SPORE GERMINATION AND LEAFY GAMETOPHYTE OF *HAPLOMITRIUM ROTUNDIFOLIUM* DEVELOPED IN CULTURE

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I. INTRODUCTION

In a previous report on the spore germination of *Haplomitrium rotundifolium* (Yang 1966), it was shown that a triangular, thalloid sporeling developed in Hoagland's solution under controlled condition, in about 87 days. Further development was not observed because of microbial contamination. Spore germination of *H. rotundifolium* and also *H. blumii* was attempted again in March, 1967. Spores were gathered from respective fresh specimens. Having been treated antiseptically, they were then planted in the nutrient solution in petri dishes. Except for a few changes which will be described later, the same technique was used previously.

The present experiment has shown that the germination of spores of *H. blumii* commenced readily, but that the later development advanced rather slowly. However, the spores of *H. rotundifolium*, harvested from Chi-tou specimens, passed through the 2-celled stage and the globose protonema to reach the triangular thalloid stage in 67 days. Further advance was a rhizomatous stage and ultimately to a leafy gametophyte. This full development of *H. rotundifolium*, from a spore to a mature plant, took place in the course of about six months.

II. MATERIALS AND METHODS

1. Materials: *Haplomitrium blumii* collected from Ali-shan on Feb. 26, 1967, by Misses F. M. Hsu and S. Y. Yao. *H. rotundifolium* collected from Chi-tou on Oct. 26, 1966 by Miss Hsu and Mr. Kao, on a trip with Dr. H. Inoue of Japan.
2. Methods:
   (1) Sterilize the mature, harvested capsules in 1:10 "Clorox" (= commercial sodium hypochloride bleach) for one minute.
   (2) Rinse with 200 ml of sterile distilled water[7].
   (3) Transfer each capsule to one sterile petri dish with 20 ml of 50% Hoagland's solution, with a pair of sterile forceps.
   (4) Crush the capsule to release the spores into the medium with the same forceps.
   (5) Place the petri dishes in a culture room at a constant temperature of 18–22°C, about 1200 lux illumination, and 8 hours of light diurnally[8].
   (6) Transfer some sporelings at the rhizomatous stage from nutrient solution to semisolid medium of 50% Hoagland's solution solidified with 1% agar, and some to the same medium but with 0.25% of activated charcoal added.

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III. OBSERVATIONS AND RESULTS

The results obtained from the present study are mainly based on the sporelings developed from Plate A of the Chi-tou collection of *Haplomitrium rotundifolium*. The main events are summarized as follows.

1. The spore germination of *H. blumii* is reported for the first time. The 2-celled stage occurred as rapidly as four days after the sowing of the spores, while in *H. rotundifolium* it took two weeks to reach that stage. But the later development of the sporelings of *H. blumii* did not proceed at the same rate as that of the latter species—most of them are still at the triangular or rhizomatous stage at the time of writing this paper.
2. In *H. rotundifolium*, the sporelings, having reached the triangular-thalloid stage, continue to grow into irregularly branched cylindrical structures about 3–4 mm. in length, in appearance like the rhizomes of a mature gametophyte. Therefore, this is called the rhizomatous stage (Pl. I, 3–4; Pl. V, 1–5). Under a Leitz dissecting microscope at 220 magnifications the cells lining the surface could be seen, and mucilage papillae were found distributed sparsely among the cells. (Pl. V. 3, 4, 5).
3. Differentiations observed in the later development of the sporelings of *H. rotundifolium:*
   a. At first, the arms of the triangular thalloid extending outwardly, along with certain cellular differentiations arising from other parts of the thallus, constitute a polybranched cylindrical rhizomatous stage (Pl. I, 3, 4; Pl. V, 1–5).
   b. The cells near the slime papillae divide and protuberances, each containing a group of cells, appear here and there on the branches, particularly toward the ends. It seems that the development at this stage does not show any definite pattern, but is rather random (Pl. V, 1–5; Pl. I, 3, 4). In the later rhizomatous stage, however, a more or less progressive development toward a specific form—a central axis with apical region and primordia—is assumed (Pl. VI. 2–4)
   c. The mucilage papillae in *H. rotundifolium* of Taiwan (or *H. blumii*) are at first one-celled (Pl. VI, 5a), initiated from margins of leaves or stems. Later, they develop into 2-celled stage but not completely septate, only narrow cuts on the sides of the cell (Pl. VI. 5b). Close examination under microscope shows mucilage pad as described by previous workers (Proskauer 1962, p. 221).
   d. Fungal association considered as the important role in the germination of *H. rotundifolium* by previous workers[10] could not be confirmed in the present experiment. Some kind of actinomycetes have been found constantly present in my cultures but they have nothing to do with the development

