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Tissue culture and regeneration of thallus from callus of *Gelidiella acerosa* (Gelidiales, Rhodophyta)

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The tissue culture of an economically important red alga *Gelidiella acerosa* (Gelidiales, Rhodophyta) included preparation of axenic material, culture of explants, subculture of excised callus and regeneration of *de novo* plants from callus in the laboratory. Sequential treatment of explants with sterile seawater consisting of 0.1% liquid detergent for 10 min, 2% betadine (with 0.5% w/v available iodine) for 5 min and 3.5% broad-spectrum antibiotic mixture with nystatin for 2 days enabled yields as high as 90% of viable and axenic explants. A prolific and rapid growth of filamentous callus on explants was observed on cut surfaces during the first month of culture. The highest level of callus induction occurred in Provasoli enriched seawater (PES) medium solidified with 1.5% agar incubated at 20–22°C and a photon flux density of 5 μmol photons m^{-2} s^{-1} with a 12:12 light–dark photoperiod. Up to 90% of the explants cultured at 5 μmol photons m^{-2} s^{-1} produced callus, whereas at 30 and 70 μmol photons m^{-2} s^{-1}, 70% and 9% produced callus, respectively. The explant culture medium with 0.5% agar content stimulated bud development in all explants, whereas higher agar concentrations (0.8%, 1.0%, 1.5%, 2.0% and 3.0%) resulted in a filamentous type of callus growth. Addition of the plant growth regulators naphthalene acetic acid and indole 3-acetic acid (auxins), and benzyl amino purine and kinetin (cytokinins) and different organic carbon supplements (glycerol, sucrose, sorbitol and mannitol) to the culture medium had no effect on callus growth or induction rate. All carbon supplies at 0.5 and 1.0 M concentration showed an inhibitory effect and most of the explants perished gradually after 2 weeks in culture. The callus mass with bud or shoot developments continued to grow when transferred to semisolid PES medium (0.2% agar w/v) on a rotary shaker. In 4 months, these shoots gave rise to 2–3 cm long plantlets of *G. acerosa*. The tissue-cultured *Gelidiella* germlings were successfully grown into full plants in the field on coral stones in 6 months.

INTRODUCTION

Tissue culture techniques have increasingly been applied to seaweeds because they facilitate development and propagation of genotypes of commercial importance (Dawes & Koch 1991; Dawes *et al.* 1993; Reddy *et al.* 2003). Furthermore, tissue culture systems have also been studied for *in vitro* production of value-added products like tocopherols and tocotrienols (Lawlor *et al.* 1990) and halogenated monoterpenes (Maliakal *et al.* 2001). Nevertheless, the successful application of tissue culture to seaweeds for either genetic improvement or applied phycology studies has been limited and lags far behind that of higher plants. The poor response of seaweeds to tissue culture techniques has largely been attributed to the fact that algal thallus structure lacks the high degree of tissue differentiation found in higher plants (Aguirre-Lipperheide *et al.* 1995). Nevertheless, recent studies on tissue culture of *Kappaphycus alvarezii* (Doty) Doty demonstrated mass production of micropropagules clonally from pigmented filamentous callus through somatic embryogenesis (Reddy *et al.* 2002; Reddy *et al.* 2003). Furthermore, the plants regenerated from tissue culture had a higher growth rate than the cultivated strain. This finding has provided the impetus for intensifying seaweed tissue culture work in our laboratory on other economically important algae to select and propagate possible elite clones as seed stock for commercial farming purpose.

