SPERMATOGENESIS IN *SELAGINELLA TAMARISCINA* (BEAUV.) SPRING

II. Opening of Antheridium, Maturation and Liberation of Spermatozoids[1]

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Abstract: The metamorphosis of spermatozoid and the formation of operculum in microgametophyte of *Selaginella tamariscina* (Beauv.) Spring are studied under EM. The distinct events during the metamorphosis are: (1) the elongation of the apical mitochondrion, (2) the separation of basal bodies and emergence of flagella, (3) the elongation of nucleus and (4) discard of excess cytoplasm.

The formation and opening process of operculum described are the significant finding of microgametophyte in heterosporous lycopods. Operculum is derived from several jacket cells. After the operculum takes off, the spermatozoids spring out in a clump. Occasionally the jacket cells give rise to the spermatids, too.

A mature spermatozoid has a twisted long nucleus, an apical mitochondrion with or without a posterior mitochondrion, a posterior plastid containing several large starch grains and two flagella. The anterior flagellum exserts from the apex of the apical mitochondrion, while the posterior one exserts from the juncture of the apical mitohcondrion and the nucleus.

INTRODUCTION

As mentioned in the previous report, most of the studies on the microgametogenesis of *Selaginella* have restricted to the light microscopic level except the Robert's (1973, 1974, 1977) (Chang and Chiang, 1991). It still remains some interrogatories because of the minute size of microgametophyte. The last stage of differentiation, metamorphism of spermatozoid, seems probably the most difficult event to deal with, since the kinetic mechanism would involve.

The spermatozoid of *Selaginella* bears two long flagella as that of *Lycopodium* and bryophytes (Pfeffer, 1871; Robert, 1974; Yuasa, 1933). Some of the spermatozoids of the bryophytes have been studied in detail, i. e., *Blasia* (Carothers, 1973), *Funaria* (Carothers and Brown, 1985), *Marchantia* (Carothers and Kreitner, 1967, 1968; Moser and Kreitner, 1970), *Polytrichum* (Paolillo, Kreitner and Reighard, 1968a, b), *Thuidium* (Rushing and Carothers, 1986). The principal constituents of the functionally mature spermatozoid of bryophytes include the spline, two basal bodies and their associated flagella, an apical mitochondrion, the nucleus, and a

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posterior plastid (Carothers, 1973). The only description of the ultrastructural survey on this genus in *Selaginella kraussiana* has revealed that the spermatozoid of this species consists of two flagella, a very long anterior mitochondrion, the nucleus, a posteriorly located plastid, and a posterior mitochondrion (Robert, 1974).

Our knowledge of ultrastructure of the biflagellate spermatozoids produced by the bryophytes, *Lycopodium* and *Selaginella* are somewhat restricted to the multilayered structure (MLS) and centrosomes (Carothers, 1973; Carothers and Duckett, 1980; Carothers and Kreitner, 1967, 1968; Moser and Kreitner, 1970; Paolillo, Kreitner and Reighard, 1968a, b; Robbins and Carothers, 1975, 1978; Robert, 1973, 1974). Robert (1977) has centered his study on nuclear metamorphism of *S. kraussiana*. Some work has been done for the multiflagellate spermatozoids of *Equisetum* (Duckett, 1973; Duckett and Bell, 1969). Most of these have emphasized on the development of MLS and nuclear metamorphism. The same are the studies on the multiflagellate spermatozoid of *Marsilea* (Myles and Bell, 1975; Myles and Hepler, 1982) and *Zamia* (Norstag, 1968, 1974).

Our previous report on the spermatogenesis of *S. tamariscina* shows the development of a mature microspore to a male gametophyte containing many spermatids (Chang and Chiang, 1991). In the present investigation, the succeeding process from spermatid to mature spermatozoid has been revealed. The formation and opening of the operculum is a significant finding to date of heterosporous lycopods. Another difference between the results of our research and those from past studies on microgametogenesis of *Selaginella* is that we represent numerous micrographs instead of merely the drawings of related structures.

MATERIALS AND METHODS

The methods for TEM and SEM studies of the endosporic gametophytes were that described in the previous paper (Chang and Chiang, 1991).

For negative staining, microspores were sterilized in 3% clorox and incubated on 1% plain agar at a 12 h/12 h light/dark cycle, 30±5°C light/25±5°C dark, no humidity control. By frequent observation with a stereo-microscope, the actively swimming mature spermatozoids were collected, stained with 2% phosphotungstic acid (PTA), pH 7.0–7.2 containing 0.5% albumin bovine serum (BSA), viewed and microphotographed on a Hitachi H-600 TEM.

RESULTS

Upon the formation of spermatozoid, the protoplasts of the individual spermatozoid are still interconnected with one another by the "cytoplasmic bridges". In the earlier stage, the "cytoplasmic bridges" are wide (Pl. I-1), as the spermatogenesis advances, the "bridges" become thinner (Pl. II-3, 4). Eventually the spermatozoids will be completely separated and surrounded by a thick electron transparent envelope (Pl. I-2, 3; III-4).

Spermatozoid exhibits rich in cellular contents, especially the mitochondria. Oil drops are also present but they are much fewer than those in the primary spermatogenous cells. In addition to the various organelles, spermatozoids are characterized by possessing the peripherally seated multilayered structure (MLS)

and a pair of basal bodies, formerly the centrioles (Pl. I-1, 3, 5). One of the mitochondria located between MLS and nucleus as well as the MLS become elongated gradually (Pl. I-2-6). Their elongation initiates the metamorphosis of spermatozoid. This peculiar mitochondrion is termed as apical mitochondrion. The longest apical mitochondrion measured in the present observation is of 4 μm (Pl. V-2). Following the onset of metamorphosis, several evident cytological changes occur subsequently, including the growth of flagella from the basal bodies, the spread and jutting out of flagella, the elongation of nucleus and discard of excess cytoplasm.

As soon as the apical mitochondrion starts to elongate, the flagella emerge from the basal bodies. One flagellum protrudues from one basal body (Pl. II). The transition zone where basal body and flagellum meet, exhibits as stellate pattern (Pl. I-4, 6). During the progressive growth of flagella, two basal bodies have moved farther from each other gradually. Finally there is a distance between two basal bodies (Pl. II-1, 3). The one near the nucleus will bear the posterior flagellum and the farther one anterior flagellum. The anterior basal body has a length of about 150 nm and stellate pattern 130 nm (Pl. I-6, II-1). The developing flagella retain coiled inside the cytoplasm for some time (Pl. I-2, 3). The separatin of two basal bodies seems probably being caused by the elongation process of apical mitochondrion. The entire length of the flagellum of a mature spermtozoid is of about 27 μm. The elongation of apical mitochondrion also leads to the flagella protrusion.

