ELECTRON AND LIGHT MICROSCOPICAL OBSERVATIONS ON THE PERINE OF THE SPORES OF SOME BOLBITIS SPECIES (FILICES)

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SUMMARY

The perine in the leptosporangiate fern genus *Bolbitis* as present in the mature sporangia either surrounds the individual spores or forms an intricate cellular structure by which the spores are interconnected. The perine appeared to consist in some species of 2 separate layers which are variously differentiated or not.

Differences in the perine do not seem to provide evidence for keeping the genera *Egenolfia* and *Bolbitis* separate, as was recently suggested by some Indian authors.

1. INTRODUCTION

When preparing a taxonomic revision of the leptosporangiate fern genus *Bolbitis*, it appeared that differences of opinion existed regarding the status of the genus *Egenolfia*. IWATSUKI (1959) had placed it in *Bolbitis*, whereas NAYAR & KAUR (1963, 1965) preferred to keep the two genera separate.

The Indian authors based their opinions on differences in the venation pattern and the structure of the perine. The venation pattern, however, showed so much variation in the species of *Bolbitis* proper that it did not seem justified to use it as a character for the discrimination of these genera. As during the taxonomic studies no other evidence had become available to support their view, a detailed investigation on the perine was undertaken.

As very little was known of the fine structure of the perine of the leptosporangiate ferns, such an investigation was also of general interest. For instance, BOWER (1923, *p. 259*) said that the plasmodium formed out of the tapetum is absorbed in the developing spores in all the more primitive ferns 'but in certain advanced types much of it may remain as a deposit on the outer wall of the spore, and is then called the "*perispore*".'. ERDTMAN (1969, *p. 39*) commented: 'It is still imperfectly known. It seems to develop after the exine proper, and forms a usually wrinkled, often more or less loosely attached covering of the mature spores'.

The present publication describes and compares the morphology of the perine as occurring in approximately mature sporangia of 4 species of *Bolbitis* (including 2 species referred to *Egenolfia* by NAYAR & KAUR). It is based upon observations with the light and the electron microscope.

2. MATERIALS AND METHODS

The species and the material investigated:

1. *Bolbitis appendiculata* (Willd.) K. Iwatsuki, s.l. – *Hennipman 3488*, N. Thailand, Mae La Noi, c. 97°55′ E 18°25′ N, 375 m alt.; *3527*, N.E. Thailand, Phu (Mt) Luang, c. 101°25′ E 17°25′ N, 750–950 m alt.
2. *B. contaminans* (Clarke) Ching ex Christensen. – *Hennipman 3536*, N.E. Thailand, Phu (Mt) Luang, c. 101°25′ E 17°25′ N, c. 950 m alt.
3. *B. sinensis* (Baker) K. Iwatsuki. – *Hennipman 3229*, N. Thailand, Doi (Mt) Chieng Dao, c. 98°55′ E 19°25′ N, c. 1425 m alt.
4. *B. repanda* (Blume) Schott. – *Hennipman 4023*, originally collected from W. Java by Prof. Dr. R. E. Holttum; cultivated at the Botanic Garden, Leiden from material in cultivation at the Royal Botanic Gardens, Kew, England.

The material mentioned under the first 3 species was collected during an expedition to Thailand (for details see HENNIPMAN & TOUW, 1966). Dried voucher specimens have been deposited at the Rijksherbarium.

The genus *Bolbitis* comprises acrostichoid ferns which produce separate sterile and fertile leaves. Pinnae of fertile leaves were taken from living material and preserved in FAPA (formalin 40%: 5; glacial acetic acid: 2.5; propionic acid: 2.5; alcohol 50%: 90) for months.

Sections used for study with the light microscope were obtained following routine histological procedures using the paraffin technique and were 5–15 μ thick. Staining was obtained using saffranin followed by fast green. Whole mounts of spores using glycerin-gelatin were studied with the light microscope from both acetolysed and non-acetolysed material. Acetolysis followed standard procedures but lasted never longer than one minute. For electron microscopy the sporangia were dehydrated through an alcohol series and embedded in Epon. Sections were stained with uranyl acetate and/or lead citrate and examined in a Philips EM300 electron microscope.

