EXPLANT AXENISATION FOR TISSUE CULTURE IN MARINE MACROALGAE

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Abstract

Unialgal explants from *Laminaria digitata*, and from a variety of red algae, were obtained by hand removing the visible epiphytes, and stirring the tissue in the presence of glass beads. Two antibiotic mixtures were found to be efficient in removing the contaminating fungi and bacteria from the algae. The procedure proved suitable as a primary step in the tissue culture of the investigated species.

Key words: unialgal, axenic, antibiotics, disinfectants, callus, regeneration

INTRODUCTION

Establishment of unialgal and axenic cultures is an important step in the culture of protoplasts, isolated cells and tissues from seaweeds. In the presence of enriched media, contaminated seaweed materials are often overgrown with bacteria, fungi or epiphytes. Bacteria and fungi affect the cultures either by competing for nutrients, by changing the physical state of the medium or by releasing substances which inhibit growth or are toxic (Berland, *et al.*, 1972). Epiphytes often show rapid growth and can completely cover the cultured tissue, resulting in unsuitable subjects for experimentation. Therefore, a variety of methods have been proposed to obtain axenic, unialgal cultures. The methods for obtaining axenic materials may be divided into three categories: treatment with disinfectants (Fries, 1963, 1970, 1984, 1985; Gusev, *et al.*, 1984, 1987); treatment with antibiotics, or combinations of treatment with disinfectants and antibiotics (Printer & Provasoli, 1968; Shephard, 1970; Gibor, *et al.*, 1980; Saga & Sakai, 1982; Zhang, 1983) and physical methods (Brown, 1982).

For obtaining unialgal materials, two main groups of methods are used in tissue culture. Physical methods include sonication (Polne, *et al.*, 1980) or simple brushing (Fries, 1970; Liu, 1987; Liu & Gordon, 1987). Chemical methods include treatment with germanium dioxide (GeO_2), which eliminates diatoms only (Guillard and Ryther, 1962; Lewin, 1966). However, as pointed out by a recent review of these techniques (McKracken, 1989), there is no universal procedure that would be applicable to every species, because each specimen has its own bacterial (Bradley, *et al.* 1988) and epiphytic flora (Ballantine, 1979).

In order to obtain axenic and unialgal explants for tissue culture of *Laminaria digitata* and several multicellular red algae, we experimented with various antibiotics, chemicals and a combination of manual and mechanical cleaning methods. Herein we present methods which resulted in axenic unialgal tissue samples from a

variety of algae. As shown by the fate of the explant in culture, these procedures proved suitable for callus induction and plant regeneration in the investigated species.

MATERIALS AND METHODS

Plant materials were collected on the shore near the Biological Station at Roscoff, in Brittany, France in December 1988. They included *Calliblepharis ciliata*, *Chondrus crispus* (gametophytes), *Gelidium pusillum*, *Gigartina teedii*, *Gigartina acicularis*, *Gracilaria lenanciforuris* (tetrasporophytes and carposporophytes), *Gracilaria foliifera* (carposporophytes), *Polyides rotundus*, *Porphyra umbilicalis*, *Pterocladia capillacea* from the Rhodophyta and *Laminaria digitata* from the Phaeophyta. Plant materials were precultured two weeks in sterile seawater supplemented with nitrogen (as $NaNO_3$, 56 mgL^{-1}) and phosphorus (as NaH_2PO_4, 8 mgL^{-1}) at 18 ℃, 12 : 12h L : D with a light intensity of 20 – 35 μmoles photon. $m^{-2}s^{-1}$. During this period, plants were thoroughly washed twice each day in sterile seawater, and visible epiphytes were removed by hand.

At the end of this adaptative period, healthy plants were washed and rinsed three to five times in steril e seawater. Plants were first agitated in sterile seawater with a 3 cm magnetic bar three times for 1 – 2min, then in the presence of glass beads (100 – 150 beads, 0.3 – 0.5 mm in diameter in a 1000 ml flask) three times for 1 mın each, and were finally rinsed thoroughly with sterile seawater. The washed plants were allowed to recover for 3 – 10 days in enriched seawater after which the treatment with glass beads was repeated, followed by another one-week recovery period. Any remaining epiphyte was manually removed with cotton or a needle under a disecting microscope. The plants were finally checked under microscope to make sure that they were epiphyte-free.