The MLS appears to be a curved sewing needle lying between two basal bodies and the apical mitochondrion. The eye containing portion is composed of four layers and represents the anterior end of this organelle as well as the cell. The longest and uppermost layer, S_1 layer is composed of an array of microtubules. The rest three layers are closely arranged with S_1 layer, but exhibit much shorter than it. In the beginning of metamorphosis, S_1 layer consists of eighteen microtubules (Pl. I-4). With continued growth, it elongates extensively and increases in the number to twenty-eight microtubules which extends to encircle the protoplast of whole well-developed spermatozoid (Pl. IV-4, 5; X-5).

Nuclear metamorphism starts simultaneously with the mitochondrial elongation. The round to ovoid nucleus elongates, finally become bending along the periphery of the spermatozoid (Pl. III-3, 5; IV-1, 4-7). The distribution of nuclear contents also alters as it continues to elongate. After a period of fibrous condition, the nuclear contents become increasingly condensed and twisted. There is a stage that those nuclear fibers look like a net, with areas of dense fibrous patterns alternate with areas of right zones (Pl. IV-2, 3). The areas of each light zone are almost identical in size and shape (Pl. IV-3). As a consequence, the netlike figure disappears, the mature spermatozoid has a long bended nucleus which is composed of uniformly distributed, parallely twisted fibrous material (Pl. IV-4; V-1). The nature of fibrous material has not been detected in the present study.

Now the flagella, MLS, elongated nucleus together with the apical mitochondrion constitute the main body of spermatid, and are located on the periphery. Rest of the cytoplasm occupies the central portion of spermatozoid (Pl. III-5; IV). Most of this cytoplasm will be cast off after the release of spermatozoids. Before the casting off of the whole cytoplasm, the protoplast of spermatozoid undergoes the profound changes. Among which are the vesiculation of some cytoplasm (Pl.

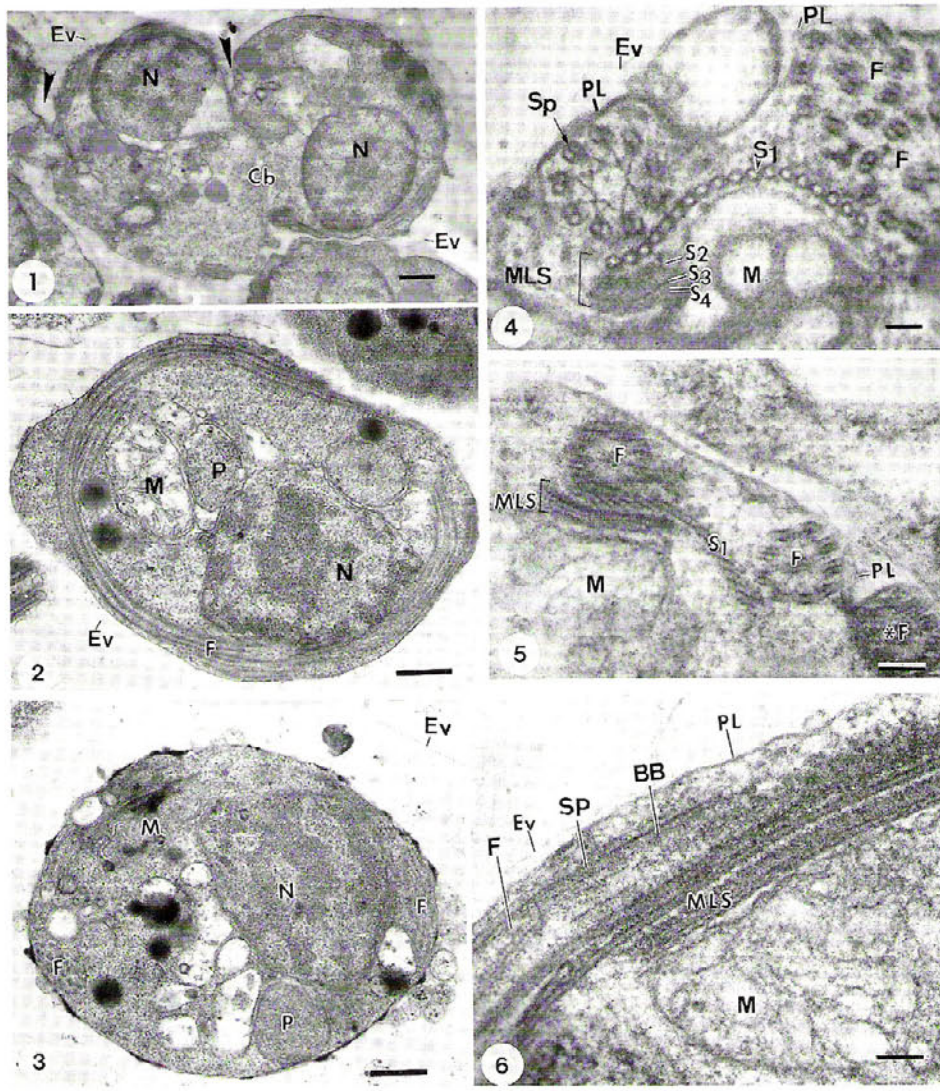

Plate I.

1: Interconnected young spermatozoids (arrowhead: cytoplasmic bridge, Cb: cytoplasmic bridge). Ev: envelope, N: nucleus.

2: Young spermatid, flagella are still embedded in cytoplasm, F: flagellum, M: mitochondrion, P: plastid.

3: The same stage as above, with flagella in pseudotransverse view.

4: A part of young spermatozoid showing the stellate pattern (Sp) of anterior flagellum in transverse view. S_1 layer of MLS has 18 microtubules. Ev: envelope, PL: plsmolemma, MLS: multilayered structure, Sp: stellate pattern, S_1: the first layer of MLS.

5: Cross section through the basal part of two flagella at the stage immediately after the jutting out of flagella. *F: the jutting out flagellum.

6: Longisection through the base of the anterior basal body, its stellate pattern, the anterior flagellum and the MLS. BB: basal body, Sp: stellate pattern.

1: bar=500 nm; 2: bar=500 nm; 3: bar=400 nm; 4: bar=50 nm; 5: bar=100 nm; 6: bar=100 nm.

