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Spore morphology and ultrastructure of the tropical moss *Helicophyllum torquatum* (Hook.) Brid. (Helicophyllaceae) in relation to systematics and evolution

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Abstract – Spores of *Helicophyllum torquatum* (Hook.) Brid. (Helicophyllaceae, a monotypic family of tropical mosses) were studied by means of TEM, SEM, and LM. Different techniques of staining were employed to reveal the sporoderm structure. The spores are isomorphic, medium-sized (ranging from 26.40 to 41.80 µm), heteropolar, subcircular amb, plane-convex, catatremate, the surface consists of granules and gemmae. The systematic significance of spore morphology of this species are being considered here for the first time. Spore characteristics are congruent with the hypothesis of an affinity of the Helicophyllaceae with acrocarpous lineages.

Helicophyllaceae / histochemistry / palynology / sporoderm / spore wall / ultrastructure

INTRODUCTION

Helicophyllum torquatum (Hook.) Brid., the only species of Helicophyllaceae, a tropical American moss family, grows on tree trunks, tabular roots and rocks. The plants are prostrate and acrocarpous, have tomentose branches and dimorphic leaves, which are organized in two lateral and one ventral series. The lateral leaves are ligulate, obtuse; ventral ones are ovate-lanceolate, shorter than the lateral ones; percurrent costa; laminar cells are hexagonal to subquadrate, papillose; capsules are cylindrical, gymnostomous; and exothecial cells are rectangular (Yano, 1979, 1984; Crum, 1994).

Fleischer (1920), Dixon (1932) and Buck & Vitt (1986) considered the Helicophyllaceae to be of pleurocarpous origin, whereas De Luna (1995) and Goffinet, Bayer & Vitt (1998) argued for affinities with acrocarpous lineages. Here we examine controversy in the light of spore morphology.

The morphological characterization of moss spores is important in systematic and evolutionary studies (McClymont, 1955; McClymont & Larson, 1964; Mueller, 1974; Boros & Jarái-Komlódi, 1975; Sorsa, 1976; Olesen & Mogensen, 1978; Brown & Lemmon, 1980, 1981, 1988; Mogensen, 1983; Carrión, Guerra & Ros 1990; Estébanez, Alfayate & Ron, 1997; Luizi-Ponzo & Barth, 1998, 1999).

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Palynological studies of the Helicophyllaceae are few. Erdtman (1957) studied the size, shape and surface of the spores. Later, Erdtman (1965) presented information about the apertural region and sporoderm stratification, which was considered "obscure". The size and the general features of the spores were superficially described in some taxonomical treatments (Yano, 1979; Crum, 1994).

The aim of the present study is the palynotaxonomical characterization of *H. torquatum*, to elucidate its relationships and support its identification in palynological samples, especially fossils.

MATERIAL AND METHODS

We obtained spores from dried specimens. A great number of exsiccates from different Brazilian Herbaria was examined (Jardim Botânico do Rio de Janeiro – RB; Instituto Anchietano de Pesquisas – PACA; Maria Eneyda P. K. Fidalgo, from Instituto de Botânica de São Paulo – SP), but only some materials from Herbarium SP had well-developed sporophytes.

Specimens examined: RB 172805, RB 172806, RB 172808, RB 225143, RB 261664, RB 323751, PACA 79856, PACA 79857, PACA 79858, SP 90267, SP 90855, SP 135217, SP 135220, SP 146884, SP 147858, SP 172056, SP 172073, SP 172075, SP 172101, SP 172242, SP 172427, SP 172462, SP 172464, SP 172521, SP 172556, SP 182252, SP 189669.
Specimens selected: Brazil: Espírito Santo, Santa Tereza, 23/XI/1982, *O. Yano et al. 4904* (SP 172427); Goiás, Caldas Novas, 8/IX/1979, *D. M. Vital 8553* (SP 147858); Goiás, Paraíso do Norte, 27/II/1974, *D. M. Vital 3011* (SP 90855*).

Spores observed with LM were prepared by acetolysis (Erdtman, 1960) and the Wodehouse (1935) method, using glycerin jelly as a mounting medium. Both techniques were modified for bryophyte spores according to Luizi-Ponzo (2001); the major modifications included: 1. Wodehouse method: the spores of each capsule were observed separately to evaluate the presence of aborted spores and other possible abnormalities. Stains were applied (basic fuchsin, and methyl green combined with eosin yellow) to reveal sporoderm stratification, 2. Acetolysis method: the spores were hydrated in distilled water for 24 h, and transferred to acetic acid for 24 h, after that, the material was acetolysed, not exceeding a maximum temperature of 85°C.

