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Time-Lapse Video Microscopy of Wound Recovery and Reproduction in the Siphonous Green Alga *Derbesia tenuissima*

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Wound recovery: Responses to various types of mechanically induced wounding were followed in the giant-celled Caulerpalean species, *Derbesia tenuissima*, using time-lapse video-microscopy. Gametophyte vesicle cells. Puncture wounding: the gametophyte cell seals the puncture in 5 min. This is followed by cycles of ruptures and sealing, ending with full recovery in 24 hrs. Cut wounding: the protoplast immediately retracts away from the wall and reforms an intact, deflated protoplast that expands to fill the original cell within 21 hrs. Crush wounding (internal). When retained within the cell wall many protoplast fragments condense, round up, and coalesce; the reconstituted protoplast expands until it attains complete recovery, filling the original cell shape in 12 hrs. Crush wounding (external). Protoplast fragments extruded from the crushed cell are more numerous and smaller taking longer to recover. Most fragments become spherical, transforming into small viable cells capable of reproduction in several days. Sporophyte filaments. Crush wounding creates many small fragments that initially condense, coalesce and then expand within the wall to restore a complete filament with normal cytoplasmic streaming within 5 hrs. **Reproduction:** gametophyte. Our culture isolates produce more females than males (30:1). Gametangia develop one day before discharge that occurs explosively (1/6 sec) at first morning light. The vesicle cell forms successive gametangia every 14 days. Sporophyte. Each sporangium develops on a lateral branch that becomes isolated by the creation of successive basal plugs. After cytoplasmic cleavage and differentiation the stephanokont spores are discharged. The spores settle quickly and germinate forming gametophyte cells.

Key Words: *Derbesia tenuissima*, gametangium & sporangium discharge, protoplast, video microscopy, wound responses

INTRODUCTION

Green algae of the order Caulerpales are multinucleate, siphonous and exhibit cytoplasmic streaming. Giant-celled species are well represented. Since these cells are vulnerable to wounding in their marine environment from sand abrasion, wave action and/or grazing efficient wound repair is critically important for their survival. The efficiency of this wound repair is indicated by the way giant-celled architecture has survived essentially unchanged for at least 400 million years (Menzel 1988).

Wound responses of various siphonous green algal species in the Caulerpales and Siphonocladales-Cladophorales complex have been well studied (for reviews see Menzel 1988). Response phenomena have been classified into three main types as follows: i) localized contraction of the cytoplasm around the wound; ii) formation of protoplasts from damaged cytoplasm; and iii) formation of a wound-plug at the wound site.

Protoplast formation and subsequent regeneration into new plants is well described in *Bryopsis* (Kim *et al.* 2001; Pak *et al.* 1991), *Microdictyon* (Kim *et al.* 2002) and *Chaetomorpha* (Klotchkova *et al.* 2003). Upon wounding, cytoplasm is extruded from the cell into seawater, fragments, and rapidly transforms into spherical protoplasts which, although essentially naked, are initially enclosed by a highly refractive, gelatinous primary envelope while they synthesise new membranes (plasmalemma and tonoplast) and cell wall. When the cytoplasm of *Bryopsis maxima* Okamura was dissociated into sub-cellular fractions, isolating the chloroplasts in one fraction, these fractions, when mixed, reassembled into numerous viable protoplasts (Kobayashi and Kanaizuka 1985). Thus, completely disrupted cytoplasm can reform into viable cells. *Derbesia* (Caulerpales) has not been studied in this context until now.

Although there are some descriptions of wound

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responses in *Derbesia* [e.g., the ability of *Derbesia* spp. to seal small puncture wounds (Ziegler and Kingsbury 1964), or to form protoplasts after wounding (Klemm 1894; Rietema 1973; Ziegler and Kingsbury 1964)], this work has not been followed up. Rietema (1973) studied the long-term development of *Derbesia marina* (Lyngbye) Solier plants derived from protoplasts created from wounded plants.

Our information on wound responses in other algal species has been gained from still images, fluorescence microscopy, transmission electron microscopy (TEM) and histochemical studies. However, these techniques do not afford a detailed understanding of the dynamics of wound responses. Such data can be obtained most readily from time-lapse video microscopy, as early work on chloroplast movement in *Derbesia tenuissima* (Moris et De Notaris) P. Crouan et H. Crouan has demonstrated (McNaughton and Goff 1990). Our work here is the first to follow these algal wound responses with video microscopy in the genus *Derbesia* which, as we will show, displays a range of wound responses.

During the diplohaplontic life cycle of *D. tenuissima* the filamentous, diploid sporophyte alternates with the morphologically different vesicular, haploid gametophyte. So different are the two that the gametophyte was originally classified as a separate genus, *Halicystis*, until it was discovered to be the haploid phase (Feldmann 1950; Kornmann 1938). Male and female gametophytes appear identical until gametangia develop, the female gametangium being dark brown to black and the male being a lighter olive-green. Gametangial formation and gamete release have been the focus of most research on *Derbesia* spp. (Page and Kingsbury 1968; Page and Sweeney 1968; Wheeler and Page 1974). Page and Sweeney (1968) investigated the pronounced, regularly recurring periodicity of gamete formation in *D. tenuissima*. Formation of gametangia displays an endogenous rhythm with a basic period of 4-5 days, which is little affected by light or temperature variation. *D. tenuissima* can also form new sporophytes by parthenogenetic development of the female gametes (Ziegler and Kingsbury 1964).

Much uncertainty and controversy exist about the exact life history seen in different isolates of *D. tenuissima* investigated by different workers. Neumann (1969, 1974) provides an excellent analysis of the life history. Whether karyogamy in an Italian strain of *D. tenuissima* occurs immediately after plasmogamy (Lee *et al.* 1998, 2000, 2001) or whether the nuclei of the sporophyte of a Yugoslavian strain are apparently haploid until sporangia form when karyogamy and meiosis occur (Eckhardt *et al.* 1986, Schnetter and Eckhardt 2000) is not clear. Eckhardt *et al.* (1986) also observed this same feature in *D. marina* from Spain and *D. novae-zealandiae* V. Chapman from Western Australia. The *D. tenuissima* culture used by Lee *et al.* (1998, 2000, 2001) was sexually reproductive and also exhibited a very low rate of parthenogenesis of the female gametes giving rise to a succession of haploid sporophytes, haploid stephanokont zoospores and female gametophytes. The Colombian *D. tenuissima* culture used by Schnetter and Eckhardt (2000) reproduced parthenogenetically and did not produce male gametophytes. It was thus permanently female. In their interpretation of the differing results derived from these two stock cultures, Lee *et al.* (2000) have proposed that the sporophyte used in Schnetter and Eckhardt's experiments may have been derived from parthenogenic female gametes, which contained only female haploid nuclei and only produced female gametophytes. If this is true, it is still to be determined which of these life cycles is the most typical for the species.

MATERIALS AND METHODS

Cultures

Three strains of *Derbesia tenuissima* were used. Strain 2773-1 was epiphytic on *Laurencia brongnartii* J. Agardh collected at White Beach, Batan I, Philippines on 22 April 1987. Strain 3440 was epiphytic on *Claviclonium ovatum* (Lamouroux) Kraft et Min-Thein collected by John Huisman, from Map Reef, Yanchep, West Australia on 15 November 1994. Strain 4303 was supplied by Sang Hee Lee, originally collected and isolated by Dr. Mitsuo Chihara from Naples, Italy in August 1990.

Stock unialgal cultures were maintained in Pyrex 100 x 80 mm deep storage dishes, containing modified Provasoli's medium (MPM/2, 10 ml enrichment per litre of natural seawater adjusted to 30 psu salinity with MilliQ water; West and McBride 1999). Subcultures for experiments were maintained in Pyrex 70 x 50 mm crystallizing dishes in the same medium. Strain 2773-1 and strain 4303 were kept at 22 ± 2°C under cool-white fluorescent light at 30 ± 5 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photon flux density with an 11:13 hrs L:D cycle. Strain 3440 was kept at 20 ± 1°C under cool-white fluorescent light at 30 ± 5 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photon flux density with an 12:12 hrs L:D cycle.

