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CRYOPRESERVATION OF THE MODEL ALGA *Ectocarpus* (PHAEOPHYCEAE)

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Abstract

The brown alga *Ectocarpus* has recently become the first fully sequenced multicellular alga and is an important biological model. Due to the large and growing number of *Ectocarpus* strains isolated and maintained by the research community, including increasing numbers of mutants, there is an urgent need for developing reliable, cost-effective long-term maintenance techniques. We report here that cryopreservation constitutes an attractive option in this respect, using a simple two-step protocol employing combined DMSO 10% (v/v) and sorbitol 9% (w/v) as cryoprotectants. This model organism appears to be remarkably robust and post-cryo recovery has been observed in all strains tested in this study. Cultures can be regenerated by the germination of cryopreserved zooids (spores), or the recovery of vegetative cells. In the latter case, dividing surviving cells may grow into the cell lumen of a neighbouring dead cell, eventually regenerating a phenotypically normal thalloidal structure.

Keywords: Barcoding, brown algae, cryopreservation, *Ectocarpus*, model organism

INTRODUCTION

The brown algal genus *Ectocarpus* (Ectocarpales, Phaeophyceae) constitutes an important biological model (37) and since the second half of the 19th century (e.g. 41), investigators have addressed a multitude of facets of its biology (3), including its sexuality and life history (25, 36), pheromones (23, 35), cell mobility and photo-/chemo-taxis (18, 26, 29), taxonomy (28, 40), biogeography (42), inheritance of organelles (37), pathologies including viruses (30, 32, 34), plasmodiophoraleans (24), oomycetes (13), fungi (33) as well as genetics (16, 27). Undoubtedly the most recent, major highlight is the publication of the annotated genome of *Ectocarpus* (4) enabling unprecedented studies at all levels, including

developmental biology (5, 6, 38). Significant for many experimental approaches, axenic cultures (31) and protoplasts (19) can be obtained in a reliable manner.

Ectocarpus has a biphasic, heteromorphic life cycle, with a diploid sporophyte generation alternating with a haploid gametophyte generation (Fig. 1). The sporophyte produces meiotic spores in unilocular (single-chambered) sporangia, which upon germination grow directly into dioecious gametophytes. These produce mitotic gametes in plurilocular (multi-chambered) gametangia. Gametes from female and male gametophytes fuse, and the resulting zygote grows into the diploid, heterozygous sporophyte thus completing the sexual life cycle. Both generations are also capable of reproducing asexually via mitotic zoids from plurilocular sporangia. Gametes, which germinate directly without fusion, develop into haploid thalli expressing sporophytic morphology, so-called partheno-sporophytes (39). These are capable of diploidisation, thus creating homozygous sporophytes (2).

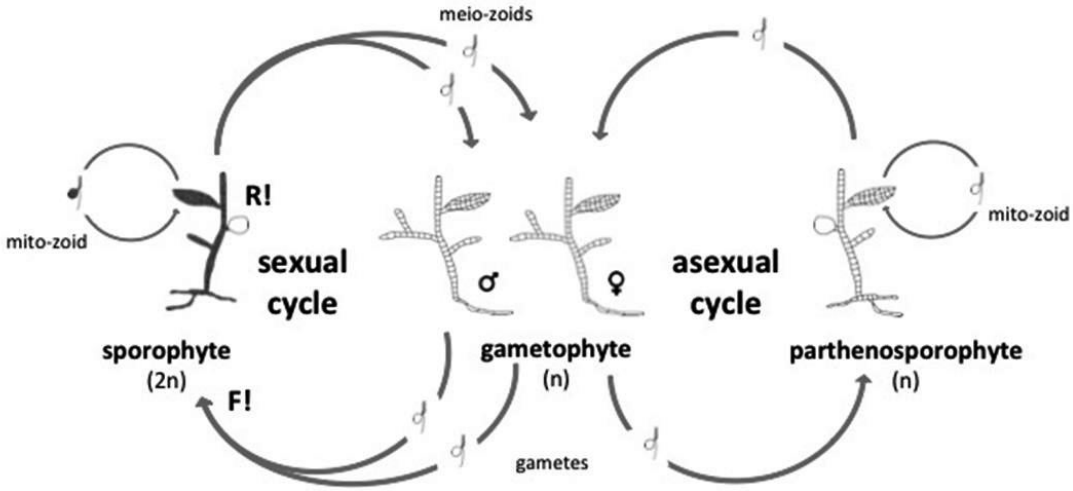

Figure 1. Schematic life cycle of *Ectocarpus*. R! - meiotic reduction; F! - gamete fusion [adapted from Peters et al. (39].

