Wulfenia **24** (2017): 125–133

Wulfenia
Mitteilungen des
Kärntner Botanikzentrums
Klagenfurt

The ultrastructure of germinating and ungerminated *Sphagnum* spores

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Summary: The ultrastructure of germinating and ungerminated *Sphagnum* spores was investigated by TEM. Germinating spores differed from ungerminated spores not only by their protoplast condition but also by the sporoderm ultrastructure. The middle layer of the intine intensely thickens on the proximal hemisphere during spore germination. It actively promotes the protoplast exit through the open laesura outside a sporoderm to form a young protonema. The granular layer which is described traditionally as an outer intine is considered as a separate acetolysis resistant layer and is compared with the endexine of pollen grains.

Keywords: *Sphagnum fimbriatum*, *Sphagnum squarrosum*, sporoderm ultrastructure, germinating spores, intine

Sphagnum mosses play an important role in ecosystems and climates of the earth. They are major components of oligotrophic swamps, bogs and tundras. Besides, *Sphagnum* and peat are the best traps for contamination products and favor good preservation of pollen and spores. In humid conditions, *Sphagnum* spores can remain viable up to three years. If the spores are stored at low temperature, they remain viable for up to 60 years (Clymo & Duckett 1986). This is why *Sphagnum* is so common in the northern climate (Glime 2015). *Sphagnum* is a stand-alone taxon. It is completely different from other mosses by many characters. Gametophytes of *Sphagnum* develop from a thalloid protonema and have a strictly defined organography with unlimited apical growth (Clymo & Duckett 1986; Ligrone & Duckett 1998). The sporoderm in *Sphagnum* is very complex (Brown et al. 2015). Even studies on the sporoderm development in two *Sphagnum* species could not solve all the problems of homologization of sporoderm layers (Brown et al. 1982a; Filina & Filin 1985b).

Five layers have been described in the sporoderm ultrastructure. The outermost layer is an electron-dense perine (Brown et al. 1982a, b, 2015) [= sculptine (Filina & Filin 1985b)]. The deeper there is an electron-translucent lamellar layer. The next electron-opaque two-layered exospore [= exine] consists of the homogeneous and thick layer B and the lighter lamellar layer A. The inner layer with acetolysis-resistant dark granules in the outer parts and in the laesura area is the endospore [= intine] (Brown et al. 1982a; Filina & Filin 1985b). The perine forms a verrucate sculpture, and the exine forms distal triradiate ridges and a pronounced cingulum (Filina & Filin 1985a; McQueen 1985; Cao & Vitt 1986; Yu-long & Pan-Cheng 1990). The study of the sporoderm during spore germination contributes to the problem of homologization of sporoderm layers in mosses and functioning of different layers and components of the sporoderm.

In this study, we have cultivated spores of two species of *Sphagnum* under different terms and conditions of storage. We compared differences in the ultrastructure of ungerminated and germinating spores. This allowed us to discuss problems of functioning of the complex sporoderm in *Sphagnum* as well as homologization of different sporoderm layers in mosses.

Materials and methods

Living samples of *Sphagnum fimbriatum* Wilson

Russia, Yakutia, Oymyakinsky District, 60°45'N, 140°59'E, August 07, 2015, Collected and determined by M.S. Ignatov, E.A. Ignatova. 15-390 [MHA].

Herbarium samples of *Sphagnum squarrosum* Crome

Russia, Khabarovsk Territory, 48°10'N, 139°21'E, July 17, 2013, Collected and determined by M.S. Ignatov, E.A. Ignatova. 13-107 [MHA].

Acetolyzed spores sample of *Sphagnum fimbriatum* Wilson

Moscow region, Sofrino. The bog near village Feodorovskaya. July 1956. Collected and determined by L.V. Kudriashov. 1541 (Palinoteca of the Higher Plant Department, Biological Faculty, Lomonosov Moscow State University).

Collection and cultivation of plant material

Spores of *Sphagnum fimbriatum* were extracted out of fresh living capsules. Spores of *S. squarrosum* were extracted out of dry capsules which had been kept in the herbarium for two years.