Gametophytes were initially isolated from the stock sporophyte cultures by pipetting young sporelings into a fresh Pyrex 70 x 50 mm crystallizing dish. To determine the sex of the gametophytes and also whether the released

gametes developed parthenogenetically, single gametophytes were further isolated by transferral into either a Pyrex 70 x 50 mm crystallizing dish, or a single well (10 ml) of a Falcon 6-well tissue culture plate. Gametophytes were also obtained by extruding the cytoplasm of a mature, vesicular gametophyte into seawater to form protoplasts (Rietema 1973) that developed into new gametophytes.

Wounding

Puncture wounds were created with either a fine Sharps No. 10 steel needle mounted in wood dowel, or a fine glass needle. Cut wounds were created with a scalpel or a single edged razor blade. Crush wounds were created with a Teflon spatula if the cell was large enough, or between two glass slides, if not. For observing protoplast formation external to the cell wall, the cell was ruptured with a suitable implement, and the cytoplasm extruded into the seawater using forceps. Where possible, the cell was wounded in the container in which it was to be filmed. If this was not feasible, the cell was either wounded in a small Petri dish or on a glass slide, in as little seawater as possible to prevent the cell from moving.

Preparation for microscopy

Samples were mounted for filming into various chambers, depending on their size, the duration of video filming and the type of microscope used. For large cells and/or long duration video work on the dissecting microscope, samples were placed in a 15 x 60 mm plastic Petri dish filled with medium. A second Petri dish bottom inserted on top created a chamber without air. This combination was sealed with Parafilm to prevent evaporation. Specimens to be followed with a compound microscope were placed in medium between two coverslips that were sealed on either side of stainless steel slides with a rectangular (15 x 25 mm) opening. The coverslips were secured using Valap (1:1:1 mixture of vaseline, lanoline and paraffin wax, melted at about 40°C). If the specimens were small enough and filming was not long term, they were sealed directly onto a clean glass slide using a coverslip and Valap. If more space was required under the top coverslip, #2 or #3 square coverslips were used as spacers and sealed onto the glass slide, just underlapping the edges of the top coverslip.

For filming of cytoplasmic streaming, tip segments of the filaments were cut with fine microdissection scissors (Roboz Surgical Instrument Company, Rockville, MD, USA), transferred to a Petri dish and allowed to recover, usually overnight. Healed segments were then transferred to the appropriate chamber for video observation.

Video microscopy

Prepared samples were examined in one of the following ways. Low magnification bright-field images were obtained on a Leica MZ8 dissecting microscope with Leica CLS150 fibre optic light supply, and imaged via a Panasonic camera (AW-E600E). Higher magnification images were obtained using a Leica DMRB or a Zeiss Universal Microscope with either Nomarski or phase-contrast optics, and imaged via a Panasonic F250 colour video camera. Time-lapse sequences were recorded on a Panasonic re-writable videodisc recorder VDR- V1000P. In order to reduce light intensity to the cell being followed, a shuttering system was sometimes used (Pickett-Heaps and West 1998). Real-time sequences were recorded on a Panasonic videocassette recorder AU-650B. For reproduction, single video frames were exported to a computer using a Targa Pro 2000 video board and capture software. Figures were assembled using Corel Draw 10 and Corel Photopaint 10.

Fluorescence microscopy

To follow cell wall formation, protoplasts were placed on poly-L-lysine coated coverslips and, at various times after formation, stained for 5 min with Calcofluor White (Fluorescent Brightener 28, Sigma-Aldrich, Castle Hill, NSW, Australia), diluted with sterile seawater to a concentration of 100 μg/ml (Kim *et al.* 2001). The protoplasts were then washed and mounted in the culture medium. Preparations were examined on an Olympus BH-2 fluorescent microscope using a UV filter. Images were captured using a Leica DC 300F digital camera.

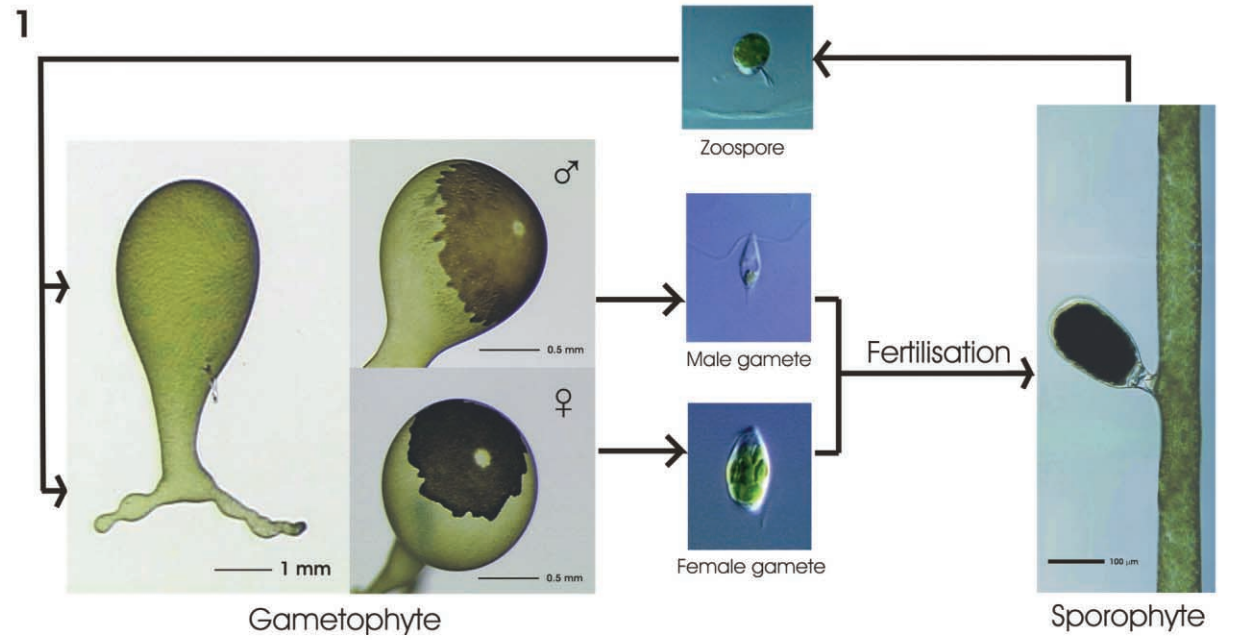
RESULTS

Gametophyte wound response

Most wounding experiments were carried out on the gametophyte because the cells are larger and more robust than those of the filamentous sporophyte (Fig. 1).

Puncture wounding

When punctured, the gametophyte protoplast (Fig. 2A) exhibited a very rapid centripetal contraction; turgor pressure was lost and the cytoplasm retracted away from the wound site. No wound plug material was extruded at the wound site (Fig. 2B). Within a minute of wounding, the broken edge of the cytoplasm darkened and became rounded. Between 1 to 2 min after wounding, the hole in