Over 330 strains belonging to the genus *Ectocarpus* (and a number of strains of related genera of the Ectocarpales) are currently lodged at the Culture Collection of Algae and Protozoa (CCAP) and the Kobe University Macroalgal Culture Collection (KU-MACC). This includes the genome-sequenced strain CCAP 1310/4 (=KU-1372) (4) as well as mutant strains, constituting an important resource for the global user community. *Ectocarpus* is one of the most comprehensively "barcoded" algal genera and the phylogeny and species level taxonomy of the strains are currently being analysed using both mitochondrial and nuclear genetic markers in a joint project between CCAP and KU-MACC.

Clearly, with the level of scientific investment in such biological resources, there is an overwhelming need to conserve these algal strains. Traditionally they have been maintained by routine serial transfer (22), under light levels suboptimal for growth (<10 µmol m^2 s^{-1} PAR) at 8^{o}C; however, this approach is labour- and resource-intensive and cannot guarantee long-term genotypic stability. Cryopreservation has been highlighted as one of the available approaches for the conservation of endangered algal taxa (1) and has been widely applied to microalgal cultures (7, 10).

To date, there have been relatively few reports on the cryopreservation of macroalgae, although approaches such as cryopreserving unicellular spores of the macroalga *Enteromorpha* (43) have proven to be successful. However, over the past three decades, work has been undertaken on marine red algae including *Gracilaria tikvahiae*, where both sporelings and apical segments of mature thalli have been successfully cryopreserved using a conventional, slow-cooling two-step approach employing DMSO as a cryoprotectant (44). Furthermore, because of its economic value as a marine aquaculture crop, there have been a number of studies published on the cryopreservation of *Porphyra yezoensis*, where two-step cooling methods employing a cryoprotectant solution composed of 10% DMSO and 0.5 M sorbitol in 50% seawater resulted in survival levels of ~60% of the conchocelis cells (20, 49). More relevant to the maintenance of the *Ectocarpus* resource has been the development of techniques for brown algal gametophytes (in particular of the Laminariales), which are filamentous and thus morphologically similar to the Ectocarpales, employing conventional two-step approaches (47, 48) and encapsulation-dehydration (45). In this study, we have applied an optimised conventional colligative, two-step cryopreservation approach, with a specific focus on monitoring cell recovery.

MATERIALS AND METHODS

Biological material and culture regime

All strains used in this study were obtained from/are available from CCAP (www.ccap.ac.uk) or KU-MACC (http://www.research.kobe-u.ac.jp/rcis-ku-macc/E.index.html) (17). All cultures were grown in Modified Provasoli (MP) medium (46). Unialgal, non-axenic *Ectocarpus* strains were transferred from standard maintenance conditions; i.e. under a 12:12 h light:dark regime (<10 µmol m^2 s^{-1} PAR) at 8ºC, to 10 ml polystyrene Petri dishes (Sterilin™, 50 mm diameter) closed with Parafilm™, and were cultivated under a 12:12 h light:dark regime (50-60 µmol m^2 s^{-1} PAR) at 15ºC. All strains employed were reproducing asexually, i.e. haploid strains were maintained as partheno-sporophytes (Fig. 1).

One to 2 weeks prior to cryopreservation, thalli were separated into smaller pieces (approx 1-2 mm in length) and were then transferred to Erlenmeyer flasks containing 100 ml of culture medium, under the same culture conditions. Culture medium was changed once a week prior to cryopreservation, to maximise culture quality.

Cryopreservation procedure

D-sorbitol (Sigma) was added to natural, filtered seawater to a final concentration of 10% (w/v). This solution was then sterilised by autoclaving (15 min, 121ºC). After cooling to room temperature, DMSO (10 ml) was added to 90 ml of the sterile D-sorbitol solution, resulting in a final concentration of cryoprotectant agents of 10% v/v DMSO and 9% w/v sorbitol. Aliquots (10 ml) of the cryoprotectant solution were then filter-sterilized into sterile Universal tubes. This was then aseptically dispensed in 1 ml aliquots into sterile cryogenic vials.