Each unopened capsule of *Sphagnum* was transferred to an eppendorf and sterilized with a fresh sterilization solution (10% hydrogen peroxide in 70% ethanol) for two or five minutes. After washing with sterile distilled water (three times by 10 minutes) spores were extracted out of capsules in a new portion of fresh water (500 µl) and were gently squeezed by tweezers. Then, capsules were removed. Suspension of spores was mixed with agar solution (about 50°C) in sterile distilled water. The final concentration of agar in suspension was 0.04%, 0.05% and 0.06%.

The spore cultivation was carried out in Petri dishes (95 mm in diameter) on a substrate (1% agar in distilled water). The drop of spore suspension (150 µl) was evenly distributed on the coverslips surface (24 × 24 mm). We placed six or seven coverslips on a substrate. The Petri dishes were enclosed with Parafilm M (PM-996, Bemis Flexible Packaging, USA) and incubated during two weeks under lighting fluorescent lamps (OSRAM L36 W/840) and a photoperiod of 16 h light to 8 h darkness at 22 ± 2°C. After two weeks of cultivating (Beike at al. 2015), coverslips with spores were fixed for transmission electron microscopy (TEM).

Transmission electron microscopy

The coverslips with spore-agar suspension were fixed for TEM with 2.5% glutaraldehyde (Glutaraldehyde 25%, TED PELLA) buffered in 0.1 M cacodylate buffer, pH 7.3 (Sodium Cacodilate, SPI-CHEM) with addition of tannic acid (0.11 g tannic acid in 110 ml fixative solution) (Mironov et al. 1994) at room temperature for 2 h, post-fixed with 2% OsO_4 (2 h) in cacodylate buffer, dehydrated in a graded series of ethanol up to 70% and contrasted with uranyl acetate, followed by dehydration through ethanol and acetone series. The dehydrated material was embedded in an Epon-acetone mixture overnight and put into pure Epon (Epon-medium, DDSA, MNA, DMP-30 as a 20/9/12/1 mixture) in an embedding mold. The coverslips were put on the embedding mold so cautiously that the spore-agar suspension touched the Epon surface without air bubbles. The material was kept in Epon for a day at room temperature and then for two days at 62°C (Mironov et al. 1994). After polymerization, the coverslips were broken off alternately placing the sample in liquid nitrogen and boiling water. Ultrathin sections of 60 nm

Table 1. The main morphological characteristics of germinating and ungerminated spores.

	Sphagnum fimbriatum germinating spores	*Sphagnum squarrosum* ungerminated spores
Protoplast		
Percentage of the area of the central lipid drop in the protoplast	43%–47%	44%–53%
Location of nucleus	Near the opened aperture	On the opposite side from the aperture
Chloroplasts	Numerous and with well-developed granum, near the opened aperture	Few and with some thylakoids in different locations
Mitochondria, EPR, Golgi apparatus and associated small vesicles	Numerous and well-developed membranous structures	Few and poorly developed membranous structures
Sporoderm		
Perine	Forms microverrucate sculpture	Forms verrucate-granular sculpture
Middle layer of intine	Very unevenly thickened	Slightly thickened and only in the equatorial area
Inner intine	Gradually turns into cell wall of the protonema	Slightly thickened and fills the gap of the laesura

were made with an ultramicrotome UC-5 Ultracut-R (Leica Microsystems). TEM was performed at the Laboratory of electron microscopy at the Biological Faculty of Lomonosov Moscow State University using an electron microscope JEM-1011 (Jeol, Japan) at accelerating voltage 80 kV, equipped with a Gatan Erlangshen-500 SW digital camera and ORIUS SC1000W digital camera with Digital Micrograph Gatan software.

Acetolyzed spores were washed out of the glycerol in distilled water and fixed with 2% OsO_4 (2 h at room temperature). Next procedures of dehydration and embedding were the same as those described above.

Results

All variants of samples of *Sphagnum fimbriatum* spores from mature unopened sporangia, which were freshly collected in the field, developed filamentous and thalloid protonemas within two weeks. Gametophores developed from some thalloid protonemas. All variants of samples of *S. squarrosum* spores from unopened sporangia from the herbarium did not develop a protonema within two weeks. The main morphological characteristics of *in vitro* cultivated spores distinguishing *S. fimbriatum* from *S. squarrosum* are summarized in Table 1.