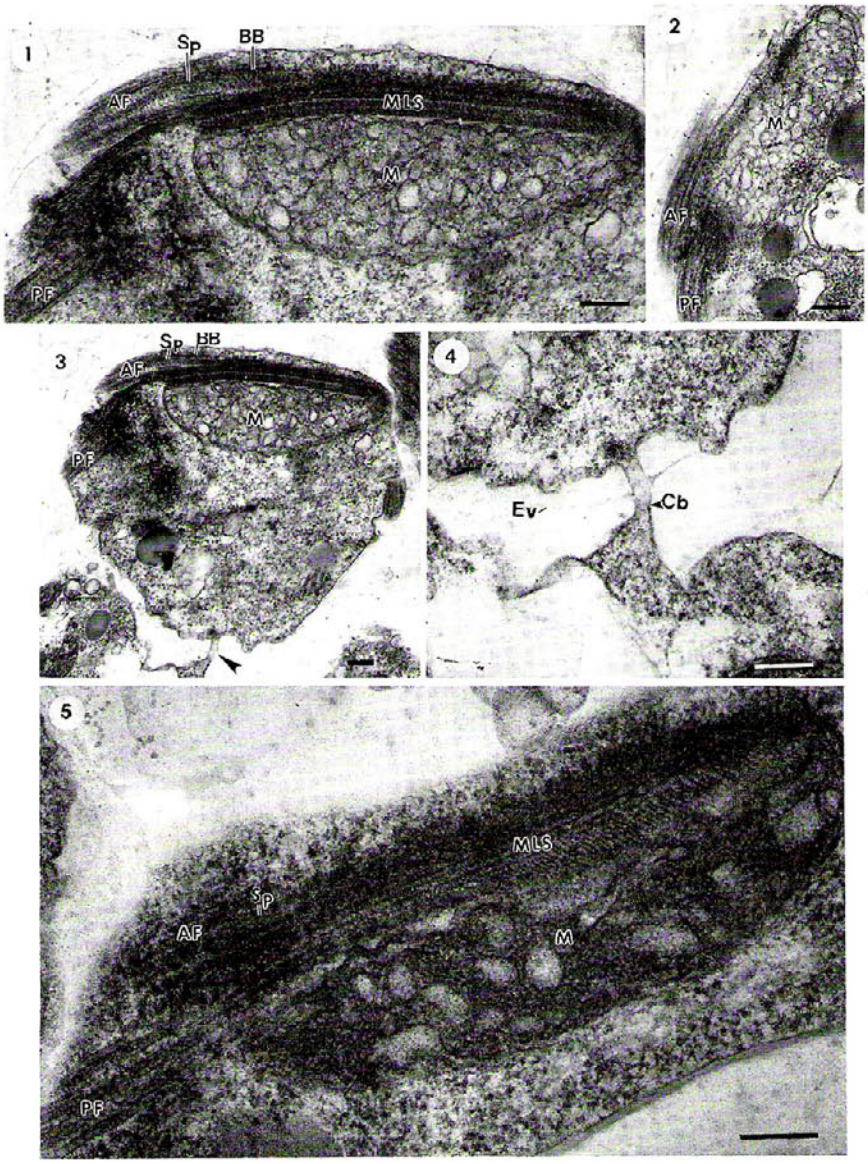

Plate II.

1: Enlarged from 3, showing the slightly elongated apical mitochondrion, note the positions of anterior and posterior flagella. AF: anterior flagellum, M: mitochondrion, PF: posterior flagellum, Sp: stellate pattern.

2: Spermatozoid with elongating apical mitochondrion, note the posterior flagellum.

3: Section through an individual spermatozoid with narrow cytoplasmic bridge (arrowhead) remained.

4: Enlarged from 3, emphasizing the narrow cytoplasmic bridge (Cb), Ev: envelope.

5: Enlarged view of an apical mitochondrion, note tangential view of the MLS and two flagella.

1: bar=200 nm; 2: bar=250 nm; 3: bar=200 nm; 4: bar=100 nm; 5: bar=200 nm.

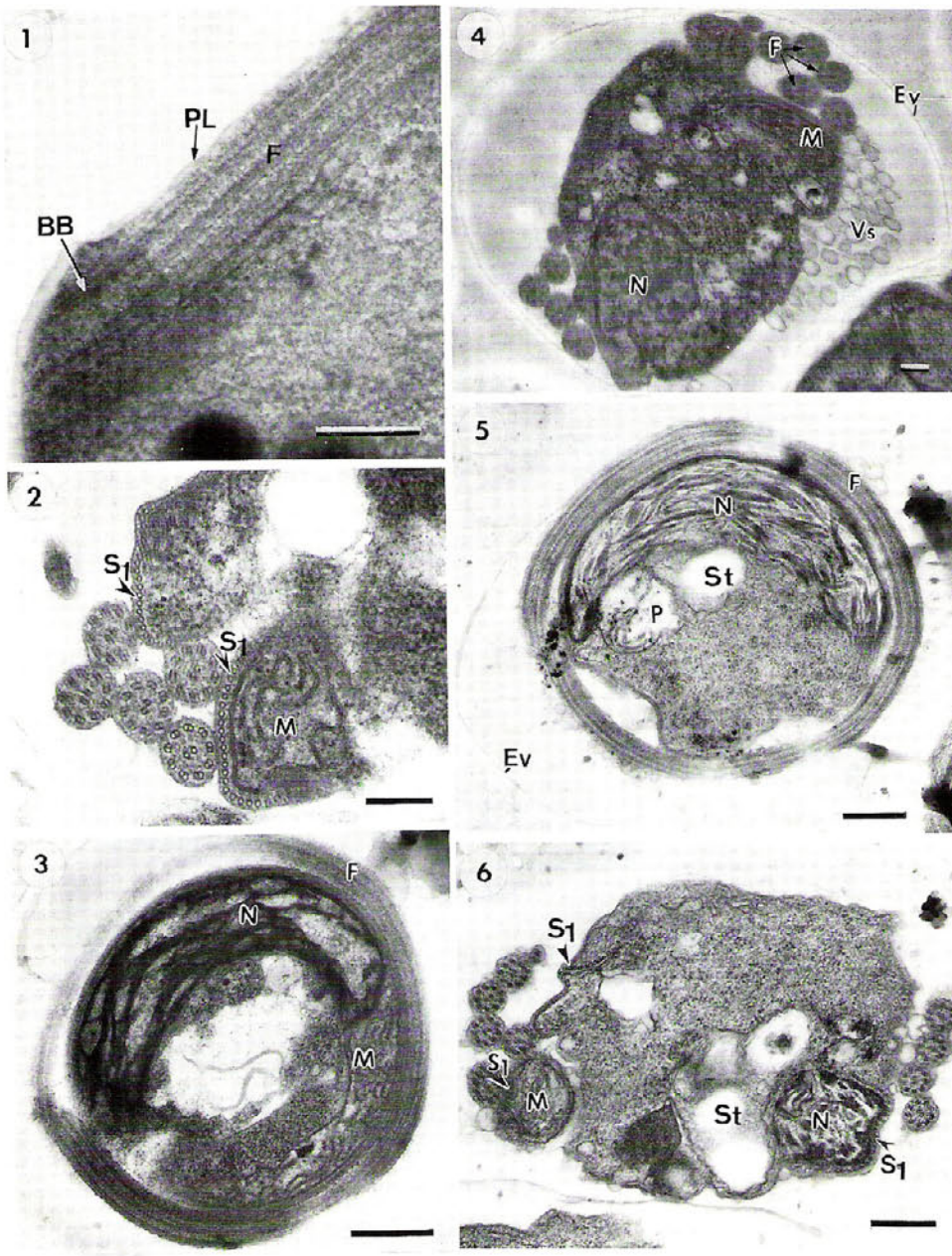

Plate III.

1: Longisection showing the connection of basal body (BB) and flagellum (F). PL: plasmolemma.

2: Two parts of a spermatozoid showing planes in cross section of S_1 layer, note its peripheral position. M: mitochondrion, S_1: the uppermost layer of MLS.