It should be remarked that the sectioning of the sporangia proved very difficult. Usually the spores became (slightly) disarranged; attempts to get entire sections of *B. sinensis* for study with the electron microscope failed.

3. OBSERVATIONS

As the application of the existing nomenclature to the structures found was rather difficult, provisional names are given. They are based on differences in texture of the membranes as seen from photographs made by the electron microscope. These differences appeared to be correlated with their position.

The term exine is applied to the relatively thick, smooth, acetolysis resistant layer that envelopes each spore. Its texture is homogeneous; the inner part often appears dark in photographs made of stained sections. The exine largely agrees with that described by LUGARDON (1965) for *Blechnum spicant*. The term outer perine is used for the acetolysis resistant layer of the released spore which has a fuzzy or granular texture. It appears as a single, undulate, closed layer which is rather rough on both surfaces, or as a much differentiated structure.

The term inner perine is used for the smooth membrane-like structure occurring just outside the exine. It may be very distinct and acetolysis resistant, or hardly perceivable and presumably not resistant to acetolysation, or even possibly absent.

1. *Bolbitis* (*Egenolfia*) *appendiculata*. – *Fig. 1B; 2C; 3A; 4F-G.* – From the study with the light microscope of cross-sections of sporangia it appeared that the outer perine is differentiated into an indistinte, closed, rather smooth shell closely enveloping the exine of each spore and a distinct cellular part that interconnects these shells. The cellular part has reticulate walls, the meshes are usually isodiametric or elongate, sometimes almost round, up to 20μ wide, the trabeculae are very narrow, up to 10 μ wide and 4–8 μ thick. When the spores are dispersed by mechanical action of the sporangium wall, the obviously very brittle, cellular part breaks irregularly. Therefore, the released spores have a crestate perine, the closed shell of the outer perine being hardly perceivable in whole mounts.

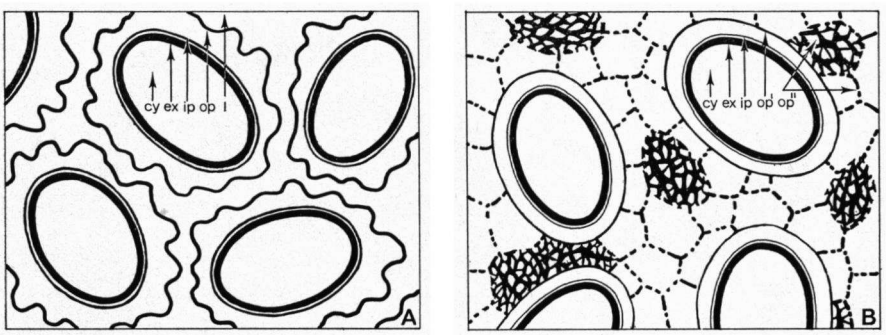

Fig. 1A–B. Schematical drawings of the spores and their perines as situated in mature sporangia. – A. *Bolbitis contaminans*. – B. *B.appendiculata* (cy: cytoplasm of the spore; ex: exine; ip: inner perine; op: outer perine; op': closed part of the outer perine; op": reticulate part of the outer perine; l: lumen of the sporangium).

Photographs made with the electron microscope always distinctly showed the existence of this rather thin shell of the outer perine, whose inner surface is much smoother than the outer one. Apart from this a rather indistinct membrane, i.e. the inner perine, situated just outside the exine, could be distinguished in some sections. No interconnections between the inner and outer perine were observed.

2. *Bolbitis contaminans*. – *Figs. 1A; 2A-B; 4A-E.* – With the light microscope it was found that the outer perines of individual spores were not connected in the mature sporangium, each spore being separately surrounded by a (strongly) undulate, rather smooth outer perine, situated at considerable distance from the exine. A thin inner perine which either closely adhered to the exine or occurred at some distance to it could be recognized in only a few places in some of the slides studied.