of the plant. When in abundance, they may cause death or unhealthy condition of the culture. Leafy gametophytes of *H. rotundifolium* have developed from medium devoid of such contamination. Moreover, when sporelings at rhizomatic stages are transferred to solid medium these fungi become disintegrated accordingly. Therefore, I conclude, in the light of the knowlege from the present experiment, no fungal association is found to be necessary in the growth and development of *H. rotundifolium* or *H. blumii.*

e. The initiation of primordia from the apical region is directly from cellular protuberances (Pl. II, 1, 2, 4; Pl. VI 1, 2). As the axis grows longer, the protuberances become separated further apart from each other (Pl. II, 4, 6). In the early stage of their development, they appear as narrow strips as viewing from lateral (Pl. II, 1, 4) and gradually they give rise to lateral leaf primordia, more or less rounded or spade-shaped (Pl. II, 2–4).

4. The formation of juvenile leafy gametophyte is, at last, completed. First, the cells of the primordia divide, thus the narrow strips increase in size, both in width and length, and become flat pieces with rounded margins. Eventually the young leaves, broadly-ovate in shape, and one layer of cells thick, are gradually formed. They are evenly spaced in a spiral pattern around the axis, and fall into three longitudinal ranks (Pl. II, 2, 3, 6; Pl. III, 1–3; Pl. VI. 3). In the meanwhile, the elongation of the axis takes place. The new gametophyte measuring only about 4.5–5 mm. in length is patterned exactly like a miniature of the parent *H. rotundifolium.*

5. Experiment has shown that sporelings kept in the nutrient solution do not develop into leafy gametophyte, but remain in the rhizomatous or triangular-thalloid-stages. Rhizomatous stages, when transferred to semisolid agar medium with or without activated charcoal can grow into upright leafy gametophytes. At the time of writing this paper, such leafy gametophytes have been obtained only in the cultures of *H. rotundifolium,* and not in those of *H. blumii.*

6. Effects of photoperiod, temperature and media on development of sporelings of *Haplomitrium:*

a. The 8-hour photoperiod and 1200 lux illumination used in the present experiment seems more favorable than 24 hours or 12 hours of light and 1200 lux illumination in previous experiments.

b. The testing of temperatures in the 18°C–20°C range did not show much effect on growth of sporelings of *H. rotundifolium.*

c. Among various tested nutrient solutions, including 50% Hoagland's solution, Knop's, Beneck's and 50% Hoagland's + GA 10^{-5} gr, GA 10^{-6}gr, and GA 10^{-7} gr.; the 50% Hoagland's solution is found to be the most favorable

for sporeling deveolopment of *H. rotundifolium*, Knop's solution showed unsatisfactory results, Beneck's is good at start but declined later.

7. In order to attempt acceleration of the growth and development of *H. rotundifolium*, shaking and centrifugation have been applied to the spores and early stages of sporelings, but no effective result was obtained from either treatment.
8. Experiments on regeneration were unsuccessful when parts of axis or rhizome-like basal portions were cut into small pieces and cultured antiseptically in 50% Hoagland's solution in 100-125 c.c. flasks. But when leaves having washed in distilled water were planted on the activated charcoal agar medium in petri-dishes, small bulbils of pin-head size were immediately initiated from cells of the leaf margin, while the central leaf cells showed no sign of growth. These bulbils enlarge and form fleshy cylinders as green as the leafy axis unlike those of *Takakia* being pale green to nearly white[10], giving rise to rhizomatous stages like those produced directly from spore germination (Pl. III, 4, 5), as previously shown by Inoue[6].
9. As seen in cross sections of the axis of *H. rotundifolium*, the cells in the core lack dense contents (Pl. IV, 3, 4) while surrounding them are several layers of cortex cells, richly supplied with conspicuous starch grains (Pl. IV, 1-3), which are also present in the leaf cells especially adjacent to the axis. Chloroplasts with pigments are hardly seen in these cells.