Gelidiella acerosa (Forsskål) Feldmann & Hamel is considered to be one of the more important sources of raw material for industrial agar production (Armisen & Galatas 1987; Roleda *et al.* 1997). In India, *G. acerosa* is the preferred source of raw material for agar production because it has higher yields of high gel strength agar (i.e. > 600 g cm^{-2}; A.K. Siddhanta, personal communication). Annually, 200–300 dry tonnes of *G. acerosa* material is harvested from natural stocks and processed for indigenous agar production (Kaliaperumal & Kalimuthu 1997). Due to the emergence of biotech industries in the country, the demand for agar has increased recently, resulting in the use of other agarophytes [*Gracilaria edulis* (S. Gmelin) P.C. Silva, *G. crassa* Harvey *ex* J. Agardh and *G. folifera* (Forsskål) Børgesen] and has also led to overharvesting of *Gelidiella acerosa* to complement the growing market demands (Kaliaperumal & Kalimuthu 1997). The efforts to restore and conserve the overexploited natural beds of *Gelidiella* have been impeded by its restricted distribution and inherently slow growth and reproduction. Consequently, the cultivation of *Gelidiella* on artificial substrata has been initiated in coastal seawaters with the aim of developing industrially viable indigenous cultivation technology for large-scale farming of *G. acerosa*.

This study was undertaken to help increase the overall pro-

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ductivity and economic prospects of field cultivation of *G. acerosa* in India, by developing *in vitro* tissue culture techniques based on clonal propagation and selecting fast-growing strains that yield high-quality phycocolloid.

MATERIAL AND METHODS

Collection of material and preparation of axenic explants

Gelidiella acerosa occurs year-round on submerged calcareous rocks in the intertidal region along the Saurashtra coast (west coast of India). Plants were collected from the Okha coast (22°28′N; 69°05′E) from April 1998 onwards, cleaned with seawater in the field, wrapped in moistened tissue towels and brought in a cool icebox to the laboratory. Healthy plants, preferably with few branches, were selected for tissue culture, and epiphytes and other microscopic organisms removed by manual brushing under a microscope. Fragments of 4–5 cm length were cultured in Provasoli enriched seawater (PES) medium (Provasoli 1968) with GeO_2 (10 mg l^{-1}) for a week in aerated flasks and maintained at 20–22°C under white fluorescent tube lights at 25 μmol photons m^{-2} s^{-1} with 12:12 light–dark photo-period unless otherwise mentioned.

After establishing a unialgal culture, the axenic explants were surface-sterilized using methods described by Polne-Fuller & Gibor (1984) and Huang & Fujita (1997a). Selected explants were cleaned gently with a brush in filtered seawater consisting of 0.1% liquid detergent (Charmy Green; Lion Corporation, Tokyo, Japan) for 10 min. Following the detergent treatment, the pieces of thallus were further treated with 2% betadine–seawater (Povidone iodine with 0.5% w/v available iodine) for 5 min, after which they were treated with a 3.5% filter-sterilized broad-spectrum antibiotic mixture with nystatin (an antifungal agent) for 48 h (Polne-Fuller & Gibor 1984). The concentration and duration of each chemical treatment as described above was optimized empirically by studying the growth of treated plant material in the laboratory using the most optimized culture conditions and growth medium under nonaxenic conditions. The efficacy of both the liquid detergent and betadine treatments in reducing the microbial growth on explants was also evaluated by counting the microbial colonies developed on ZoBell agar plates by serial dilution (Oppenheimer & ZoBell 1952). The sterility of explants as processed above was eventually confirmed by growing on ZoBell agar plates for at least 2 weeks in a bacterial incubator.

Explant culture and callus induction

After sterilization, explants were cut into 5 mm segments using sterile surgical blades in autoclaved seawater, wiped with sterilized filter papers to remove sterilant and mucilage from the cut ends and transferred to the explant culture medium. Fifteen explants per plate were inoculated on solidified PES medium with 1.5% Bacto agar (Difco, Detroit, MI, USA) and grown initially at 5 μmol photons m^{-2} s^{-1} for 4–5 weeks for production of callus. The excised callus was then subcultured in both liquid as well as solidified PES medium as described above. During subculture, the callus was transferred to fresh medium once every 2 months whereas the liquid medium was replenished every week. All culture dishes were maintained at 30 μmol photons m^{-2} s^{-1} to allow further callus growth and morphogenesis. After removal of the callus, the explants were transferred to fresh culture medium to allow regrowth of callus.

