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Germination and growth of somatic cells of Philippine strains of *Kappaphycus alvarezii* (Doty) Doty (Solieriaceae, Rhodophyta)

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Abstract. Cell culture is immensely useful in strain improvement efforts for commercially important seaweeds. However, application of this technique in *Kappaphycus alvarezii*, a popularly cultured carrageenophyte still needs to be explored. Somatic cells from three Philippine strains of *Kappaphycus alvarezii* were isolated from different thallus segments (apical, middle, and basal) and tissue types (cortical and medullar by dissociating softened tissues. The effects of culture media, irradiance, photoperiod and temperature on the growth of vegetative thalli were determined. *Kappaphycus alvarezii* cells exhibited a monopolar vegetative growth pattern - a protuberance formed at one pole of the cell within 24 to 48 h. Green filaments emerged from the buds on the 18th to 20th day of culture reaching a maximum length of 1.72 mm in 77 days. Although germination was highest (5.89%) in cells cultured in f/2 + 4 ppm Indole-3-butyric acid (IBA), no marked effect of IBA on specific growth rate (SGR) was observed. Maximum SGR (4.30 % day-1) was obtained in cells cultured in f/2 without IBA at 20 µmol $m^{-2}s^{-1}$ and 14L: 10D photoperiod. Excepting sterile seawater (SSW), SGR generally decreased with increasing IBA level in medium. Highest germination growth was from cortical cells of apical segments cultured at low irradiance (20 µmol m^{-2} s^{-1}), low temperature (20°C) and longer duration of light exposure (14L: 10D photoperiod). This study demonstrated the feasibility of producing *Kappaphycus alvarezii* plantlets from coalescence of cells. However, optimization of conditions for propagation of cells and growth of plantlets to seedstock size (ca. 50-100g) in growth hormones other than IBA is recommended.
Key Words: cell culture, monopolar growth pattern, *Kappaphycus alvarezii*.

Introduction. Red seaweeds reproduce asexually through specialized cells called carpospores. However, they also exhibit totipotency. That is, the individual cell, as well as pieces of tissues, behave as a zygote or a spore and may form a whole new organism if isolated from the parent individual. Thus, among red algae, development or progression of a single cell to a multicellular organism, and eventually to maturity, is not limited to spores or zygotes (Waaland 1990). This behavior complements with the complex sexual processes and sporangial structures characteristic for this group, probably to offset the lack of flagellated reproductive and vegetative structures.

Single-cell technology or the ability of single cells to generate differentiated plantlets is immensely useful in strain improvement efforts for commercially useful seaweeds. It would make bypassing of sexuality possible, thus maintaining a pure gene pool. In genetic manipulation, desirable trait can be transferred from one species to another by fusion of vegetative cells (Cheney 1999). Desired mutations can also be induced and specific isolates cloned through isolation and regeneration of viable somatic cells.

Polne-Fuller & Gibor (1987) reported development of new thalli via callus-like growths from isolated cells of a number of green, brown and red seaweeds. Tait et al (1990) were also able to isolate and culture a range of cell lines obtained from tissues of *Porphyra umbilicalis*. In the present, however, reports on the application of this technology to *K. alvarezii*, a popularly cultured carrageenophyte in the Philippines, is scanty.

This study explores the application of single cell technology in the production of germlings as precursors of *Kappaphycus alvarezii* seedstocks. The primary aim is to determine the conditions for germination and growth of *Kappaphycus alvarezii* somatic cells. Specifically, the effects of the plant growth hormone Indole-3-butyric acid (IBA), different culture media, irradiance levels, photoperiod and temperature on germination and growth of somatic cells of different cell ages (apical, middle, and basal) and cell types (cortical and medullar) were investigated.

Materials and Methods

Effects of IBA levels, culture media, irradiance and photoperiod on cell germination and growth. Thalli of healthy *Kappaphycus alvarezii* (green giant strain collected from Bohol, Philippines) were cleaned thoroughly in sterile seawater (SSW) using a soft brush. The epiphyte-free thalli were blot-dried in paper towels and the middle parts of the main axes, representing the intermediate region between young and old tissues, were cut into 2-5 cm segments. The seaweed pieces were soaked in SSW with antibiotic solution (0.25 mg mL^{-1} chloramphenicol + 0.25 mg mL^{-1} ampicillin) for a week or until the tissues have softened.

The cells were dissociated by scraping the softened thallus with the blunt side of a sterile scalpel in a Petri dish filled with SSW. The cells were strained through 500 μm nylon sieve to remove large tissues. The density of the cells in the suspension was then estimated using a Sedgewick rafter counting chamber and was determined as: cells mL^{-1}= (ave. no. of cells/volume of transect) x spell out (CF), where CF= concentrated sample/volume of sample (Azanza & Aliaza 1999).