   Goebel[5] believes that the central cells destitute of granules have to do with conducting but the writer thinks contrarily, they may function as supporting elements while the outer cortex may serve for conducting as well as storage. *Haplomitrium rotundifolium* of Taiwan has a larger core and narrower cortex than *H. blumii* of Indonesia, which Campbell[1] reported has a definitely smaller core and broader cortex consisting of more than one kind of tissue.

IV. DISCUSSION AND CONCLUSION

1. Vegetative gametophytes of *Haplomitrium rotundifolium* have been raised from spores antiseptically in the laboratory. The spore germination of *H. blumii*, for the first time reported here, has been carried so far only to early thalloid stages and not yet to the leafy gametophyte stage, but they proceed quite steadily reaching rhizomatous stage approximately two months later than that of *H. rotundifolium*; and they remain at that stage for several months afterward without showing further changes in this study.

   However, the culture is still green and viable and continous observation should be made. Any result showing advancement toward the completion of a leafy gametophyte, will be reported in another paper.
2. The life cycle of *H. rotundifolium* as manifested in the present experiment, from spore to gametophyte, consists of 4 distinct stages:

(1) The 2-celled to globose protonema stage.
(2) The triangular-thalloid stage.
(3) The rhizomatous-branched stage.
(4) The leafy gametophyte stage.

3. Spores from only one of the 17 capsules of *H. rotundifolium* tested (in March 1967) developed into leafy gametophytes, while the rest revealed young sporelings only, and advanced no further (Table 1).

Table 1. Experiments on the germination of spores of *H. rotundifolium* and *H. blumii*

Date of sowing	Number of plates	Stages of germination						Remarks
		Approx. rate of germ.(%)	In liquid medium				On semi-solid medium	
			No germ. (%)	Stage I (%)	Stage II (%)	Stage III (%)	Stage IV (%)	
March 20, 1966	5			*	**	0	0	% not recorded, other results refer (19)
June 2, 1966	5			*	**	0	0	
Feb. 25, 1967	one flask (B)	0						Contaimination and discarded
Mar. 5, 1967	Plate A (R)	93.0	7.0	24.3	27.8	20.9	20.0	Many of Plate A (R) reached stage IV when transferred to semi-solid medium.
	Plate B (B)	97.4	2.6	96.8	0.6	0	0	
	Plate C (B)	92.0	8.0	88.8	3.2	0	0	
	Plate D (B)	93.3	6.7	93.3	0	0	0	
	Plate E (B)	97.7	2.3	82.1	5.2	0.4	0	
Mar. 22, 1967	Plate A_1 (B)	93.4	6.6	59.7	10.7	23.0	0	17 plates of spores tested, only A_1 & B_1 showed development.
	Plate B_1 (B)	92.3	7.7	60.7	8.8	2.8		

a. Stage I. The 2-celled to globose protonema stage.
Stage II. The triangular-thalloid stage.
Stage III. The rhizomatous-branched stage.
Stage IV. The leafy gametophyte stage.
b. (R) — Spores of *H. roundifolium*
(B) — Spores of *H. blumii*
*=moderate amount
**=more than half of the cultures develop into Stage II
0=no germination or certain particular stage not reached.

4. The possible reasons for reaching the formation of leafy gametophyte from the spores of only one particular capsule and not all others, may be due to differences in certain internal factors, such as the genetic make-up, degree of maturity, enzymatic effect, etc. within the spores. The age of spore may also play an important role. External factors seemed to bring little influence on the growth and development of the sporelings.

