Arctoa (2012) **21**: *63-69*

SPORODERM ULTRASTRUCTURE IN *ANTHOCEROS AGRESTIS* PATON

УЛЬТРАСТРУКТУРА СПОРОДЕРМЫ *ANTHOCEROS AGRESTIS* PATON

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Abstract

The sporoderm ultrastructure in *Anthoceros agrestis* Paton is unique. The wall of mature spores consists of granules varying in size and shape, and does not have any homogeneous or lamellar layers. The electron-lucent sporopollenin, which forms granules of the exosporium, is comparable to that in other spore-bearing plants (mosses, liverworts and Pteridophyta) in its electron density, while it is different in structure. Electron-dense substances in the gaps between the exosporium granules are resistant to acetolysis and are probably sporopolleninous.

Резюме

Спородерма *Anthoceros agrestis* Paton характеризуется уникальной ультраструктурой. Оболочка зрелых спор построена из разнообразных по размеру и очертаниям гранул и не имеет гомогенных или ламеллятных слоев. Спорополленин основного, гранулярного, компонента оболочки по электронной плотности, но не по строению, сопоставим со спорополленином экзоспориев других споровых растений. Электронно-темные включения между гранулами основного компонента оболочки сохраняются после ацетолизной обработки спор и, вероятно, являются спорополлениновыми.

KEYWORDS: *Anthoceros*, exosporium, hornworts, sporoderm ultrastructure

INTRODUCTION

Hornworts represent a monophyletic group, whose phylogenetic position among the land plants has been hotly debated in recent decades. Latest molecular data suggest that hornworts are sister group to vascular plants (Beckert *et al.*, 1999, Samigullin *et al.*, 2002; Shaw & Renzaglia, 2004, Qui *et al.*, 2006; Troitsky *et al.*, 2007; Stech *et al.*, 2003), and that has aroused considerable interest to their morphology. Data on the spore morphology and ultrastructure are particularly pertinent because they allow comparisons with fossil material, including even the earliest land plants (Taylor, 2003; Taylor *et al.*, 2011). Macro- and microfossils of hornworts are supposedly dated to Early Cretaceous (Jarzen, 1979; Taylor at al., 2009).

Recent phylogenetic studies indicate that the phylum Anthocerotophyta includes two classes: Leiosporocerotopsida Stotl. & Crand.-Stotl. and Anthocerotopsida Jancz. ex Stotl. & Crand.-Stotl. (Duff *et al.*, 2007; Renzaglia *et al.*, 2009). The former class comprises the only species, *Leiosporoceros dussii* (Steph.) Hässel. According to the molecular data, the species is very distinct from other Anthocerotophyta (Duff *et al.*, 2007; Renzaglia *et al.*, 2009). It is characterized by unusually small psilate spores and a unique disposition of cyanobacteria in the thalli (Villarreal & Renzaglia, 2006). Other members of the phylum are referred to the latter class and are grouped into four families: the Anthocerotaceae Dumort. (*Anthoceros* L., *Folioceros* D.C. Bharadwaj and *Sphaerosporoceros* Hässel), the Notothyladaceae Müll. Frib. ex Prosk. (*Notothylas* Sull. ex A. Gray, *Phaeoceros* Prosk., *Paraphymatoceros* Hässel), the Dendrocerotaceae (Milde) Hässel (*Dendroceros* Nees, *Megaceros* Campbell, *Nothoceros* (R.M. Schust.) J. Haseg., and *Phaeomegaceros* Duff, J.C. Villarreal, Cargill & Renzaglia) and the Phymatocerotaceae Duff, J.C. Villarreal, Cargill & Renzaglia (*Phymatoceros* Stotler, Doyle & Crandall-Stotler). *Anthoceros*, *Folioceros* and *Sphaerosporoceros* constitute a monophyletic group, separated from other members of the Anthocerotophyta (Duff *et al.*, 2003; Shaw & Renzaglia, 2004; Duff *et al.*, 2007).

