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# Strain selection and cell isolation of *Ulvaria oxysperma* (Kuetz.) Bliding (Chlorophyta) for net cultivation

Donald F. Kapraun & Stan G. Sherman
*Department of Biological Sciences, University of North Carolina, Wilmington, NC 28403-3297, USA*

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**Abstract**

North Carolina and Bermuda isolates of the green seaweed *Ulvaria oxysperma* (Kuetz.) Bliding were grown in cross-gradient light-temperature culture to determine the effects of varied photon fluence rates, temperature and photoperiod on their growth and reproduction. Results indicated significant genetic-based variation between the two populations.

Spheroplast were obtained from the vegetative thallus of the North Carolina isolate following enzymatic digestion with abalone gut extract and maceration. Single cell isolates developed new cell walls in 2–3 days and attached to nylon net substrate. Cultured isolates regenerated into juvenile plants with normal development and morphology.

Results are discussed in terms of the suitability of this economically important species for land-based mariculture.

## Introduction

Considerable success has been achieved during recent decades in developing techniques for single cell isolation and regeneration to improve agricultural plants (Cocking, 1983). In contrast, cell isolation and tissue culture research in seaweeds is a relatively new field and lags far behind that of land plants (Cheney, 1986; Cheney *et al.*, 1986). Only recently have attempts been made to apply this technology to marine algal mariculture needs such as simplification of propagation and improvement of genetic stock (Polne–Fuller *et al.*, 1984; Saga *et al.*, 1986). Nevertheless, viable protoplasts and spheroplasts have now been isolated from species of multicellular red, green and brown seaweeds (Millner *et al.*, 1979; Saga *et al.*, 1986; Cheney, 1986). However, identification of procedures for initiating subsequent regeneration of macrothalli has been more elusive (Xue–wu and Gordon, 1987; Polne–Fuller & Gibor, 1987). Notable success has been achieved with species of *Porphyra* (Zhao & Zhan, 1981; Tang, 1982; Polne–Fuller & Gibor, 1984; Fujita and Migita, 1985; Chen, 19860 and *Monostroma* (Zhang, 1983; Saga & Sakai, 1984) which are the most important cultivated seaweeds in Japan (Ohno, 1972; Nisizawa *et al.*, 1987).

Continuing efforts to encourage the utilization of marine algal resources in North Carolina currently center on strain selection and isolation of single cells for seeding of mariculture nets with *Ulvaria oxysperma*, which is closely related to

*Monostroma* (Bliding, 1968), and *Porphyra carolinensis*. Previously, field and laboratory investigations were conducted on these and other tropical and warm temperate species to provide basic information on their phenology as well as details of life history and karyology (Kapraun & Flynn, 1973; Kapraun & Luster, 1980; Freshwater & Kapraun, 1986; Kapraun & Freshwater, 1987; Kapraun & Lemus, 1987).

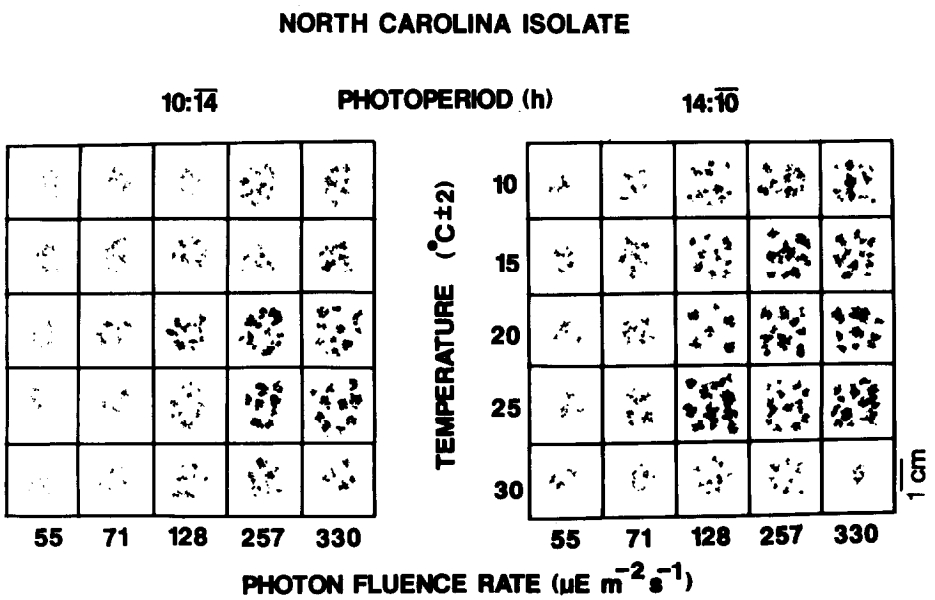
The present communication describes the process of selecting *Ulvaria oxysperma* strains appropriate for growth in a land-based irrigation mariculture system (Rheault & Ryther, 1983; Lignell & Pedersén, 1986). Information is provided as well for procedures employed to isolate spheroplasts, induce their attachment to commercial nylon nets, and to initiate their development into normal leafy blades. Results of parallel investigations on *Porphyra carolinensis* will be the subject of a separate report.