5. The leafy gametophytes produced from the present experiment are extremely small, about 4.5–5 mm. in length and 0.5–1 mm. in width of the stem. The total plant is about 1/5 of a typical mature gametophyte. The occurrence of such a dwarfed gametophyte in the present experiment resembles what Ward[17] has reported on an aberrant gametophyte induced from *Polytrichum commune* whose height and width are 5 mm, and 1.0 mm. respectively.
6. Although small, the cultured leafy gametophytes may seem to be, yet they show normal polarity and leaf arrangement[15].
7. The transfer of sporelings in the rhizomatious stages from liquid (50% Hoagland's solution) to semi-solid medium (50% Hoagland's solution+1% agar+0.25% activated charcoal) may have stimulated growth and the initiation of erect leafy axes from the rhizomatic axes.
8. A study on the anatomy of the axis of a mature gametophyte selected from the culture, reveals a distinct structure of the stem as seen in a cross section. There is a hyaline of central strand surrounded by several layers of cortical cells, richly supplied with starch granules. They usually aggregated in plastids.
9. The sporelings of *Haplomitrium* have undergone several stages (from protonema of globose, triangular-thallose, rhizomatous-branches) before they reach the leafy gametophyte. Rhizoids have not been observed throughout all the four stages of development. This is definitely a special pattern of sporelings occurred only in *Haplomitrium*; both *H. rotundifolium* and *H. blumii* went through the same processes, in spite of the fact, the latter have not yet reached the final, gametophyte stage at the end of this study.
10. The phylogeny of *Haplomitrium* may have some relationships with some mosses (*Sphagnum*) because of its erect appearance of the leafy gametophyte and the triangular-thalloid stage in the sporeling; and its early globose protonema resembles *Anthocerotales* (*Dendroceros, Megaceros*)[11], except the latter develop rhizoids immediately after their formation.

The absence of rhizoids in *Haplomitrium* is similar to the attribute of *Takakia*[12] but the sporeling development of the latter has not been reported at present. One may conclude, therefore, that *Haplomitrium* can be considered as a unique plant possessing some moss-like attributes and some liverworts particularly, in their sporeling development.

It is currently thought by some, that *Haplomitrium* is an intermmediate form between liverworts and mosses, and this can be warranted by the results of this study.

In closing, it should be mentioned, that *H. rotundifolium* has been reported previously[19] in 4 places: Yang-ming Shan, Chi-tou, Alishan and Taiping-shan in Taiwan. Two new localities are added at Luan-shan, Haw-lien, petrophilous, alt. 700, and Ta-tung Shan, alt. 1,000, Taiwan by Dr. C. C. Hsu, Aug. 16, 1967 and Mr. M. T. Kao, Nov. 12, 1967.

V. SUMMARY

1. The leafy gametophyte of *Haplomitrium rotundifolium* has been developed from the germination of its spores in Hoagland's solution under controlled condition in the course of about six months.
2. Some of the 4 distinct stages of development in the life cycle of *H. rotundifolium* revealed from the present experiment seem to correlate to that of other bryophytes. The triangular thalloid stage is so much like the protonema of *Sphagnum* and the early globose stage resembles that of *Dendroceras* according to Nehira's report[11]. This may lead some one to interprete that Calobryales are probably intermediate forms between some mosses and liverworts.
3. The leafy gametophytes in culture are grown excellently well in plates on agar media with activated charcoal, approaching, approximately 1/3 of the size of an ordinary plant.
4. The germination of the spores of *H. blumii* although showed steady progress reaching rhizomatous branch stage but none of them indicate any further advance than this.
5. The significant results from this study include: (1) the completion of the leafy gametophyte of *H. rotundifolium* from controlled laboratory and (2) the occurrence of rhizomatous branch stage in *H. blumii;* both are first report in science.

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EXPLANATION OF FIGURES

PLATE I

Fig. 1. Globose protonema ×150.
Fig. 2. Triangular-thalloid stage ×60.
Fig. 3. Rhizomatous stage ×1 (taken from a plate culture in Hoagland's solution), about 67 days old.
Fig. 4. Ditto, on activated charcoal agar ×1, 70 days old.
Figs. 5, 6. Later rhizomatous stages. 5. ×50. 6. Much enlarged, showing one branch apex with developing primordia ×70.