Light microscopy (LM) images and descriptions of spores have been published for some modern hornwort species (Jarzen, 1979). Spores from Maastrichtian sediments are similar to extant hornwort taxa and particularly attributed to species of *Phaeoceros* (Jarzen, 1979; Archangelsky & Villar de Seone, 1996). However, LM data alone sometimes is not sufficient for unequivocal spore identification as well as for phylogenetic conclusions. Scanning electron microscopy (SEM) is actively applied as it provides plenty micromorphological characters useful for taxonomy and identification, since horn-

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worts have a rather uniform morphology of both gametophyte and sporophyte (Hässel de Menéndez, 1990, Zhang & Wu, 2006; Renzaglia *et al.*, 2009). For example, the sporoderm sculpture of *Phaeoceros* species was shown to be taxonomically significant (Hässel de Menéndez 1990; Cargill & Fuhrer, 2008; Crandall-Stotler *et al.*, 2008). However, there are only brief references concerning sporoderm ultrastructure studied through transmission electron microscopy (TEM) of the group (Taylor *et al.*, 2002), and few published ultramicrographs, including one of the closely related species *A. punctatus* (Ajiri & Ueda, 1976; Brown & Lemmon, 1990; Renzaglia *et al.*, 2009; Villarreal & Renzaglia, 2006; Villareal *et al.*, 2004, unpublished thesis). It is clear that sporoderm ultrastructure in the Anthocerotophytae differs essentially from the studied liverworts and mosses: there is no tripartite lamellae except a very ephemeral TPL in Leiosporoceros (Renzaglia *et al.*, 2009), no perine (Brown & Lemmon, 1986; Estebanez *et al.*, 2006; Filina &Filin, 1985; Renzaglia *et al.*, 1997; Steinkamp & Doyle, 1979, 1984; Tryon & Lugardon, 1991).

The aim of this study was to explore the general morphology of mature spores of *Anthoceros agrestis* Paton as well as their sporoderm surface and ultrastructure for broad comparison with the earlier published results, and to document the obtained data with LM, SEM and TEM micrographs.

MATERIAL AND METHODS

Thalli with capsules of *Anthoceros agrestis* were collected by Dr. V.R.Filin in September, 2008 nearby Lucino village, at the Zvenigorod Biological Station, Zvenigorod District, Moscow Province, and by Dr. M.V. Remizova and Prof. D.D.Sokoloff in September, 2009 in the garden-patch, Klimovsk, Moscow Province.

The capsules were detached from the thalli, cut along and fixed in freshly made 2.5 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.3). A part of fresh material was acetolyzed according to the standard technique (Hesse *et al.*, 2009). Morphometry and visualization of acetolyzed spores were made from glycerin jelly slides for LM using a Leica DME and DFC290 camera. Twenty spores were measured for their equatorial diameter, polar axis, length of laesura rays, exine thickness, lumen diameter and spine height. Mean, minimum and maximum values are given. Acetolyzed spores were also prepared for TEM.

For TEM, capsules were fixed in glutaraldehyde and acetolyzed spores were post-fixed in 2% solution OsO_4 for 10 hours at 4°C. Then the material was transferred to 70% ethanol through a series of different concentrations of ethanol, and contrasted by saturated solution of uranyl acetate in 70% alcohol at 4°C (10 hours). The material was dehydrated through a gradual transition to absolute ethanol and placed in a mixture of acetone and absolute alcohol, then in pure acetone and in a mixture of Epon resin and acetone. Dehydrated specimens were finally embedded in Epon mixture (Weakley, 1975). The material was kept for 24 hours at room temperature. Then the material was curated for 48 hours at 62°C. Ultrathin sections of the specimens (60 nm) were made with an ultramicrotome (Leica Ultracut-R). The sections were stained with uranyl acetate (Geyer, 1974). The sections were studied and images were taken using a JEM-1011 TEM (80 V) in the Laboratory of the electron microscopy of Biological faculty of Lomonosov Moscow State University. Also spore sections were done from mature spores that appear from the cracked top of capsules, and they were compared with acetolyzed mature spores. Mature spores without special treatment were mounted on SEM stubs on nail polish. The stubs were coated with gold and examined in CamScan in the same laboratory. The terminology used for the spore ultrastructure follows Traverse (2007).