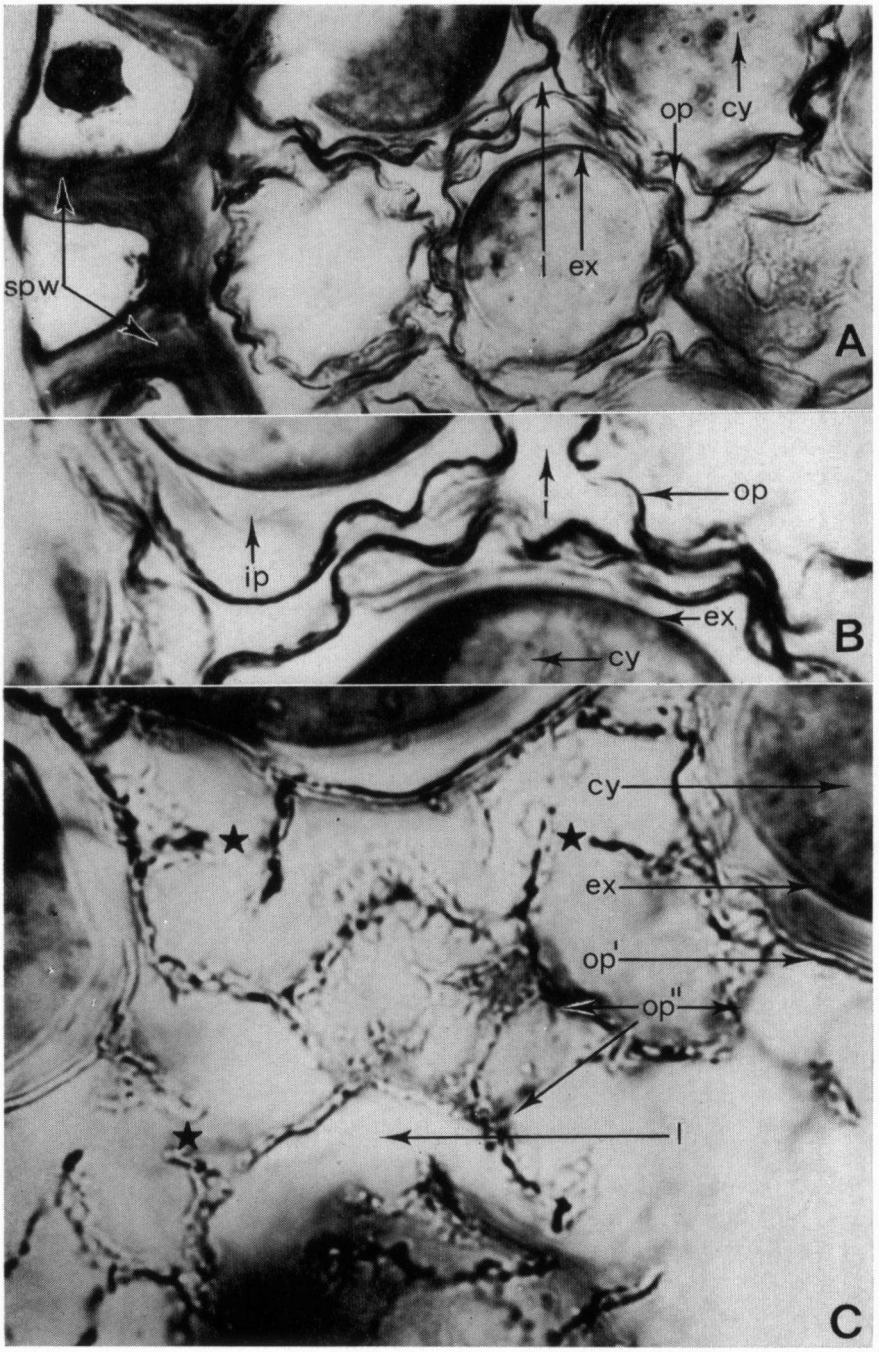

Fig. 2A–C. Photographs made with the light microscope of cross-sections through sporangia with approximately mature spores. – A–B. *Bolbitis contaminans*: A: configuration of the spores within the sporangium; B: detail of the outer perine surrounding some adjacent spores (A: c. 350 ×; B: c. 800 ×). – C. *B. appendiculata*, detail of the reticulate part of the outer perine which connects individual spores (c. 2,000 ×) (★: places where the reticulate part of the outer perine has been ruptured during sectioning; for further explanation see fig.1).