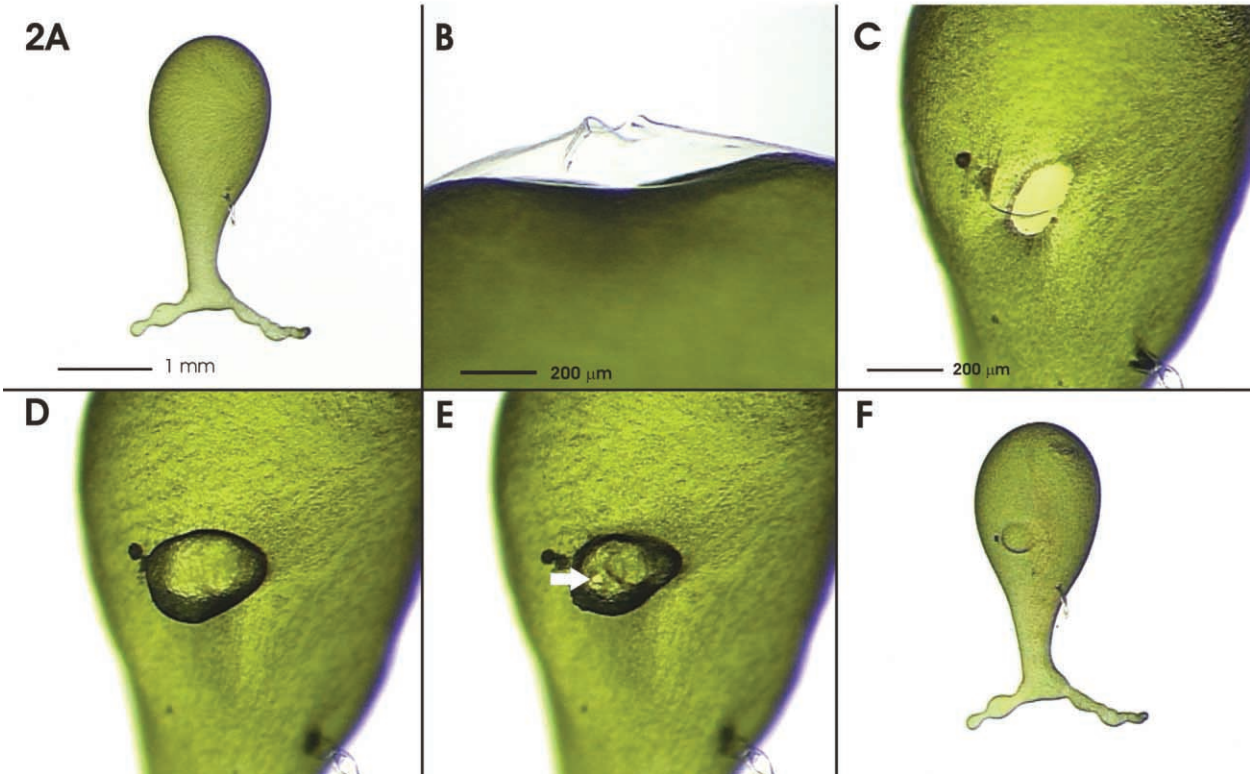


Fig. 1. Diplohaplontic life cycle of *Derbesia tenuissima*. (Note: Figure is composed of images taken from all three strains of *D. tenuissima*).

Fig. 2. *Derbesia tenuissima* gametophyte (3440): response to puncture wounding. (A) Intact gametophyte cell. (B) Puncture wound viewed from side. (C) Centripetal closure begins 1 to 2 min after wounding. (D) Cytoplasm ballooning out through the hole in the cell wall around 4 min after wounding. (E) Distending cytoplasm ruptures again at the initial wound site (arrow). (F) Fully recovered cell (approx. 24 hrs later).

the cytoplasm began to constrict centripetally (Fig. 2C). Within 4 to 5 min, the hole in the cytoplasm was closed whereupon turgor pressure began to build in the vacuole again, causing the protoplast to balloon through the hole in the cell wall (Fig. 2D). When the distension became too great the protoplast ruptured again (Fig. 2E). These ruptures varied in position and were much smaller than in the initial wound; they were just big enough to release the pressure and allow the expanded protoplast to collapse partially. These small ruptures were immediately sealed, and the cycles of sealing and rupture continued with diminishing intensity. The interval between the expansions and contractions was variable, ranging in one recovering cell from 23 to 341 sec. A time-lapse sequence revealed small, localised contractions occurring in the cytoplasm surrounding the constricted wound site, just after the wound had closed. All cells fully recovered from puncture wounding (Fig. 2F) after about 24 hrs, and most were able to form gametangia later.

Cut wounding

When vesicular gametophyte cells were cut open, the response was also a centripetal contraction. The entire cut edge of the protoplast constricted, pulling away from the wall (Fig. 3A). Once the wound had closed (Fig. 3B), the entire cytoplasmic mass retracted towards the basal end of the cell (Fig. 3C) before the pressure in the vacuole increased, causing expansion of the deflated protoplast (Fig. 3D). The cytoplasm around the wound site became much darker, presumably due to the migration of chloroplasts to the wound area (Fig. 3D). As with the puncture wounding, a series of pressure fluctuations followed, visible as expansions and retractions of the protoplast (Fig. 3E). Throughout wound repair, the cytoplasm was actively streaming and thin cytoplasmic bands of organelles moved across the field in waves. A new cell wall was eventually formed over the protoplast and 21 hrs after wounding the cell appeared to be fully repaired, although somewhat smaller than the original (Fig. 3F). In time, the repaired cell developed into a full sized gametophyte.

Crush wounding

When the cell's cytoplasm was broken apart by crushing with forceps, numerous fragments of various sizes were created within the cell wall (Fig. 4A). Quite often the cytoplasm in the rhizoidal base was less disrupted than in other parts of the cell, allowing it to close centripetally, and form a vacuole. The fragments within the cell wall condensed and became rounded. The largest quickly developed a vacuole and began to expand at 35 to 40 min after wounding. Eventually the expanding fragments met and fused to form a single protoplast (Fig. 4B).

By this stage the protoplast had again come into contact with the cell wall, and as turgor pressure in the vacuole increased, the protoplast began to re-inflate. The protoplast burst and retracted every time the pressure became too great, and the cell deflated a little before the pressure increased again. These pressure fluctuations created an impressive spectacle in time-lapse and caused the whole protoplast to move with a rocking motion. In one experiment this pressure variance occurred 33 times before the cell was fully re-inflated. As with the puncture wound, the cycle intervals were variable, for example between 2 and 13 min and most commonly around 5 min. Complete sealing of the cytoplasm in this cell took 3 hrs, and complete recovery occurred in 12 hrs (Fig. 4C). Cytoplasmic streaming was restored before the cell was fully re-inflated.

Protoplast formation after extrusion into seawater

When the cytoplasm was extruded from the wall into seawater, it fragmented into small pieces (Fig. 5A). Within 2 to 10 min, depending on their size, the fragments contracted and became spherical (Fig. 5B-C). The larger fragments took longer to become spherical than the smaller ones. The cytoplasm underwent extensive reorganisation by folding and rolling, causing the protoplast to rotate. Once rotation had stopped, contractions ran around the edge of the protoplast, usually alternating in opposite directions. In one protoplast, contractions lasted from 40 to 96 sec. A primary envelope, which was a highly refractive sheath, developed around the protoplasts (Fig 5D). High magnification revealed vesicles moving within this envelope. These vesicles increased in size towards the outer edge of the envelope (Fig. 5E-F).

Protoplast cell wall formation

A time-course staining experiment was conducted with protoplasts formed by extruding cytoplasm into seawater containing Calcofluor White (Fig. 6A). The images show that deposition of cell wall material at the edge of the primary envelope did not begin until 2-3 hrs after formation (Fig. 6B-C). The cell wall material became smoother and thicker over time (Fig. 6D-E) and by 20 hrs the protoplasts were well covered by a new cell wall (Fig. 6F).