Germinating spores of *Sphagnum fimbriatum*

Protoplasts of germinating spores possess a large central lipid drop that fills nearly a half of the area of the protoplast in the sections (Fig. 1: 1–5). The nucleus is located near the central lipid drop on the side where the sporoderm breaks and the protoplast comes out (Fig. 1: 3). Many lipid droplets are observed throughout the protoplast; they are particularly numerous near the plasma membrane. A part of osmiophilic globules has a multilamellar structure (Fig. 1: 7, 8). Chloroplasts and mitochondria occur much more rarely in the cell. Chloroplasts with well-developed grana and thylakoids with starch grains are located on the periphery of the cell part

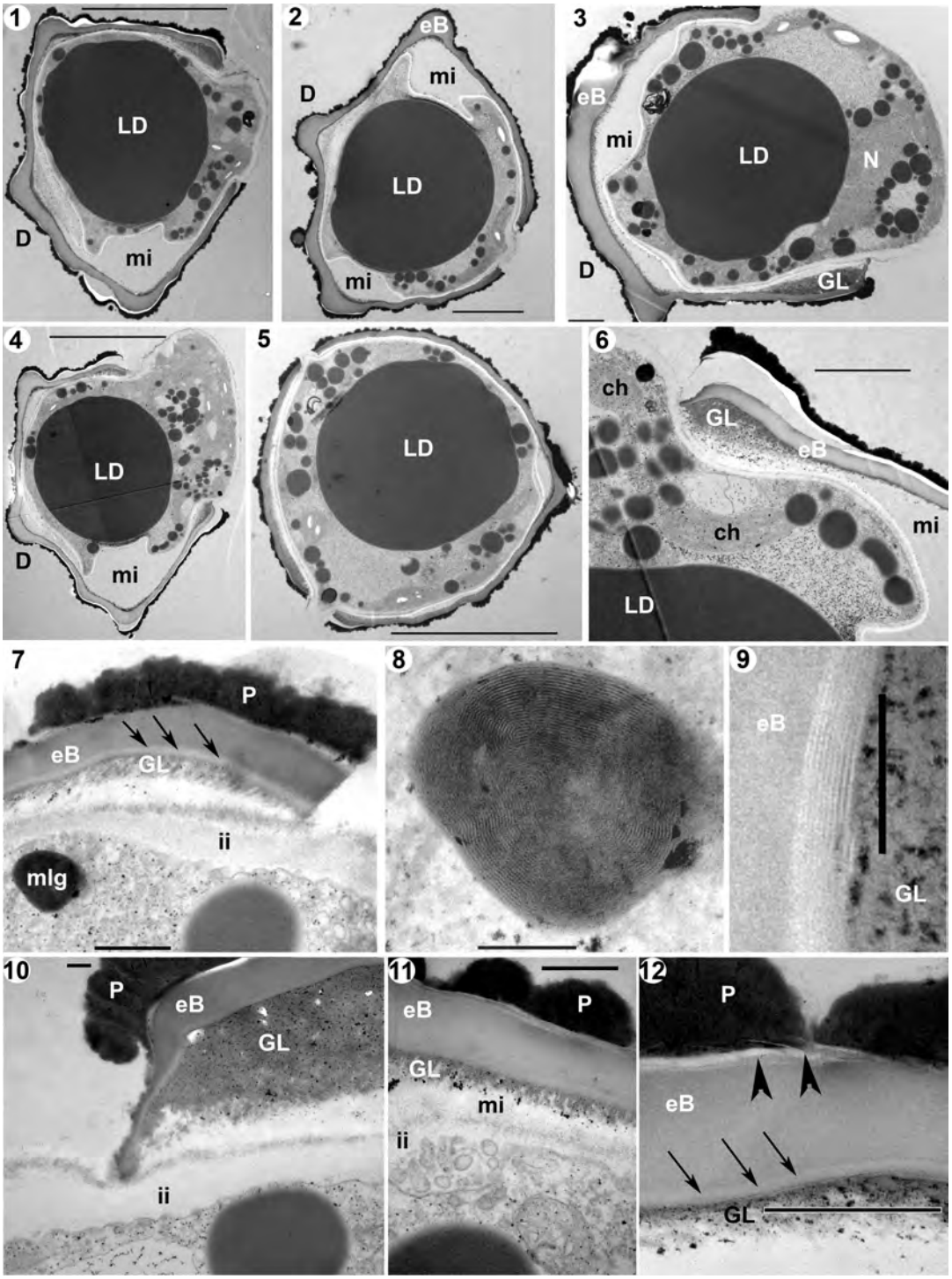

Figure 1. The ultrastructure of germinating spores of *Sphagnum fimbriatum*. TEM. 1, 2, 3, 4 – section of a whole spore in equatorial view; 5 – section of a whole spore from polar view; 6 – part of spore near the opened laesura, the outer granular intine and middle intine are thicker; 7 – part of sporoderm near rupture, note that all the layers are not thickened; 8 – multilamellar globule, part of 7; 9 – part of exine layer A; 10 – details of sporoderm in opened laesura with thicker granular layer (outer intine); 11 – undulate plasma membrane and small vesicles near thickened middle intine; 12 – details of sporoderm with clearly visible exine layer A (black arrows) and translucent layer (black ▶

that extends outwardly from the sporoderm (Fig. 1: 6). Small mitochondria tend to be situated in the surface part of the cytoplasm, so they are mostly located under the plasma membrane. An endoplasmic reticulum (ER), Golgi apparatus and associated small vesicles predominantly fill the part of cytoplasm that is free from the lipid droplets and that faces the broken sporoderm. Particularly many vesicles are located under the plasma membrane near gaps in the exine and perine and towards the thickened middle intine (Fig. 1: 11). This is caused most probably by the cell wall development of the growing protonema and the thickening of the middle layer intine.