PLATE II

Fig. 1. Rhizomatous stage approaching to the formation of gametophyte ×28.
Fig. 2. A leafy gametophyte showing linear apical primordia and mature leaves radially placed on the axis, about 8 months old ×20.
Fig. 3. Leafy gametophyte with well formed leaves 6½ months old ×28.
Fig. 4. Young gametophyte, showing protuberance, young primordia in narrow strips and ovate lateral leaves, 5½ months old ×28.
Fig. 5. Young gametophytes arising from rhizomatous branches ×20.
Fig. 6. Ditto, in older stage with more leaves developed on the axes ×20.

PLATE III

Figs. 1-3. Heads of fully developed gametophytes with spreading leaves, showing the exact character of the wild type ×20.
Figs. 4, 5. Regeneration of leaf. 4. Small bulbils initiated from leaf margin. 5. Ditto, in older stage, and much enlarged, a. rhizomatous branches developed from the bulbils. b. the old leaf.

PLATE IV

Fig. 1. Enlarged cortical cells showing granules ×600.
Fig. 2. Portion of cortex showing cells filled with starch granules ×150.
Fig. 3. C. S. of stem showing central strand "a" surrounded by cortex ×70.
Fig. 4. Ditto, showing initiation of a young leaf and distinct nuclei at the apex ×70.

PLATE V

Figs. 1-5. Rhizomatous stage, showing irregularly cylindrical branches arising from triangular-thalloid stage. Drawn with camera lucida under a dissecting microscope.
Figs. 3-5. Showing mucilaginous papillae distributed among surface cells.
Figs. 1, 3, 5. Showing progressive differentiation of axis into branches and apical regions.

PLATE VI

Fig. 1. Cells in apical region of a young gametophyte, showing primordia, each with a terminal slime papilla ×370.
Fig. 2. Apical portion of a young gametophyte with primordia ×245.
Fig. 3. Ditto, in more mature stage ×70.
Fig. 4. Gametophytes arising from rhizomatous branches ×70.
Fig. 5. Cells from axis showing slime papillae ×370. (Figs. 1-5 drawn with the aid of camera lucida)