In order to determine optimum conditions for obtaining axenic materials, plants were submitted to various anti-bacterial treatments as follows: 1) 70% (v/v) ethanol for 30s, 1, 3 and 5 min; 1% and 2%(v/v) sodium hypochlorite, for 10 min (red algae)and 15 min (*L. digitata*) or 1 to 10% (v/v) Betadine (iodinated polyvinyl pyrrolidone)for 5 or 10 min; 2) treatment with a 1 – 5% Betadine solution for 5 min followed by thorough rinsing with sterile seawater and culture for 3 – 10 days in Provasoli enriched seawater (PES, Provasoli, 1968) containing various combinations of antibiotics (Table 1); 3) thorough washing with sterile seawater under a laminar flow-hood (ADS Laminaire HR 16) followed by incubation in the antibiotic solutions listed in Table 1.

To assay for sterility, the treated plant materials were washed in sterile seawater for two to three times to remove disinfectants or antibiotics, and cut into 1 – 2 mm pieces which were incubated on solid or in liquid sterility test media (Table 2).

After a two-week preculture period in PES medium (Provasoli, 1968) to recover from anti-bacterial treatment, axenic plant materials were cut into 1 – 2 mm fragments and plated on Petri dishes containing La medium plus 1% or 1.5% Difco Bacto-agar. Petri dishes were observed at intervals to monitor the various forms of development of the explants. When appropriate, the explants which had grown calluses or callus-like structures were subcultured in liquid or on

solid medium. All cultures were kept at 18 ℃, with a photoperiod of 12 : 12, L : D and a light intensity of 25–45 μmoles photon $m^{-2}s^{-1}$. Manipulation of the explants was performed under a laminar flow-hood, in autoclaved media with filter-sterilized vitamin and nutrient stock solutions.

Table 1 Stock antibiotic solutions for explant axenisation in marine algae.

Antibiotics	A1	A2	A3	A4
Penicillin G	1g	2.5g	/	/
Streptomycin sulfate	1.5g	1.5g	2g	2g
Chloramphenicol	0.5g	2.0g	/	/
Carbenicillin	0.01g	/	/	/
Gentamacin sulfate	0.25g	0.65g	/	/
Neomycin sulfate	0.1g	/	/	0.1g
Kanamycin	0.15g	/	/	/
Nystatin	0.02g	0.42g	0.5g	/
Ampicillin	/	1g	/	/
Erythromycin	/	/	1.5g	1.5g
Rifampicine	/	/	0.02g	0.05g
Sulfamethoxipyridazine (SMP)	/	/	/	0.5g
DDW	100ml	100ml	100ml	100ml

Antibiotics were purchased from Sigma (St Louis, MI, USA). Stock antibiotic solutions were diluted with 10 volumes of PES medium for treating plant materials.

Table 2 Sterility test media

A) Solid test medium (modified from Gibor, *et al.*, 1980)	
PES medium	1000ml
Agar	5g
Glucose	0.5g
Sucrose	1g
Yeast extract	5g
Nutrient broth	8g
Vitamin stock solution[a]	1ml
B) Liquid test medium	
PES medium	1000ml
Glucose	2.5g
Sucrose	2.5g
Yeast extract	5g
Nutrient broth	5g
Vitamin stock solution[a)]	1 ml
C) Vitamin stock solution[a)]	
Myo-inositol	100 mg
Nicotinic acid	50mg
Glycine	20mg
Folic acid	5 mg
DDW	100 ml

a) tetrasporophytes

RESULTS AND DISCUSSION

1. Establishment of unialgal and axenic plant materials.

Stirring the plants in the presence of glass beads in sterile seawater achieved the removal of most epiphytes, including single-celled algae. Unialgal plant materials were obtained with frequencies of over 80–90% from all algae tested. No unicellular algae were found later in culture. In particular, this treatment eliminated diatoms, obviating treatment with germanium dioxide (GeO_2), a chemical found to inhibit or reduce the growth of some macroalgae (Hopkin & Kain, 1978; Markham & Hagmeier, 1982). The above treatment proved especially useful in precleaning highly-branched species such as *Pterocladia capillacea* and *Gelidium pusillum*. Although it partially damaged the algal tissue, no harmful side-effects were observed on the explants cultured *in vitro*. In some species, such as *Gracilaria foliifera*, callus formation only occurred in the wounded part of the explants (Pl. I, Figs. 1 and 2). Establishment of unialgal cultures required 3 to 4 of the above treatment, combined with careful hand-removing of the remaining epiphytes and intermediary 3 to 10 day-culture periods. Periods of culture between each cleaning allowed fronds to recover from treatments, and also allowed remaining epiphytes to grow so that they were easily found and removed at the next cleaning.