The sporoderm consists of seven wall layers (Fig. 1: 6, 7, 10) and it is the most complex structure found in bryophytes (Brown et al. 2015). The outermost layer, an electron-dense perine, is composed of closely packed granules with often visible electron-opaque narrow gaps. Granules of perine form a microverrucate spore surface. Deeper, a scaly layer of translucent material is observed. This layer is composed of three to six lamellas, which extend into the perine and the exine. A delamination of perine occurs most easily along this layer. The gray two-layered exine is located deeper. The outer exine layer B is unevenly thickened; it forms the relief of the distal spore hemisphere, an equatorial thickening and distal ridges. The inner exine layer A is composed of eight light and dark regularly alternating lamellae. They are of the same thickness all over the exine and are clearly seen only in sections made perpendicularly to the lamellae (Fig. 1: 9). Electron-translucent three-layered intine is adjacent to the protoplast. The outer intine contains electron-dense acetolysis-resistant granules and laminae. The outer intine is thin all over the sporoderm except for the laesura region. It thickens greatly along the trilete mark and forms ridges of the laesura (Fig. 1: 6, 10). The middle layer of intine is thickened very unevenly. It is thinner than the outer granular intine on the distal hemisphere and unevenly thickened in the equatorial area and on the proximal hemisphere. It is thin near the trilete mark. The outer and middle intine layers are broken in an open laesura as it is also observed in the exine and perine. Only the innermost intine is continuous and gradually transformed into the cell wall of the growing protonema. This layer is thin all over the spore perimeter but it becomes 1.5–2 times thicker than the inner intine layer in the area of the cell wall outside the sporoderm.

Ungerminated spores in *Sphagnum squarrosum*

Protoplasts of ungerminated spores possess a large central lipid drop that fills about half of the area of the protoplast in the sections (Fig. 2: 1, 2). In addition, there are many small lipid droplets dispersed in the free space of the cell between organelles and a central lipid drop. The nucleus is located closely to the central lipid drop and contrariwise to the side of the sporoderm rupture and laesura (Fig. 2: 2, 3). There are only few chloroplasts and mitochondria. Chloroplasts have few membrane structures. ER, Golgi apparatus and small vesicles are detected poorly.

