AN ATTEMPT TO CREATE AIR SACS IN SPORES?
ON THE UNUSUAL SPORE STRUCTURE IN MOSS ENCALYPTA LONGICOLLIS

Svetlana V. PolevoVA1, AndreY V. Moiseenko2, Maria A. Kolessnikova3,
Darya A. Ashikhimina1 & Michael S. Ignatova1,3

Abstract

An empty perine processes covering spore surface are discovered in Encalypta longicollis, being likely the first known case of the gas-filled structures in moss spores. TEM images perform a diversity of variants of inner structure of these processes, showing a progressive decomposition of their inner material, from spongy to lacunose and finally resulting with a cavity in their middle up to half of their volume. The decomposition is associated with the spreading of osmiophilous compound, which fills the spongy parts of perine and spreads along a flexuose channels that are terminated in areas where perine material shows maximal decomposition. Encalypta longicollis is characterized by outstandingly large spores, 55–85(–95) μm in diameter, whereas in other species of the genus spores are 9–40(–50) μm. The gas-filled space reduces the spore density, probably facilitating its ability for dispersal by water currents.

KEYWORDS: bryophyte, spore, perine, exine, dispersal, TEM

INTRODUCTION

Plants differ from animals in disability to cross space in short time, as almost all of them are tightly rooted in a fixed place. Therefore, the diaspore dispersal ability is crucial for plant, allowing population expansion and finding individuals for breeding. Seed plants have enormous diversity of adaptation for pollen and seed spreading, especially by means of animals, while spore plants are dependent mostly of air currents for spore dispersal and water medium for spermatozoid delivery to archegonia. Moss spores are anemochorous with the only two known exceptions for zoochory by Splachnaceae (Koponen, 1990; Marino, 2009; McCuaig et al., 2015) and Schistostega (Ignatov & Ignatova, 2001). Despite recent literature reported a number of cases of moss spore carriage by birds (Lewis et al., 2014; Chmielewski & Eppley, 2019)

1 – Lomonosov Moscow State University, Faculty of Biology, Leninskie Gory Str. 1-12, Moscow 119234 Russia – Россия, 119234, Москва, Ленинские Горы, д. 1 стр. 12, Московский государственный университет, биологический факультет. E-mails: svetlanapolevova@mail.ru
2 – Lomonosov Moscow State University, Laboratory of electron microscopy, Leninskie Gory Str. 1-32, Moscow 119234 Russia – Россия, 119234, Москва, Ленинские Горы, д. 1 стр. 32, Московский государственный университет, Лаборатория электронной микроскопии. E-mails: postmoiseenko@gmail.com
3 – Tsitsin Main Botanical Garden, Russian Academy of Sciences, Botanicheskaya Str., 4, Moscow 127276 Russia – Россия 127276 Москва, Ботаническая 4, ГБС РАН.
and small mammals (Barbé et al., 2016), nothing is still known about the efficiency of this way of dispersal. We may just hypothesize that it is highly underestimated, judging from the distributional patterns of some species.

The dependence of moss spore dispersal ability on the spore size was in the focus of several studies. The size 20–25 μm is usually considered as an important limit, so smaller spores have a limitless ability to spread, whereas the larger ones seem to be too heavy for this (Hedenäs, 2012; Johansson et al., 2014).

Large spores in mosses usually occur in obligatory epiphytic lineages (Hedenäs, 2012) and in epigeios species of ephemeral life strategy (Furness & Hall, 1981) or growing in xeric environments. In the molecular phylogenetic tree of Encalyptaceae (Ignatov et al., 2016) the basal position is kept by Bryobrittonia (with spores 7 μm in diameter) and E. streptocarpa (with spores 9–15 μm), while in most species of Encalypta spores are 25–40 μm, and in E. brevipes up to 50 μm. The outstandingly large spores of E. longicollis, 55–85 (–95) μm, are difficult to link to above mentioned factors: this species is terrestrial and it grows in non-ephemeral habitats.