Based on the sterility tests, the ethanol (70% for 0.5, 1, 3 and 5 min treatment) and the sodium hypochlorite (1 or 2% for 15 min with *Laminaria digitata*, and 10 min for red algae) did reduce bacterial numbers. However, as pointed out already by Liu (1987) and Bradley *et al* (1988), both chemicals were not disinfectants suitable for the algae tested in these experiments, since the treated tissues eventually died in further culture. Treatment with Betadine at 5% (v/v) concentration in sterile seawater for 5 min was not toxic to any tested species and did reduce the number of bacteria However, it did not remove all bacteria, even in combination with antibiotic mixtures A1 and A2 (Table 1).

Complete axenisation was achieved by incubating the plant materials in the antibiotic solutions A3 and A4 (Table 1). This indicates that erythromycin and rifampicin are of special interest for the preparation of axenic materials from Rhodophyta and Phaeophyta. A similar result was recently reported by Bradley *et al*. (1988). In contrast, the A1 and A2 antibiotic mixture solutions (Table 1) could remove most, but not all bacteria. This agrees with the report by Berland & Maestrini (1969) that some marine bacteria are resistant to many antibiotics. Pretreating plant materials with 5% Betadine was found useful in reducing baterial numbers. This pretreatment, however, could be replaced by washing and rinsing plant materials in sterile seawater under a laminar-flow hood, as axenic materials were thus obtained.

As shown in Table 3,, the optimal and maximal durations of the treatment largely depended on the species under investigation. This variation may be accounted for by differences in the bacteriral strains associated with the various algal species investigated, or by differences in the localization of bacteria. Electron microscopy of *Porphyra umbilicalis* callus revealed that bacteria could be present within the callus cell walls, as well as on the surface of the thallus (unpublished

data). The maximal possible exposure to antibiotics was only slightly longer than the exposure time required to kill the contaminant microorganisms (Table 3). This illustrates that the side-effects of antibiotics on the growth and development of algae must be taken into account. Such effects have already been largely documented. For example, Darveau and Lynch (1977) reported that the antibiotic, prodigiosins, has special activity against certain blue-green and green algae. Antibiotics have been found to be excessively toxic to *Acetabularia* (Shephard, 1970). In land plant tissue, cell and callus cultures, some antibiotics were shown to inhibit the RNA and DNA synthesis and other systems (Kavi Kishor & Mehta, 1988; Satos & Salema, 1989). Therefore, it was necessary to allow the obtained axenic plant materials to recover by culturing for at least two weeks before using the tissues for further experiments.

Table 3 Optimal (o) and maximal (m) duration of treatment with antibiotic solutions (in days) for the axenisation of various marine macroalgae

Species	A3		A4	
	o	m	o	m
Chondrus crispus	5	7	4	7
Calliblepharis ciliata	3	5	3	5
Gelidium pusillum	4	5	4	5
Gigartina teedii	4	6	3	5
Gigartina acicularis	4	6	3	5
Gracilaria lenanciforuris a)	7	9	7	9
Gracilaria lenanciforuris b)	7	9	7	9
Gracilaria folifera a)	7	9	6	9
Polyides rotundus	6	8	5	8
Porphyra umbilicalis	7	10	7	12
Pterocladia capillacea	7	9	6	8
Laminaria digitata	6	8	5	7

a) tetrasporophytes, b) carposporophytes

It is also noteworthy that the liquid sterilization test medium was more sensitive than the solidified medium. Explants taken from the same treatment seemed bacteria free when planted onto the solid medium, whereas they proved to be contaminated in the liquid medium. It is likely that a liquid medium is more favorable for bacteria to grow or recover from the antibiotic treatment. This result stresses that different physical states of test media may lead to different conclusions. We, therefore, recommend using both a solid and a liquid medium. The solid medium offers a convenient way to detect contamination by eye and the liquid medium is more appropriate to check for complete sterility.

2. Tissue culture of *Laminaria digitata*, *Chondrus crispus* and other red algae.

The suitablility of these procedures as preliminary cleaning steps for tissue culture was investigated with sporophytes of *Laminaria digitata* and with various red

algae.