The sporoderm consists of seven layers (Fig. 2: 3–6). The electron-dense perine is thin with large granules and granule clusters that form a verrucate spore surface. The translucent layer consists of three to four lamellae along which a delamination of the perine easily occurs. The electron-gray two-layered exine is composed of an unevenly thickened outer exine layer B and inner lamellar exine layer A. The exine layer B forms the spore relief and is eroding in the center of the laesura. The exine layer A consists of eight light and dark regularly alternating lamellae, which are of the

▶ arrowhead). ii – inner intine, mi – middle intine, GL – granular layer (outer intine), P – perine, eB – exine layer B, arrows – exine layer A, ch – chloroplast, LD – lipids drop, mlg – multilamellar globule; N – nucleus. Scale bars = 10.0 µm (1, 4, 5); 5.0 µm (2); 2.0 µm (3, 6); 0.5 µm (7, 11, 12); 0.2 µm (8, 9, 10).

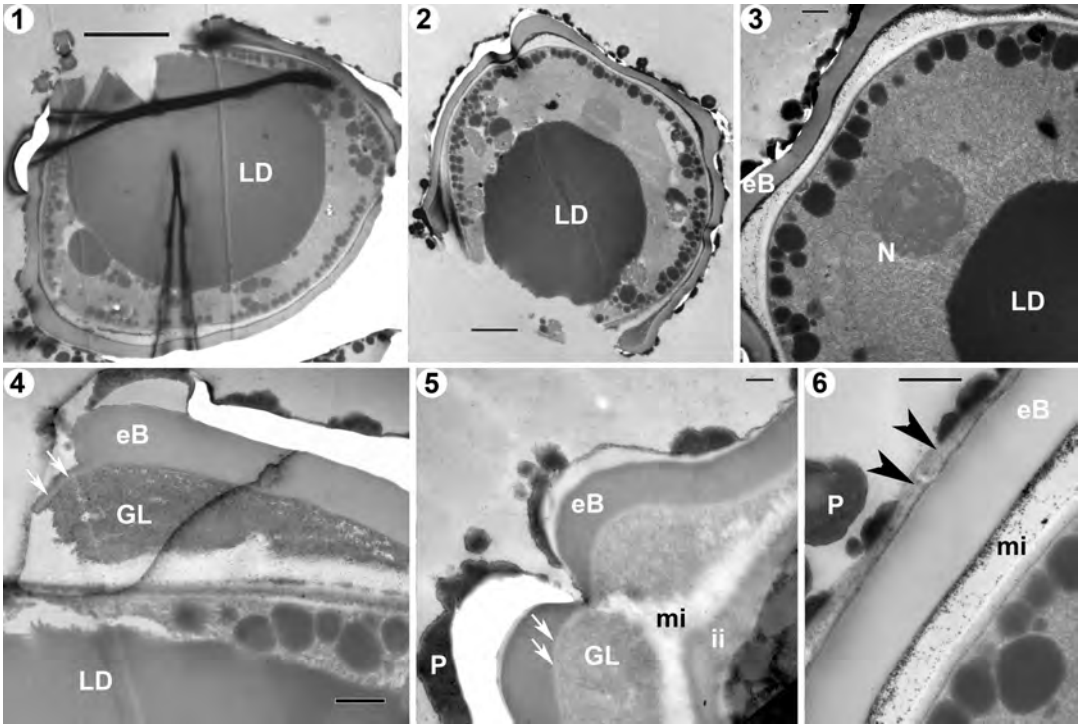

Figure 2. The ultrastructure of ungerminated spores in *Sphagnum squarrosum*. TEM. 1, 2 – sections of a whole spore; 3 – part of spore with nucleus; 4 – part of opened aperture with clearly visible exine layer A (white arrows); 5 – intact aperture with clearly visible exine layer A (white arrows); 6 – parts of sporoderm without thickened layers with translucent layer (black arrowhead). ii – inner intine, mi – middle intine, GL – granular layer (outer intine), P – perine, eB – exine layer B, N – nucleus, arrowhead – translucent layer, LD – lipid drop. Scale bars = 5.0 µm (1); 2.0 µm (2); 0.5 µm (3, 4, 6); 0.2 µm (5).

same thickness all over the exine. They are clearly seen only in sections made perpendicularly to the lamellae. The three-layered intine corresponds well to earlier descriptions (Brown et al. 1982a, b, 2015; Filina & Filin 1985b). The outer intine contains electron-opaque acetolysis-resistant granules and laminae. It thickens greatly only under the trilete mark and forms the ridges of the laesura (Fig. 2: 4, 5). The middle intine layer is slightly thickened only in the equatorial area. The inner intine is thin all over the sporoderm, slightly thickened under the laesura and fills the gap of the laesura.