Spores in Encalypta were comprehensively illustrated with SEM images by Horton (1983). However, some neglected features of this species remained undescribed. In the course of its peristome studies (Ignatov et al., 2018) with TEM, we found holes in large outgrowths on spore surface of E. longicollis. Bryological literature call them “papillae”, while palynological terminology uses “clavae”, “baculi”, “verrucae”, “granules”. We will use in the following text “processes”, because the shape of perine sculpture elements in question is extremely diverse, from low hummocks to high clavae, though the cavity formation seems to be principally identical in all of them. The description of cavities in the perine processes of E. longicollis and an attempt to characterize their development based on available material is in the focus of present study.

MATERIAL AND METHODS

Sampling. For TEM studies, specimen from Yakutia, Ignatov & Ignatova 17-675 (MHA9025160) was used. To be certain that it is not exceptional, we compare this collection with other Asian specimens from Yakutia (Ignatov & Ignatova 16-1145, MHA) and Tyva (Pisarenko 0p04896, MHA).

LM. Observations and photography were done under Olympus CX–41 microscope with digital camera Infinity 2–2.

SEM. Scanning electron microscope observations were done with the SEM Jeol 6380 for specimens coated by gold without additional preparation.

TEM and Tomography. Recently opened capsules collected in Yakutia were fixed shortly after collecting in 2.5% glutaraldehyde in 0.05M PBS. Further steps were done after several weeks or few months. Specimens were post-fixed with 1% osmium tetroxide in PBS, pH 6.8, for 6 hours. Then material was dehydrated through an ascending ethanol-acetone series to 100% acetone. After that samples were embedded in araldite 6005 medium, according to the manufacturer’s protocol. Sections of 50 nm thick were done with a Leica-5 ultratome. Sections were examined under JEM-1011 TEM (Jeol, Japan) at 80 kV and a CCD ORIUS SC1000W under control of GATAN Digital Micrograph in the Laboratory of electron microscopy at the Faculty of Biology of Lomonosov Moscow State University.

Electron tomography

Electron tomography data were acquired using JEOL JEM-2100 transmission electron microscope, equipped with 200 kV LaB6 electron gun and Gatan Ultrascan 1000FTXP 2k CCD camera. 250 nm sections were mounted into Jeol 21311HTR high-tilt holder, which allows tilting up to 70°. However, we tilted the sample only to 60° due to relatively thick sections.

Electron inelastic scattering in a tilted stained section causes images to look “foggy” and blurred. To minimize this effect, we used Gatan GIF Quantum ER energy filter in EFTEM mode with 20 eV energy-selecting slit, positioned at zero-loss peak and filtering out inelastically scattered electrons. These electrons also may cause the section to shrink in direction perpendicular to the section plane. To prevent this process from happening during data acquisition, we pre-irradiated the sample at acquisition illumination conditions for 5 minutes.

SerialEM software was used to carry out tomography data acquisition workflow. The dataset consists of 145 images, taken with 1 deg tilt step at 1500 EFTEM magnification yielding a 0.9 nm pixel size.

Electron tomography was reconstructed with IMOD software. We did not use colloidal gold fiducials, but we got a fine frame alignment with the IMOD patch-tracking algorithm. FBP algorithm was used for the reconstruction. After data trimming and squeezing final three-dimensional section reconstruction has dimensions 1.53 × 1.48 × 0.23 μm.

Visualization was made with UCSF Chimera software and IMOD 3dmod software (see Supplementary material: http://arctoa.ru/ru/Archive-ru/28_2/Encalypta_movie.mp4).

Images from the Z-stacks were contrasted by stacking some of them using the software package HeliconFocus 4.50 (Kozub et al., 2008). The original files for stacking are available as Supplementary material 2.

RESULTS

Light microscopy (Figs. 1A–B).

Spores brownish, spheric to ellipsoidal from polar views, in lateral view low convex distally and low conic proximally, (50–)55–85 (–95) μm in diameter. Sporoderm thick and dark brown, with inapparent stratification. Processes on spore surface are spheric, 1–3 μm, or ovate, 8×2.5 μm. The latter occur near equatorial zone of proximal side, being somewhat radially arranged (1A, E). Processes on the distal hemisphere are more uneven, irregular in shape and irregularly arranged. Some processes, especially along the spore visible edge, are...
Encalypta longicollis: an attempt to create air sacs in spores?