Table 1. Enumeration of bacterial and fungal colonies of explants treated with different chemical sterilants. Values in parenthesis indicate fungal colonies.[1]

Treatment	Dilution			
	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Untreated	UC	UC	UC	47 (13)
Detergent	UC	UC	68 (10)	20 (6)
Betadine	46 (6)	18 (6)	15 (4)	12 (4)
Detergent + betadine	16 (5)	11 (4)	8 (2)	0 (2)

[1] UC, uncountable (formed a thin film of bacterial growth all over the surface).

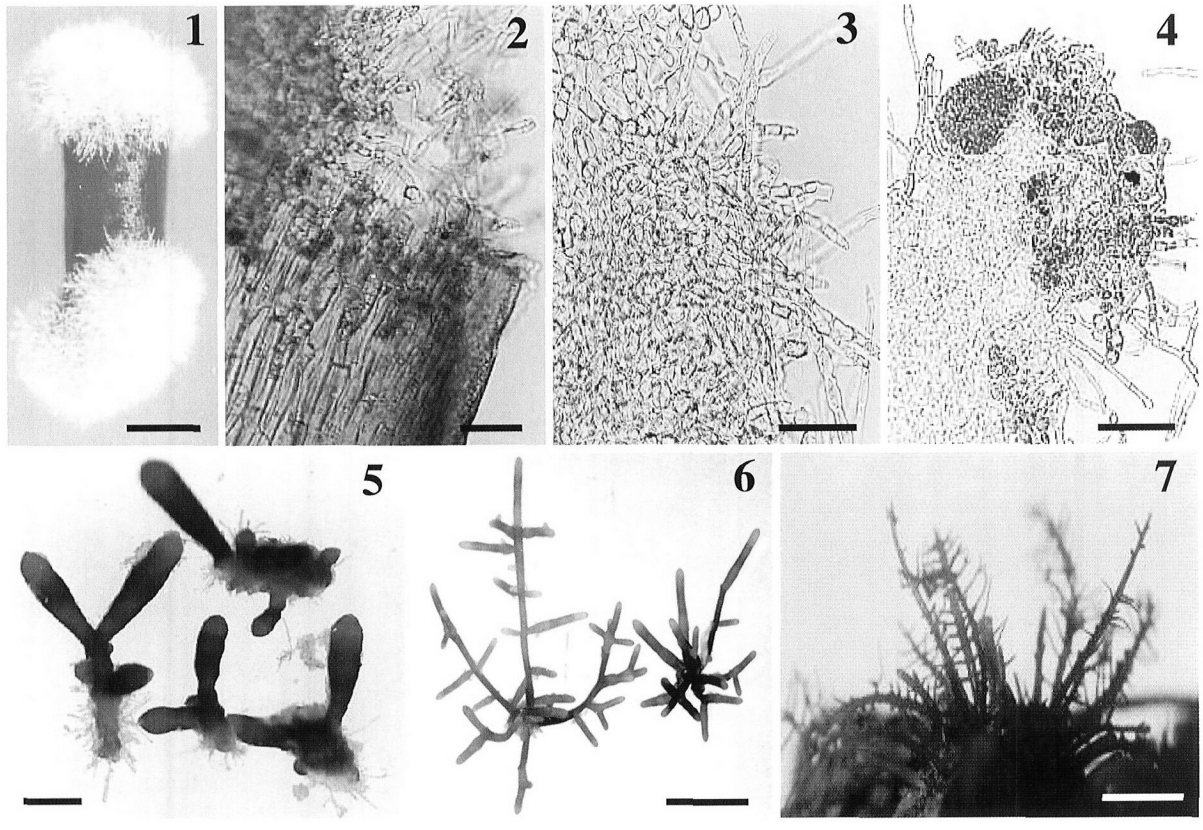
Optimization of light, carbon source and growth regulators to obtain a high callus induction rate

Optimal light intensity for maximum callus induction was determined by culturing the explants under 5, 30 and 70 μmol photons m^{-2} s^{-1} using compatible culture conditions. The effect of different sources of organic carbon on callus induction was tested by culturing explants in medium supplemented with glycerol, sucrose, sorbitol and mannitol at 0.1, 0.5 and 1.0 M. The effect of growth-promoting hormones such as the auxins naphthalene acetic acid (NAA) and indole 3-acetic acid (IAA) and the cytokinins benzyl amino purine (BAP) and kinetin on callus induction was tested using 0.1 or 1.0 mg l^{-1} of each or in combination.