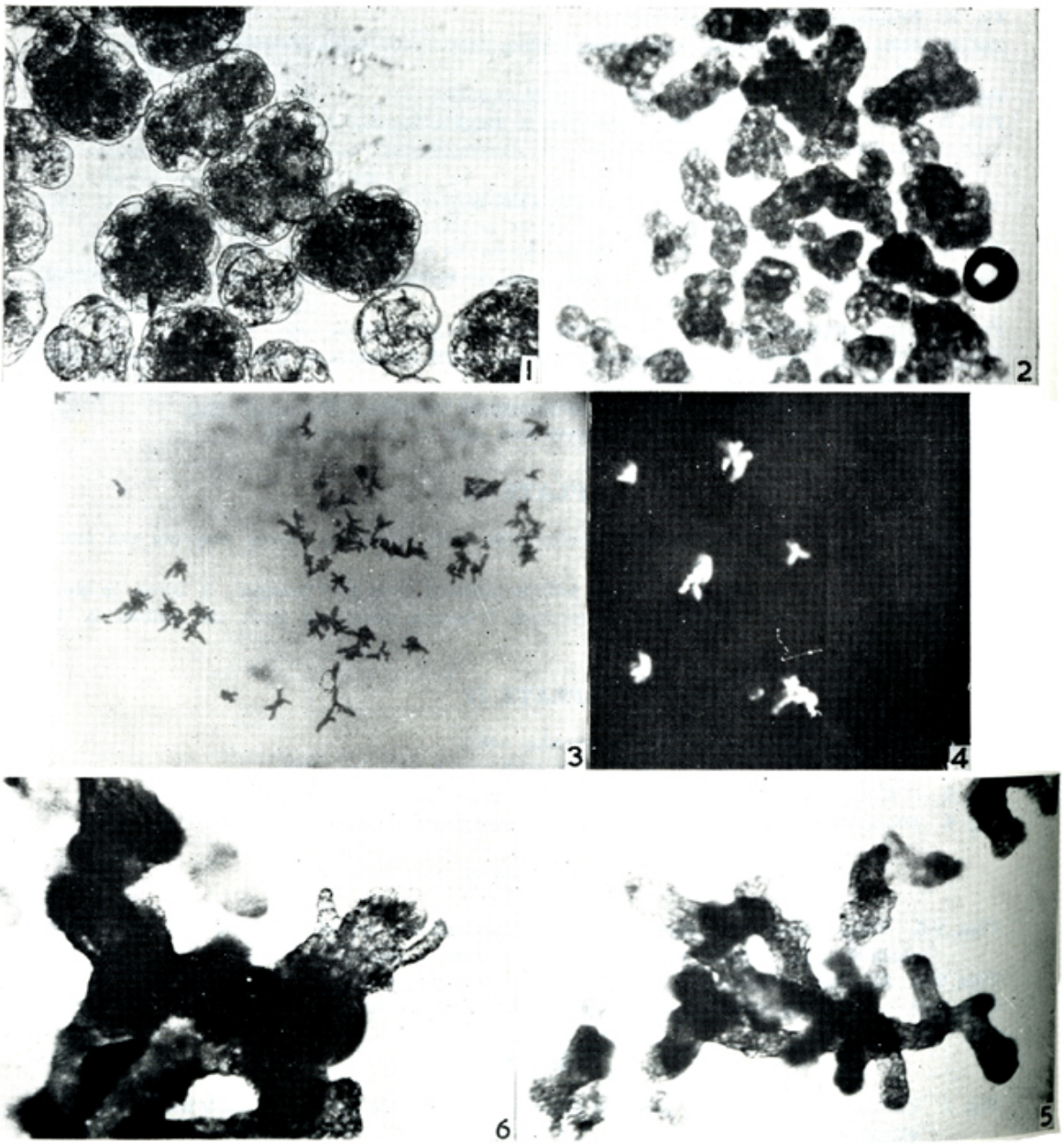
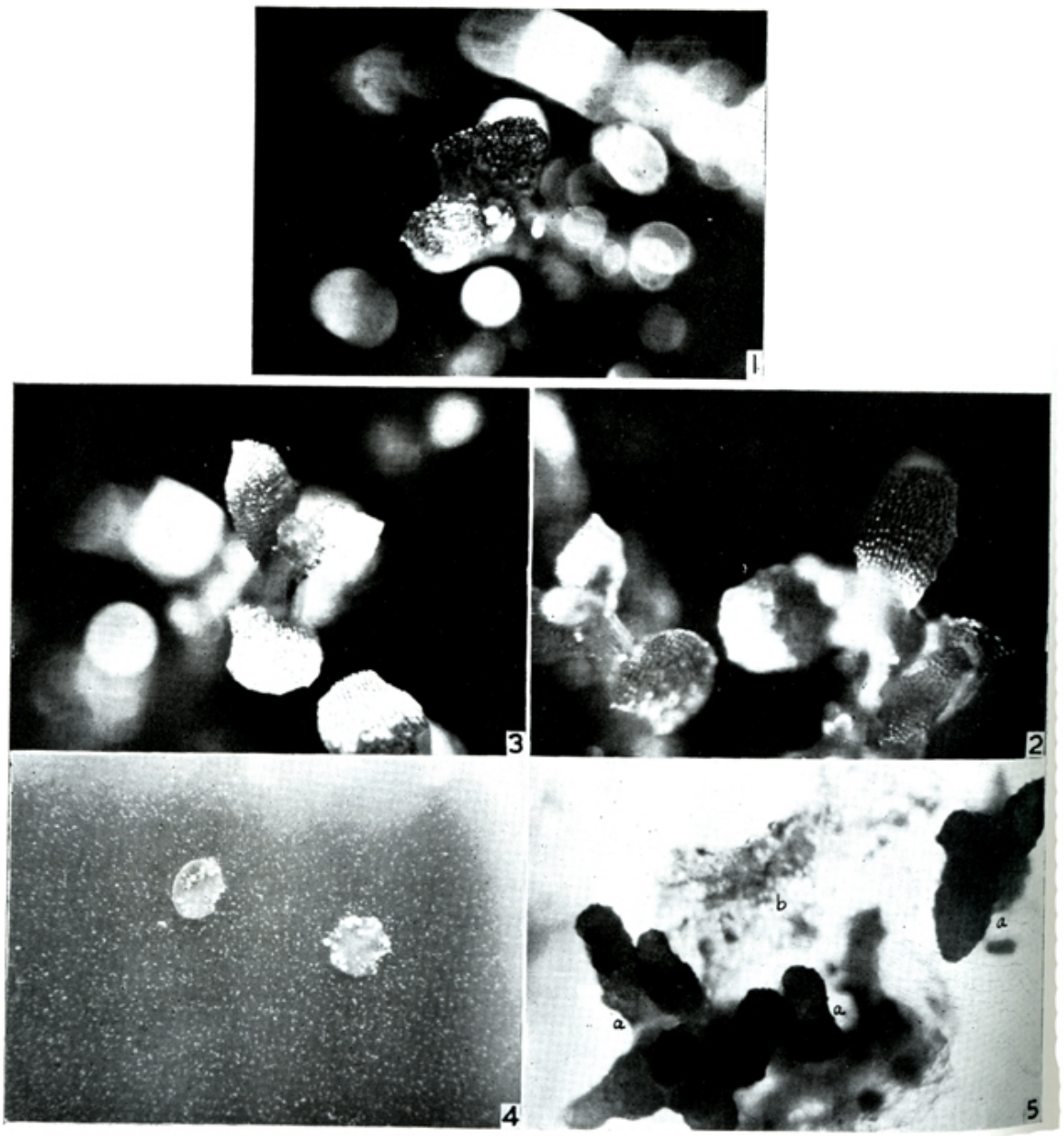