RESULTS

LM. Spores are trilete, rounded-tetrahedral and dark brown. The polar axis (P) is 38.9 (36.8–41.3) μm; the equatorial diameter (E) is 52.2 (45.7–59.5) μm; P/E=0.75 (0.67–0.84). The outlines are circular to sub-circular from the polar view (amb) and elliptic from the equatorial one (Fig. 1: *3, 6, 7*). The proximal hemisphere is flat to dihedral. The laesura rays have straight, thickened margins, which extend to the equator (Fig. 1: *1, 4, 5*). The laesura rays are of the same length, about 18.3 (14.8–22.0) μm. The thickenings turn into equatorial rim on extremities of the laesura rays. The sporoderm is approximately 1.8–2.2 μm thick, but is too dark and sculptured to measure its thickness accurately in transmitted light (Fig. 1: *7*). The spores are echinate distally and wavy proximally.

The distal face is coarsely reticulate with lumina of hexahedral or irregular outlines. Lumina are up to 7.1 (5.8–9.6) μm. There are high spines with curved tops on the furcation of the muri (Fig. 1: *9*). The spines are 3.3 (2.4–4.4) μm high; they have irregularly curved tips and give the spores a shaggy appearance. The proximal face is also coarsely reticulate but without any processes on the muri (Fig. 1: *2, 8, 9*). The lumen shape is slightly extended from the pole to the equator (Fig. 1: *4, 5*). The rim diverges from thickened margins of a bordered laesura resembling tree branches.

SEM. The distal face is coarsely reticulate and echinate. The lumina have more or less rounded outlines (Fig. 2: *2*). The muri are thick, low and scabrate with narrowly conical spines (Fig. 2: *2*). The spines are slightly distorted, often with several (two or three, rarely four) tips, which are irregularly curved. The lumen bottom is smooth with small granules of different sizes.

The proximal face is coarsely reticulate. The lumina are oval and extended from the pole to the equator. Thick muri are in contact with a thickened bordered laesura forming an equatorial rim. The lumen bottom and the muri are scabrate, occasionally with granules of various sizes.

TEM. The endosporium (intine) is very thin, light, microgranulate, and somewhat thickened under the laesura (Fig. 3: *2, 3, 4, 7*). The exosporium (exine) is massive,