The callus was excised from the explants after 30, 45 or 60 days using a sterile surgical blade, after which the callus was subcultured in both semisolid (0.2% agar) and liquid PES medium on an orbital rotary shaker (100 rpm) and maintained under the culture conditions used to obtain axenic cultures. After growing to 2 cm in length in the laboratory, the propagules were carefully placed on a 2–3 mm nylon thread and wound around a small piece of coral stone, which was then put in a fishnet bag and transferred to the sea for further propagation. The field cultivation experiments were carried out at Krusadai Island (9°16′N; 78°8′E) in the Gulf of Mannar.

RESULTS

The simultaneous treatment of explants with 0.1% detergent for 10 min and 2% betadine for 5 min resulted in almost total elimination of bacterial colonies compared to the untreated control (Table 1). The betadine treatment significantly reduced bacterial contamination of explants to as low as 12 colonies at 10^{-6} dilution, compared to detergent (20 colonies) and the control (> 45 colonies). The bacterial growth at lower dilutions of untreated explants was intense and formed a thin film of bacterial growth over the whole surface of the agar plate. However, fungal contamination was unaffected in all treatments, indicating the need for inclusion of fungicidal agents to the axenization protocol. Treatment of explants with broad-spectrum antibiotics supplemented with nystatin for 48 h following the detergent and betadine treatments enabled the production of viable axenic explants free of microbial contami-



Figs 1–7. Callus induction and regeneration of *de novo* plants from pigmented callus of *Gelidiella acerosa*. Scale bars = 100 μm (Figs 2, 3), 200 μm (Fig. 4) or 1 mm (Figs 1, 5–7).

Fig. 1. Explant with callus on both cut ends after 30 days culture.
Fig. 2. Longitudinal section of explants showing the origin of callus development.
Fig. 3. Morphology of filamentous callus cells.
Fig. 4. Development of embryo-like formations from subcultured callus 10 days after transferring to liquid culture.
Fig. 5. Proliferations of multiple shoots from an excised callus mass after 30 days in liquid culture.
Fig. 6. Growth of 30 days old juvenile plants with rhizoids regenerated from subcultured callus in aerated culture flasks.
Fig. 7. Field grown tissue culture plants 5 months after transplantation.

nants in *G. acerosa*. This protocol consistently rendered a high frequency of axenic explants without affecting the callus development potential. Preliminary laboratory culture studies to evaluate the potentially lethal effect of the above chemical treatments on the growth of plants also showed no deleterious effects.

Callus induction, subculture of excised callus and regeneration of young plantlets through buds has been successfully accomplished in *G. acerosa* (Figs 1–7). The explants showed a filamentous type of outgrowth from the cut ends (Fig. 1), mostly from the cortical and medullary regions (Fig. 2), from 4 days onwards. In some cases, filamentous callus growth was observed over the whole surface of the explant, and occasionally both callus and bud formation was also noticed. After 20 days, the entire cut surface of the explant was covered with filamentous outgrowth that formed a mound with filamentous growth on its surface. The cross section of mounds (filamentous callus-like growth) contained either irregular cell masses or closely intermeshed branched filaments, depending on the stage of growth (Fig. 3). The filaments were uniseriate, mostly unbranched and apical in growth, and contained pigmented cells of varying sizes in chains. The apical cell was small, triangular in shape and had dense cytoplasm. The width of a filament was 7.5–10 μm and cell length ranged from 25 to 50 μm. The cells at the base of the filament were larger. The 40 day old callus measured 2–2.25 mm in diameter.