Protoplast development inside the original gametophyte cell wall

When the cytoplasm was extruded from the gameto-

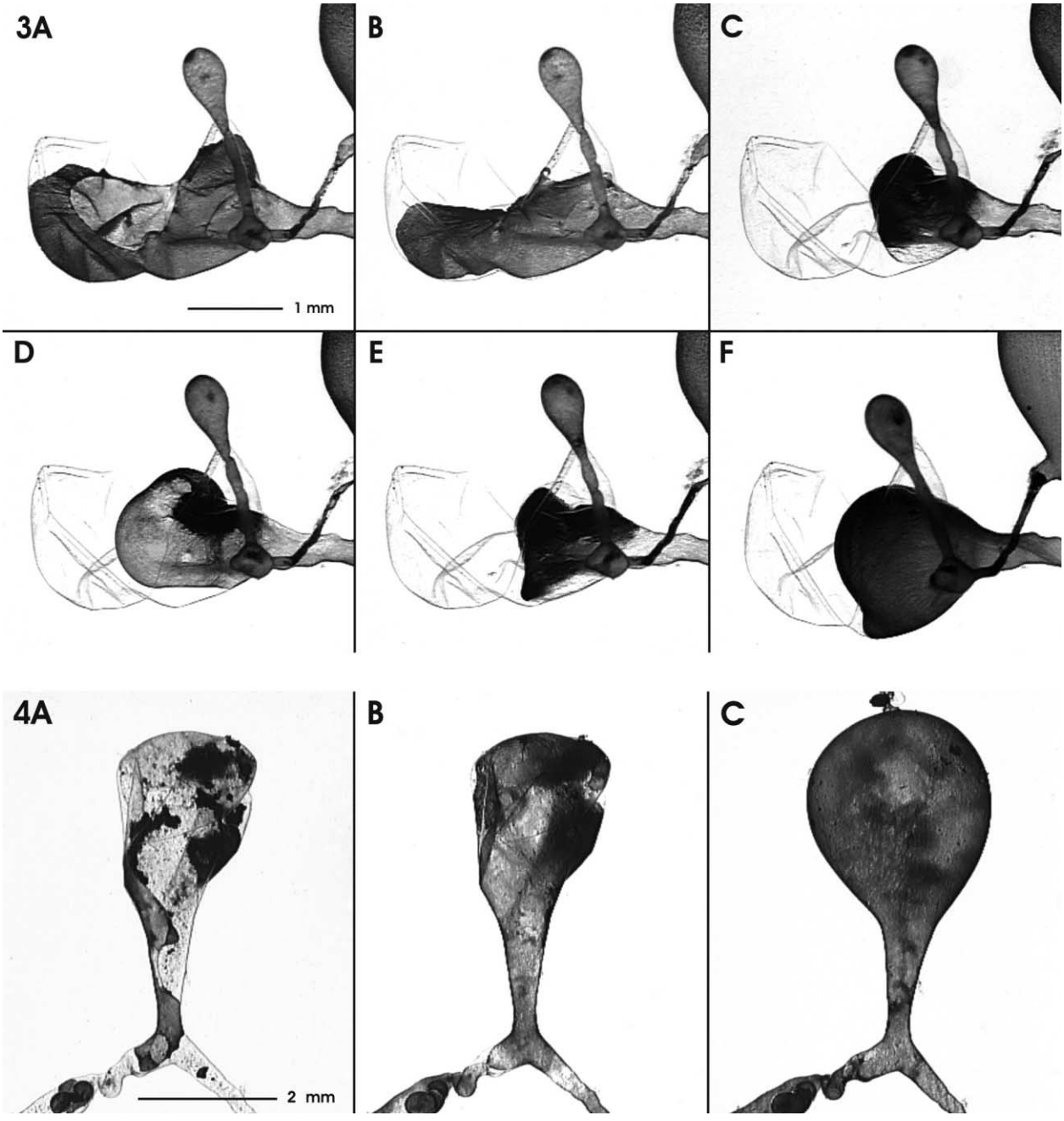


Fig. 3. *Derbesia tenuissima* gametophyte (2773-1): response to cut wounding. (A) Cut gametangial lobe with edge of cytoplasm beginning to constrict centripetally and pull away from cell wall. (B) Wound in cytoplasm has closed. (C) Cytoplasm has retracted and condensed. (D) Expansion of protoplasm. The cytoplasm around the wound site is very dense. (E) Retraction as protoplast ruptures. (F) Recovered cell 21 hrs later.

Fig. 4. *Derbesia tenuissima* gametophyte (2773-1): response to crush wounding. (A) Crushed cell with fragmented cytoplasm, shortly after wounding. (B) Expanding fragments have fused and formed a single protoplast. (C) Recovered cell, 12 hrs after wounding.

phyte and some fragments were left inside the wall, these also readily formed protoplasts (Fig. 7A), which usually expanded more rapidly than external protoplasts. Expanding internal protoplasts did not develop a thick primary envelope like external protoplasts, but tended to elongate elliptically before rapidly expanding radially. They readily fused with others nearby (Fig. 7B), creating larger protoplasts which continued to expand and fuse until they reached the cell wall. Expansion occurred more readily in protoplasts that remained in the rhizoidal base, or were trapped within a fold of the cell wall and somewhat protected from the external environment.

Two protoplasts retained close to the cut in the cell wall and therefore the external environment, started out behav-

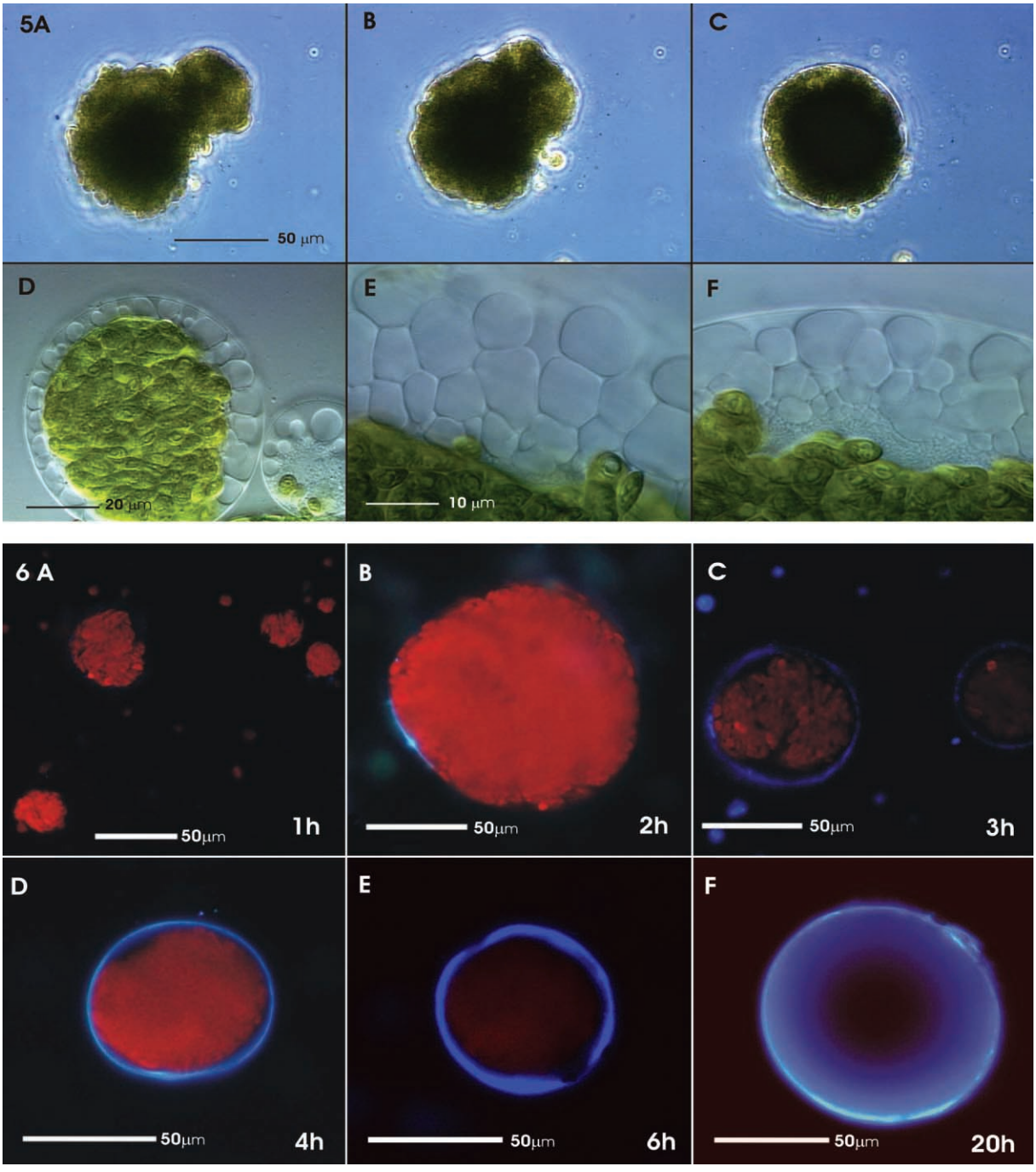
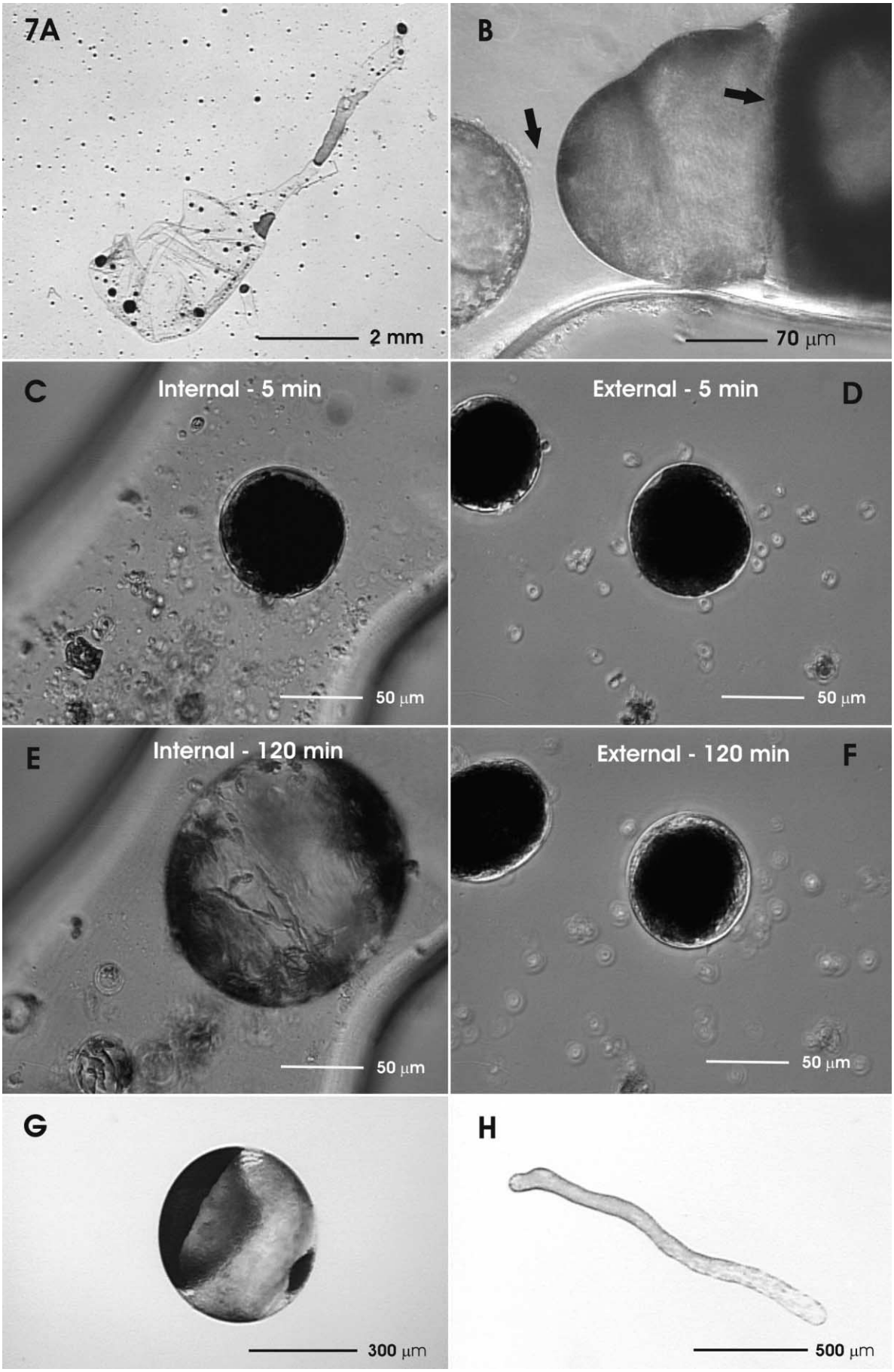