Fig. 1. Encalypta longicollis spores. A–B: LM images. A: Total views of putatively proximal hemisphere: ovate sculpture elements are somewhat radially arranged closer to the equator (cf. with lateral view in “E”), while close to poles the sculpture elements are smaller and highly heterogeneous in size; some processes, especially along the spore visible edge, are with light center (corresponding to cavities shown in Fig. 2 and partly further). B: Total views of putatively distal hemisphere: processes are more uneven, irregular in shape and irregularly arranged. C–I: SEM images. C, D: putatively proximal face with ovate processes closer to equator; E: lateral view, showing part of proximal hemisphere near equator, with ovate processes (cf. with “A”); F: disintegrating spore tetrad, showing different ornamentation between hemispheres; G–I: spore ultrasculpture, showing mixture of larger processes and smaller granules (G), less granulose areas (H) and tetrad scar area (I). J–K: TEM images. J: exine split into two lamellae and outer lamella strongly curved and partly interrupted; aperture slit is arrowed. K: total sporoderm ultrastructure (I: intine, E: exine, P: perine).
with light center in all studied specimens.

**Scanning electron microscopy** (Figs. 1C–I).

Spores from herbarium collections are strongly modified, with concavities, especially at dorsal side. Equatorial zone looks more rigid, forming a ring conspicuous by its bold surface. Otherwise the surface is granulose, the processes being highly diverse. Most of them are spheric, 1–3 μm, but some are ovate, up to 6(–8) μm long and they form radial pattern (Fig. 1E) in the sub-equatorial part of proximal hemisphere. Small granules, 0.2–1.0 μm, are irregularly scattered between and sometimes upon perine processes (Fig. 1G–I). Otherwise the spore surface is rough, being covered by still smaller round elements of 50–200 nm.

**Transmission electron microscopy** (Figs. 1J–K, 2, 3, 4).

Intine is ca. 1 μm thick (Fig. 1K), electron light, homogeneous, occasionally looking fibrillose, similar all around spore in available sections.

Exine is 0.2–0.3 μm thick (Fig. 1K, slightly darker than intine, homogeneous, sharply (Figs. 1K, 3A) or more or less gradually (Fig. 3B) differentiated from intine. It is solid, or split into two layers (Fig. 3H, I), separated by electron-light medium and in this case reaching 0.6–0.7 μm in thickness. Fig. 1J illustrates once found exine slit, likely the aperture.

Perine is especially diverse and complicated in *Enchytraea longicollis*. The continuous dark layer covering spore all around, excepting aperture (Fig. 1J), is 0.2 to 1.0 μm thick, but with processes up to 4 μm high and 4 μm long (Fig. 2A); occasionally they are 6 μm long, likely representing ovate processes seen in Figs. 1A, E.

Smaller globules, 100–200 nm, with lighter central part, are scattered among perine processes (Fig. 2C, D, E, F). They may confluenase with these processes, which is apparent by lighter round bodies within the matrix of processes (arrows): their size and color are similar to inner medium of small globules. The border of darker outer layer and inner part of small globules is marked by narrow ring of more electron dense material (Fig. 2C), which is also apparent around light bodies within large perine processes (Fig. 2E). Confluence events are at places apparent by a concavity on the perine process filled by small globule (Fig. 2F, arrow).

Ovate globules similar to perine processes on spore surface occur also on the surface of spore sac (Fig. 2G–I). They have the same electron density, shape and size, and sometimes also a light inclusions, indicating confluence events and, conclusively, enlarging by means of them (Fig. 2I). However, we never saw any cavities even on the earliest stages of their formation (Fig. 2D) in the globules attached to spore sac.

Some perine processes (Figs. 2A, B, D) have homogeneous content, but most of them have middle and proximal part looking decomposed to various extent, from slightly heterogeneous (Fig. 2D, E), to lacunose (Fig. 3I), having cavities with cavernose sides (Fig. 3F, G, J) and finally having large cavities with almost smooth inner walls (Fig. 3L, M).