With *Laminaria digitata*, axenic explants excised from above the meristematic zone of the plant were grown on solid La medium. Calluses were obtained with a frequency of about 2% of explants after 30－45 days of culture. Callus formation occurred at the cut edge of the tissue. As shown in Pl. I, Fig. 3, cells along the cut edge underwent cell elongation and enlargement, followed by cell division which resulted in callus formation. After a further 60 days of culture in the solidified medium, multicellular filaments formed from the peripheral cells of the callus (Pl. I, Fig. 4). When transferred into liquid La medium and broken in small pieces, filaments grew rapidly, giving rise to masses of intertwined filaments which could be multiplied by mechanical fractionation. That such filaments are vestiges of aposporic gametophytes, and also reported by others on tissue culture of *Laminaria digitata* (Micalef and Cosson, personal communication), *Laminaria japonica* (Yan, 1984), *Laminaria saccharina* (Lee, 1985) and *Ecklonia radiata* (Lawlor *et al.*, 1988).

In *Chondrus crispus* axenic explants (1－2 mm in length)were excised from the whole plants, with the exception of the holdfasts. After 80－90 days of culture on La medium solidified with agar, about 1.5% of the explants formed callus-like structures at the cut end of the tissue (Pl. I, Fig. 5). When the callus-like structures with their origin tissue were transferred to liquid medium, they grew very slowly and did not release single cells, as expected from former experiments with *Pterocladia capillacea* (Liu & Gordon, 1987). After a further 5－6 months of culture on solid medium, the callus-like structures gave rise to new plantlets (Pl. II, Fig. 7), a result similar to that reported by Chen (1982). When the explants that did not bear calluses were incubated in liquid La medium, less than 1% of the explants gave rise to filamentous structures originating from the medullary layer of the explant. Such filaments were colored at the base and colorless in their apical part (Pl. I, Fig. 6). They are reminiscent of the filaments obtained in tissue culture of *Eucheuma alvarezii* and *E. uncinatum* (Polne-Fuller & Gibor, 1987) and *Agardhiella subulata* (Cheney, *et al.*, 1987). However, these filamentous structures did not develop further under our culture conditions over the next six months of culture.

Callus and callus-like structures capable of plant regeneration were obtained from a variety of other red algae using axenic explants prepared according to the procedures described here. They included *Porphyra umbilicalis* (Pl. II, Fig. 8) *Pterocladia capillacea* and *Gelidium pusillum* (Pl. II, Figs. 9 and 10).

In conclusion, axenic explants were obtained from several multicellular algae using a simple cleaning procedure which combines stirring in the presence of glass beads and incubation with antibiotic solutions A3 and A4. Most of the explants survived in culture and, depending on the species under investigation, low but significant proportions regenerated plantlets or gave rise to callus or callus-like structures. This provided opportunities to study in more detail the differentiation or dedifferentiation processes involved with micropropagation of macroalgae.

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References

Ballantine, D. L., 1979. The distribution of algal epiphytes on macrophyte hosts offshore from La Parguera, Puerto Rico. *Bot. Mar.* **22**: 107–111.

Berland, B. R. and S. Y., Maestrini, 1969. Study of the bacteria associated with marine algae in culture. II: Action of antibiotic substance. *Mar. Bio.* **3**: 334–335.

Berland, B. R., D. J. Bonin, and S. Y., Maestrini, 1972. Are some bacteria toxic for marine algae? *Mar. Bio.* **12**: 189–193.

Bradley, P. M., D. P. Cheney, and N., Saga, 1988. One step antibiotic disk method for obtaining axenic culture of multicellular marine algae. *Plant Cell, Tissue and Organic Culture* **12**: 55–60.

Brown, L., 1982. Production of axenic cultures of algae by an osmotic method. *Phycologia.* **21**: 408–410.

Chen, L. C. M., 1982. Callus-like formation from *Irish moss. Biol. Bull. nat. Taiwan Normal Univ.* **17**: 63–67.

Cheney, D. P., A. H.Luistro, and M. Bradley, 1987. Carrageenan analysis of tissue cultures and whole plants of *Agardhiella subulata. Proc. Int. Seaweed Symp.* **12**: 161–166.

Darveau, R. P. and D.L. Lynch, 1977. The antibiotic activity of prodigiosins against certain blue-green and green algae. *Phycologia.* **16**(3): 349–351.

Fries, L., 1963. On cultivation of axenic red algae. *Physiol. Plant.* **16**: 695–708.

Fries, L., 1970. The influence of microamounts of organic substances other than vitamins on growth of some red algae in axenic culture. *Br. Phycol. J.* **5**: 39–46.