Fig. 5. *Derbesia tenuissima* gametophyte (2773-1): protoplast formation. (A) Irregular fragment of cytoplasm, extruded into seawater. (B) Cytoplasm condenses and rounds up. (C) A spherical protoplast, 10 min after extrusion. (D) A primary envelope containing vesicles appears around the protoplasts. (E) & (F) Vesicles increase in size towards the outer edge of the envelope.

Fig. 6. *Derbesia tenuissima* (4303): protoplasts at various developmental stages stained with Calcofluor White. (A) 1hr & (B) 2 hrs: a faint wall deposit on one side of the protoplast. (C) 3 hrs: uneven deposition of cell wall material. (D) 4 hrs & (E) 6 hrs: cell wall material becomes thicker and more evenly distributed. Red chloroplast autofluorescence is visible. (F) 20 hrs: protoplast is well covered by a new cell wall. The wall thickness and the Calcofluor fluorescence obscure the chloroplast autofluorescence.

ing like internal protoplasts, but soon stopped expanding and instead condensed again and developed a thick primary envelope, like the external protoplasts. Two protoplasts of the same initial diameter of 70 μm, one internal (Fig. 7C) and the other external (Fig. 7D), were filmed over the same time period. The internal protoplast expanded rapidly, and had doubled in size to 150 μm diameter within 120 min (Fig. 7E). It then fused with a larger expanding protoplast


7A
2 mm
B
70 μm
C
Internal - 5 min
50 μm
External - 5 min
D
50 μm
E
Internal - 120 min
50 μm
External - 120 min
F
50 μm
G
300 μm
H
500 μm

at around 140 min after formation. The controlled pattern of expansion was also seen in other expanding, internal protoplasts, where the protoplast had a clear girth and dense cytoplasm at each pole. In contrast, the external protoplast did not expand over the same time period, but instead developed a thick primary envelope (Fig. 7F).

Development of protoplasts formed by extrusion

Each crushed vesicular cell produced over one hundred protoplasts that developed into new gametophytes. Protoplasts derived from the one cell and kept in the same culture dish did not always develop the same morphology. After 13 days some remained spherical (Fig. 7G), some became clavate (not shown) and some became elongated (Fig. 7H). Protoplasts only 13 days old were capable of producing gametangia in synchrony with the endogenous rhythm of gametangial formation exhibited by the mature undamaged gametophytes (Fig. 7G). However, they were unable to release the gametes, possibly due to their small size, and instead the gametangial mass detached from the cytoplasm and sank to the bottom of the vacuole, as sometimes occurred in the larger normal gametophyte cells. The gender of the gametophytes resulting from protoplasts strictly depended on the gender of the parent cell i.e. protoplasts derived from a female cell developed into female gametophytes while those from a male cell developed into male gametophytes.

Sporophyte filament wound response

Whilst following the wound responses in the sporophyte proved more difficult than in the gametophyte, some interesting observations were made. When sporophyte filaments were crushed between two slides, the cytoplasm within the filaments fragmented into numerous condensed segments. These segments filled the width of the filaments, with collapsed sections of cell wall between them (Fig. 8A). Initially the segments condensed further, and the bent and twisted filaments began to straighten themselves. After about one hour the protoplasts started expanding, causing the filaments to further straighten and the collapsed sections of cell wall to re-inflate (Fig. 8B). In sections where the cell wall had not collapsed, cytoplasmic strands often stretched between the protoplasts as they approached each other (Fig. 8C). The protoplasts expanded along the length of the filament until they met and fused to re-form a continuous cytoplasm (Figs 8D-F). There appeared to be no apparent order to the movement of the chloroplasts within the segments prior to fusion, but shortly afterwards, normal chloroplast movement along the filament was fully restored. When a sporophyte filament was cut, the cytoplasm immediately retracted and sealed itself. A material with similar optical qualities to that of the primary envelope surrounding protoplasts appeared at the cut end, on the outside of the cytoplasm, and, like the primary envelope of protoplasts, developed round bodies that looked like vesicles (Fig. 8G). Once the cytoplasm had healed the filaments grew out through the cut end (Fig. 8H), and developed into a new vegetative shoot tip. Septations/plugs were formed in some of the crushed filaments (Fig. 8I).

Reproduction: gametophyte and sporophyte

In the normal life cycle of *Derbesia tenuissima*, gamete and spore release coincidentally invoked responses similar to wound responses. The mature gametophytes of 2773-1, whether in an isolation dish or within a colony, produced gametangia concurrently every 4-5 days, as is characteristic of this species (Page and Kingsbury 1968; Page and Sweeney 1968). The female gametangium was dark brown and the male gametangium was a pale, olive green (Fig. 1). In this strain, almost all the gametophytes were female. Only two male gametophytes were observed amongst 60 single-celled isolates from zoosporelings, a female to male ratio of 30:1. The gametangia first appeared in the cell's dark period, at least 24 hrs prior to release. Each gametangium had one or more release pores. During the 12 hrs prior to release, the cytoplasm in the gametangium became distinctly wrinkled (Fig. 9A). Gametes were generally released at the onset of the photoperiod, around 7:00 a.m., although we observed many gametophytes that had released earlier. Other workers reported that release was triggered by light (Page and Kingsbury 1968; Page and Sweeney 1968; Lee *et al.* 2002, 2003). Gametes were discharged explosively, in $1/6^{th}$ of a second (Fig. 9B). To catch this moment (Fig. 9B) required long periods of video taken in real time. Gamete discharge left a large clear area in the cytoplasm under the pore (Fig. 9C), which was rapidly closed, thus re-forming the continuous cytoplasm of the

Fig. 7. *Derbesia tenuissima* gametophyte (2773-1): protoplast development. (A) A crushed gametophyte showing protoplasts forming both inside the cell wall and in the seawater surrounding the cell. (B) Expanding protoplasts fuse when they contact each other (arrows). (C) Protoplast inside cell wall (internal), 5 min after formation. (D) Protoplast in seawater (external), 5 min after formation. (E) Internal protoplast, 120 min after formation. (F) External protoplast, 120 min after formation. (G) A spherical 13 day-old protoplast with gametangium. (H) An elongate 13 day-old protoplast.