Whole *Ectocarpus* thalli were transferred to 15 cryogenic vials (Greiner bio-one) containing 1 ml of the above cryoprotectant solution and incubated for 15-30 min at room temperature under ambient light prior to cryopreservation. Samples were then cooled, employing a controlled-rate cooler (Planer plc, Kryo 360-3.3), starting at +20ºC, cooling at 1ºC min^{-1} to -40ºC. Samples were then removed and immediately plunged in a Dewar containing liquid nitrogen. The cryovials containing the samples were subsequently stored in the CCAP cryostore: three were allocated for viability testing, 10 vials stored in the working

cryo-bank and two vials stored in the back-up cryostore, as advised in Day *et al*. (9), in either liquid nitrogen (-196ºC), or in the nitrogen vapour phase, at <-135ºC.

Recovery procedure and viability assessment

To test for viability, three vials were removed from cryostorage, transferred to the laboratory in liquid nitrogen and rapidly warmed in a water-bath pre-warmed to +40ºC. Immediately after melting of all the ice, the vials were transferred to a laminar-flow cabinet where, using standard aseptic techniques, thalli were washed once in culture medium before being transferred to a Petri dish with 10 ml of fresh MP medium. This washing step was introduced to remove most of the cryoprotectants and organic materials released from lysed *Ectocarpus* cells, thus reducing potential bacterial overgrowth. If necessary (i.e. there was significant bacterial growth), the washing step was repeated after 1 or 2 weeks in culture. In these cases, the original Petri dish was retained in order to check for germlings, which might have originated from zoids released after rewarming. In all cases, directly after rewarming, Petri dishes were wrapped in aluminium foil and thalli were incubated for 12-24 h at 15ºC in the dark (to avoid photo-oxidative induced injuries), before exposure to increasing light levels and, finally, standard culturing conditions [12:12 h light: dark regime (50-60 µmol m^2 s^{-1} PAR) at 15ºC], where material was incubated for a further 6-8 weeks. Due to the multicellular, three-dimensional structure of the *Ectocarpus* thallus and the propensity of some strains/samples to produce mitotic zoids capable of regenerating thalli, conventional viability assays involving cell or colony counts were unsuitable. Strains were considered viable when regrowth could be observed, i.e. cells survived the treatment and regenerated phenotypically normal thalli, in all three Petri dishes.

The effects of cryopreservation, post-treatment survival and recovery of cells were observed under the light microscope using either a Leica Labovert inverted microscope, or a Zeiss Axiovert 200 (equipped with phase-contrast and Nomarski DIC optics). Images were acquired using a Leica DFC320 Camera with the Leica IM50 Version 5 software.

RESULTS

On being cryopreserved, most strains of *Ectocarpus* suffered injuries, resulting in cell death; however, occasionally some strains e.g. *Ectocarpus* sp. CCAP 1310/92 were apparently completely unaffected (Table 1). In all the strains studied, it was possible to distinguish between live and dead cells on day 1 after rewarming. In apparently undamaged, surviving cells the plastid membranes appeared intact and cells had the characteristic golden-brown pigmentation of the Phaeophyceae (Fig. 2A), whilst in severely injured/dead cells the contents were coagulated and of greenish colour (Fig. 2A). None of the strains studied was axenic and small numbers of rod-shaped bacteria could be observed associated with the algal cells in both treated and control cultures. Within 2 days, active recovery of vegetative cells was observed, with some surviving cells "germinating" into the cell lumen of neighbouring dead cells (Fig. 2B). This occurred through the connecting cell plate, rather than penetration of their own cell wall and was achieved by "pushing" a protuberance of their cell membrane into one of the adjoining cells prior to cell division (Fig. 2B, C). Furthermore, the number of rod-shaped bacteria associated with both live and dead cells had increased.

Table 1. Post-rewarming recovery of exemplar *Ectocarpus* strains belonging to different genotypic groups.

Genotypic group[1]	Strain no.	Species	GenBank accession no.	Ploidy[2]	Post cryopreservation recovery[3,4] (days)			
					1	3	7	14
1a	CCAP 1310/38	*E. siliculosus*	FR668738	H	+	+	++	+++ V
1b	CCAP 1310/44	*Ectocarpus* sp.	FR668744	P	++++	++++	++++	++++ + V&Z
1c	CCAP 1310/4	*Ectocarpus* sp.	Genome strain	P	+/-	+	++	+++ V
2d	CCAP 1310/32	*Ectocarpus* sp.	FR668733	P	+++	++	++	+++ V
3b	CCAP 1310/30	*Ectocarpus* sp.	FR668731	P	++	+++	++++	++++ V
4	CCAP 1310/92	*Ectocarpus* sp.	FR668784	P	++++ +	++++ +	++++ +	++++ + V&Z
5b	CCAP 1310/17	*E. fasciculatus*	FR668720	P	+	++	+++	++++ V&Z