The diameter, in polar view, was measured for 100 spores (the reference specimen – indicated by an * in the list of specimens examined and in Table 1), or 30 spores (comparison specimens), using LM. The measurements (Table 1) were obtained from acetolysed spores, contained in at least five slides, in a maximum period of seven days after the acetolysis (Salgado-Labouriau, 1973). Mean (M), standard deviation (S), standard error (S_M), range (X_{min}-X_{max}), confidence interval (CI) and variability (V) are presented. The mean value of the polar (P) and equatorial (E) axes, in an equatorial view, are also shown in Table 1.

The mean value of perine, exine, and intine thickness from non-acetolysed spores, as well as exine thickness from acetolysed ones, are also presented and were obtained from 10 measurements.

The description of the external surface was complemented with SEM observations. After acetolysis, the spores contained in the assay tube were washed with distilled water and stored in a bottle containing 70% ethyl alcohol. Afterwards, about three drops of the sediment in the bottle were placed in the MEV stub. The dried stub was then coated with a layer of gold (*ca* 20 nm) and then the spores were analyzed.

For TEM observations, the spores were fixed in 2.5% glutaraldehyde and rinsed four times in a buffer solution. Post-fixation was made in osmium tetroxide 1%; three hours later, the spores were dehydrated in an alcohol series, embedded in Spurr resin, and heated at 70°C for 48 hours. After that, ultrathin sections (65-70 nm) were cut, and the material was stained with uranyl acetate and lead citrate.

The palynological terms follow Barth & Melhem (1988) and Punt *et al.* (1994).

RESULTS

Spore characterization

The spores are isomorphic, medium-sized (Table 1), heteropolar, subcircular amb, plane-convex, catatremate, the surface consists of granules and gemmae.

In LM (Figs 1-5), the perine exhibits isolated granules and gemmae on the distal (Fig. 4) and equatorial faces (Figs 1, 2), fused in some parts of the proximal surface (Fig. 5). This is more evident under SEM observations (Figs 6-9), which reveal irregular perine plates and perforations (Fig. 9). Perine processes measure about 0.82 µm in the distal pole, and 0.78 µm in the proximal one.

Exine thin, psilate, measuring 0.58 µm in the distal pole, and 0.50 µm in the proximal one. After the acetolysis method, the exine in the distal pole measures about 0.55 µm.

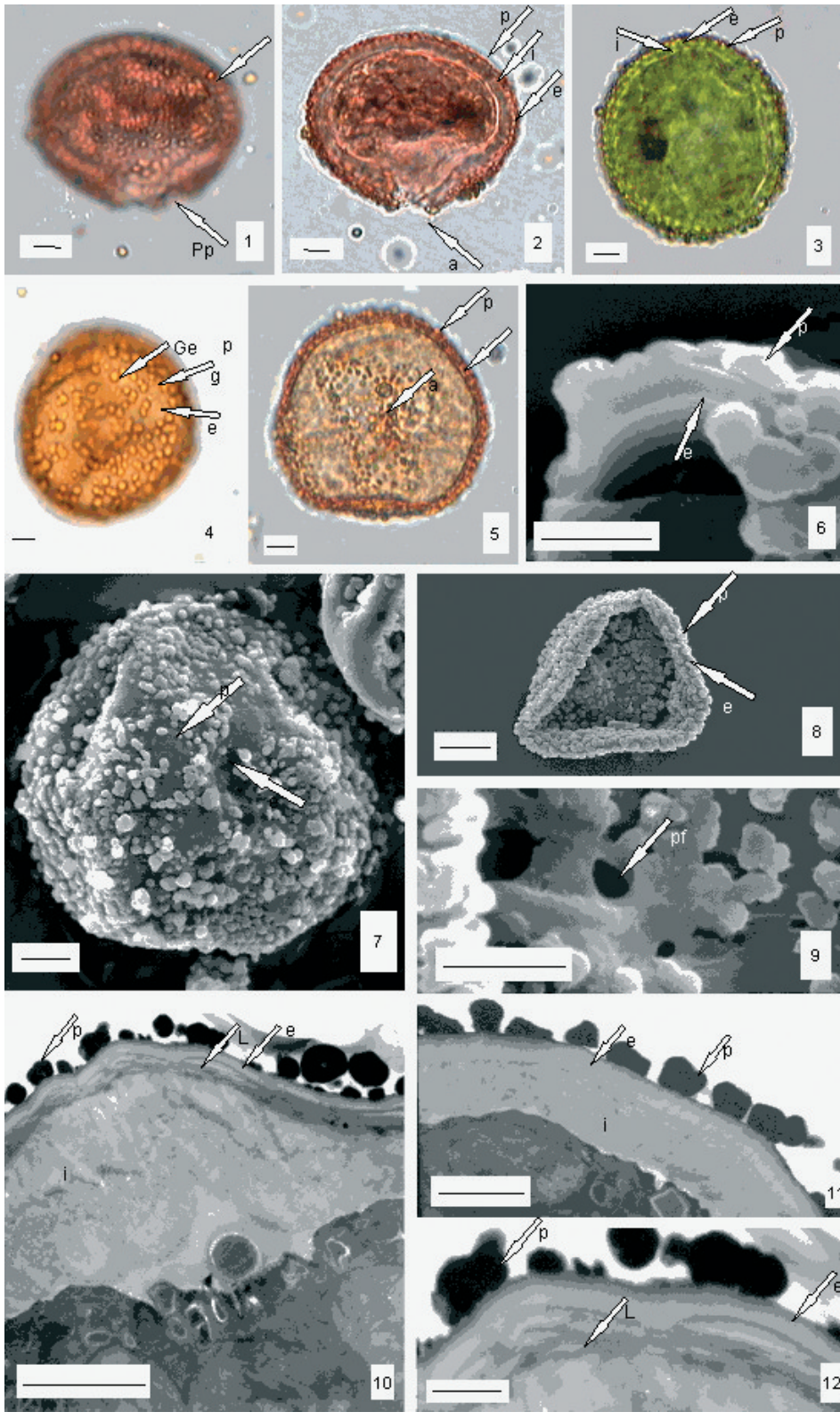
The intine varied from 2.24 µm in the distal pole to 2.82 µm in the proximal pole. It was stratified and its inner portion is thinner and brighter than the external one. Observations of immature spores revealed absence of the inner portion of the intine.