3, 4: Longi- and cross sections of the coarse-fibrous stage, note the extremely long apical mitochondrion (M) in 3. Ev: envelope, F: flagellum, M: mitochondrion, N: nucleus, Vs: vesicles.

5, 6: Longi- and cross sections of the fine-fibrous stage. P: plastid, St: starch grain.

1: bar=200 nm; 2: bar=200 nm; 3-5: bar=500 nm; 6: bar=400 nm.

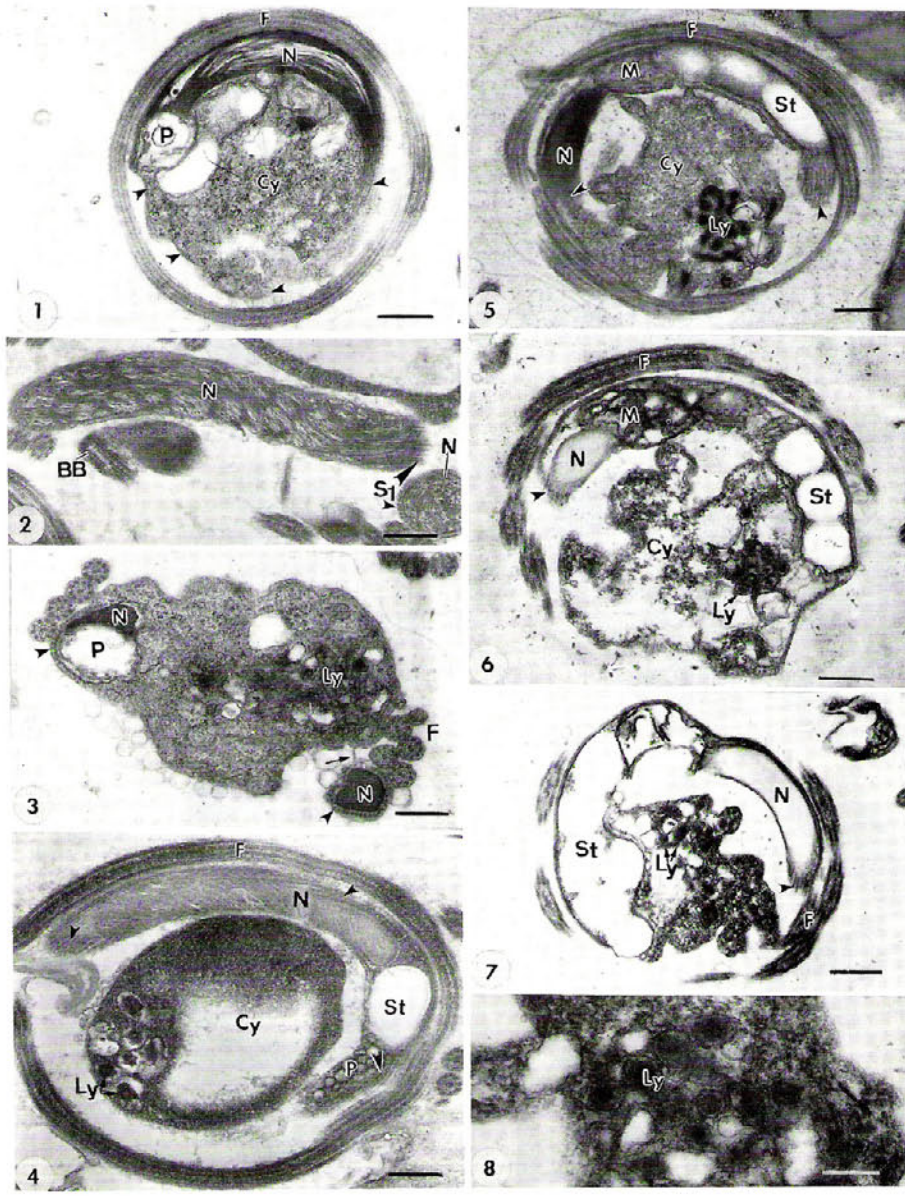

Plate IV.

1-4: Showing the series of sequential changes of nucleus (Fig. 3: cross section, others: longisection), nuclear fibers become thinner, arrow in 3 indicates the frail linkage of cytoplasm and nucleus, arrowheads indicate S_1 layer. BB: basal body, Cy: cytoplasm, F: flagellum, Ly: lysosome, N: nucleus, P: plastid, St: starch grain.

5-7: Showing the sequential changes of cytoplasmic inclusions; note the elimination of cytoplasm as a whole; disintegrating organelles; gathering lysomes (Ly); conspicuous starch (St) in Figs. 5-7.

8: Lysomes (Ly) bounded in their own membranes.

2-4: bar=400 nm; 5: bar=450 nm; 1, 7: bar=500 nm; 6: bar=440 nm; 8: bar=200 nm.