Discussion

The two studied species of *Sphagnum* do not differ in the sporoderm development until mature sporoderm (Brown et al. 1982a; Filina & Filin 1985b). There are only differences in the sculpture, which we have also noted. The difference of spore surface (microverrucate in *S. fimbriatum* and verrucate in *S. squarrosum*) is of taxonomic significance (Filina & Filin 1985a; Cao & Vitt 1986).

The ultrastructure of germinating spores and ungerminated spores of *Sphagnum* differs markedly in the condition of the protoplast and sporoderm structure (Fig. 4). The protoplast of the germinating spores in *Sphagnum* is actively functioning with well-developed bi-membrane and single-membrane components. The cell tends to get out of a tight sporoderm and all active

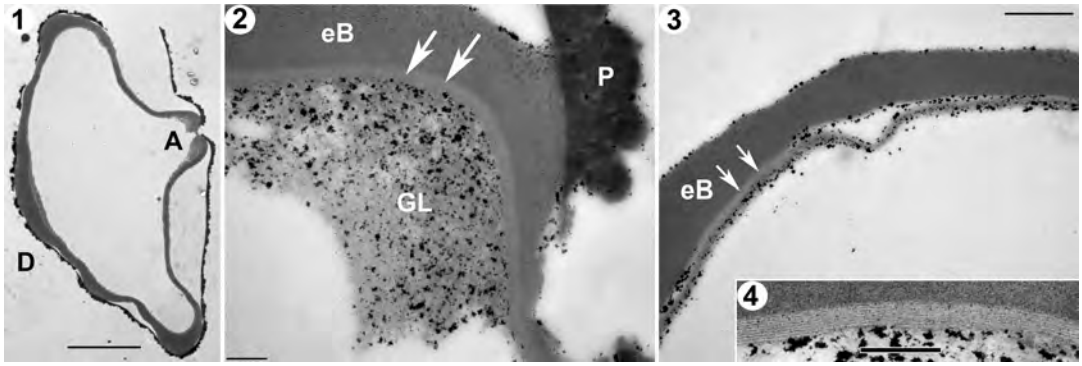

Figure 3. The ultrastructure of acetolyzed spores in *Sphagnum fimbriatum*. TEM. 1 – section of a whole spore; 2 – part of intact sporoderm in the laesura with clearly visible exine layer A (white arrows); 3 – part of sporoderm with separated granular layer with clearly visible exine layer A (white arrows); 4 – part of A exine layer. A – aperture, D – distal pole, GL –granular layer, P – perine, eB – exine layer B, arrows – exine layer A. Scale bars: 5.0 µm (a); 0.2 µm (b, d); 0.5 µm (c).

organelles move to the opened aperture (Fig. 1: 6). A large central lipid drop in ungerminated spores occupies a somewhat smaller area in sections than it does in germinating spores (Table 1). This is an expected result, because the lipid is consumed in the process of germinating cell activity. However, these results are not statistically significant, because spores were investigated in insufficient amount. Lipid drops in *Sphagnum* spores can be observed in sporocytes (Filina & Filin 1985a). In sections of mature spores, there are only a few lipid drops and they occupy a large part of the spores (Brown et al. 2015). Apparently, in spore development the small lipid drops are combined into a large drop and in spore germination the contrary is true as it was described for some mosses (Olesen & Mogensen 1978). Dynamics of the content of spare lipids in the life cycle in *Physcomitrella* was described in detail by Huang et al. (2009). However, in this case, the central lipid drop is hardly used during germination. Protoplasts of the ungerminated spores of *Sphagnum* seem to be in a dormant state in spite of the broken sporoderm. The nucleus is located far from the aperture region. Chloroplasts with few membrane structures are sparse as well as mitochondria. Other cell membrane components are unclear. However, these differences are quantitative.