Fries, L., 1984. Induction of plantlets in axenically cultivated rhizoids of *Fucus spiralis. Can. J. Bot.* **62**: 1616–1622.

Fries, L., 1985. Propagation of *Fucus* (Fucales, Phaeophyta)by hairs with trichothallic growth. *Phycologia.* **24**: 481–484.

Gibor, A., M., Polne, M. Biniaminov, and M., Neushul, 1980. Exploratory studies of vegetative propagation of marine algae: procedure for obtaining axenic tissue. *Proc. Int. Seaweed Symp.* **10**: 587–593.

Guillard, R. R. L. and J. H., Ryther, 1962. Studies on marine planktonic diatoms I. *Cyclotella nana* Hustedt and *Detonula confervacea* (Cleve)Gran. *Can J Microbiol.* **8**: 229–239.

Gusev, M. V., A. H., Tambiev, N. N Kirikova, *et al*, 1984 ..Obtaining of axenic explants from marine algae thallus. *Izv. Acad. Nauk USSR ser. Bol.* **5**: 761–721.(in Russian)

Gusev, M. V., A. H. Tambiev, N. N. Kirikova, *et al.*, 1987. Callus formation in seven species of agarophyte marine algae. *Mar. Biol.* **95**: 593 –597.

Hopkin, R. and J. M. Kain, 1978. The effects of some pollutants on the survival growth and respiration of *Laminaria hyperborea. Est. Coast. Mar. Sci.* **7**: 531–553.

Kavi Kishor, P. B. and A. R. Mehta, 1988. Effect of Rifampicin and Gentamycin on growth, organogenesis and carbohydrate metabolising enzymes in callus cultures of *Tobacco. Phytomorphology.* **38**(4) 327–331.

Lawlor, H. J., J. A. McComb, and M. A. Borowitzka, 1988. The Development of Filamentous and Callus-like Growth in Axenic Tissue Culture of *Ecklonia radiata* (Phaeophyta). *In*: Algal Biotechnology. Staler *et al.* Eds., Elsevier Appl Sciences, pp. 139–150.

Lee, T. F., 1985. Aposporous gametophytes formation in stipe explants from *Laminaria saccharina* (Phaeophyta). *Bot. Mar.* **28**: 179–185.

Lewin, J., 1966. Studies metabolism in diatoms V. Germanium dioxide, a specific inhibitor of diatom growth. *Phycologia* **6**: 1–12.

Liu, X. W., 1987. Tissue, Cell and Callus Culture of *Pterocladia* and *Porphyra* Seaweeds. MSc Thesis, Victoria Univ. of Wellington, New Zealand. 86 pp.

Liu, X. W. and M. E. Gordon, 1987. Tissue and cell culture of New Zealand *Pterocladia* and *Porphyra* species. *Proc. Int. Seaweed Symp.* **12**: 147–154.

Markham, J. W. and E. Hagmeier, 1982. Observation on the effects of germanium dioxide on the growth of macro-algae and diatoms. *Phycol.* **21**(2): 125–130.

McKracken, I. R., 1989. Purifying algal cultures. A review of chemical methods. *Proc. N. S. Inst. Sci.* **38**: 145－168.

Polne-Fuller, M. and A. Gibor, 1987. Calluses and callus-like growth in seaweeds: induction and culture. *Proc. Int. Seaweed Symp.* **12**: 131－138.

Polne, M., A. Gibor, and M. Neushul, 1980. The use of ultrasound for the removal of marine-algal epiphytes. *Bot. Mar.* **23**: 731－734.

Printer, I. and L. Provasoli. 1968. Heterotrophy in subdued light of 3 chrysochromulina species. *Bull. Misoki Mar. Bio. Inst., Kyoto Univ.* **12**: 25－31.

Provasoli, L., 1968. Media and Prospects for the Cultivation of Marine Algae. *Proc. Jpn. Conf. Hacone.* pp. 63－75.

Saga, N. and Y. Sakai, 1982. A new method for pure culture of macroscopic algae, the one step selection method. *Jap. J. Phycol.* **30**: 40－43.

Santos, I. and R. Salema, 1989. Penicillins and activity of nitrogen metabolism enzymes in plant tissue cul· Ture. *Plant Science.* **59**: 119－125.

Shephard, D. C. 1970. Axenic Culture of Acetabularia in a Synthetic Medium. In: Methods in Cell Physiolo· gy. Ed. Prescott, D. M. **IV**: pp. 49－69.