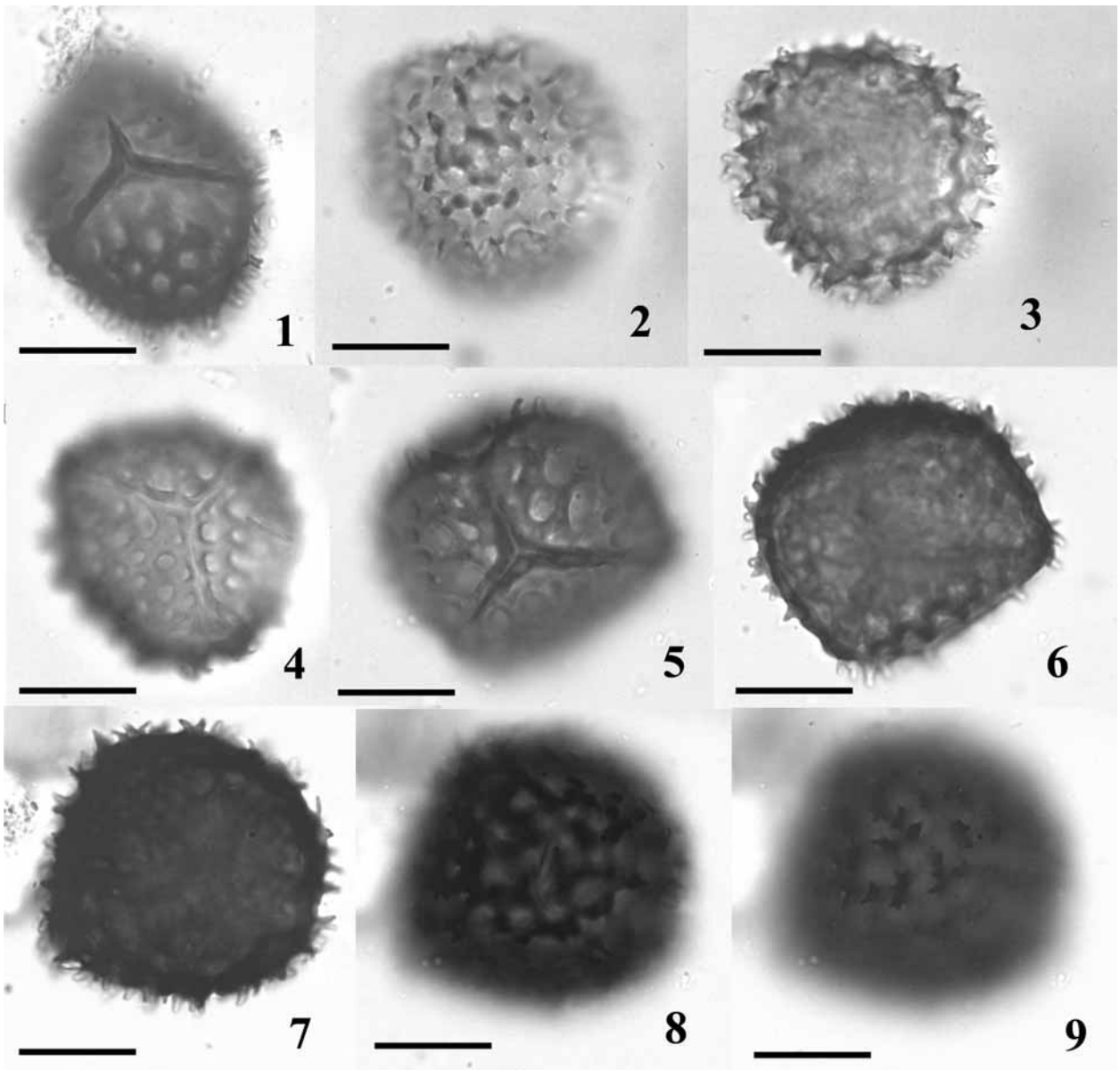
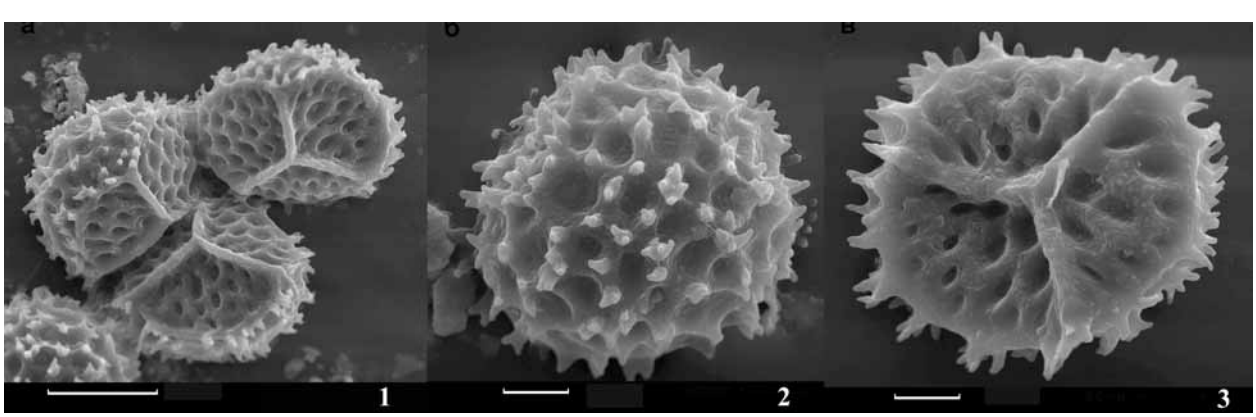


Fig. 1 (above). LM. *Anthoceros agrestis* spores. 1, 4, 5: proximal hemisphere with a trilete laesura; 3, 6, 7: optical section, sporoderm thickness is poorly discernible; 2, 8: distal hemisphere; 9: branchy tips of spines. Scale bars 20 μm.

Fig. 2. (below). SEM. *Anthoceros agrestis* spores. 1: disintegrating spore tetrad; 2: distal face with spines connected by ridges; 3: proximal face with a trilete laesura and oval lumina. Scale bars: 30 μm for 1, 10 μm for 2-3.