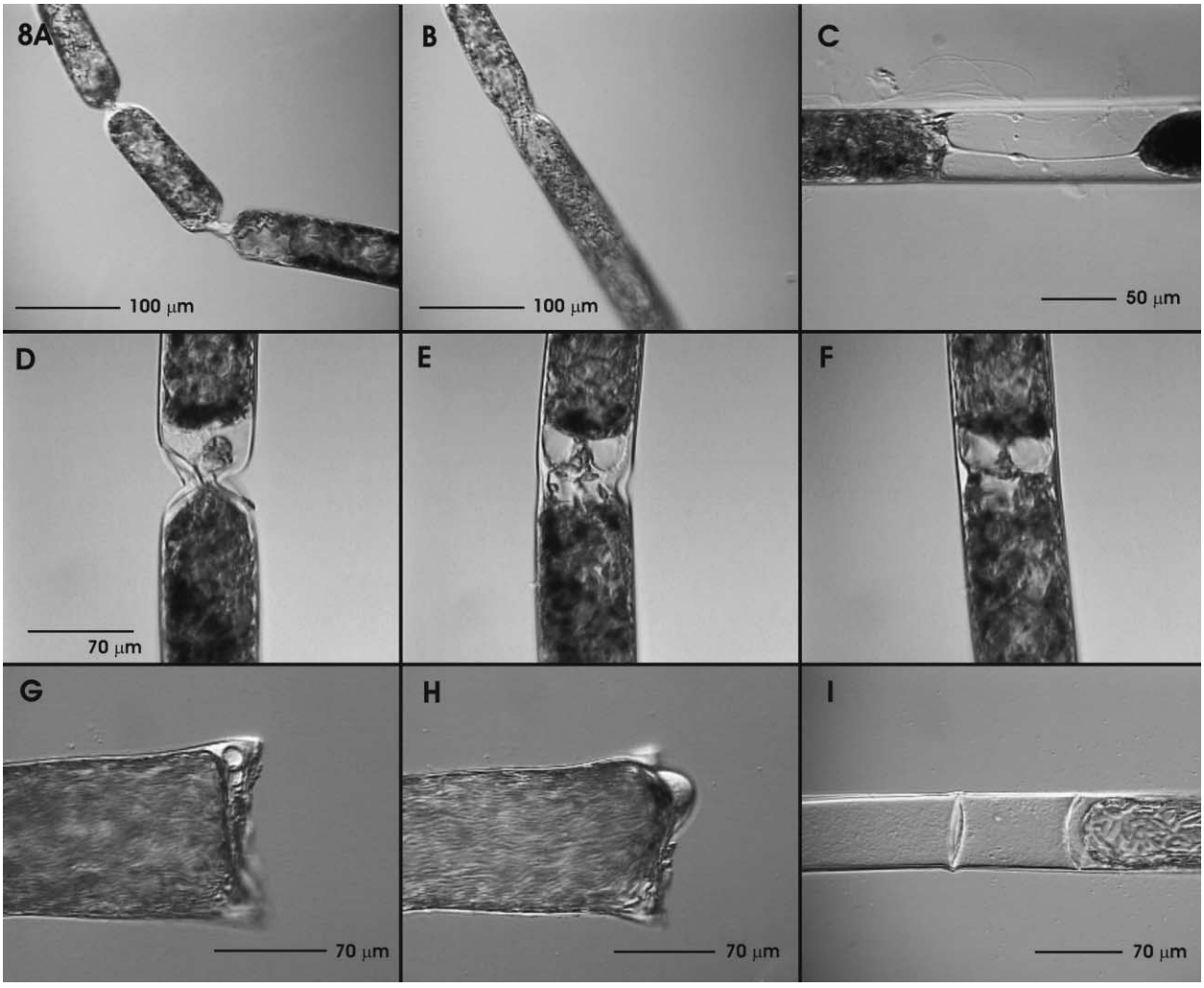


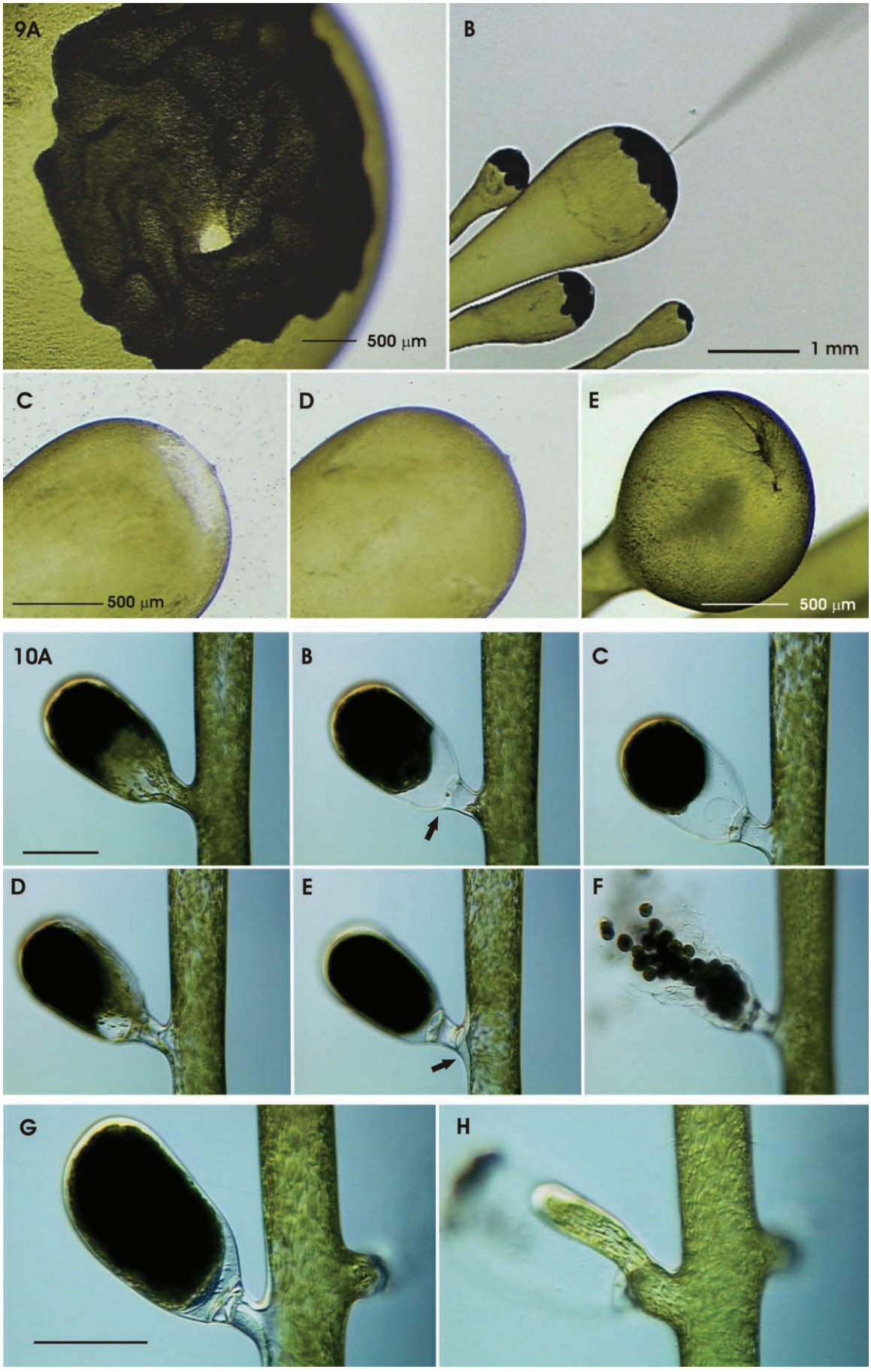
Fig. 8. *Derbesia tenuissima* sporophyte (2773-1): response to wounding. (A) A crushed filament containing condensed protoplasts, separated by collapsed cell wall. (B) The protoplasts expand and fuse, straightening the filament in the process. (C) Cytoplasmic strand joining two protoplasts as they expand towards each other. (D-F) Shortly after the protoplasts fuse and the filaments straighten, cytoplasmic streaming is restored. (G) A cut filament showing the sealed cytoplasm and the refractive "plug" material. (H) A healed filament beginning to grow out of the cut end. (I) Septations sometimes formed in a recovered crushed filament.

gametophyte cell (Fig. 9D). A distinct post-release scar remained in the cytoplasm for at least 2 hrs after discharge (Fig. 9E).