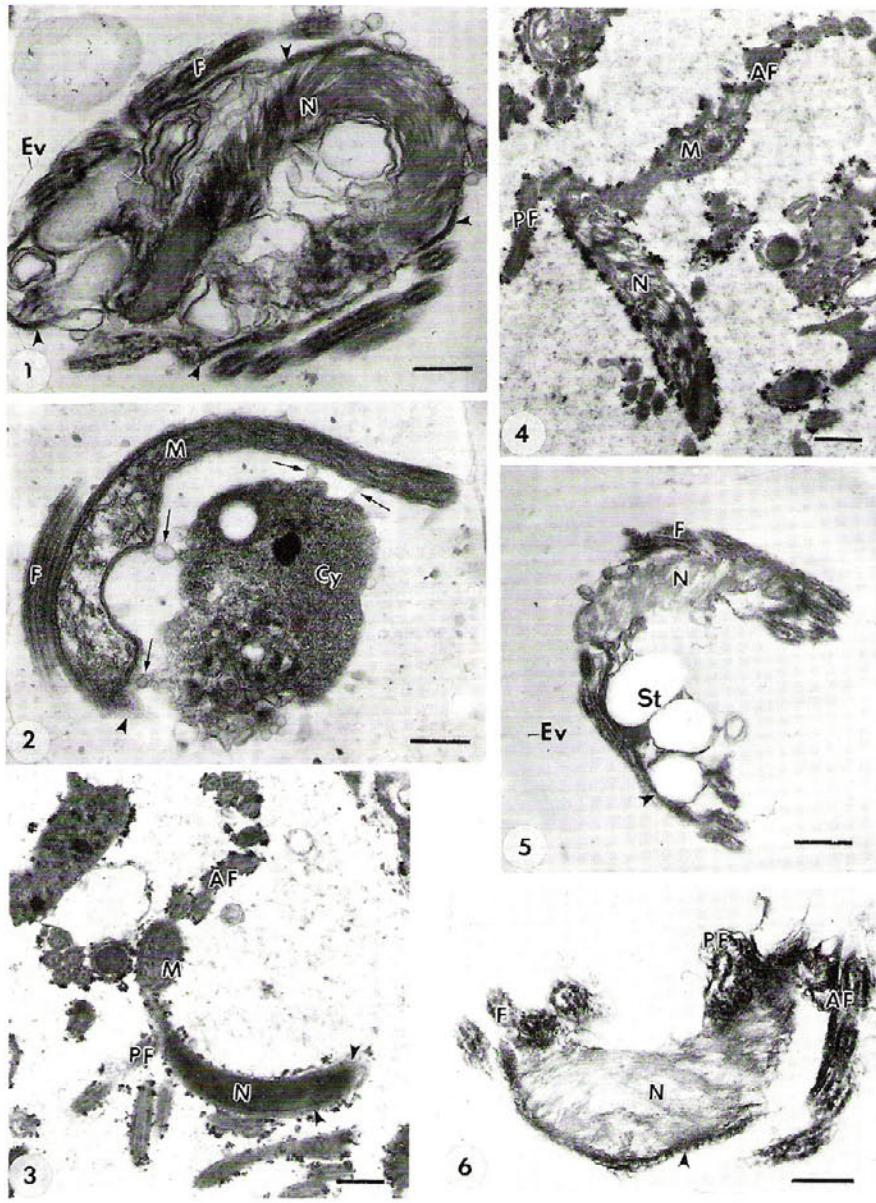

Plate V.

1: Spermatodzoid with twisting nucleus (N). Ev: envelope, F: flagellum. All the arrowheads in this plate indicate S_1 layer.

2: Spermatozoid with apical mitochondrion (M), also showing the frail connection (arrows) of cytoplasm (Cy) and apical mitochondrion.

3, 4: Two longisectional views of the anterior portion of spermatozoid.

5: The posterior portion of a spermatozoid.

6: Another view of the anterior portion. AF: anterior flagellum, M: mitochondrion, N: nucleus, PF: posterior flagellum, St: starch grains.

1, 2: bar=400 nm; 3-6: bar=500 nm.

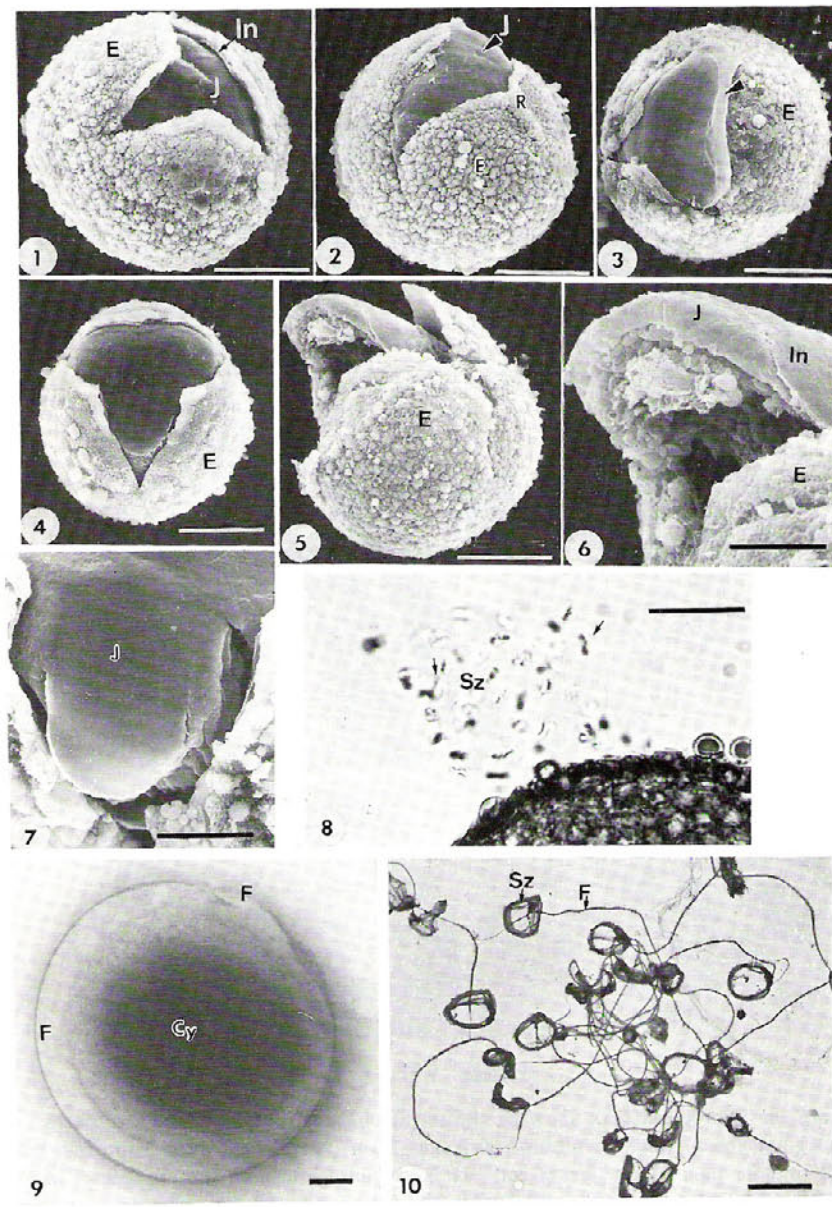

Plate VI.

1-5: The SEM view showing the opening of operculum. E: exine of microspore, J: jacket cells of microgametophyte, arrowheads: the protruding jacket cell in process of forming operculum.

6: Enlarged from 5.

7: Enlarged from 4.

8: The newly spring out spermatozoids (Sz) under LM, arrows indicate envelope, LM.

9, 10: Negative staining spermatozoids. Cy: cytoplasm, F: flagellum, Sz: spermatozoid.

1-5: bar=25 μm; 6, 7: bar=10 μm; 8: bar=100 nm; 9: bar=100 nm; 10: bar=5 μm.