About 5 mL cell suspensions were dispensed in each of the 48 sterile Petri dishes (16 treatments x three replicates) containing either SSW (33 ± 1 ppt) or f/2 enriched with IBA at 0, 4, 8 and 12 pm. Cells were cultured in the dark and at 60 μmol $m^{-2}s^{-1}$ in a lightbench under two 40-watt fluorescent white lamps at 12L: 12D photoperiod and at 24 ±1°C. Cultures were enriched with culture media once a week. Germination rate (GR) was determined using the following formula: GR = (no of germinated cells/initial cell density) x 100. Filament length was measured on the 43rd day of culture using the ocular micrometer eyepiece (4x) of an inverted microscope.

Plantlets grown in the preceding experiment were further cultured for 34 days in two growth media (f/2 and SSW enriched with 0, 4, 8, 12 ppm IBA), at two levels of irradiance (20 μmol m^{-2} s^{-1} and 60 μmol m^{-2} s^{-1}), two photoperiods (14L: 10D and 10L: 14D) and at 24 ± 1°C. Specific growth rate (SGR) was determined as percent growth day^{-1} using the equation: SGR = Ln (L_2/L_1) x t^{-1}, where t is time in days, L_1 is the initial length and L_2 the length on day t (Orduña-Rojas & Robledo 1999).

Germination and growth of cells from different tissue types, thallus age and cultivars. Epiphyte-free thalli from apical, middle and basal portions of three strains of *Kappaphycus alvarezii* (Bohol, green giant; strain L and Bohol Bisaya) were cut into 2-5 cm segments and were soaked in antibiotic solution (Chloramphenicol + Ampicillin concentration/dosage) for about a week until the tissues have softened. Cells from different thallus regions were dissociated separately in SSW and strained through 200 μm and 500 μm sieves. The cell density per ml was estimated as previously described.

Subsequently, isolation and culture of cells from different tissue types were conducted. Cortical and medullar cells from apical segments of the cultivar and thallus region that yielded the highest rate in the preceding experiment were isolated. The apical

thalli were washed in SSW to remove epiphytes and then soaked in 75 mL SSW + 25 mL antibiotic solution until the tissues have softened. The soft cortical tissues were placed in a Petri dish filled with SSW and slightly scraped off using the blunt side of scalpel. The medullar tissues were lightly mashed using a stirring rod.

The cells were cultured in f/4 (+ 4 ppm IBA) in sterile vials and placed in a lightbench at 20 μmol photon m^{-2} s^{-1}, 14L: 10D photoperiod and at 20°C, 25°C and 30°C. Germination rates were determined after 2 weeks and the germlings were further grown for 20 days.

Statistical analysis. The experiments were conducted in multifactor CRD. Data were tested for homogeneity of error variances before analysis using unvaried ANOVA to determine significant differences among mean values. If significant differences were detected, post analysis was made using Duncan's Multiple Range Test (DMRT) at α=0.05 using the SPSS software (v. 10.0).

Results

Effects of IBA levels, culture media, irradiance and photoperiod on cell germination and growth. Formation of protuberance or bud at one pole of the cell was observed within 24h to 48h in cells cultured in nutrient-rich (f/2) medium with IBA. On the 10th day, the buds became brown and nearly spherical and either remained large or further developed into small clumps of brownish cells (Figure 1). The filaments which emerged from the brownish mass formed green radiating disks in about 18 to 20 days. In 32-34 days, the original brown mass of cells developed into basal discs of filaments which tend to overlap and congregate.

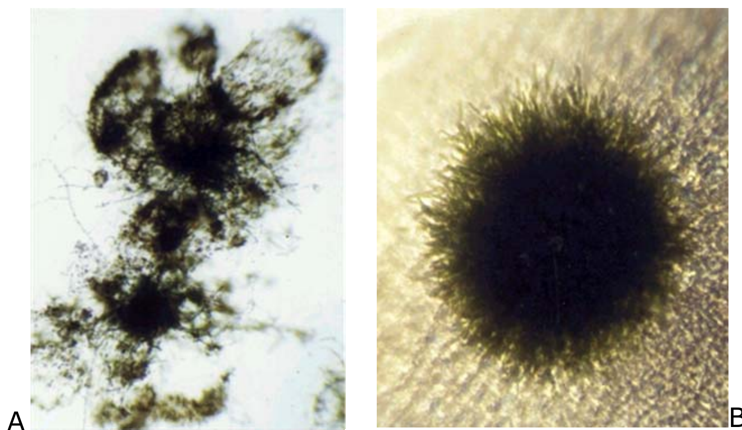

Figure 1. Germinating *Kappaphycus alvarezii* cells, filaments emerging from cells (A) and filaments radiating from a germinating disk (B).

Generally, germination rate was lower in cells cultured in SSW and highest (5.89%) in f/2 + 4 ppm IBA at 60 μmol photon m^{-2} s^{-1} (Table 1).Cells cultured in the dark did not germinate. Filaments were thick and green in richer media, but were brown, unbranched and threadlike in cells cultured in SSW (Figure 2). At 43 days of culture, filament length was highest (1.02 mm) in f/2 +4 ppm IBA cultured at 60 μmol photon m^{-2} s^{-1} (Table 1) but numerical differences observed between different levels of IBA, culture media and irradiance were not statistically significant.