## Material and methods

*Sources of isolates.* *Ulvaria oxysperma* (Kuetz.) Bliding was collected from St. George's West, Bermuda, 20 May 1986 and from Wrightsville Beach, North Carolina, 15 January 1987. Unialgal cultures were maintained in enriched seawater medium (VSE) (Freshwater & Kapraun, 1986).

*Culture experiments.* Photon fluence rate, photoperiod and temperature effects on growth and reproduction were studied using a cross-gradient ligt-temperature apparatus which permitted the simultaneous culture of the isolates in 25 combinations of the two parameters (Fig. 1). Culture vessels were inoculated with 8–10 uniseriate (10–14 day old) germlings. Inoculation procedures and culture conditions have been previously described (Freshwater & Kapraun, 1986). Experiments in 10:14 and 14:10 light : dark cycle were conducted with temperatures ranging from 10–30 ± 2 °C (Fig. 1). Photon fluence rates ranged from 50–330 $\mu Em^{-2}s^{-1}$ and were measured with a Lamba I. Cor. PAR (Photosynthetic Active Radiation) quanta meter.

*Preparation and purification of spheroplast.* Single cells were isolated only from freshly collected North Carolina *Ulvaria oxysperma* specimens.




*Figs. 1–2.* Growth responses of *Ulvaria oxysperma* to photon flux densities and temperature regimes under 10:14 and 14:10 h LD photoperiods. Fig. 1. North Carolina isolate after 14 days. Fig. 2. Bermuda isolate after 28 days.

Vegetative thallus from the central blade portion was removed and washed in a 1% v/v seawater-soap (Sparkleen, Fisher Sci. Co.) solution three times to remove surface debris and epiphytes. Frond segments were then soaked for 2 min in 1% Betadine in seawater (Chen, 1986). Cleaned tissue was cut into < 10 mm pieces and blotted dry. 1.0 g fresh weight was placed in an Ehrlmeyer flask containing 25 ml enzyme solution. This consisted of 30 mg/ml abalone gut extract (Sigma Chem.) in 0.6 M sorbitol and sterile VSE (pH 6.0, salinity 35‰). The flask was then placed on a low speed shaker at room temperature (24 °C) for 30 minutes. Algal tissue was removed from the enzyme solution and washed in three changes of VSE with 0.6 M sorbitol (SWS) and the pH adjusted to 7.8. Blade portions were placed in a glass grinder with SWS solution to which chloramphenicol (Sigma Chem.) had been added at the rate of 1.0 mg/ml and macerated for 3 minutes. This mixture was filtered through a 120 $\mu$m nitex nylon net (Tetko, Inc.) and incubated at 20 °C for 24–48 hours. Cells were collected by centrifugation at 400 × *g* for 3 minutes and the resulting pellet resuspended in 10 ml VSE with chloramphenicol.