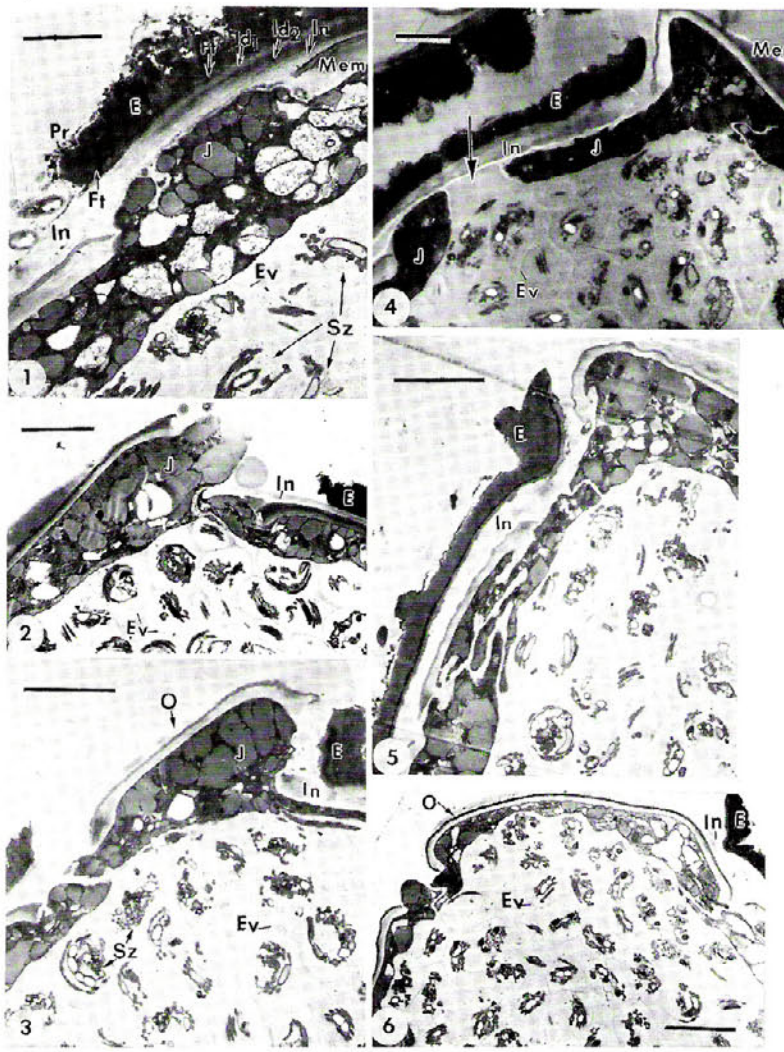

Plate VII.

Sections through the operculum (O), showing the serial changes of the related structures.

1: Exine (E) and the common membrane of jacket layer (Mem) have been ruptured, but intine (In) still intact; part of the jacket cell (J) is protuding through the rupture of membrane, black arrows indicate spermatozoids (Sz), Ev: envelope of spermatozoid. Ft: foot layer, Id_1: indexine-1, Id_2: indexine-2.

2: Intine splits, jacket cells (J) protrude above the exine (E). Spermatozoids are enclosed in their envelopes individually.

3: A newly formed operculum (O).

4: Partition (arrow) between two jacket cells (J).

5: Jacket cell adjacent to the operculum begin disintegrating.

6: Another operculum, note the adjacent jacket cells. E: exine of microspore, In: intine of microspore, J: jacket cell, Mem: common membrane of jacket, O: operculum, Sz: spermatozoid.

1, 4: bar=2.5 μm; 2, 3, 5, 6: bar=5 μm.

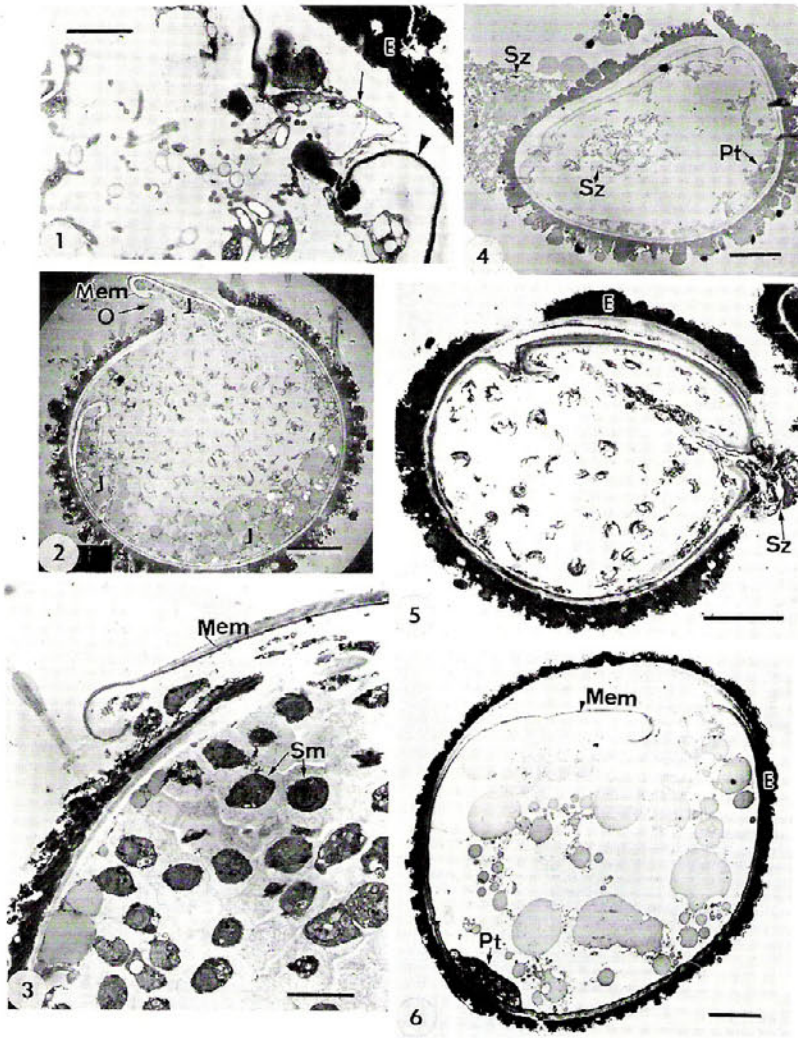

Plate VIII.

Liberation of spermatozoids (Sz).

1: Arrow shows the spermatozoids (Sz) and the accompanying slime-like residue of jacket cells, arrowhead: common membrane of jacket layer.

2: Lifting operculum (O), jacket cells (J) are disintegrated.

3: The developing spermatids (Sm) enclosed in their envelopes are expelled by this microgametophyte, the jacket cells have already been disintegrated.

4: A later stage of expelling spermatozoids (Sz), outside the microgametophyte is a clump of expelled spermatozoids intermingled with residue of jacket cells; note the common membrane of jacket layer and the intact prothallial cell (Pt).

5: Clumps of spermatozoids (Sz) are sprining out, jacket layer is almost empty.

6: Prothallial cell (Pt) persists after all the spermatozoids are expelled.

E: exine of microspore, J: jacket cell, Mem: common membrane of jacket, O: operculum, Pt: prothallial cell, Sz: spermatozoid.

1: bar=2.5 μm; 2: bar=5 μm; 3: bar=500 nm; 4-6: bar=10 μm.