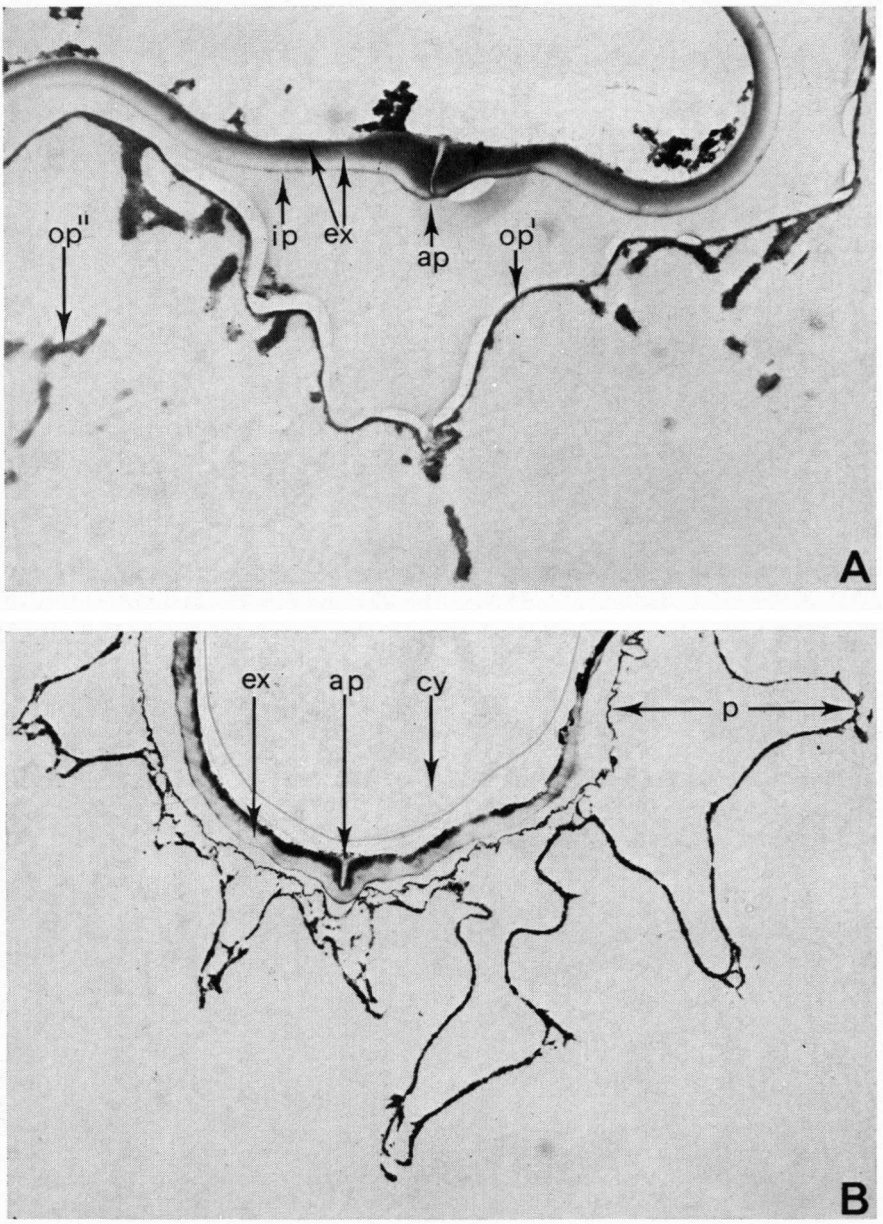

Fig. 3A–B. Photographs made with the electron microscope of cross-sections through spores. – A. *Bolbitis appendiculata* (c. 7,800 ×). – B. *B. sinensis*, the cytoplasm retouched (c. 10,000 ×) (ap: aperture; p: perine; for further explanation see fig.1).