[1]Genotypic grouping according to Stache-Crain *et al.* (42), A.F. Peters (personal communication): Results of nrRNA gene sequence comparisons suggest these genotypic groups to be separate species [Peters *et al*. (40), Heesch, unpublished data]. [2]Putative ploidy: H = heterozygous sporophyte; P = partheno-sporophyte. [3]Culture/ thallus health status: completely dead (-); occasional live cells (+/-); ~5% of cells alive (+); 5- 25% cells alive (++); 25-50% cells alive (+++); >50% cells alive (++++); phenotypically normal culture (+++++). [4]Recovery from vegetative cells (V); recovery from zoids (Z); recovery from both zoids & vegetative cells (V&Z).

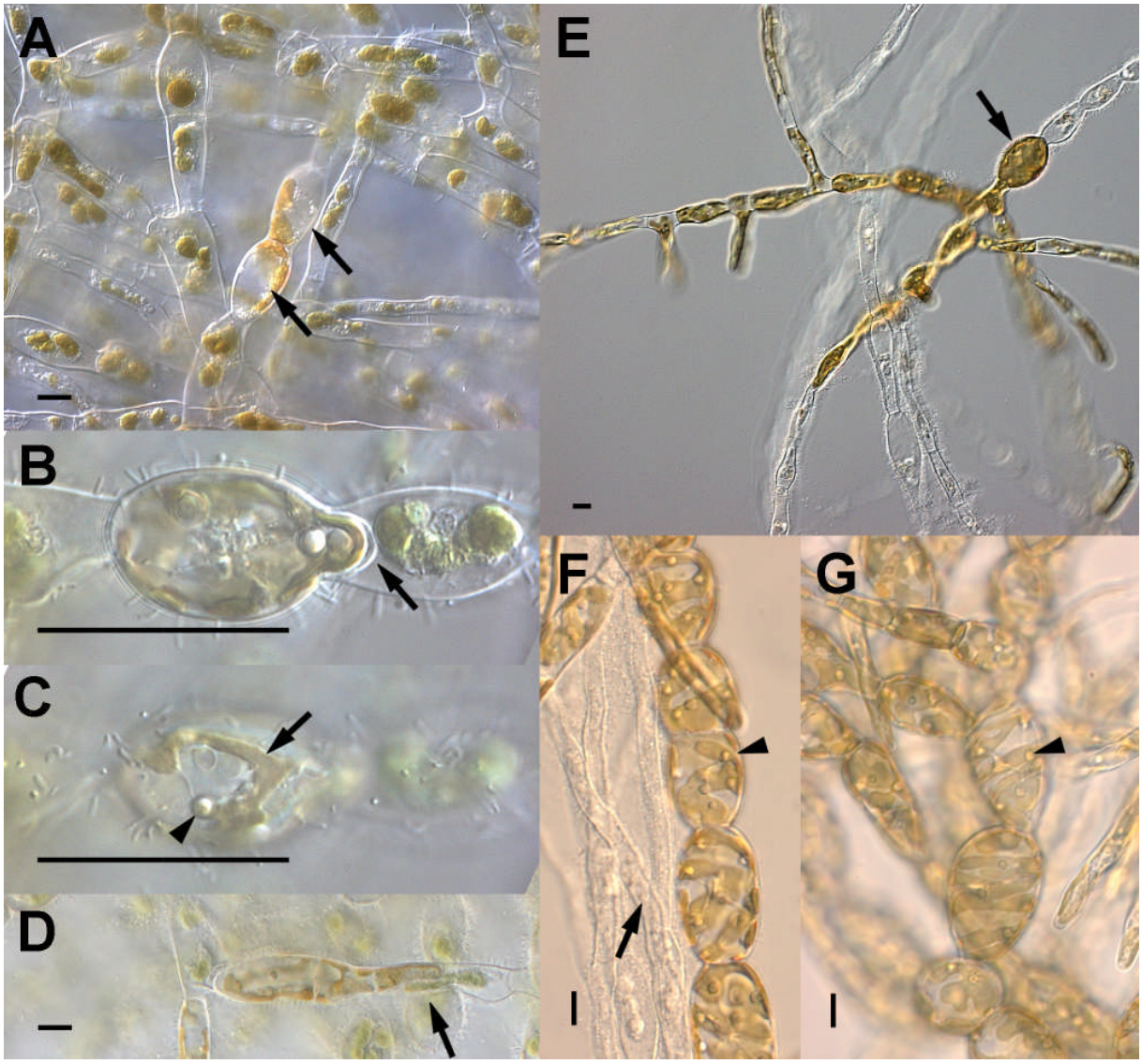

Figure 2. Post-cryopreservation recovery of *Ectocarpus* sp. CCAP 1310/4: **(A)** Day 1 after rewarming - two surviving cells (arrows) contain intact plastids with golden brown pigmentation; **(B)** & **(C)** Day 2 after rewarming. - surviving cell growing into the cell lumen of a neighbouring dead cell (B; arrow), intact plastid in live cell (C; arrow) with a pyrenoid (C; arrowhead); **(D)** Day 4 after rewarming - a three cell long filament growing within the cell walls of neighbouring dead cells (arrow); **(E)** Day 11 after rewarming - a surviving cell (arrow) has grown into a filamentous, branched, multicellular thallus; **(F)** Day 14 after rewarming - normal cells with ribbon-shaped plastids containing protruded pyrenoids (arrowhead), and colourless dead cells (arrow); **(G)** Untreated control sample with ribbon-shaped plastids containing protruded pyrenoids (arrowhead). (all scale bars 10 µm).

By day 4, dead cells could be clearly distinguished from live cells and, although most dead cells were still greenish in colour, some, particularly smaller, cells had bleached. Many surviving cells had developed into two-three cell filaments (Fig. 2D). While surviving cells could be either round or elongated, with a length to width ratio in the range 1.2 - 3.8 (n = 11) for *Ectocarpus* sp. strain CCAP 1310/4, the new filaments always consisted of long thin cells. By day 4 there were large numbers of rod-shaped bacteria in all samples, which were

especially abundant on/ associated with dead (white) cells. Between day 4 and 7 the new filamentous cells started to develop branches.