The excision of callus from the explants generally caused wilting of filaments in the callus mounds, requiring more than a week to recover and regenerate fresh filaments. The process of recovery started with a deep red pigmentation and the regeneration of branched white filaments over the surface. After 4 weeks of subculture, all excised calli developed lobes or spherical buds from the basal surfaces touching the nutrient agar medium. The buds apparently developed from some cells that differentiated into somatic embryo-like formations and subsequently produced a morphotype similar to tetraspore development (Fig. 4). The transfer of such budded calli to semi-solid PES medium (0.2% agar) enabled the rapid morphogenesis and regeneration of buds (Fig. 5) into young plantlets similar to the original plants used for tissue culture. After culturing for 3–4 months, the buds developed into 2–3 cm long plantlets (Fig. 6) in lab cultures. The tissue-cultured *Gelidiella* germlings became well attached on coral stones in field cultivation and successfully grew into full plants in 6 months (Fig. 7).

The callus induction rate of explants varied under different irradiances. Explants cultured at 5 μmol photons m^{-2} s^{-1} showed a positive response with a rate of callus induction as

Table 2. Optimization of agar content in explant culture medium to maximize callus induction rate in *Gelidiella acerosa*.

Agar concentration (%)	Callus induction rate (%)[1]
0.5	BD[2]
0.8	86[3]
1.0	95[3]
1.5	90
2.0	88
3.0	78

[1] Mean values of 100 explants counted on 10th day after inoculation of explants.
[2] Bud development.
[3] Occasional bud development observed.

high as 90%, whereas 30 and 70 μmol photons m^{-2} s^{-1} resulted in 70% and 9% production, respectively. At higher light intensities, both callus and explants started bleaching gradually after 10 days. The culture of explants under blue, red, green or yellow light or in darkness did not favour callus induction (data not shown).

No callus formation was observed in explants cultured on PES medium solidified with a combination of 0.5% each of agar and carrageenan. Nevertheless, in 0.5% agar, all explants showed bud formation but without any callus development (Table 2). Callus induction was observed only in explants cultured on 0.8% agar or above. Bud formation in explants was reduced with increasing agar content in the explant culture medium. Maximum callus induction was observed in 1.0–1.5% agar (more than 90%). Use of different sources of organic and inorganic carbon in the explant culture medium did not enhance the rate of callus formation. All carbon sources used at 0.1 M concentrations showed limited callus induction rate (50%) and growth compared to the control without carbon sources (87%). At higher concentrations, the explants bleached after 10 days in culture. Similarly, there was no obvious effect of hormones on callus induction compared with the control (Figs 8, 9). However, auxins (NAA and IAA) at 0.1 mg l^{-1} concentration showed a callus induction rate similar to that of the control, compared to treatment with cytokinins alone or in combination with auxins.

All subcultured calli produced plantlets irrespective of their harvesting period. Reinduction of callus on explants was observed in all explants when they were grown in PES medium at 5 μmol photons m^{-2} s^{-1} irradiance with 1.5% agar at 20–22°C.

Germlings in field cultivation grew to a maximum length of 4 cm in 6 months from the initial plantings. All tissue culture plants were characterized by a profuse branching pattern and some had as many as 88 branches (Table 3).