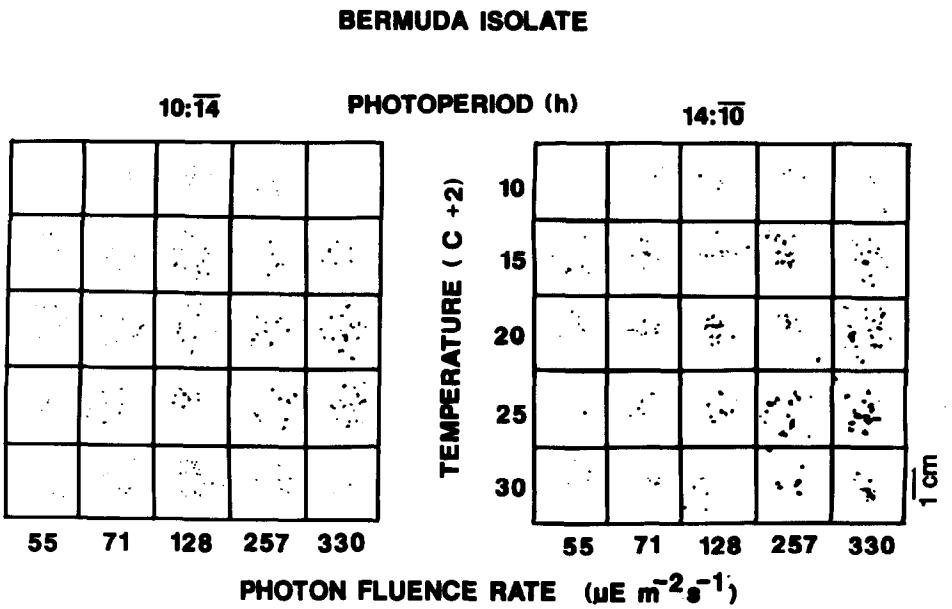
*Assessment of spheroplast viability.* Viability of liberated cells was assessed by the ability to 1) acculumate the vital stain neutral red (0.04% w/v) (Stadleman & Kinzel, 1972) and 2) exclude the mortal stain Evans blue (0.025% w/v) (Kanai & Edwards, 1973). Calcofluor (0.01% in SWS) was used to check for cell wall material on the isolated cells (Hughes & McCully, 1975; Millner *et al.*, 1979), and as a further test of their viability (Marchant & Fowke, 1977). Cells were stained for 5 minutes before determining spheroplast yield and viability with an AO Brightlinehemocytometer (Fisher Sci. Co.). Calcofluor staining was observed with an Olympus BH2–RFK fluorescence microscope.

*Cell attachment and regeneration.* Cells in SWS suspension, were diluted with VSE to 25–35 × $10^2$ cells/ml and placed in a culture vessel with squares of commercial nylon fish net (Memphis Net and Twine Co., # 9, 0.5 in. mesh). Attachment rate was determined by removing 4–6 pieces of net from the cell medium at 1-day intervals and incubating in VSE. Cell regeneration and plantlet development were monitored for 5–6 weeks.

## Results and discussion

*Cross-gradient culture.* Growth responses of *U. oxysperma* isolates from North Carolina and Bermuda in cross-gradient light-temperature culture under 10:14 and 14:10 photoregiems are shown in Fig. 1 and 2. Vegetative development was optimal in both photoperiods tested between 15–25 °C, and relatively reduced at 10 and 30 °C. Growth was positively correlated with photon flux densities, with apparent saturation at 257–330 $\mu Em^{-2} s^{-1}$ (Figs. 1 and 2). In both isolates, reproduction by biflagellate swarmers occurred in all photoperiod, temperature and photon flux density combinations, but was especially pronounced in 14:10 h photoregimes at 25 and 30 °C. The reduced vegetative development observed in these same culture conditions is therefore most likely attrubutable to blade erosion following sporulation rather than to inhibition of growth. Similar results have been reported in other marine algae (Edwards, 1969; Kapraun & Luster, 1980; Amsler, 1985; Freshwater & Kapraun, 1986) where higher temperatures increased reproductive effort at the expense of vegetative growth.

Growth and reproduction data obtained for *U. oxysperma* in cross-gradient culture were used to predict optimal periods for growth in nature. Coastal marine habitats in the vicinity of Wrightsville Beach typically experience an annual water temperature range in excess of 20 °C, ranging from 8 to 28 °C (Freshwater & Kapraun, 1986). Consequently, by extrapolating results from culture, where growth was best between 15 and 25 °C, *U. oxysperma* mariculture should be most successful during spring (March–May) and fall (September–November). Although the North Carolina isolate can survive the ambient temperatures of winter (c. 10 °C), minimal growth rates




would be expected unless supplemental heating is provided.