By Day 11, surviving cells could be distinguished from new cells by their darker pigmentation and round shape: the average cell size (length to width) ratio of surviving cells on Day 11 was 1.4 (1.2 - 1.7; n = 5) for *Ectocarpus* sp. CCAP 1310/4. In most cases "germination" was unidirectional (Fig. 2E); however, surviving cells were occasionally observed to be the origin of more than one or two filaments. After 2 weeks (day 14) filaments composed of round new cells were observed (Fig. 2F), which were virtually identical to untreated cells (Fig. 2G), with the ribbon-shaped plastids and protruded pyrenoids characteristic for the genus *Ectocarpus*.

In some strains including *E. fasciculatus* CCAP 1310/17; *Ectocarpus* sp. CCAP 1310/27; *Ectocarpus* sp. CCAP 1310/44 and *Ectocarpus* sp. CCAP 1310/92, germination of cryopreserved zoids, which subsequently formed phenotypically normal thalli was observed. In some strains, e.g. *Ectocarpus* sp. CCAP 1310/27, germination of zoids inside the plurilocular sporangia was occasionally observed. This also resulted in the regeneration of a normal culture.

By applying the technique described in this study, to date, >50 *Ectocarpus* strains have been successfully cryopreserved in the CCAP, and ~120 strains in KU-MACC. Although absolute levels of cellular survival have not been quantified for all the strains tested, minimum survival levels in the range 25-50% of the cells in a thallus were required to ensure rapid recovery of a vegetative thallus and to avoid rapid over-growth by bacteria. At present, an on-going rolling programme is underway to cryopreserve the remaining ~200 *Ectocarpus* strains in CCAP and their closest relatives.

DISCUSSION

Cryopreservation offers an alternative maintenance technique to conventional serial sub-culture for algae. In cells stored at -196°C, normal metabolic reactions cease (15), offering the possibility of long-term, stable storage of viable cells, measured at least in decades. It has been demonstrated that cryopreserved microalgal cell lines may be preserved with no significant reduction in viability after >22 years storage (10), but to date the primary focus within the protistan community has been on the conservation of unicellular algal taxa.