The dark grey perine material is at places totally homogeneous, as in most part of perine elements in Figs. 2D, but in many processes their proximal parts are spoty with electronically dark “drops”, which can be assumed as a spongy tissue with holes filled with osmiophilous substance (Fig. 3A, E). Such areas often have flexuose channels (or bundles of thin channels), filled by osmiophilous compound and connecting a relatively more decomposed areas with the exine (Figs. 3D, F, G).

At places osmiophilous compound fills more or less large space between exine and perine (Fig. 3A, B). Channels in spongous areas wherever apparent, are perpendicular to exine, spreading inside processes (Fig. 3C, D). Cavities, interpreted as the later stage of perine (or its primary material) decomposition, keep the same shape, “mushroom-shaped” or “Pharaonic sceptre-shaped” (Figs. 3 F, G, H, 4A). Cavities in the central part of processes (e.g. Fig. 3J) or otherwise disconnected from exine (Fig. 3K), can be explained by the section position. Note that some processes, especially larger ones, have curved zone of decomposition and can be interpreted as at first growing upright and then curved and fell on spore surface (Fig. 3H).

The studied material represents numerous stages of cavity formation, showing that the smaller is the cavity, the less smooth is its outline. The earlier stages look as heterogeneities with irregular small lacunes (Fig. 3I and Fig. 4); later “alveolar” or “cavernose” sides of cavities occupy progressively thinner layer (Fig. 3J); and finally, in the processes with the largest cavities, their inner walls are almost perfectly smooth (Fig. 3L, M). Figs. 2A, B, D represent a similar series at lower magnification. In all cases we observed, the processes with fully developed cavities had minimal thickness of their “outer wall”, i.e. the process of perine material decomposition has its fixed limit, usually 0.7–0.8 μm thick.

In few sections, the cavities in processes were filled by embedding medium (Fig. 3K), likely due to cracks in their walls (unseen in pictures), but in most caseed the cavities were empty.

**Tomography application**

The images from tomographic studies allow us to discern a narrow channel across exine, which has a continuation in perine (Fig. 4A). Nothing osmiophilous is seen in intine as a continuation of this path.

In addition to section images, the 3D reconstruction illustrates the shapes of cavities, outlining relatively large reservoirs around the area of progressive decomposition. Such reservoirs always surround this area, although never have immediate contact with most electronically light spots (Fig. 4, SM1).

**Discussion**

The empty processes, similar to above described, seems were never observed in spores of bryophytes. The
Encalypta longicollis: an attempt to create air sacs in spores?

Fig. 2. TEM images of Encalypta longicollis sporoderm ultrastructure, A–B: total views, showing perine processes, some with more or less developed cavities in their central part. C–F: Smaller globules, 100–200 nm, with lighter central parts, showing their variation in size and position and confluence with larger perine processes, apparent by lighter round bodies within the matrix of processes (arrows): their size and color are similar to inner medium of small globules; the border of darker outer layer and inner part of small globules is marked by narrow ring of more electron dense material (C), which is also apparent around light bodies within processes (E); at places the confluence events are apparent from a concavity on perine process (F, arrow). G, H, I: remnants of spore sac wall, where cells are strongly decomposed; large globules on their surface are somewhat similar to processes over spore wall in shape, size and electron density, but lacking inside any cavities and even heterogeneity; small globules with lighter central zone are similar to those in C–E, and looks confluence with larger globules (cf. E and I, arrows).
Fig. 3. TEM *Eucalypta longicollis* sporoderm ultrastructure showing variation at the join of exine and perine at places where the cavities are forming within perine processes. Osmiophilous compound is accumulated along the border of perine and exine (A, B, C, D), fills the spongy proximal part of perine and forms flexuose channel (C, D), conducting osmiophilous compound, associated with decomposition of perine material within processes is observed, starting from small lacunes (H), and further resulting in expanded space (D, E, F, J), contacting exine immediately (E), or through kind of channels (F, G). In perine processes with smaller cavities, the inner walls of cavities are strongly eroded or porose, indicating still expanding volume of cavities (H, I), while in processes with largest cavities the inner cell walls are nearly smooth (L, M). Exine at places split into two layers (B, H, I), separated by electron-lighter material. In “K” the cavities in processes on the left are filled by embedding medium, while the central part of process on the right is partly empty.
Fig. 4 TEM *Encalypta longicollis* sporoderm ultrastructure; Helicon Focus’ stack of 30 sequential tif images 1,15 nm thick (arbitrary color). Part of intine (I), exine (E) and proximal part of perine (P). The most prominent channel across exine (seen partly closer to intine) continues in perine as apparent flexuose channel filled by osmiophilous material, terminated in several pools. Osmilphilous material is also accumulated at the border of exime and perine, where the channel to perine starts. Note the differentiation of perine from homogeneous distal part to granular proximal part, where small less osmiophilous granules (darker in this figures) are rather evenly embedded in somewhat electron denser perine matrix. Closer to channel of osmiophiophilous compound various stages of perine erosion is observed (cf. Fig. 3). See also Supplemetary material.