Replicate cross-gradient culture experiments demonstrated a consistent and significant difference between vegetative development in the North Carolina and Bermuda isolates. While cultures of the former produced blades 6–8 mm in diameter within 14 days under optimal conditions, the maximum size of blades formed by the Bermuda isolate after 28 days was 3–4 mm in diameter. These varied growth patterns under identical culture conditions strongly suggest that these *U. oxysperma* isolates represent discrete populations with genetically based physiological variations, i.e. ecotypes. Similar variations in growth and reproductive responses have been documented for regional populations of red algae (Edwards, 1970, 1971; Kapraun, 1978a, 1978b, 1979).

In the case of the economically important seaweed *U. oxysperma*, these findings suggest that a systematic screening of regional isolates in culture could greatly benefit attempts to select strains with desirable characteristics for mariculture.

*Spheroplast isolation. Ulvaria oxysperma* cells isolated in the enzyme solution were initially oval to pear-shaped, but gradually became spherical (Fig. 3). Tests with neutral red (Figs. 3 and 4), Evans blue and Calcofluor indicated that approximately 90% of these cells were viable. Calcofluor staining viewed with a fluorescence microscope showed that isolated cells retained some cell coating external to the plasmalema, and should therefore be referred to as spheroplast (Adamich & Hemmingsen, 1980). Typical yield of viable cells was 25–35 × $10^4$ cells/ml from 1 g of tissue. These results compare favorably with yields reported for *Enteromorpha, Monostroma* and *Ulva* (Saga, 1984).

Previous reports of single cell isolation from seaweeds have cited the necessity of using axenic tissue as bacteria thrive on the enzyme-osmoticum solution (Berliner, 1981; Zhang, 1983; Polne-Fuller & Gibor, 1984, 1987; Polne-Fuller *et al.*, 1984; Polne-Fuller *et al.*, 1986; Chen, 1986; Wue-wu & Gordon, 1987). However, successful techniques have been developed for isolation of single cells from unialgal (but not bacteria free) tissue which are more rapid and still result in high levels of viability (Sage, 1984; Saga *et al.*, 1987).

Since the objective of the present study was to use isolated cells for propagation in a mariculture system rather than to initiate tissue cultures or cells for somatic hybridization, we attempted to