In this study, genotypically different strains of the filamentous model alga *Ectocarpus* were subjected to a standardised two-step protocol. A preliminary pilot-study undertaken using a cooling rate of 1ºC min^{-1} to -40ºC, prior to plunging in liquid nitrogen, employing DMSO (10%) as cryoprotectant proved unsatisfactory, with only a quarter of the strains tested withstanding the procedure (Yamagishi & Kawai, unpublished data). Although there is no data available for this alga, it may be assumed that sorbitol acted as a non-permeating cryoprotectant, which may assist in the dehydration of the algal cells prior to cryopreservation. On employing the cryoprotectant mix and protocol used in this study, all the test strains in the pilot study recovered (Yamagishi & Kawai, unpublished data).

As has been reported elsewhere (12, 14), damage to chloroplasts by low temperatures and cryopreservation had a major impact on the degree of survival in *Ectocarpus*. Injuries could be clearly observed after rewarming, where deterioration of ultrastructure could be observed including loss of classical, ribbon-like plastids and coalescence of the cellular contents within damaged/killed cells. Additionally, oxidative stress resulted in degradation of the main accessory photosynthetic pigment fucoxanthin, leading to a colour change from classical golden brown to green. Although no physiological assays were undertaken, it is probable that the combination of physical injury and physiological stress reduced the photosynthetic

capacity of the surviving cells, which subsequently recovered: this has previously been reported for the cryo-sensitive alga *Euglena gracilis*, where photosynthetic capacity increased from 17% ± 3% of the control levels 24 h after rewarming to 48% ± 8% 48 h after rewarming (11). In this study, active growth can be presumed to be "fuelled" by the active photosynthesis of the surviving and new cells within the thallus. Where cells had obvious gross injuries after rewarming, subsequently bleaching of the chlorophyll occurred, with rupturing of the cell membrane and release of most of the cellular contents. This release of organic material stimulated a bacterial growth, which increased during the experimental period. Where little injury occurred, e.g. in *Ectocarpus* CCAP 1310/92, bacterial levels were not appreciably greater than in normal healthy *Ectocarpus* cultures; however, in strains where significant levels of cell death occurred, i.e. in those where <50% of the original cells withstood the cryopreservation procedure, bacteria grew rapidly to the extent that in order to ensure successful revival/regeneration, the thalli needed to be transferred to fresh MP medium on day 7.

Sporophytes (including partheno-sporophytes) of *Ectocarpus* can be distinguished from the gametophytes by the presence of a prostrate basal system of branched filaments consisting of two types of cells, elongated cells ("E" cells), and round cells ("R" cells), from which sparsely branched erect filaments branch (21). Gametophytes, in contrast, develop directly from the meiotic zoid into a rhizoid and richly branched erect filaments (39). An initial hypothesis, prior to the initiation of this study, was that *Ectocarpus*, which has relatively large cells (commonly ~30 x 50 µm) with large vacuoles, would be particularly susceptible to intracellular ice formation during the cooling process. The authors speculated that most regeneration would be due to the germination of zoids, rather than from vegetative cells. Unexpectedly, regeneration from zoids, although routinely observed in some strains, did not form the main contribution to the regeneration of a normal healthy culture; this appeared to primarily derive from the recovery of the vegetative cells within the thallus. Moreover, in this study, there seemed to be no clear pattern of survival: surviving cells were found among the large "R" as well as the smaller "E" cells. However, between day 3 and day 11, the average cell length to width ratio decreased from 2.4 to 1.4, suggesting that most surviving cells turned into "R" cells. That elongated cells differentiate into round cells is in accordance with the observations of Le Bail et al. (21), who demonstrated that, during their early development, young *Ectocarpus* sporophytes showed apical growth in the elongated cells at the end of filaments, while the "E" cells within the filament progressively differentiated into round cells. Branching occurs preferentially in the round cells, while the development of erect filaments in sporophytes is limited to round cells (21, 39). The above may allow for rapid regeneration of the alga if and when it is subjected to significant environmental stress, such as a neap tide where exposure to the atmosphere could result in cell death within the thallus.

In conclusion, the development of cryopreservation methods is critical to the conservation of algal genetic resources/biodiversity for fundamental and future genomic studies. They are also crucial to the rapidly developing algal biotechnology sector, where the current focus on algal biofuels has stimulated considerable interest in the exploitation of algae. As with other organisms, irrespectively of whether wild type or mutant strains are employed, there is a requirement in any industrial process to ensure the genotypic and phenotypic stability of master stock-cultures. This will necessitate the application, or development, of robust, reproducible cryopreservation procedures (8).

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