DISCUSSION

Successful seaweed tissue culture requires the development of viable axenic explants capable of producing callus that develops into whole normal functional plants. Most of the sterilization protocols followed for obtaining axenic explants either affect the viability of the explants, or result in low callus induction rate or poor callus growth (Gusev *et al.* 1987; Polne-Fuller & Gibor 1987a; Evans & Butler 1988; Aguirre-Lipperheide & Evans 1991, 1993). Polne-Fuller & Gibor (1984, 1987a, b) demonstrated the establishment of axenic explants from a wide range of seaweeds by (1) removing epiphytes ultrasonically; (2) treating the surface with chemical sterilants; and (3) sequentially exposing explants to broad-spectrum antibiotics. In our study, the treatment of explants successively with detergent, chemical disinfectants (like betadine) and

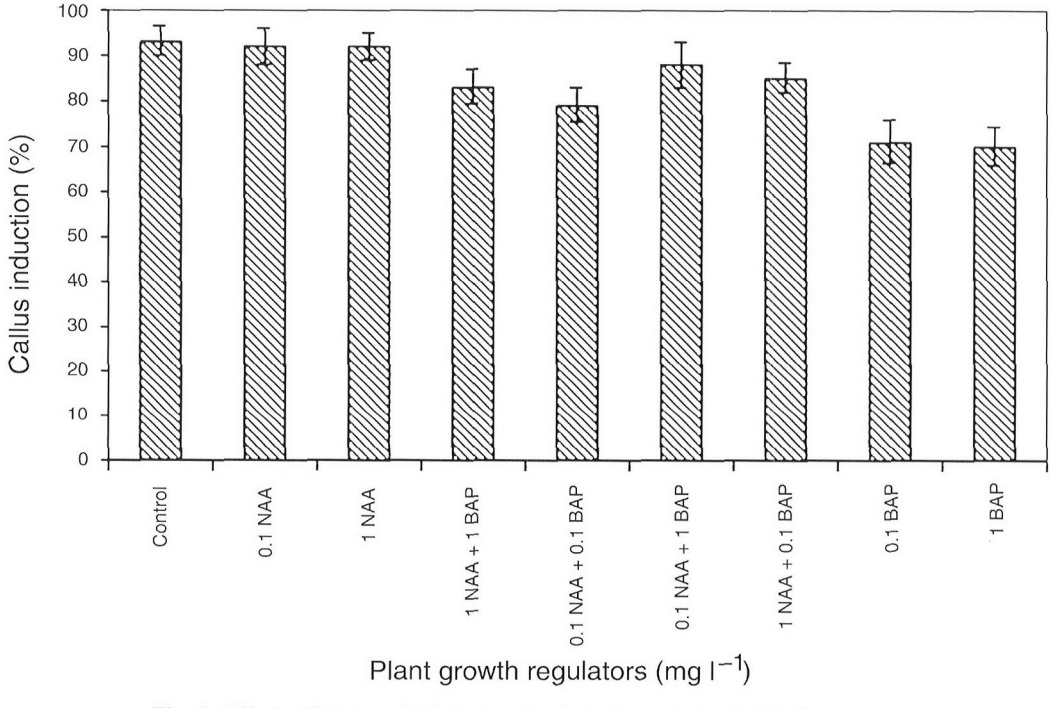


Fig. 8. Effect of NAA and BAP on callus induction rate in *Gelidiella acerosa*.