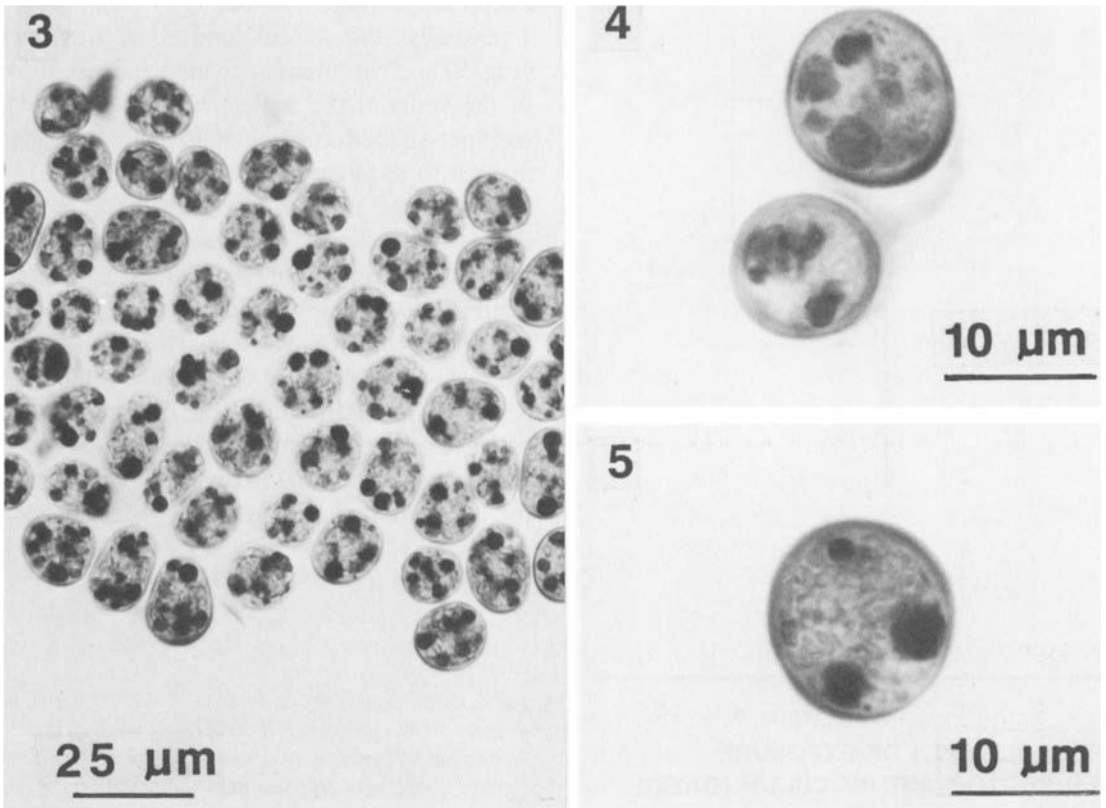
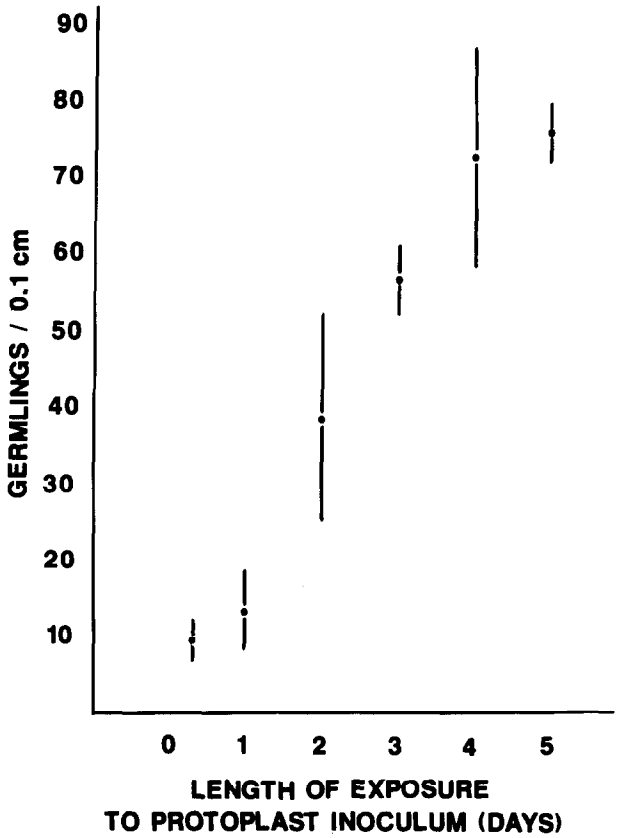



*Fig. 3–5.* Spheroplast isolation in *Ulvaria oxysperma*. Fig. 3. Monostromatic tissue after incubation in SWS showing release of cells from margin. Fig. 4. Released cells with cell wall remnants after staining with neutral red. Fig. 5. Isolated cells stained with neutral red following enzyme treatment and maceration.

modify these unialgal procedures for *Ulvaria*. Initially, an osmoticum with high concentrations (< 1M) of sorbitol (Millner *et al.*, 1979; Saga, 1984) or manitol (Cheney *et al.*, 1986) was used. Despite the use of antibiotics, contamination resulted in significant reduction of cell viability. A resuspension and enzyme medium was finally developed with 0.6 M sorbitol and antibiotic concentrations sufficient to give high levels of viability in cell suspensions, even after extended periods of incubation (24–48 hours). Once cells were transferred to VSE for net seeding, sufficient cell wall had formed so that an antibiotic is no longer required.