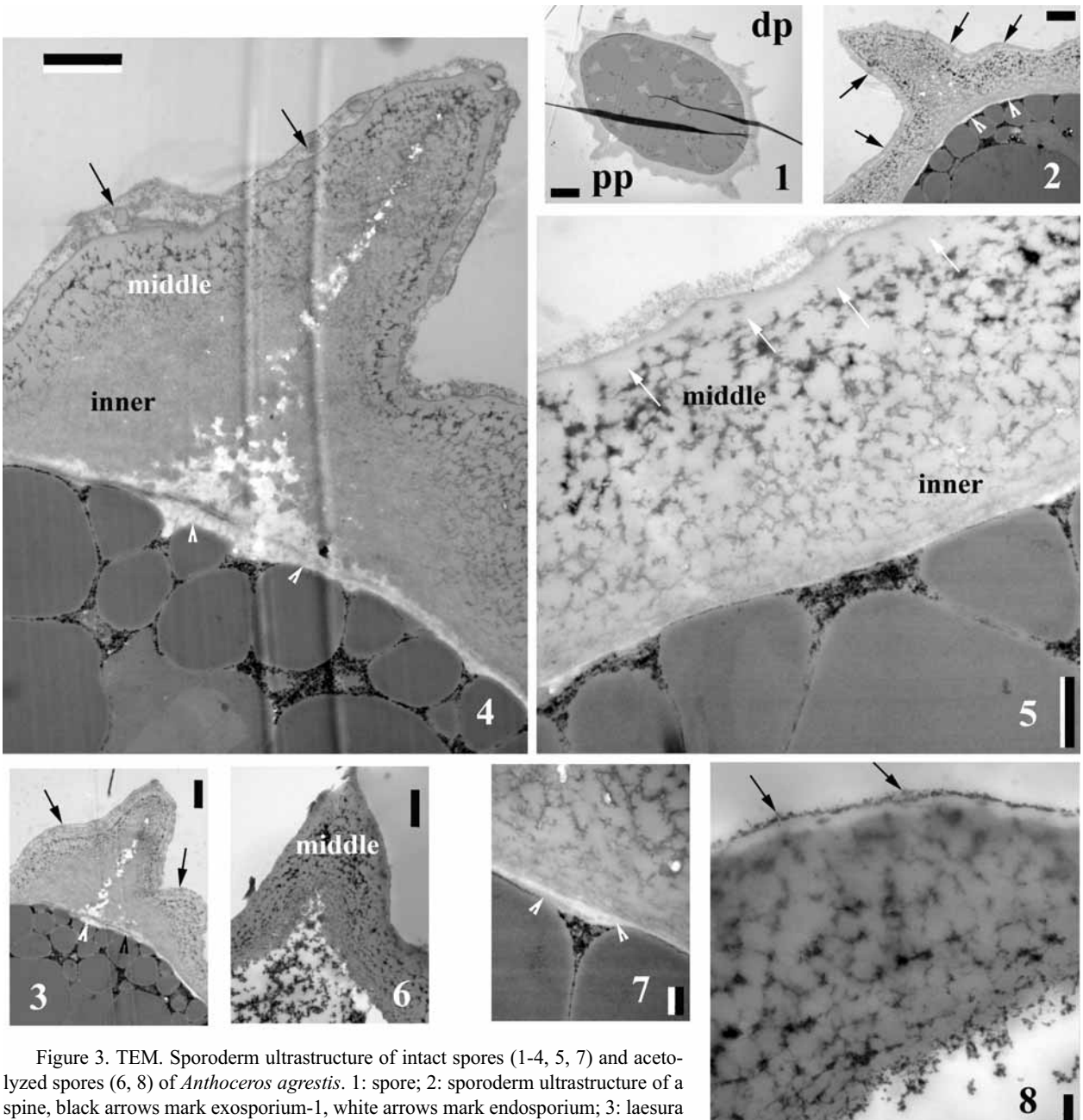


Figure 3. TEM. Sporoderm ultrastructure of intact spores (1-4, 5, 7) and acetolyzed spores (6, 8) of *Anthoceros agrestis*. 1: spore; 2: sporoderm ultrastructure of a spine, black arrows mark exosporium-1, white arrows mark endosporium; 3: laesura slit in the central part; 4: slit in the laesura connection; 5: structure of exosporium-2, showing inner and middle strata, and outer stratum (tecum) arrowed; 6: spine of acetolyzed spore; 7: absence of lamellose elements in the innermost sporoderm, white arrows mark endosporium; 8: sporoderm ultrastructure of acetolyzed spores, black arrows mark exosporium-1. dp – the distal pole; pp – the proximal pole with laesura slit; inner – inner exosporium stratum; middle – middle exosporium stratum; outer – outer exosporium stratum. Scale bar: (1) 5 μm; (2, 3, 4, 6) 1 μm; (5) 0.5 μm; (7, 8) 0.2 μm.

granular, of a variable thickness; it forms spines and muri (Fig. 3: *1*). There are two layers: outer exosporium-1 and inner exosporium-2. The exosporium-2 has three strata, which differ in their structure and gradually turn one into another (Fig. 3: *2, 3, 4, 5*). The inner stratum is made of small granules with very narrow and electron-translucent gaps between them (Fig. 3: *5, 7*). The inner stratum is strongly thickened in the spine bases and laesura. Inner margins of the laesura are uneven and look torn (Fig. 3: *3, 4*). The leasura slit reaches the sporoderm surface only in the contact of three laesura rays on the proximal pole. The middle exosporium-2 stratum is made of rather large granules with wide gaps between them filled with electron-dense substance. This is the thickest layer in the spine region. Exteriorly large