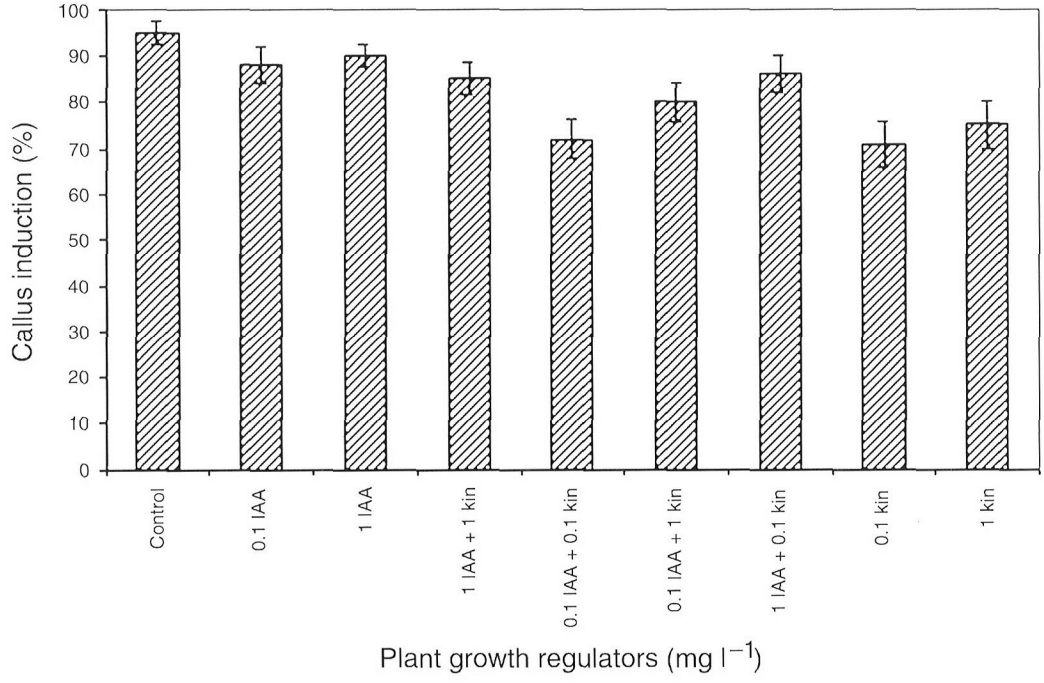


Fig. 9. Effect of IAA and kinetin on callus induction rate in *Gelidiella acerosa*.

broad-spectrum antibiotics with a fungicidal agent (nystatin) enabled us to produce a high frequency of viable axenic material without affecting the callus development and morphogenetic potential of explants.

The development of filamentous outgrowths from cut surfaces, particularly from both cortical and medullary portions, of explants has been reported from a number of red algae (Cheney *et al.* 1987; Polne-Fuller & Gibor 1987b; Bradley & Cheney 1990; Butler & Evans 1990; Robaina *et al.* 1990; Yokoya *et al.* 1993; Huang & Fujita 1997a, b; Reddy *et al.* 2003). The callus in *Gelidiella* was also of the filamentous type and developed primarily from cortical and medullary tissues, though some studies did report callus development in *Gelidiella* from medullary tissues (Dawes & Koch 1991) or cortical portions only (Polne-Fuller & Gibor 1987a). Though there is no experimental evidence of functional differences between cortical and medullary tissue in callus formation, the bud formations in *Gelidiella* were found to develop more frequently from cortical portions of the explants. The occurrence of buds (sometimes with limited filamentous callus formation) directly from cut surfaces of explants in the present study could presumably be explained by their development from pre-embryonic determined cells programmed for embryonic differentiation, as in higher plants (Sharp *et al.* 1980). The abundant growth of filamentous outgrowths from both cut ends of explants in the present study could be due to the consequences of wound healing together with a change of habitat, as well as to the competence of explants for regeneration. Conversely, Robledo & Garcia-Reina (1993) observed apical callus formation in response to physical abrasion in *Solieria filiformis* Kützing grown in air-turbulent cultures.

The frequency of callus formation in seaweeds is highly inconsistent and reported to be dependent on several factors, including the physiological state of the thallus, the season of collection of thallus and the source of the explant from the thallus (Fries 1980; Kawashima & Tokuda 1990; Huang & Fujita 1997b). Tissue culture studies, especially for agarophytes, have been sporadic and have often reported very low callus induction rates. For example, Gusev *et al.* (1987) reported 15% callus induction for *Gelidium vagum* Okamura and 4% for *Gracilaria verrucosa* (Hudson) Papenfuss, whereas Kaczyna & Megnet (1993) recorded 30% for that species. In another study, Polne-Fuller & Gibor (1987a) showed 0.5% and 0.6% callus induction, respectively, with *G. papanfussii* I.A. Abbott and *G. robustum* (Gardner) Hollenberg & I.A. Abbott. Liu & Gordon (1987) achieved 12% in *Pterocladia capillacea*

Table 3. Growth data of four tissue-cultured germlings of *Gelidiella acerosa* in field cultivation.