In the sporophyte, the developing sporangia were isolated from the main filament in a two-part septation process involving events similar to a wound response. During the initial stages of sporangial development, the cytoplasm was continuous between the sporangium and the filament (Fig. 10A). In the early hours of the morning the cytoplasm was cleaved in two at the point where the sporangium

Fig. 9. *Derbesia tenuissima* gametophyte (2773-1): gamete ejection. (A) Mature female gametangium just minutes before release. (B) The gametes are ejected within $1/6^{th}$ of a second (filmed in real time). (C) A large void is left in the gametangial space post-release. (D) Organelles migrate back into the void to re-form the continuous cytoplasm. (E) A post-release scar remains at the gametangial site for about 2 hrs after discharge.

Fig. 10. *Derbesia tenuissima* sporophyte (2773-1): sporangial development and spore release. (A) Cytoplasm is continuous between developing sporangium and filament. (B) Cytoplasm is violently cleaved, as the first incomplete septation is formed (arrow). (C) Cytoplasm in filament balloons into sporangial space through a hole in plug. (D) Sporangial protoplast and filament fuse and cytoplasmic streaming into the sporangium is restored. (E) Cytoplasm is cleaved again, and a second septation formed with refractive material building up inside the filament section at the base of the plug (arrow). (F) Zoospores released at 07:00 a.m. (G) The septation process was filmed a second time. (H) Lateral branch growing out through the base of a recently released sporangium. Scale bar = 100 μm.


9A
500 µm
B
1 mm
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G
H

joins the filament. When viewed in time-lapse, this action appears as a violent jerking of the filament with enough momentum to shift the sample downwards in the field of view. This initial separation of the cytoplasm was rapidly followed by the formation of a plug at the base of the sporangium (Fig. 10B). While the rounded segment of sporangial cytoplasm retracted towards the dome of the sporangium, the cytoplasm of the filament ballooned into the empty sporangial space through a small aperture remaining in the plug (Fig. 10C). This incomplete initial septation occurred both times the process was observed. The filament protoplast and the sporangial protoplast then fused, and organelles began streaming in and out of the sporangium again (Fig. 10D). It is this fusion of protoplasts that bears similarity to protoplast fusion in wounded cells. Final cleavage of the protoplast occurred when a second septation was formed, completely separating the sporangium from the filament (Fig. 10E). At this stage refractive material, optically similar to the primary envelope of the gametophyte protoplasts and the material also seen within wounded sporophyte filaments, appeared below the septation site. This refractive material gradually disappeared and cytoplasmic streaming was restored in the main filament. Just prior to their discharge (Fig. 10F), the mass of zoospores within the sporangium retracted. A separate sequence filmed in real-time showed the zoospores within the sporangium displaying the first tentative movement of their stephanokont flagella just before discharge. The flagella rapidly became more active until the top of the sporangium burst, and the zoospores actively swam out. A second sporangial septation was filmed, and although the septation plug remained intact, thus protecting the main filament during spore release, it appeared to form a weak region in the cell wall which enabled the growth of a lateral vegetative branch out through the base of the empty sporangium (Figs 10G-H).

Observations of chloroplast movement

The lenticular chloroplasts of *Derbesia tenuissima* (2773-1) generally had only one pyrenoid. Time-lapse video microscopy revealed an interesting pattern of chloroplast movement in the sporophytic filaments. The cytoplasm in the tip of a filament contained chloroplasts, which oscillated backward and forward. A single chloroplast oscillated in the same section of cytoplasm for over 9 hrs before it moved out of field. The shortest distance it moved was 10 μm, whilst the longest was 67 μm. The speed varied between 0.83 $\mu m \cdot sec^{-1}$, and 6 $\mu m \cdot sec^{-1}$ with a mean of 2.78 $\mu m \cdot sec^{-1}$. Chloroplast movement throughout the filament of *Derbesia* fitted the description of that in *Bryopsis*, consisting of "*simultaneous stops, reversals, gatherings towards a common centre and subsequent dispersals*" (Menzel and Schliwa 1986a, b). When chloroplast movement was recorded *en masse* it appeared to surge in waves along the filament. Although time-lapse sequencing of chloroplast movement in the gametophyte vesicles was not extensive, a short recording suggested a pattern of chloroplast movement similar to that in the sporophyte. This type of movement would generate the unusual striations seen moving through the cytoplasm of the gametophyte when recorded at low magnification.

DISCUSSION

Time-lapse video microscopy of *Derbesia* has revealed a wide range of dynamic wound responses, including centripetal contraction and protoplast formation. This technique also revealed striking rhythmic streaming in the *Derbesia* cytoplasm, and chloroplast oscillations in the filament tips; phenomena that were not observed in the time-lapse study of McNaughton and Goff (1990) on chloroplast movement in *Derbesia*. Video microscopy also enabled us to capture both gamete and spore release and the cellular changes leading up to these reproductive events.

Wound healing by centripetal contraction

The remarkable ability of *Derbesia* to recuperate so rapidly following traumatic injury was quite unexpected, and there can be little doubt as to its importance for the long-term survival of the species. The centripetal contraction of the protoplast following puncture wounding was unexpectedly rapid (10 min) in *Derbesia* when compared with the Siphonocladalean species *Ventricaria ventricosa* (J. Ag) Olsen et West (Nawata *et al*. 1993) and *Valonia utricularis* (Roth) C. Agardh (Satoh *et al*. 2000), where this same response can take up to 60 min. This rapid response serves to seal small wounds and maintain cellular integrity.

Caulerpalean cells exhibit cytoplasmic streaming driven by the actin cytoskeleton (Menzel 1987; Menzel and Elsner-Menzel 1989, Menzel and Schliwa 1986a, b). Although actin is present in Siphonocladalean cells (Satoh *et al*. 2000), they do not exhibit such cyclosis. It may be the inherent motility of the Caulerpalean cytoskeleton when compared to that of the Siphonocladalean that enables the more rapid contraction responses in *Derbesia*. Dramatic alteration of the cytoskeleton must occur to drive the cytoplasmic motility that results from wounding, for example the reorganisation of the actin cytoskeleton at a wound's edge in the

Siphonocladalean genus *Valonia* as revealed by the striking immunofluorescence images obtained by Satoh *et al.* (2000). Similar experiments conducted on *Derbesia* gametophyte cells would be worthwhile. Anti-cytoskeletal drugs (cytochalasins to inhibit actin, oryzalin to inhibit microtubules and/or BDM to inhibit myosin) could also be utilised to determine which cytoskeletal systems are involved in wound recovery.

Protoplast formation

The alternate wound response to centripetal contraction is protoplast formation, which in *Derbesia* cells is employed in response to more extensive disruption to the cytoplasm. The formation of protoplasts is clearly an important mechanism for ensuring some, if not most, of the cytoplasm is preserved so as to be able to generate viable new plants. The effectiveness of protoplast formation (and thus wounding itself) as a means of vegetative propagation is clearly evident and is an important survival strategy.

Transformation of fractured cytoplasm into viable protoplasts in seawater is a remarkable phenomenon. Immediately after extrusion, the cytoplasm is disrupted into numerous fragments, which are no longer protected by a cell wall. To prevent the inclusion of foreign bodies, the forming protoplasts must possess some mechanism for organelle recognition (Kim *et al.* 2002, 2006; Pak *et al.* 1991). In some siphonous green species, lectin-carbohydrate complementary systems could facilitate organelle recognition and aggregation during protoplast formation. A lectin, named 'bryohealin', which has an affinity for N-acetyl-D-glucosamine and N-acetyl-D-galactosamine has been isolated from the vacuolar sap of *Bryopsis plumosa* (Hudson) Agardh (Kim *et al.* 2006). FITC-labelled lectins identified carbohydrates with N-acetyl-D-glucosamine and N-acetyl-D-galactosamine sugar moieties on the chloroplast membranes (Kim *et al.* 2006). Similar carbohydrates have been identified on chloroplasts of *Microdictyon umbilicatum* (Velley) Zanardini (Kim *et al.* 2002), although a compatible lectin has not yet been identified in this species. Fusion events are common in the wound responses of *Derbesia*. Fragmented segments of cytoplasm in both the sporophyte and gametophyte are capable of fusing to re-form motile cytoplasm and expanding protoplasts also fuse when they meet. Recognition of self is important in these events and, as has been proposed for the recognition and aggregation of protoplast organelles, lectin-carbohydrate systems could facilitate this recognition. *Derbesia tenuissima*, which readily forms protoplasts, could be useful for further investigations into these recognition systems.