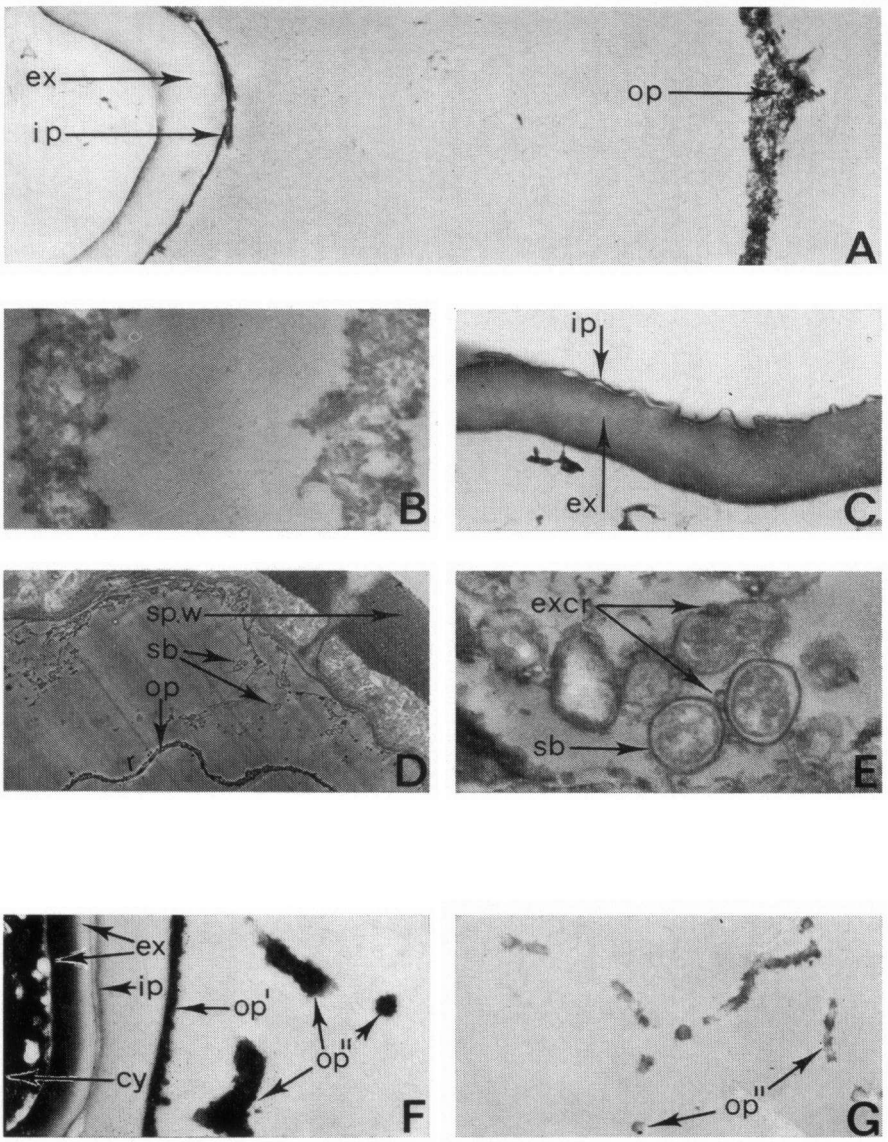

Fig. 4A–G. Photographs made with the electron microscope of cross-sections through sporangia. – A–E. *Bolbitis contaminans*: A: detail of the acetolysis resistant layers surrounding the spore's cytoplasm (c. 10,000 ×); B. detail of the outer perine of two adjacent spores (c. 25,000 ×); C: detail of exine and inner perine (c. 15,000 ×); D: spherical bodies scattered near the sporangium wall (c. 1,500 ×); E: detail of the spherical bodies with their excrescences (c. 10,000 ×). – F–G. *B. appendiculata*: F: detail of layers surrounding the spore's cytoplasm (c. 12,500 ×); G: detail of the reticulate part of the outer perine which connects the spores (c. 6,000 ×) (excr.: excrescence of the spherical bodies; sb: spherical bodies; for further explanation see fig.1).

Under the electron microscope the perine proved to be distinctly double-layered. The undulate outer perine was closed with a rather rough surface, of a texture comparable to that found in *B. appendiculata.* The inner perine which is relatively distinct always closely adhered to the exine in the cross-sections studied. No interconnections were found between the inner and the outer perine. Apart from these two layers, almost spherical, acetolysis resistant bodies could be demonstrated in the sporangium. They showed a distinct outer membrane, sometimes with a few, almost round excrescences surrounding a far less distinct inner part, and might be relevant structures regarding the formation of the acetolysis resistant materials. They were almost always found near a possible residual wall of the tapetum lying close to the sporangium wall.