Under TEM (Figs 10-12), the exine was homogenous in the distal pole (Fig. 11), and stratified in the center of the proximal pole (Figs 10, 12), where we observed a lamellar structure. The intine was thick in the proximal pole, especially in the central region, providing the intine with a heterogeneous aspect (Fig. 10); it was thinner in the distal pole, and exhibited a discrete stratification (Fig. 11).

The morphological variations observed on the proximal surface, including perine organization, reduction of homogenous exine thickness, presence of an exine lamellar portion, and increased intine thickness, characterize the apertural region and the heteropolarity of the spores.

Table 1. Morphometric data of the diameter (D), in polar view, and polar (P) and equatorial (E) diameters, in equatorial view, of *Helicophyllum torquatum* spores (in µm).

Material	*P*	*E*	*D*				
			(X_{min}-X_{max})	M±s_M	S	95% CI	V (%)
D. M. Vital 3011*	28.60	37.40	(30.80-41.80)	35.40±0.25	2.46	34.91-35.89	6.95
O. Yano *et al.* 4904	25.64	35.20	(26.40-39.60)	32.34±0.60	3.28	31.12-33.56	10.14
D. M. Vital 8553	24.20	34.84	(26.40-39.60)	32.41±0.69	3.79	31.00-33.82	11.69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DISCUSSION AND CONCLUSIONS

Erdtman (1957, 1965) characterized spores of *H. torquatum* as medium-sized and having a doubtful apertural region. The exine was interpreted as being divided into nexine and sexine. Yano (1979) and Crum (1994), using different preparation techniques, also described the spores of *H. torquatum* as medium-sized. Here, we confirm a) the occurrence of an apertural region by LM and stains, and by TEM through observations of sporoderm features at the proximal pole, and b) the medium size of the spores (Table 1), based on the definition of Erdtman (1952) for this size class (from 25 to 50 µm).

Intine stratification was more evident in the proximal pole, where the intine was thicker. This stratification was confirmed by the texture variation under TEM. McClymont & Larson (1964) were the first to report a stratified intine in mosses, in *Archidium alternifolium* (Dicks. ex Hedw.) Schimp. (Archidiaceae Schimp.) spores. The same stratification was demonstrated by Mueller (1974) in *Fissidens limbatus* Sull. (Fissidentaceae Schimp.). More recently, Estébanez *et al*. (1997), studying species of *Grimmia* Hedw. (Grimmiaceae Arnott), showed its wide distribution among different taxonomic groups and the need to carefully evaluate intine stratification in a phylogenetic context.