*Spheroplast attachment and regeneration.* Results of attachment experiments indicate that cells were able to attach to nylon net within 3 hours after harvest in resuspension medium (Fig. 6). Attachment rate was highest during the initial 48 hours of net seeding, and gradually decreased. Additional experimentation will be required to determine optimal germling density and, thus, cell concentration of inoculum and length of seeding. However, preliminary results suggest that most attached germlings were able to develop into leafy thalli at densities up to 5 per linear mm of net (Fig. 7).

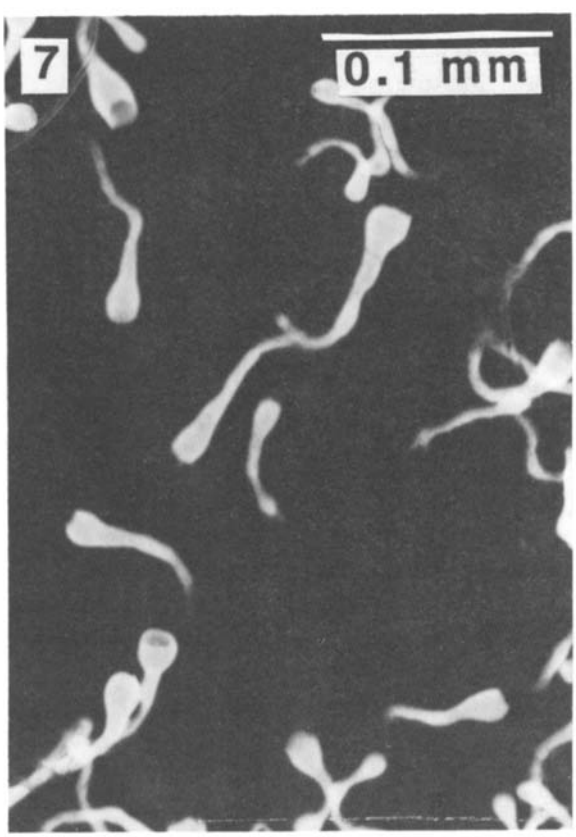
*Ulvaria oxysperma* germlings have a unique and characteristic developmental sequence (Bliding, 1968; Kapraun & Flynn, 1973). Swarmers released from marginal cells (Fig. 11) germinate to form an erect filament which is initially uniseriate,

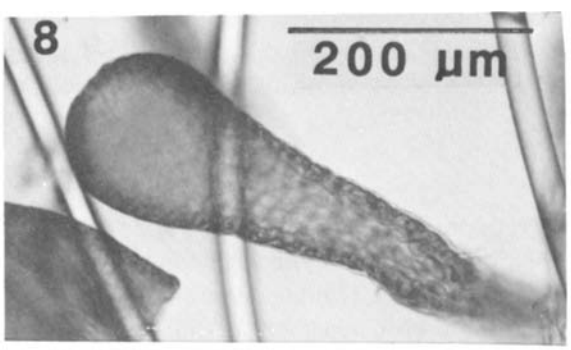


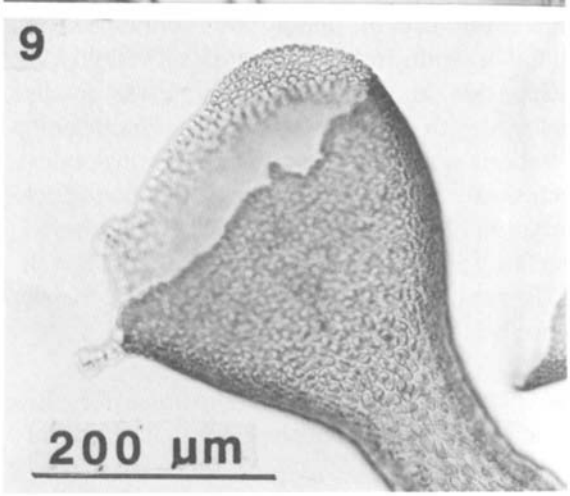

becoming multiseriate and then tubular (Fig. 8). Eventually the distal end becomes swollen (Fig. 9) and ruptures following the decomposition of the walls of the upper cells. The lobes of the trumpet-shaped tube then expand laterally and grow into monostromatic blades (Fig. 10).