3. *Bolbitis (Egenolfia) sinensis.* – *Fig. 3B.* – Under the light microscope the layers which are presumed to represent the outer perine seemed to consist of an inner shell that more or less follows the shape of the exine and a densely folded, equally distinct, outer part the proximal sinuses of which fuse with the inner shell. The folds of the individual perines are for the greater part connected by low crestae occurring on the distal sinuses. Several scattered, minute spines are present over the entire outer surface of the perine.

Attempts to obtain sections of sporangia for study with the electron microscope remained unsuccessful. Therefore, it was not possible to confirm the observation with the light microscope that the low distal crestae on the folds of the outer perine of individual spores are (partly) continuous. From sections of not completely mature spores it appeared that the perine is indeed divided into an inner and an outer layer. These are not fused by the proximal sinuses of the undulate part but are connected by a multitude of septae which are especially abundant near the distal sinuses and where the two layers are nearest to each other. An inner perine could not be demonstrated but might well be present.

4. *Bolbitis repanda.* – The data obtained from the study of sections with the light microscope were in accordance with those obtained for *B. sinensis.* Therefore, no material was studied with the electron microscope.

4. DISCUSSION

4.1. The value of the perine for a proper delimitation of the genus Bolbitis.

The direct cause for the undertaking of this study was to verify in our material NAYAR & KAUR's (1965) statement that, i.a., the perine can be used as a character for discriminating between the fern genera *Bolbitis* and *Egenolfia.* According to these authors (1963, *p. 94*) the perine 'is characteristically reticulate in *Egenolfia,* and variously folded and mostly psilate in *Bolbitis*'. In a later publication (1965, *p. 143*) the perine was described more in detail, that of *Egenolfia* as having 'a reticulately ornamented surface with the corners of the reticulations often prominently thickened: the pattern is very faint in *E. bipinnatifida*' (a taxonomic synonym of *Bolbitis sinensis* (Baker) K. Iwatsuki), and that of *Bolbitis* as being not reticulate but closed.

Observations on cross-sections of spores with the light microscope had already indicated the possibility that these statements might not be correct. It could be shown that the perine in *B.* (*Egenolfia*) *appendiculata* is not reticulate throughout but differentiated into a closed shell next to the exine and a reticulate cellular part that interconnects these shells. Under the electron microscope both layers appeared to have the same texture. The innermost surface of the shell, however, is smoother than the other surfaces of the outer perine.

Prominently thickened corners in the reticulate part of the perine, as reported and drawn by NAYAR & KAUR (1963, *t. 28 fig. 14*) for *Egenolfia asplenifolia* (a taxonomic synonym of *B. appendiculata*), were never found.

Besides, it appeared that in other species ascribed to *Egenolfia* by them a reticulate perine was found to be completely absent. The perine of *B.* (*Egenolfia*) *sinensis* appeared under the light microscope comparable to that described for *B. repanda*. A photograph published by them (1963, *t. 27 photo 8*) and ascribed to *B. sinensis* belongs presumably to a specimen of *B. appendiculata* s.l. (!). The very faint reticulated pattern reported for the perine of *Egenolfia bipinnatifida* might be the wrong interpretation of the light-microscopical image of a perine with many small spines on its exterior surface.

The differences of the perine do not seem to provide a reliable argument for separating *Egenolfia* from *Bolbitis* as delineated by IWATSUKI (1959).

4.2. General considerations.

In the course of studies of spores present in more or less mature sporangia, with the use of several techniques, some unexpected properties of the perine were discovered.

Firstly, the spatial distribution of the mature perine. It was found that what is here provisionally called 'outer perine' could present itself either as an undulate, closed, rather rough envelope around each individual spore (*B. contaminans*), or as a much more differentiated structure, consisting in *B.* (*Egenolfia*) *appendiculata* of an indistinct ellipsoidal shell surrounding the spore and a cellular part by which the shells surrounding the individual spores are interconnected. The delicate, reticulate cellular part breaks irregularly when the spores are dispersed.

Secondly, the perine is not a simple structure. It was found to be distinctly double-layered in *B. contaminans* and *B. appendiculata* and to consist of an outer and an inner perine which appeared to have a different texture as seen from photographs made with the electron microscope: the inner perine being membrane-like and the outer perine much more irregular in texture and morphologically variously differentiated.