B–E: TEM, The ultrastructure of perine at early stages of formation of cavities. B and C: osmiophilous compound fills inner part of perine where it has porose or spongy texture; D: cavity in perine process, surrounded by porose layer enriched with osmiophilous inclusions; Note that osmiophilous inclusions are always present near places looking eroded.
mentioned observation may be explained by uneven deposition of sporopollenin precursors. In the process of precipitation and/or polymerization of sporopollenin on such initial particles different kinds of perine are forming. Additional material for perine production is precipitated, immuring small globules with lighter cores and forming rather resistant to degradation outer perine of 0.7–0.8 μm thick. Along with the complex perine formation, the degradation of the substance of initial particles occurs in the central and proximal parts of processes, cavities are forming and their contents is replaced by air.

The fact that globules on the spore sac of a putatively same electron dark material never have cavities, and the coincidence of the largest channels in perine with tiny channels in exine suggest that the cavity formation is a complex and somehow spore-regulated process, but its details remain for further investigation.

The maximally similar patterns among the published illustrations are the following. Takakia spores, described by Renzaglia et al. (1997) have thickening of exine in a shape on high murrnocks, thus some paradermal sections would find round sections of processes of election dark perine material with election light middle part. Seems a somewhat similar bicolor sections of consolidated datrls in the perine of ferns Cheilanthes and Notholaena were illustrated by Tryon & Lugardon (1991).

Perine in Pterygoneurum and Ptychomitrium in some sections shows kind of holes, but in that case the perine elements are irregular in outlines, partly fused, i.e. having little in common with Encalypta longicollis case.

* * *

The rather regular structure of empty processes in spores of Encalypta longicollis and especially solid outer perine of about the same thickness indicate a possibly adaptive significance, so the plant maintains such a structure. Obviously, the gas filled structures reduce the spore density to facilitate its ability to spread. However, the profit for the air transportation seems to be too low for spores of such size. At the same time, the ability to float might be more interesting to consider. Horton (1983) mentioned that “noteworthy feature of the habitat of E. longicollis is the consistent association of populations with moist situations”. We also collected it near wet cliffs and similar places. The minor reduction of density for spores, which are usually sinkable (Skripnikov, pers. correspondence, based on observation of several species), may retain them floating on the surface and provide a sufficient advantage. The experiments on the efficiency of such “life vest”, however, require studies using living material, as spores from herbarium collections and even opened capsules are deformed.

ACKNOWLEDGMENTS

This work was accomplished with the use of scientific equipment of Electron Microscopy laboratory, Faculty of Biology at Lomonosov MSU (Shared Research Facility “Electron Microscopy in Life Science” and Unique scientific installment “3D Electron Microscopy and spectroscopy”). We thank RFBR for support of the present study, the projects 19-04-00976 and 18-04-00971.

LITERATURE CITED

BARBÉ, M., É.E. CHAVEL, N.J. FENTON, L. IMBEAU, M.J. MAZEROLLE, P. DRAPEAU & Y. BERGERON. 2016. Dispersion of bryophytes and ferns is facilitated by small mammals in the boreal forest. – Ecology 23(3–4): 67–76. DOI: 10.1080/11956866.2016.1259917.