Thickness of the compacted exine is less at the proximal pole when compared to the distal pole, but observations under TEM indicate that the thickness of the lamellar region is variable. The presence of the proximal lamellar region showed a relationship with the reduction of the exine thickness observed under LM, and its greater fragility in that region of the spore, indicated by the presence of broken areas, including in preparations using the Wodehouse (1935) method, as modified by Luizi-Ponzo (2001). These data can confirm the observations of Olesen & Mogensen (1978), which indicated the fading of the proximal lamellar region when the spores suffer volume alteration, and Mogensen (1983), who suggested that the increase intine thickness noted could increase the speed of the exine rupture from some hours to few minutes.

Besides exine fragility, morphological features, including increase in intine thickness, and structural alterations of perine elements, allow the proximal apertural area in the spores to be described. This observation corroborates findings identifying the presence of apertural regions, and not only scars, in moss spores (McClymont, 1955; Erdtman, 1957, 1965). They demonstrated structural alterations on the sporoderm surface. Moreover, McClymont & Larson (1964), Boros & Jarái-Komlódi (1975), Sorsa (1976), Olesen & Mogensen (1978) and Brown & Lemmon (1980, 1981) indicated the occurrence of a thin area of exine, concomitant with a thickening of the intine and external morphological alteration.

Figs 1-12. *Helicophyllum torquatum* (Hook.) Brid. **1-5. Spores observed by LM.** (1-3. Prepared by the Wodehouse method. 1-2. Stained with basic fuchsin. 4-5. Prepared after acetolisys) **1.** Equatorial view, surface, **2.** Optical section. **3.** Employing methyl green combined with eosin yellowish, distal view, optical section. **4.** Polar view, surface. **5.** Proximal view, optical section. **6-9. Spores observed by SEM. 6.** Sporoderm structure of a fractured spore. **7.** Distal view, granulated surface. **8.** Proximal view, granulated surface. **9.** Irregular perine plates and perforations. **10-12. Spores observed by TEM. 10.** Proximal view, sporoderm structure. **11**. Distal view, sporoderm structure. **12**. Proximal view, detail of the lamellar structure. (Scales: Figs 1-5, 7: 5 µm – Figs 6, 9-11: 2 µm – Fig. 8: 10 µm – Fig. 12: 1 µm. **a** = apertural region, **e** = exine, **Ge** = gemmae, **g** = granules, **i** = intine, **L** = lamellar structure, **p** = perine, **pf** = perforation, **Pp** = proximal pole. From *D. M. Vital 3011* (SP).

Mogensen (1983) summarized the types of apertural regions so far described for moss spores, indicating three different morphological patterns. Subsequent studies pointed to different morphological variations in the apertural regions of moss spores (Brown & Lemmon, 1988; Carrión *et al*., 1990; Estébanez *et al*., 1997; Luizi-Ponzo & Barth, 1998, 1999).

The apertural region of the spores of *H. torquatum* showed characteristics similar to those demonstrated by Olesen & Mogensen (1978) for the spores of *Ceratodon purpureus* (Hedw.) Brid. (Ditrichaceae Limpr.), mainly in the intine thickness and stratification, which was interpreted by the author as an indication of the periodicity of deposition of this sporoderm layer. This morphological pattern of the apertural region was presented by Mogensen (1983) as a variation of the *Funaria*-type, the most frequent in moss spores, in which the intine thickness increase is not accompanied by stratification.

In the past, the relationship between *H. torquatum* and pleurocarpous taxa was postulated by authors who argued its proximity with Racopilaceae Kindb. (Fleischer, 1920; Dixon, 1932). This placement was not accepted by Vitt (1984), but, later, Buck & Vitt (1986) joined both families, on the basis of dimorphic, deeply rolled leaves and prostrate habit, interpreting them as pleurocarpic. De Luna (1995) showed that *H. torquatum* is acrocarpous and suggested that its affinities were with Hedwigiaceae Schimp. This hypothesis was adopted in the recent classification of mosses by Buck & Goffinet (2000). Here, we confirm the acrocarpous condition of the species, according to the concept proposed by La Farge-England (1996), as indicated by De Luna (1995).

The palynological characteristics of *Racopilum* P. Beauv. (Racopilaceae), which has small and inaperturate spores (Erdtman, 1965), are very different from the *H. torquatum* spores described here. On the other hand, the results of Erdtman (1957, 1965) and Boros & Jarái-Komlódi (1975) demonstrated that some Hedwigiaceae species have heteropolar spores, with a proximal apertural region. These characteristics, also present in Helicophyllaceae spores, indicate an affinity with the acrocarpic lineage. However, in *H. torquatum*, while the spores are heteropolar, the surface is formed by granules and gemmae that form plates of irregular outline on the proximal face, where isolated perforations are observed, and there is external evidence of the apertural region. The results confirm the taxonomic relevance of spore morphology and indicate the importance of the inclusion of palynological data in future phylogenetic analysis.

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