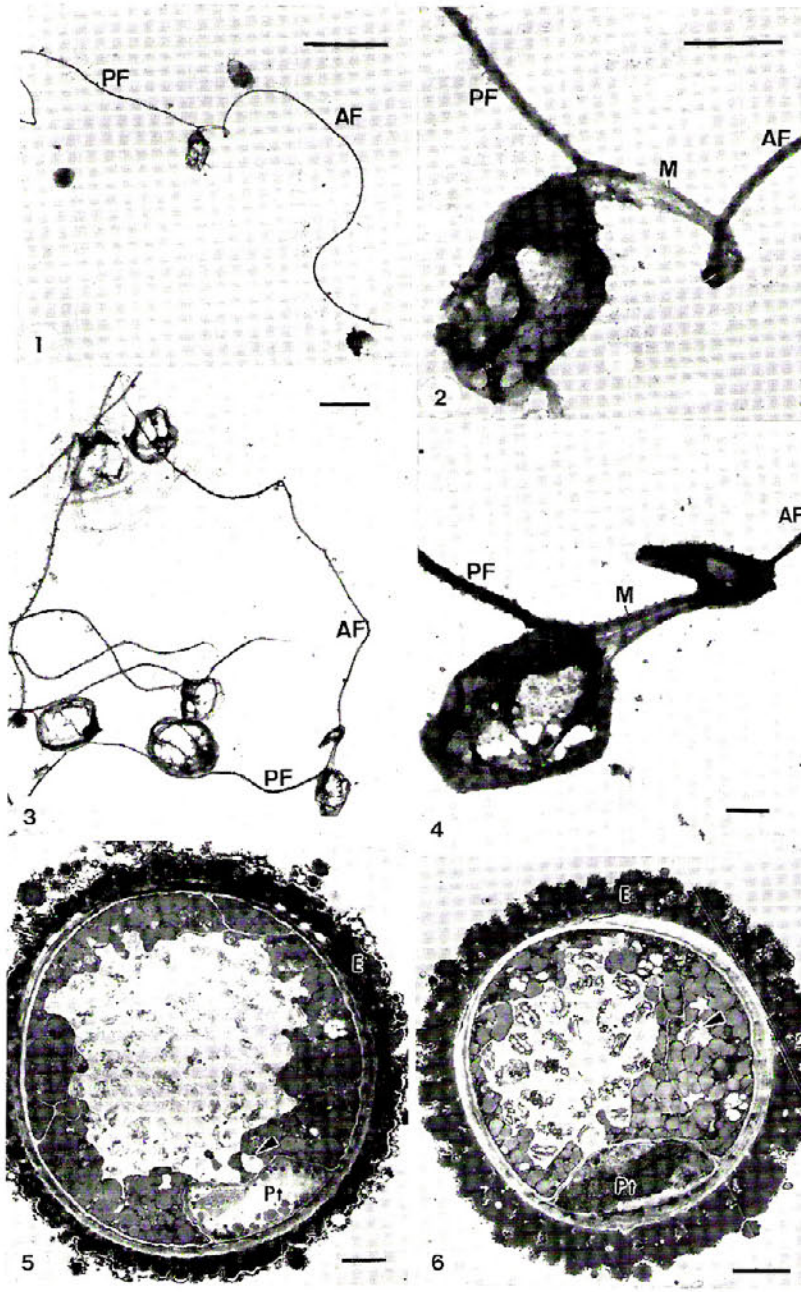

Plate IX.

1-4: Negative stained spermatozoids; 2, enlarged from 1; 4, enlarged from 3; note the apical mitochondrion (M), the anterior flagellum (AF) and posterior flagellum (PF). The nucleus and the posterior plastid are somewhat twisted so they show a loop-like figure under the stain. AF: anterior flagellum, M: apical mitochondrion, PF: posterior flagellum.

5, 6: Showing the spermatozoids (arrowheads) produced in jacket layer.

1, 3: bar=5 μm; 2, 4: bar=500 nm; 5, 6: bar=5 μm.

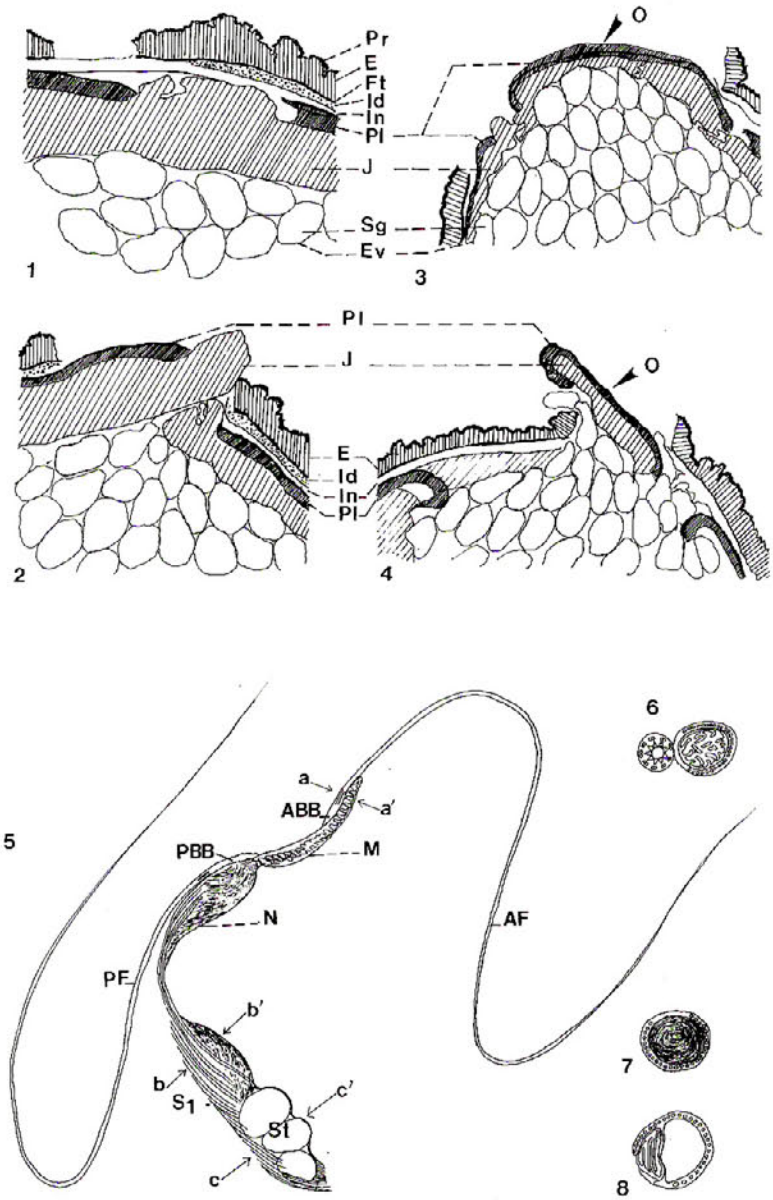

Plate X.

1-4: Drawings showing the forming process of operculum. Ev: envelope of spermatogenous cell, E: exine of microspore, Ft: foot layer of microspore, Id: indexine of microspore, In: intine of microspore, J: jacket cell, O: operculum, Pl: plasmolemma of microspore (e.g. common membrane of jacket layer), Pr: perine of microspore, Pt: prothallial cell. 1: from Pl. VII-1; 3: from Pl. VII-6; 4: from Pl. VIII-2).

5: The schematic diagram of a mature spermatozoid in longisection.

6-8: The cross sections correspond to the small letters in fig. 5. a-a': fig. 6, the stellate pattern and apical mitochondrion (M). b-b': fig. 7, the nucleus (N). c-c': fig. 8, the posterior plastid and the starch grain (St) containing in it.