granules of outer stratum are fused in a solid tectum (Fig. 3: *5*). A thin, interrupted layer of exosporium-1 is located at the outside (Fig. 3: *2, 3, 4, 8*). The exosporium-1 is made of small granules, which are slightly more electron-dense than the granules of exosporium-2. Large granules occa-

sionally occur among small granules. The large granules are of different sizes and similar to the exosporium-2 in their electron density.

The endosporium is lost after acetolysis; some peculiarities of different exosporium parts are preserved, including electron-dense substances in the gaps between exosporium granules (Fig. 3: *6, 8*).

Discussion

Spore morphology has long been used in taxonomic studies of hornworts (Hasegawa, 1984, 1993; Campbell, 1981, 1982 a, b, 1984, 1986; Hässel de Menéndez 1989; 1990). The color, size and sculpture of spores are important. *Anthoceros* s.str. is characterized by dark spores with a distinct triradiate mark. LM characteristics combine the features of sculpture and texture and give a lot of important information. Such characters of the surfaces as processes (spines, spinules, papillae, mamillae and bacula), proximal pits and reticulum, and strips along the laesura are used in diagnoses of the Anthocerotopsida taxa. Smooth or sculptured surface along the laesura was the main feature for classification of spore types in *Anthoceros* (Bharadwaj, 1960, Asthana & Srivastava, 1991). There are however certain incongruence between views in LM and SEM. For example, smooth stripe along lesurae in *Anthoceros fusiformis* Austin and *A. caucasicus* Steph. are distinct in LM, but it is not as pronounced as *A. erectus* Kashyap or *A. bharadwajii* Udar & A.K. Asthana in SEM views.