Results of the present investigation indicate that spheroplasts from the vegetative central thallus area retain a high degree of tcipotency, exhibiting a developmental sequence as detailed above. In contrast, spheroplast from blade margins often undergo a division sequence after resuspension. Although these cells remain viable, they were not observed to regenerate. A similar difference in potential to divide has been reported for cells isolated from various areas of *Porphyra perforata* J. Ag. blades (Polne–Fuller & Gibor, 1984).

*Fig. 6.* Germling density on nylon net following varied lengths of exposure to spheroplast inoculum suspension. Data points = number of germlings/ 0.1 cm determined from counts on 5 nets. Vertical lines indicate SD.



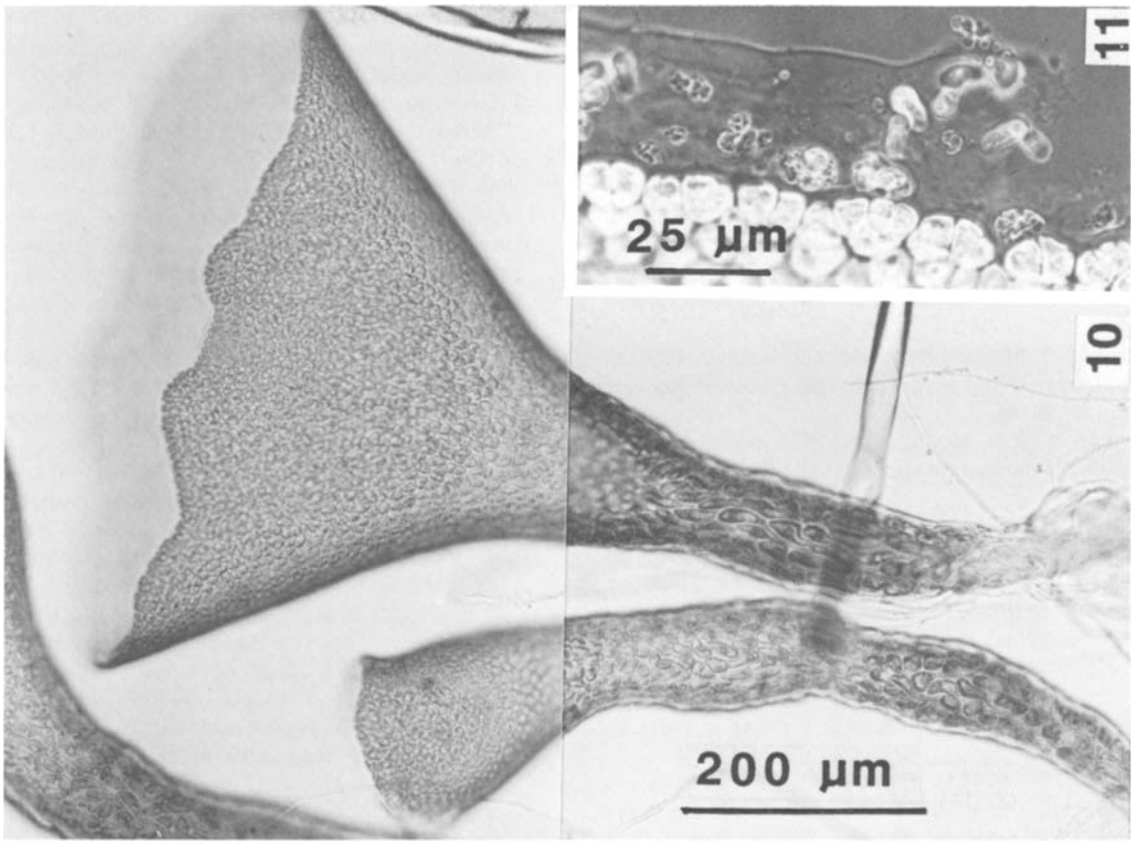



*Fig. 7–11.* Developmental stages of *Ulvaria oxysperma* on nylon net in VSE. *Fig. 7.* Germling development and density pattern 14 days after inoculation. *Fig. 8.* Hollow, monostromatic tube with inflated distal end. *Fig. 9.* Tubular thallus following rupture of distal end. *Fig. 10.* Trumpet-shaped monostromatic thallus after 21 days. *Fig. 11.* Release of biflagellate swarmers from marginal cells.

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