BROWN, R.C., B.E. LEMMON, M. SHIMAMURA, J.C. VILLARREAL AGUILAR & K.S. RENZAGLIA. 2015. Spores of relictual bryophytes: diverse adaptations to life on land. – Review of Palaeobotany and Palynology 216: 1–17.

CARRIÓN, J.S., M. J. CANO & J. GUERRA. 1995. Spore morphology in the moss genus Pterygoneurum Jar. (Pottiaceae). – Nova Hedwigia 61: 481–496.

CHMIELIEWSKI, M.W. & S.M. EPPLEY. 2019. Forest passerines as a novel dispersal vector of viable bryophyte propagules. – Proceeding of the Royal Society B. Biological Sciences 286(1897): 20182253. doi: 10.1098/rspb.2018.2253.

ESTÉBANEZ, B., T. YAMAGUCHI & H. DEGUCHI. 2006. Ultrastructure of the spore in four Japanese species of Ptychomitrium Furrn. (Musci). – Grana 45(1): 61–70. DOI: 10.1007/s10713-006-5557-2.

FURNESS, S. B. & R. H. HALL. 1981. An explanation of the intermittent occurrence of Physcomitrium sphaericum (Hedw.) Brid. – Journal of Bryology 11: 733–742.

HEDENÄS, L. 2012. Morphological and anatomical features associated with epiphytism among the pleurocarpous mosses—one basis for further researchon adaptations and their evolution. – Journal of Bryology 34: 79–100.

HORTON, D.G. 1983. A revision of the Encalyptaceae (Musci) with particular reference to the North American taxa Part II. – Journal of the Hattori Botanical Laboratory 54: 351–532.

IGNATOV, M.S., V.E. FEDOSOV, A.V. FEDOROVA & E.A. IGNATOVA. 2016. On the systematic position of Discculum (Bryophyta). – Acta 25(2): 278–284.

IGNATOV, M.S. & E.A. IGNATOVA. 2001. On the zoocory of Schistostega pennata (Schistostegaceae, Musci). – Acta 10: 83–96.

IGNATOV M.S., U.N. SPIRINA, M.A. KOLESNIKOVA, D.A. ASHIKHMINA, E.A. IGNATOVA & S.V. POLEVOVA. 2018. Peristome development pattern in Encalypta poses a problem: what is the primary peristomial layer in mosses? – Acta 27(1): 1–17. DOI: 10.15298/acta.25.01.

JOHANSSON, V., N. LÖNNELL, S. SUNDBERG & K. HYLANDER. 2014. Release thresholds for moss spores: the importance of turbulence and sporophyte length. – Journal of Ecology 102: 721–729. doi: 10.1111/1365-2745.12245.

KOPONEN, A.K. 1990. Entomophily in the Splachnaceae. – Journal of the Linnean Society 104: 115–127.

KOZUB, D., V.KHMELIK, YU. SHAPOVAL, V. CHENTSOV, S. YATSENKO, B. LITOVCHENKO & V. STARYKH 2008. Heicon Focus Software. http://www.heliconsoft.com.

LEWIS L. R., R. ROZZI & B. GOFFINET. 2014 Direct long distance dispersal shapes a New World amphiptheris disjunction in the dispersal-limited dung moss Tetraplodon (Bryopsida: Splachnaceae). – Journal of Biogeography 41: 2385–2395. DOI:10.1111/jbi.12385.

MARINO, P. 2009. The ecology and fly dispersed dung mosses (family Splachnaceae): manipulating insect behaviour through odour and visual cues. – Symbiosis 47: 61–76.

McCUAIG, B., S.C. DUFOUR, R.A. RAGUSO, A.P. BHATT & P. MARINO. 2015. Structural changes in plasids of developing Splachnum ampullaceum sporophytes and relationship to odour production. – Plant Biology (Stuttgart) 17(2): 466–473.

RENZAGLIA, K.S., K.D. MCFARLAND & D.K. SMITH. 1997. Anatomy and ultrastructure of the sporophyte of Takakia ceratophylla (Bryophyta). – American Journal of Botany 84: 1337–1350.

TRYON, A.F. & B. LUGARDON. 1991. Spores of the pteridophyta: surface, wall structure, and diversity based on electron microscope studies. – New York, Springer Verlag. 648 pp.