Yan, Z. M., 1984. Studies on tissue culture of *Laminaria japonica* and *Undaria pinnatifida*. , *Hydrobiologia.* **116/117**: 314－316.

Zhang, D., 1983. Study on the protoplast preparation, culture and fusion of somatic cells from two species of Green algae — *Ulva linza* and *Monostroma angicava* Kjellm. *J. Shandong coll. Oceanology.* **13** (1): 57－65.

Liu & Kloareg: Explant axenisation for tissue culture in marine macroalgae

PLATE I

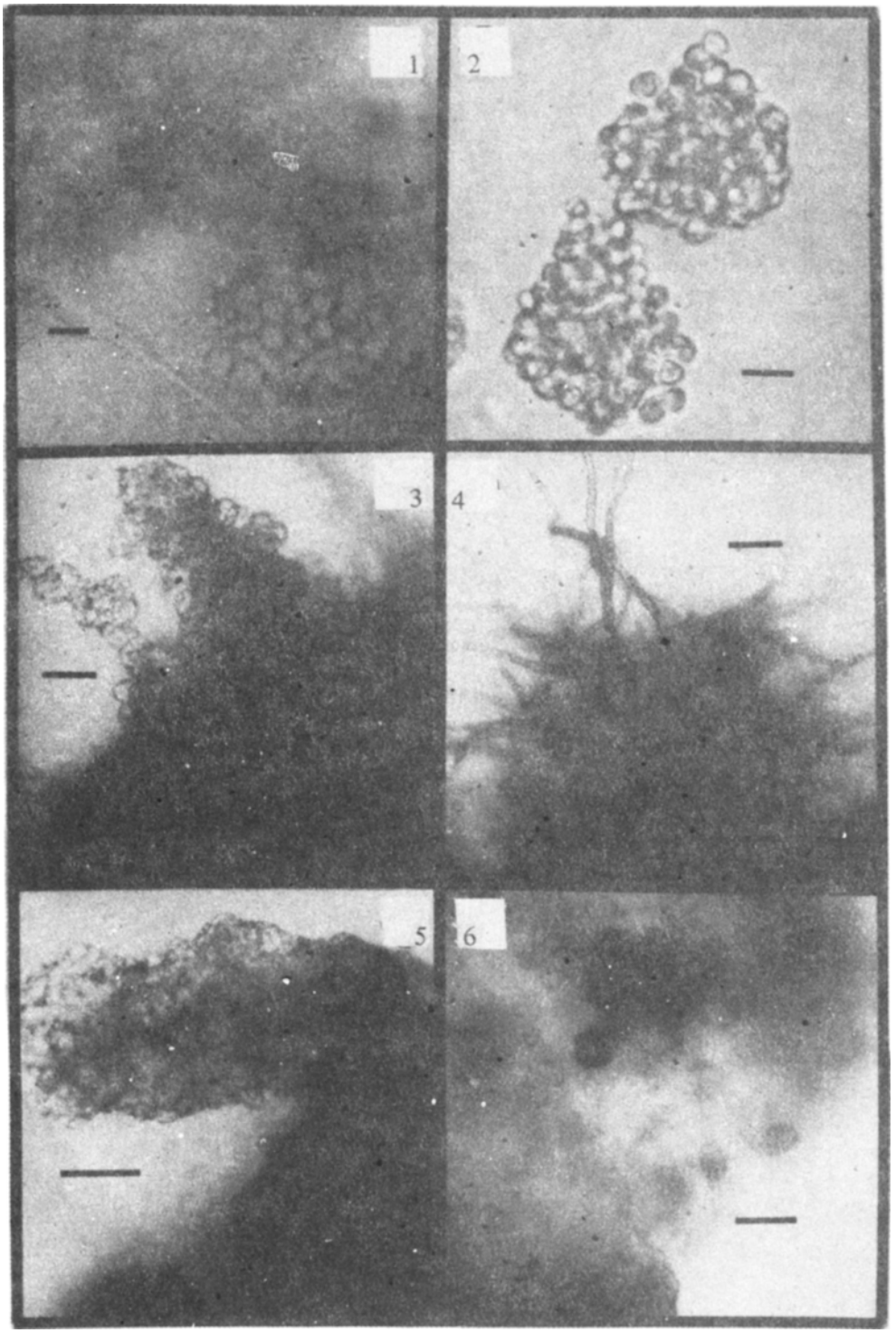

PLATE II