet-Ast (1975) described it as granular. As this layer precedes the pectocellulosic intine (PAS-positive and fluorescent after Calcofluor White), it is possible that sporopollenin precursors and polysaccharides (except cellulose) are synthesized simultaneously during its formation, as shown in different bryophytes like *Phaeoceros carolinianus* (Renzaglia *et al*. 2020) and *Sphaerocarpus* (Renzaglia *et al.* 2015).

Immunocytochemistry techniques are necessary for precise identification of the pectins in the spore walls of *Riccia*, such as the immunogold used in *Anthoceros* by Renzaglia *et al.* (2020), which also found callose in its composition. These techniques could shed more light on the composition and distribution of pectins within the sporoderm layers.

Intine layer

The intine, the innermost layer of the spore wall in *Riccia* species, is the only pectocellulosic layer (PAS-positive, fluorescent after Calcofluor White, and stained with Alcian Blue). Our findings are consistent with the descriptions made for *R. glauca* (Beer 1906) and *R. frostii* (Black 1913) and align with the sporoderm studies in other *Riccia* species (Steinkamp & Doyle 1979; Thaithong 1982). The intine fills the discontinuities in exine 3, aligning with the ridges of the outer layer of exine 1. This is an unreported characteristic in *Riccia* and had yet to be observed in previous studies of any *Riccia* species, including *R. curtisii* (Jovet-Ast 1975) or *R. cavernosa* (Thaithong 1982, Jovet-Ast 1966).

Evolutionary implications

The maintenance of spores in a tetrad configuration in *R. curtisii* might be a plesiomorphic character in Ricciaceae, suggesting that this species is more ancestral (Brown *et al.* 2015). Conversely, the free spherical shape observed in *R. plano-biconvexa* may represent a derived trait. Our results hint at a possible evolutionary progression in the thickness of exine 3 among the studied species, with *R. curtisii* showing thinner exine 3 and *R. plano-biconvexa* displaying thicker exine 3. Additionally, the spatial separation between exine 1 and exine 2 increased with the cavities or spaces, suggesting a probable adaptation of the spore to the ability to float (Glime 2017).

Based on the results of the cytochemical tests used in this study, exine 1 and 2 share a structural organization (presence of lamellae) and similar chemical properties, different from those found in exine 3. Thus, we categorized exine 1 and 2 as constituting an "ectexine" and exine 3 as an "endexine" (Faegri & Iversen 1964; Punt *et al.* 2007; Brown *et al.* 2015; Renzaglia *et al.* 2015). Further cytochemical analyses of the sporoderm of other bryophytes (*sensu lato*) should be performed to gain a broader understanding of the possible evolution of the chemical composition of these layers.

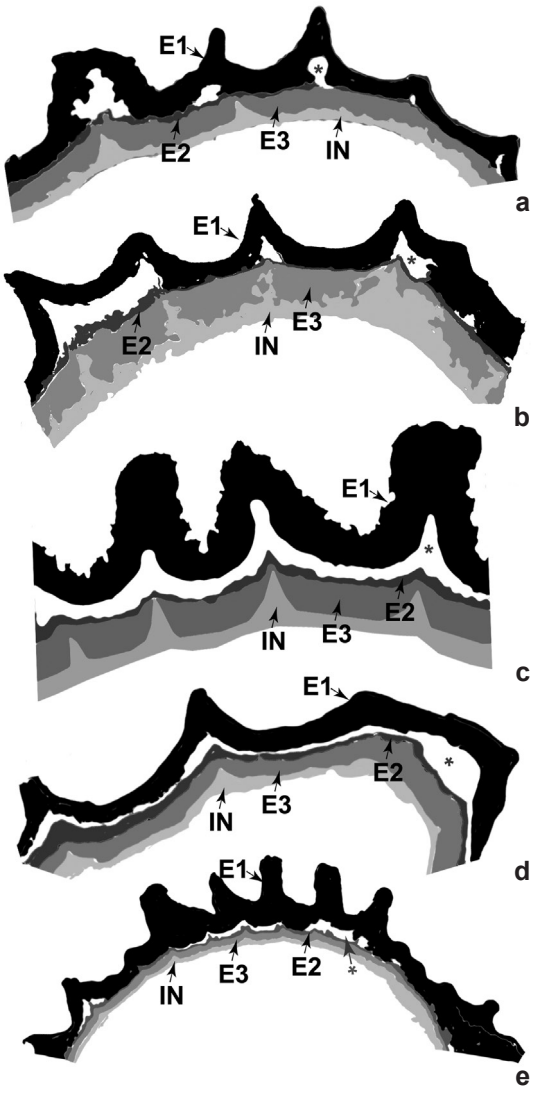

Figure 7 – a-e. Schematic drawings of the stratification of the spore wall in *R. plano-biconvexa* (a) *R fruchartii* (b), *R. cavernosa* (c), *R. stenophylla* (d), and *R. curtisii* (e), showing the four layers of the sporoderm: exine 1 (black), exine 2 (dark grey), exine 3 (gray) and intine (light grey). The cavities between exine 1 and 2 are marked with an asterisk. (E1 = exine 1; E2 = exine 2; E3 = exine 3).

Our observations reveal distinct variations in spore shapes and ornamentation among the studied species, highlighting the importance of these features in taxonomic identification.

1. We followed a modified terminology inspired by previous works on *Riccia* and pollen grains. We designate the outermost layer of the sporoderm as "exine 1", which forms ridges, tubercles, and areolae on the spore surfaces. This layer displays a characteristic color, possibly due to a yet unidentified chemical compound, and exhibits staining patterns similar to sporopollenin, a biopolymer associated with the colonization of land by plants.

2. Beneath exine 1, we identified a thin layer termed "exine 2", which had been previously overlooked in light microscopy-based studies. Cytochemical tests helped reveal the presence of this layer, and we noted a mixture of polysaccharides and sporopollenin in its composition. The specific function of exine 2 remains unknown, but its detection adds a new layer of complexity to the spore wall structure.

3. The innermost layer of the sporoderm, termed "exine 3", displayed a diverse chemical composition comprising a combination of sporopollenin and polysaccharides. The fibrillar structure of exine 3 aligns with previous descriptions, but its relationship with the subsequent pectocellulosic intine provides new insights into the spore wall's architecture.

4. The intine, the innermost layer of the spore wall, is the only exclusively pectocellulosic layer and fills the discontinuities present in exine 3, aligning with the ridges of exine 1. Further studies employing immunocytochemistry techniques could shed light on the composition and distribution of pectins within the sporoderm layers.

5. The tetrad arrangement of spores observed in *R. curtisii* suggests a possible plesiomorphic character in Ricciaceae. In contrast, the free spherical shape of *R. plano-biconvexa* may be a derived characteristic. This observation, along with the variation in the thickness of exine 3 among the studied species, hints at a potential evolutionary trend in the sporoderm structure.

6. Our results highlight the importance of employing cytochemical tests to study the spore wall, as they enable a deeper understanding of each layer's structural and chemical properties.

7. This study provides valuable insights into the sporoderm structures of different *Riccia* species, offering a foundation for future investigations in liverwort spore wall evolution and taxonomy.

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Data availability statement

In accordance with Open Science communication practices, the authors inform that all data are available within the manuscript.

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