According to SEM data, only spores of *Leiosporoceros dussii* are smooth, without any ornamentation. Spores of other hornworts bear processes on the distal face and often on the whole surface. A similar sculpture is described in papillate spores of *Nothoceros* and *Megaceros*. The spores bear many verrucae on the distal face and some verrucae in the center of the contact area, between the rays of the laesura (Shaw & Renzaglia, 2004; Zhang & Wu, 2006; Villarreal *et al.*, 2004). Spores of *Phaeomegaceros fimbriatus* have a verrucate contact area and a rugulate or vermiculate distal face with 4-11 depressions (Villarreal & Renzaglia, 2006). The vermiculate sculpture is known also for spores of *Notothylas, Phaeoceros* and *Dendroceros*. Spores of *Dendroceros crispatus* (Hook.) Nees. are multicellular unlike those of other Anthocerotophyta and have verrucate to granulate vermiculate surface (Schuette & Renzaglia, 2010). The sculpture of *Notothylas* spores is either tuberculate or vermiculate, with mamillae or papillae (Hasegawa, 1979). Spores of *Phaeoceros* are vermiculate or reticulate in the contact area and bear spines or verrucae on the distal face (Shaw & Renzaglia, 2004; Zhang & Wu, 2006; Cargill & Fuhrer, 2008). Spinules are present in the sculpture pattern of *Folioceros* and *Anthoceros* as well. Spores of *Folioceros* do not have a clear tetrad mark though the sculpture slightly differs on the distal and proximal faces (see Bharadwaj, 1960). Spines, verrucae and rod-like sculptural elements are larger on the distal face. The processes are smaller and more densely arranged on the proximal face (Zhang & Wu, 2006; Villarreal *et al.*, 2004). Spores of some *Anthoceros* bear large spines on the distal face and a distinct trilete mark (suture) on the proximal one. Spines of the proximal face can differ considerably among different species (Zhang & Wu, 2006; Hässel de Menéndez, 1990).

The analysis of SEM data reveals that the ornamentation of proximal spore face can be either similar to or different from that on distal spore face. There is one exception, where these types occur within the same species. In case of *A. subtilis* Steph. some pictures of Asthana & Srivastava (1991, Tabl. 41: 1-6) illustrate this species as having similar ornamentation, while in others the distal and proximal spore faces have obvious differentiation (l.c., Tabl. 40: 4,5,6). Otherwise, all other species belong to one type.

The similar ornamentation is characteristc of *A. cavernosus* Steph., *A. orizabensis* (Steph.) Hässel, *A. tuberculatus* Lehman & Lindenb., *A. venosus* Lindenb. & Gottsche (Hässel de Menéndez, 1990), *A. macounii* M. Howe (Ignatova *et al.*, 2010; Hässel de Menéndez, 1990), *A. angustus* Steph., *A. alpinus* Steph., *A. bharadwajii, A. erectus, A. macrosporus* Steph., *A. subtilis* (part) (Asthana & Srivastava, 1991).

The different distal and proximal spore faces occur in *A. fusiformis*, *A. hispidus* Steph., *A. lamellatus* Steph., *A. patagonicus* Hässel, *A. peruvianus* Steph., *A. scariosus* Austin, *A. simulans* M.A. Howe (Hässel de Menéndez, 1990), *A. muscoides* Colenso (Campbell, 1982 b), *A. agrestis* (our data), *A. laminiferus* Steph. (Campbell, 1982 b), *A. punctatus* L. (Villarreal et al, 2004, Asthana & Srivastava, 1991), *A. crispulus* (Mont.) Douin, *A. pandei* Udar & A.K. Asthana, *A. subtilis* (part) (Srivastava & Asthana, 1987).

Within both types, there is a variation. The spore surface of some species bear large spines or knobs, while in others it is lamellate to papillate. The further classification needs special study, including the variation that may involve the single species, e.g. *Anthoceros macounii* has larger spines in SEM pictures of Ignatova *et al.* (2010), but smaller in those given by Hässel de Menéndez (1990).

These sculpture types are in agreement with the LM data and, without prejudice, with molecular phylogenetic studies (Villareal *et al.*, 2010).

The sporoderm in all investigated hornwort taxa is granulate, without any lamellae. There is no perisporium because these plants do not have a tapetum in the capsules. The sporoderm consists of several strata of the exosporium and a single stratum of the endosporium (intine). The outer exosporium is homogeneous; the inner one is granulate (Renzaglia *et al.*, 2009; Taylor et al., 2002; Villarreal et al., 2004).

The thinnest exosporium of *Leiosporoceros dussii* contains a very thin granular layer. The exosporium of

Dendroceros crispatus is granulate and vermiculate (Villarreal et al., 2004; Schuette & Renzaglia, 2010). The exosporium of *Folioceros fuciformis* (Mont.) D.C. Bhardwaj has inner homogeneous and outer granulate-vermicular parts (Villarreal et al., 2004).