The qualitative difference is the ultrastructure of the inner sporoderm layer, the intine. The middle intine is greatly developed in the germinating spores of *Sphagnum* (Fig. 1: 1–4). Some parts of this layer have a thickness that is significantly greater than that of other sporoderm layers combined. All sporoderm layers in the ungerminated spores of *Sphagnum* correspond in the structure and thickness to those of the described mature spores of *Sphagnum* (Filina & Filin 1985b). Brown et al. (1982a, b, 2015) described a two-layered intine, although they probably did not distinguish middle and inner intine.

Only sporopollenin-containing layers survive acetolysis. The perine saves its structure after acetolysis. The translucent layer disappears most easily. This layer is not detected even after partial acetolysis. As a result, the perine falls off from the spore easily (Fig. 3: 1). Exine layers A and B are resistant to acetolysis. Lamination of layer A is even better visible in acetolyzed spores than in intact spores (Fig. 3: 4). The granular layer (outer intine) persists as a separate layer composed of granules and laminae (Fig. 3: 2). In some places, it can delaminate from the exine (Fig. 3: 3). During sporoderm development, this granular layer initiates in the aperture region

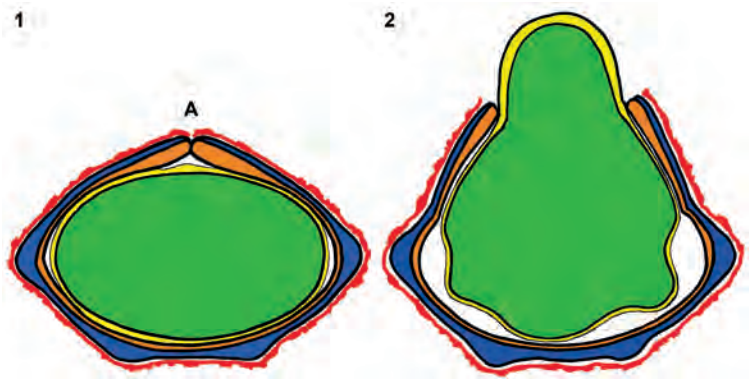

Figure 4. The diagrams of sporoderm ultrastructure in *Sphagnum*. 1 – ungerminated spore; 2 – germinating spore. A – aperture, red – perine, blue – exine, orange – granular layer, white – middle intine, yellow – inner intine, green – protoplast.

immediately after the formation of the exine layer A and before initiating the exine layer B (Brown et al. 1982a; Filina & Filin 1985b), similarly to initiation of the endexine in pollen development. These facts make it possible to compare the exine layer A and outer granular intine of *Sphagnum* with lamellar TPL-layer of *Oedipodium* (Polevova 2015). The exine layer A of *Sphagnum* corresponds to the lamellar TPL-layer in the equatorial area of *Oedipodium*. The outer granular intine of *Sphagnum* corresponds to the TPL-layer on the distal hemisphere and laesura of *Oedipodium* (see Fig. 15 in Polevova 2015).

Monroe (1968) described the thickening of cell walls after two weeks of germinating of *Funaria hygrometrica* spores. Standard histochemical tests for polysaccharides, proteins and lipids were made on *Polytrichum commune* and *Funaria hygrometrica*, yielding further evidence to the interpretation based on the ultrastructure and sequence of germination phases (Olesen & Mogensen 1978). However, in the described cases, the sporoderm (intine) has changed only in the aperture. Thickening of the intine on the proximal hemisphere agrees well with the protoplast activity in germinating spores of *Sphagnum*. This is clearly seen as the plasma membrane possesses a wavy profile near the thickened middle intine and the opened laesura, there are many subsurface vacuoles. Here, the growth of the cell wall occurs. The considerable thickness of the middle intine probably promotes passive displacement of the protoplast in *Sphagnum* spores outside the sporoderm to the active life at the expense of photosynthesis. Thus, the sporoderm plays an active role in the germination of *Sphagnum* spores and is not only the passive protection of the dormant spores from adverse environmental effects during dissemination.

Acknowledgements

We are grateful to E.A. Ignatova and M.S. Ignatov for providing plant material for this study and to Dr V.R. Filin, Dr N.P. Matveyeva, Dr N. Zavialova and Dr M. Tekleva for the interesting discussion about the sporoderm ultrastructure. The work was supported by the RSF grant 14-50-00029.

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