The variation in the morphology of the perine, as shown above, is perhaps not unique. For instance, BRAGGIO (1966) gave a well-illustrated description of the morphology of the spores of davallioid ferns. Unfortunately he did not comment upon his findings. From his excellent drawings of, e.g., the spores of *Oleandra*, it appears that the perine surrounding the spiny exine is either a shallowly undulate, closed sphere (*Oleandra costaricensis* Maxon, op. cit., *fig. 7*)

or crestate with incidental (*Oleandra madagascarica* Bonap., op. cit., *fig. 3*) to many (*Oleandra welwitschii* (Baker) Pichi-Serm., op cit., *fig. 4*) perforations. I suppose that the perine (partly) interconnects the individual spores in the mature sporangia of species of which the dispersed spores show a crestate perine.

Crestate or spiny as well as undulate perines are found in species of one genus (i.a. *Dryopteris*). Even a distinct perine might not be formed at all, as was shown by HANNIG (1911) for some *Athyrium* taxa and by HARRIS (1955) for some *Blechnum* species. The latter's studies showed that 'pending a thorough investigation, there are cases where the ordinary techniques do not enable one to decide with certainty "whether a perinous coating is present or not".' (HARRIS 1955, *p. 17*).

A comparable kind of variation in the morphology of the perine is also present in some fossils. Mr. J. Muller drew my attention to a paper by POTONIÉ (1965) which amongst others deals with the spores of the *Sphenophyllales* (*Sphenopsida*). One of his arguments for proving that what he calls perine does not belong to the exine, applies to the orientation of the perine in the sporangia. He found that 'Die als Perispor aufgefassten Bildungen sind bei den noch im Sporangium lagernden Sporen manchmal miteinander verwachsen oder ineinander verschränkt.' (POTONIÉ 1965, *p. 30*). He also commented upon the morphological differences of the perine (*p. 29*) 'Einmal hat es einen regelmässigen polygonalen Querschnitt und trägt ein sehr tief- und weitlumiges Reticulum (*Sphenophyllum dawsoni*). Ein andermal ist es fast so unregelmässig geformt wie zerknittertes Papier und zeigt kegelförmige, unregelmässige Ausbeulungen verschiedener Grösse und Richtung (*Bowmanites trisporangiatus*)'. Both species represent *Sphenophyllaceae* with trilete spores.

I would emphasize that the species of *Bolbitis* studied are taxonomically closely related. Therefore, the marked differences in the perine surrounding the monolete spores are supposed to be caused by slight genetical differences which strongly affect or determine the interactions of haploid and diploid materials during spore maturation.

A detailed investigation on the genesis of the wall(s) surrounding the spore's cytoplasm is necessary to obtain a correct nomenclature of the layers. For instance, what has been called exine ('Exospor') in *Polypodium aureum* by Hannig, a species that lacks a perine, might well be very different from the exine described above for *Bolbitis*. Hannig, who treated the spores with different chemicals, found that the exine consisted of three layers. From other literature and from my own observations on spores it is supposed that a study of the sporogenesis, using cytochemical and electron microscopical techniques, may well be a great help for the correct interpretation of the light microscopical image and may further provide new data which will permit a better delimitation of the larger taxa of the Pteridophytes in particular.

ACKNOWLEDGEMENTS

The author feels much obliged to Mr. J. Muller, palynologist at the Rijksherbarium (Director: Prof. Dr. C. G. G. J. van Steenis) for stimulating and fruitful discussions and for critically reading the MS; to Drs. W. J. de Priester, Department of Cell Biology, Zoological Laboratory, Leiden, who kindly supplied the information on the spores as studied with the electron microscope. Valuable technical assistance was received from Mr. G. de Leeuw and Miss I. Heres.

The larger part of the material used for this paper was collected during a trip to tropical Asia made possible through grants from the foundations 'Treub Stichting' and 'Treub Mij.'. One of the species studied was carefully grown by Mr. A. Mulder thanks to facilities supplied by the authorities of the Hortus Botanicus, Leiden (Director: Prof. Dr. W. K. H. Karstens).

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