Megaceros flagellaris (Mitt.) Steph., *Nothoceros* sp., *Phaeoceros carolinianus* (Michx.) Prosk. and *Notothylas orbicularis* (Schwein.) Sull. have a typical sporoderm ultrastructure: outer homogeneous (E1) and inner granular exosporium (E2). There is a distinct boundary between the exosporium layers. *Phaeomegaceros fimbriatus* (Michx.) Prosk. and *Anthoceros punctatus* are characterized by thin, homogeneous exosporium (E1), large, granular exosporium (E2) and indeterminate limit between them. *Phaeoceros carolinianus*, *Notothylas orbicularis* and *Anthoceros punctatus* possess an inner translucent layer (E3) (Villarreal *et al.*, 2004).

Our data are in good correspondence with the previous studies. As a rule, the sporoderm ultrastructure in *Anthoceros* includes outer homogeneous, inner granular exosporium and inner translucent layers. I distinguish three strata in exosporium-2. The term stratum is most suitable since the strata gradually transform into each other. The outer exosporium-2 is the agglomeration of granules resulting in a tectum. The middle and inner strata of exosporium-2 differ from each other by presence/absence of electron-dense material. This material is seen in ultramicrographs of the sporoderm in *Anthoceros punctatus* in the inner part of the exosporium (Villarreal *et al.*, 2004, unpublished thesis). The electron-dense material is sporopolleninous because it does not change after acetolysis. The sporopollenin in *Anthoceros* preserved after acetolysis is of two types: electron-translucent sporopollenin of large granules, and electron-dense one between the granules. I have detected external exosporium-1, which is the result of late deposition of sporopollenin, representing a possible analogue of the perisporium. Comparison of sporoderm ultrastructure in *A. agrestis* and *A. punctatus* (Ajiri & Ueda, 1976; Villarreal *et al.*, 2004) revealed no differences between the species. Endosporium in pictures of Ajiri & Ueda (1976) does not allow comparison with our data, however Villareal *et al.* (l.c.) describe a thicker endosporium comparing with what we observed. However this difference need additional check as it is not clear how this character is affected by the stage of development: the data presented by Villareal *et al*. (l.c.) material might represent a slightly later stage of maturation comparatively with our spores taken from open capsule, not spores spread out of capsule.

The most distinctive feature of the sporoderm ultrastructure of *Anthoceros* is low electron density of the main component of the wall (exosporium granules) and high electron density of the substance which fills gaps between the granules. The perisporium, which is usually electron dense in all studied bryophytes, as well as in all other spore-bearing plants, is lacking in *Anthoceros* as there is no tapetum in its capsules (Brown & Lemmon, 1986; Estebanez *et al.*, 2006; Filina &Filin, 1985; Renzaglia *et al.*, 1997; Steinkamp & Doyle, 1979, 1984; Tryon & Lugardon, 1991). Another characteristic feature of *Anthoceros* ultrastructure is complete absence of any lamellar formations in mature sporoderm (Heckman, 1970; Taylor, 2003; Taylor *et al.*, 2011). However, this needs to be additionally checked in the ontogenetic study of the sporoderm because some structures can be masked in the mature sporoderm during its development.

CONCLUSIONS

Anthoceros has a unique sporoderm ultrastructure. The wall of mature spores in *Anthoceros agrestis* (both layers of the exosporium) consists of granules of different sizes and shapes, and does not have any homogeneous or lamellar layers. The electron-lucent sporopollenin, which forms granules of the exosporium, is comparable to the exosporium of other spore-bearing plants in its electron density, while it is different in structure. Electron-dense substances in the gaps between exosporium granules are resistant to acetolysis and are probably sporopolleninous. In their electron density (but not in morphology and topography) they correspond to the perisporium of bryophytes.

ACKNOWLEDGMENTS

Sincere thanks go to V.R.Filin, D.D.Sokoloff, and M.V.Remizova (Biological Faculty, MSU) for the plant material, to V.R.Filin for the useful critical comments, to A.G.Bogdanov (Laboratory of Electron Microscopy, MSU) for technical assistance on SEM and TEM, and to J.C. Villarreal for helpful criticism and supplying rare publications.

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