Time-lapse video microscopy revealed unexpected activity in protoplasts as they recovered after being extruded into seawater. The contractions at the beginning of their recovery appear indicative of the activity of an actin cytoskeleton, but further studies using fluorescence microscopy and inhibitive drugs are necessary to confirm this possibility. The images of dynamic vesicles in the primary envelope constitute the first report of this that we are aware of. These vesicles appear consistent with TEM images of vesicles in the primary envelope of *B. maxima* (Pak *et al.* 1991). Their origin, composition and role are unknown; however they could be involved in exocytosis of cell membrane or cell wall material.

Formation of a new cell wall is essential for the viability of protoplasts. The cell wall of a *Derbesia* gametophyte is composed mostly of xylan, with some cellulose (Hoek *et al.* 1995). Our experiments using Calcofluor White showed the gradual deposition of cell wall material, beginning around 3 hrs after wounding. Calcofluor White is not specific, and stains a range of polysaccharides, including β-D-glucans, xyloglucan and substituted celluloses (Wood 1980), even chitin (Ruzin 1999) and some synthetic fibres (Waaland R., 2003, personal communication). Therefore, the basic composition of the developing protoplast cell wall cannot be determined from this test alone.

Different morphologies displayed by the young *Derbesia* protoplasts in this study (Figs 7G-H) are consistent with earlier observations of Rietema (1973) which showed that protoplasts derived from cytoplasm in the rhizoidal base of a gametophyte cell developed an elongate shape, whereas those derived from cytoplasm in the vesicular top became spherical. This observation suggests that the different shaped protoplasts in our study were derived from different regions of the original cell. We found that only the spherical young protoplasts developed gametangia (Fig. 7G). If it is true that they derived from the vesicular cytoplasm, while the elongated ones came from the base, this indicates a degree of cytoplasmic determination within these single giant cells. The difference in behaviour between *Derbesia* protoplasts formed internally and externally is also of interest. Whether the presence of the gametophyte cell wall creates a chemical or mechanical difference that induces the different responses is unknown. Simple experiments could determine which of these two possibilities is true.

With regard to protoplast formation the Caulerpalean *Derbesia* gametophyte behaves quite differently to some morphologically similar Siphonocladalean species, such as *Ventricaria ventricosa* (Nawata *et al.* 1993; Shihira-Ishikawa

1987) and *Boergesenia forbesii* (Harvey) J. Feldman. When a *Boergesenia* or *Ventricaria* cell is cut open, the cytoplasm slowly breaks apart to form numerous protoplasts. However, when a *Derbesia* gametophyte is cut in half, the hemispheres of cytoplasm remain intact while the broken edge of the cytoplasm contracts centripetally like a drawstring bag. This difference in immediate wound responses does not appear to have a phylogenetic basis since the *Derbesia* type of response does occur in some Siphonocladalean species, such as *Ernodesmis verticillata* (Kützing) Børgesen and *Valoniopsis* sp. (Martin E., 2003, unpublished data). However, the difference in the response of *D. tenuissima* and *Ventricaria ventricosa* may be a manifestation of the underlying differences in cytoplasmic structure between the two (McNaughton and Goff 1990; Shihira-Ishikawa 1992; Wheeler and Page 1974). Another key difference in cellular behaviour between these two morphologically similar species is the asexual reproduction via 'segregative cell division', exhibited by *Ventricaria*, and lacking in *Derbesia*. During segregative cell division, common in most Siphonocladalean species, the cytoplasm of intact cells fragments to form protoplasts in the course of normal development (i.e. not the result of wounding) (Bøergesen 1913, Enomoto and Okuda 1981, Fritsch 1945, Okuda *et al.* 1997), resulting in the distinct reticulation, also characteristic of the *Ventricaria* wound response. The resulting spherical protoplasts later develop into new vegetative plants. Species that undergo this segregative cell division (i.e. *Ventricaria*) usually exhibit protoplast formation as their main response to being cut open. However, in *Derbesia* and *Bryopsis*, both species that lack segregative cell division, protoplasts are readily formed only when the cytoplasm is mechanically broken up and exposed to seawater. Segregative cell division may have evolved from a wound healing adaptation (Graham and Wilcox 2000). A relationship between life cycle processes and wound healing is supported also by the time-lapse observations of sporangial development, displaying violent cleaving and re-fusion of the cytoplasm that is so similar to the responses of protoplasts in crushed *Derbesia* cells.

Plug formation

No visible plug is extruded from the wound upon damaging the *Derbesia* gametophyte, although other Caulerpalean species such as *Caulerpa* spp. and *Bryopsis* spp. extrude a conspicuous, gelatinous wound plug immediately on wounding (Burr and West 1971; Dreher *et al.* 1978; Dreher *et al.* 1982). However, plug material can be found in *Derbesia* at the base of the sporangia, in the gametangial release pore and as a result of mechanical injury to the sporophyte filaments (Menzel, 1979, 1980; Lee *et al.* 2003). In the sporangial septation sequences we obtained, a dense layer of highly refractive material with similar optical qualities to the primary envelope of the protoplast appears briefly between the plug and the filament. The two part septation process we observed in the formation of two plug layers at the sporangial base is like that seen by Menzel (1980). We also observed wound plug-septations commonly in the wound repair of sporophyte filaments and these are similar to those observed by Menzel (1980) in *D. tenuissima*. This material, like the primary envelope, may be involved in either repair of the damaged cell membrane, or synthesis of new cell wall at the plug site. Pak *et al.* (1991) noted that the primary envelope of *Bryopsis* protoplasts looked similar to the extruded wound plugs when viewed by TEM.

Life history observations

Although this project was not intended to be a comprehensive study of the life history of *D. tenuissima*, we were able to make some other observations when growing the cells for the wound response experiments. The rhythmic synchronicity of the gametangium formation, the ability for the female gametes to develop parthenogenetically and the higher incidence of female gametophytes to male concurs with earlier observations of the *D. tenuissima* strain from the Mediterranean (Page and Kingsbury 1968; Page and Sweeney 1968; Ziegler and Kingsbury 1964). With a ratio of 30:1, the Philippines strain used in this study had an even higher incidence of female gametophytes to male than the two females to one male ratio found in the Mediterranean strain used by Ziegler and Kingsbury (1964). Despite the above similarities, a key difference observed in the Philippines strain (2773-1) is that under these culture conditions many gametes were released from the gametangia prior to the onset of the light period. This may negate the hypothesis proposed by Wheeler and Page (1974), that the release pore was broken down by light activated enzymes to the point where the internal turgor pressure burst the pore open, causing release.

The life histories of this Philippine strain, the strain used by Lee *et al.* (1998, 2000, 2001, 2002) and the strains used by Eckhadt *et al.* (1986) and Schnetter and Eckhardt (2000), are different. Lee's strain produces both male and female gametes, while the female gametes have very low frequency of parthenogenesis in which haploid sporophytes arise (Lee *et al.* 1998, 2000). The Colombia strain of Schnetter reproduces parthenogenetically and does not produce

male gametophytes, the Yugoslavian isolate reproduces sexually and has both male and female gametophytes. The isolates used in our current study add more information about the matter but do not resolve which is the most 'typical' life history for *D. tenuissima*. It is quite likely that life histories, genetics, physiological processes and many other aspects of reproductive biology vary among the many populations of *D. tenuissima* occurring around the world. It is also likely that molecular investigations will reveal many cryptic species within a *D. tenuissima* complex.

Conclusion

The dynamic events captured by video microscopy are valuable in showing the range and complexity of behaviour associated with *D. tenuissima* wound responses. These responses hint at the involvement of cytoskeletal components such as actin, and suggest further avenues for research. Data from immunofluorescence microscopy, TEM and histochemical analysis should allow a better understanding of these responses, and the time-lapse sequences obtained allow for more specific interpretation of these data.

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