III-4; IV-3), the accumulation of lysosomes (Pl. IV) and the disintegration of rest organelles. The degrading cytoplasm is connected with the main body of spermatozoid by several frail vesiclized strands (Pl. V-2). Finally these strands break down, all the cytoplasmic residues are eliminated and lead to the completion of the metamorphism. A mature spermatozoid contains a pair of flagella, an apical mitochondrion, a long twisted nucleus, with or without a posterior mitochondrion and a posterior plastid with three to five large starch grains. The flagella are extruded from both ends of the apical mitochondrion, i. e., the anterior flagellum extrudes from the apex of the apical mitochondrion and the posterior flagellum from the juncture of it and the nucleus (Pl. II; V-3, 4). Each structure in the spermatozoid can be clearly distinguished on the negative-staining material (Pl. IX-1-4).

As the microgametogenesis proceeds, the kinetic movement together with the increasing volume of the whole male gametophyte cause the wall of microspore to split along the trilet ridge. Consequently the gametophytic tissue protrude above the surface of spore wall (Pl. VI-1). Before splitting, the microspore wall can be recognized as perine, exine, foot layer, endexine-1, indexine-2 and intine (Pl. VII-1; X-1). Exine is of 1.2-2 μm in thickness. It splits first. While the plasmolemma of the original microspore which is now the common outer membrane of the entire jacket cells splits and the jacket cells in proximal pole become protruded, the intine still remains intact (Pl. VII-1). Subsequently, the intine ruptures, too. Thus gives a way for jacket cells to protrude above the trilet (Pl. VII-2, 3). The plate of protruding jacket cells act as an operculum and is torn along the opening line of spore wall (Pl. VI-2-4). Meanwhile, the residual jacket cells become separated from each other (Pl. VII-3-4), some of them gradually disintegrate into slime-like structure, and intruding into the developing spermatozoids (Pl. VIII-1, 2). The separation and disintegration of jacket cells are attended with the rupture of intine, the spermatozoids spring off in a clump (Pl. VIII-4, 5). As the disintegration of the jacket cells proceeds, the operculum is lifted and taken off (Pl. VIII-2). Ultimately, all the jacket cells are disintegrated and expelled, leaving the common membrane and some residues of slimes (Pl. VIII-5, 6). The jacket cells in some specimens are found to give rise to spermatozoid (Pl. IX-5, 6). The prothallial cell retains its figure even after all other structures within the spore wall disappear (Pl. VIII-4, 6).

The newly released spermatozoids are still enclosed in their individual envelope (Pl. VI-8). The flagella appear to be coiled on the periphery of the cytoplasm (Pl. VI-9). A short time after the elimination of whole residual cytoplasm, the envelope bursts, the long tapering flagella stretch out and brings about the dispersal of spermatozoids rapidly.

Majority of spermatozoids within the same gametophyte are very well synchronized for stages of development. Accidentally, the spermatids which are still in the process of development are released. Whether these spermatids can undergo metamorphosis to attain maturity or not has not been studied.

DISCUSSION

Selaginella and *Lycopodium* are known to be the only two living genera in vascular plants which produce biflagellate male gametes. As described elsewhere, biflagellate sperms are commonly present in all members of bryophytes as

well as some algae. As expected, the present result shows that its spermatozoids appear to be similar in the basic organization, even the structure of MLS.

In change to a long twisted nucleus from an ovate or round one, evidence present here shows that the nuclear material undergoes a profound differentiation. It is difficult to give an feasible explanation for this phenomenon merely based on the morphological evidence obtained here. The same situation has occurred in *S. kraussiana* (Robert, 1977) and some bryophytes (Carothers, 1973; Carothers and Duckett, 1980; Paolillo *et al.*, 1968a, b). On the other hand, the nucleus in spermatozoid of *Lycopodium cernuum* has been found to be ovoid in outline in spite of its closely associated taxonomic affinity with *Selaginella* (Robbins and Carothers, 1978). Since the case in *Lycopodium cernuum* is the only study for this genus in nuclear morphology of spermatozoid, more survey in other species is needed here to elucidate the development of these stages in lycopods. With the exception of the shape of nucleus, the structure and orientation of centrosome in spermatozoid of *Lycopodium cernuum* are in accord with those of spermatozoid in *Selaginella* and numerous bryophytes (Carothers, 1973; Carother and Duckett, 1980; Paolillo *et al.* 1968a, b; Robbins and Carothers, 1978; Robert, 1977).

Within the specimens we examined, some spermatozoids possess a posterior mitochondrion located between nucleus and posterior plastid, some do not. It is interesting that among those numerous small mitochondria how the apical mitochondrion elongate and persist. Though the mitochondrial fusion is known in animal spermatogenesis (Favard and Andre, 1970) and in zoospore formation in some fungi (Cantino and Mack, 1969), it still lacks of evidence to explain the difference between the apical mitochondrion and other short ones which become coalescent in *S. tamariscina.*

The behavior of jacket cells in *S. tamariscina* is partly disagreement with the studies by most of the earlier workers citated above. The jacket cells in *S. kraussiana* have been believed that they function as nutrients and have been absorbed by spermatogenous cells, since they have disintegrated into slime during the spermatogenesis (Belajeff, 1885; Millardet, 1869; Slagg, 1932). In *S. martensii* and *S. caulescens*, Pfeffer (1871) described that there was a thin layer of slime between antherozoid mother cells and the old spore wall, but he did not explain how the slime originated. Pfeffer has found that all the jacket cells were primary spermatogeous cells.

As revealed in the present study, the function of jacket cells can not be simply be categorized as nutrient tissue. The jacket cells are morphologically and functionally specialized into at least two clearly parts: (1) proximal "operculum", (2) a larger amount of distal ordinary "tapetal jacket layer". The presence as well as the components of operculum in *Selaginella* are described as the first report of such structures in lycopods. Though the transformation of the jacket cells into spermatozoids has been seen in a few specimens, it seems not the main destination of this structure.

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萬年松雄配子之生成

II. 精細胞成熟的過程

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摘　　要

本文於電子顯微鏡下觀察萬年松 *Selaginella tamariscina* (Beauv.) Spring 精細胞成熟的過程：變態及「囊蓋」(operculum) 的形成。變態包括下列變化：(1)前端粒線體的延長、(2)基體 (basal bodies) 的分離及鞭毛的伸展、(3)細胞核的延長、(4)剩餘細胞質的排除。

在產生異型配子的石松族中，雄配子體形成過程中「囊蓋」的形成與開啓爲本研究之創見。「囊蓋」由一些保護層細胞組成，當「囊蓋」掀開後，精細胞成團湧出。偶亦見保護層細胞發育爲精細胞。

成熟的精細胞具有細長而扭曲的細胞核、一個前端粒線體、有或無一個末端的粒線體、一個內含數顆大澱粉粒的末端色素體、及兩根鞭毛。前鞭毛自前端粒線體的先端伸出細胞外，後鞭毛自前端粒線體與細胞核之間伸出。