Dates on which growth measured	Length of plants 1–4 (cm)				Number of branches on plants 1–4			
	1	2	3	4	1	2	3	4
26 Jul. 2002	1.0	0.8	0.3	0.5	7	10	8	7
30 Aug. 2002	1.1	1.3	1.2	0.7	16	17	13	11
26 Sep. 2002	1.7	1.8	1.9	1.2	19	18	23	9
28 Oct. 2002	2.0	2.2	2.3	1.1	36	33	41	13
28 Nov. 2002	3.0	2.6	2.5	0.5	56	61	77	2
27 Dec. 2002	3.5	2.8	3.8	1.2	59	63	86	11
27 Jan. 2003	3.8	4	3.9	1.5	66	70	88	14

(Gmelin) Bornet. In order to achieve consistency in callus induction rate and growth, various culture media supplements (including growth-promoting substances) and carbon supplies (organic and inorganic) have been examined using optimized culture conditions (Bradley & Cheney 1990; Dawes & Koch 1991; Kaczyna & Megnet 1993). The high percentage of callus induction observed in *Gelidiella* could be due to its genetic nature, in addition to the culture medium and conditions that were employed.

The agar concentration in culture media has been reported to affect morphogenesis in higher plants (Debergh 1983). Robaina *et al.* (1990) studied the effects of solidity and osmolality of explant culture medium and reported that the solidity of the culture medium plays an important role in the development of either bud or callus formation in *Gelidium versicolor* (Gmelin) Lamouroux, *Grateloupia doryphora* (Montagne) Howe and *Laurencia* Nägeli. All *Gelidiella* explants cultured in 0.5% agar showed 100% bud formation, whereas the same organism cultured in 0.8–3.0% agar showed callus development under identical culture conditions. The osmolality of the culture medium seems to play a crucial role in determining bud or callus development.

Supplementation of explant culture medium with different carbon supplies did not affect callus growth or induction rate. The bleaching effect of explants after 2 weeks in media with > 0.1 M glycerol, sucrose, sorbitol or mannitol suggests some toxic effect resulting from nonmetabolism of the carbon in the culture medium. McLachlan (1977) described a toxic effect of mannitol on the growth of *Fucus edentatus* de la Pylaie in boron-deficient conditions. Another possibility is that mucilaginous compounds, or the unfavourable pH resulting from the liberation of acidic polysaccharides from cut surfaces, may inhibit the action of the enzymes involved in sugar metabolism (Jacobi 1962).

The exogenous supply of plant growth regulators like IAA, NAA (auxins) and BAP and kinetin (cytokinins), individually or in combination, to explant culture medium showed no obvious effect on callus induction rate or size in *Gelidiella*. Nevertheless, auxins (NAA and IAA) at both of the concentrations tested showed callus induction and growth rates similar to that of the control. This was not the case for cytokinins, individually or in combination with auxin. Further studies are required to illustrate how growth-promoting substances promote callus induction and growth selectively in some algae.

The observed ability of explants to grow additional callus after the excision of the original callus offers the opportunity to increase the rate of callus production and dispense with the expensive and time-consuming production of fresh axenic explants. The new callus developed is similar in every respect to the original callus and so the same explants can be used repeatedly without the need for new ones.

All four of the germlings transferred to the field survived successfully and grew to fully functional plants with fruiting bodies. The thallus became as thick as that of wild plants within 15 to 20 days of transfer, became attached to the coral stone and started branching in all directions. After 6 months, the plants attained maturity by bearing several stichidia. Studies are underway to generate more biomass clonally from tissue-cultured progeny to evaluate the quality and quantity of agar produced.

The foregoing account describes *in vitro* tissue culture methods for micropropagation and field cultivation of tissue culture progeny of *Gelidiella,* which is an important source of bacteriological grade agar in India. The persistence of excised callus in subculture observed in this study provides a new source of material for cell and protoplast studies which in turn offer an impetus for genetic improvement of economically important seaweeds.

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