PLATE I

PLATE II



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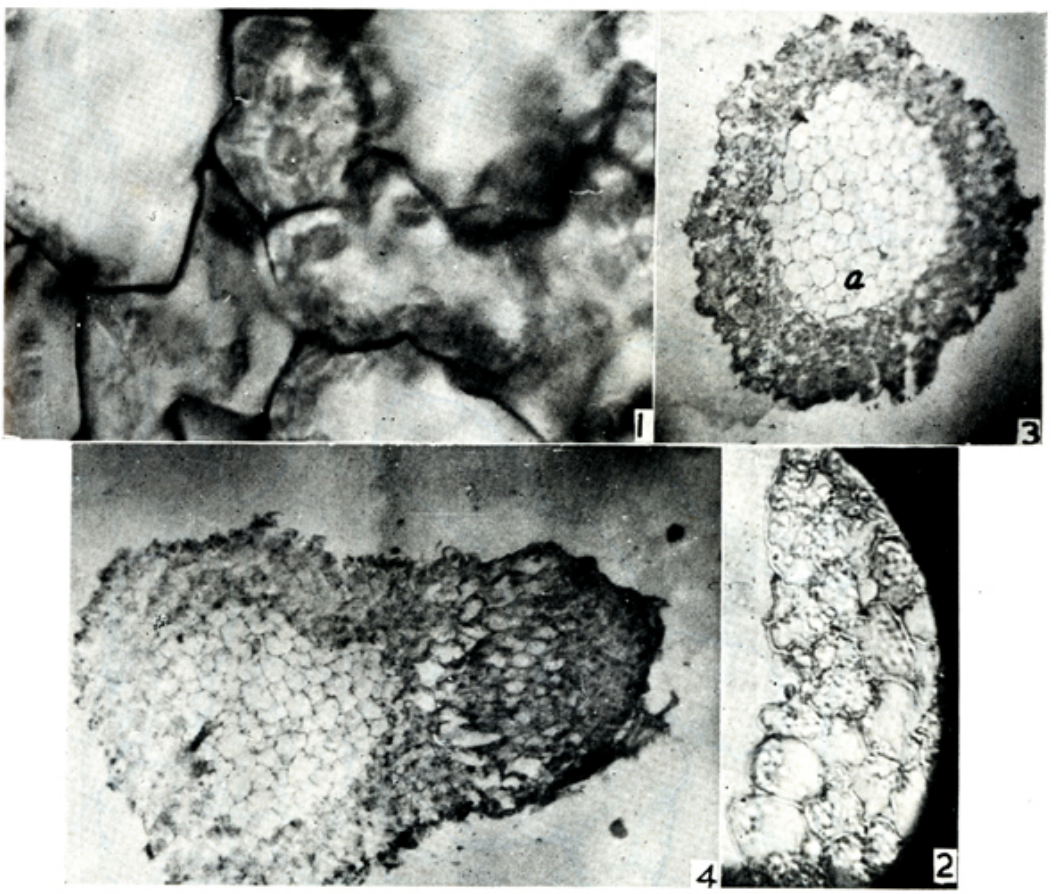

PLATE IV

PLATE V

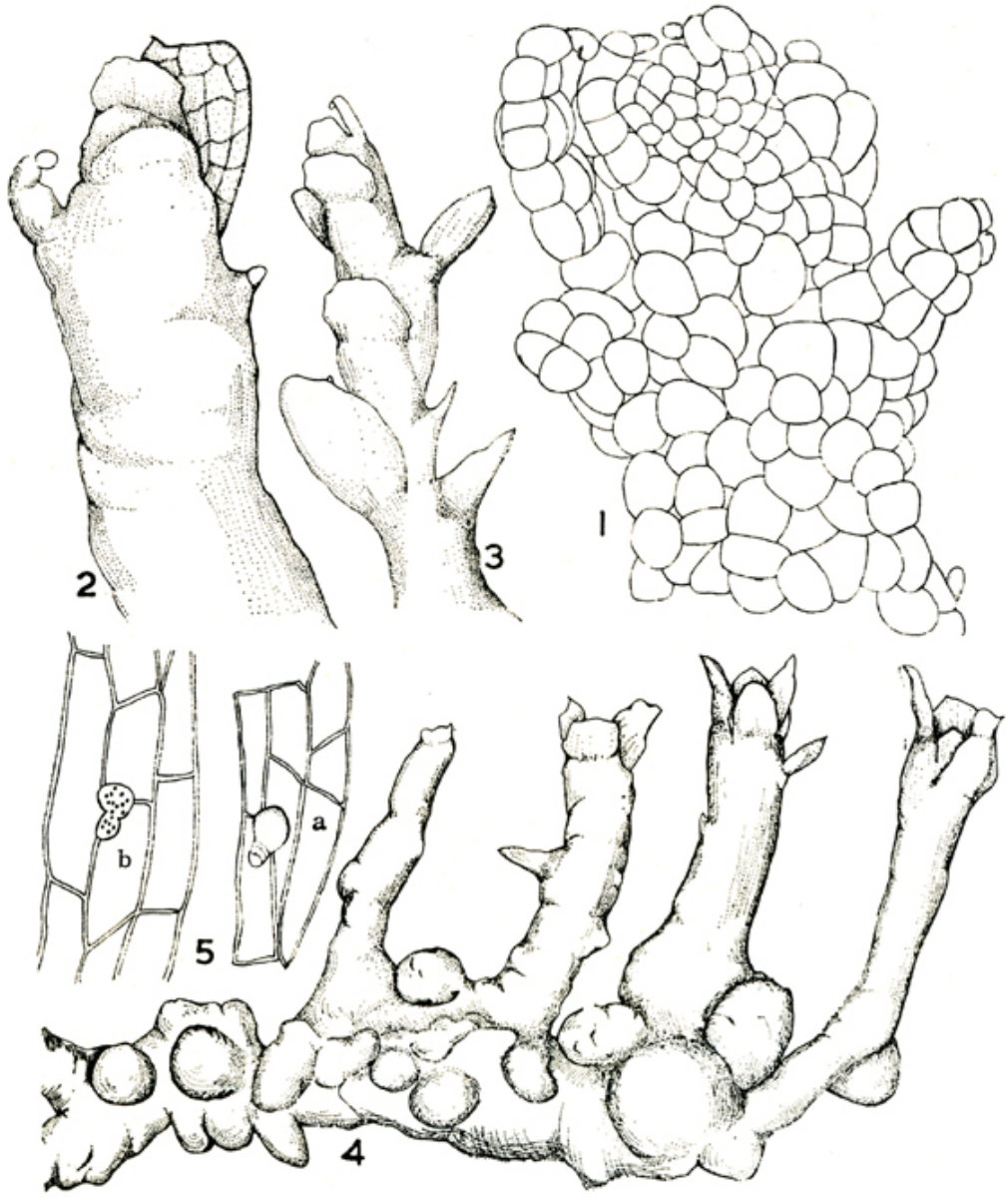

PLATE VI