Table 1

Germination rate and filament length of cells from middle segment of *Kappaphycus alvarezii* (Bohol, giant green) cultured at 24 $\pm$ 1°C, 60 μmol photon m^{-2} s^{-1} and 12L: 12D photoperiod in f/2 and SSW enriched with different levels of Indole-3-butyric acid (IBA).

IBA level (ppm)	Germination rate (%)		Filament length (mm)	
	f/2	SSW	f/2	SSW
0	2.72	0.16	0.30	0.41
4	5.89	1.92	1.02	0.26
8	3.14	1.60	0.48	0.27
12	3.28	0.96	0.34	0.22

Specific growth rate (SGR) was highest (4.30%) in *Kappaphycus alvarezii* cells cultured in f/2 without IBA, at 14L: 10D photoperiod and at 20 μmol photon m^{-2} s^{-1} (Table 2). Regardless of duration of light exposure, SGR of cells cultured in SSW at 60 μmol photon m^{-2} s^{-1} generally decreased with increasing IBA levels. But at 20 μmol photon m^{-2} s^{-1}, SGR of cells cultured in SSW increased with increasing IBA levels. At 77 days, filament lengths of germlings ranged from 0.18 mm to 1.78 mm.

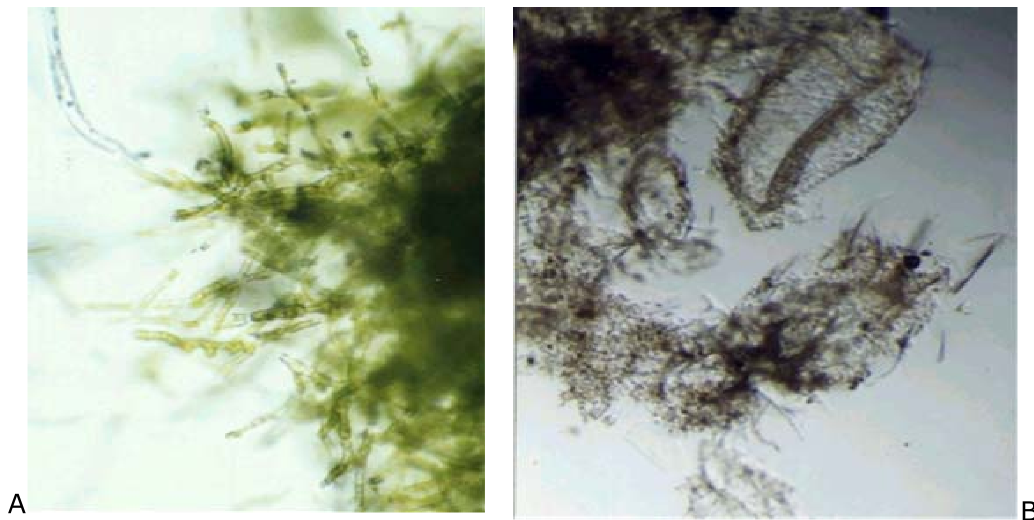

Figure 2. Filaments arising from *Kappaphycus alvarezii* cells cultured in F/2 (A) and in SSW (B) enriched with 4 ppm IBA.

Table 2

Specific growth rate (SGR) and filament length of *Kappaphycus alvarezii* (Bohol, giant green) cells cultured for 77 days at 24 ± 1°C at various irradiance and photoperiod in culture media enriched with varying levels of Indole-3-butyric acid (IBA)

Photo period	IBA level (mm)	SGR (%)				Filament length (mm)			
		60 μmol photon $m^{-2} s^{-1}$		20 μmol photon $m^{-2} s^{-1}$		60 μmol photon $m^{-2} s^{-1}$		20 μmol photon $m^{-2} s^{-1}$	
		f/2	SSW	f/2	SSW	f/2	SSW	f/2	SSW
14L:10D	0	3.38	1.52	4.30	1.37	1.00	0.89	0.98	0.90
	4	2.69	1.34	2.46	0.65	1.15	0.95	1.56	0.41
	8	2.58	-0.45	2.36	1.80	1.71	0.57	1.78	1.45
	12	2.24	-0.36	1.59	3.49	1.41	0.56	1.17	1.95
10L:14D	0	2.15	3.58	1.82	0.88	1.62	1.48	0.64	0.71
	4	1.40	0.70	1.17	1.36	1.53	0.53	0.75	0.91
	8	1.35	0.30	-0.31	2.39	1.10	0.80	0.50	1.44
	12	1.39	0.20	-2.53	2.67	0.67	1.07	0.18	1.23

Germination and growth of cells from different tissue types, thallus age and cultivars. Generally, germination was high in cells obtained from young tissues (apical segments) than from mature tissues (middle and basal segments). Germination rate was highest (8.7%) in cells from apical segment of Bohol Bisaya cultured in f/4 (Figure 3). Apical cells from different strains appear to have different nutrient requirements. Cells from segments of Bohol giant and strain L exhibited higher germination rate in f/2 and F media, respectively.

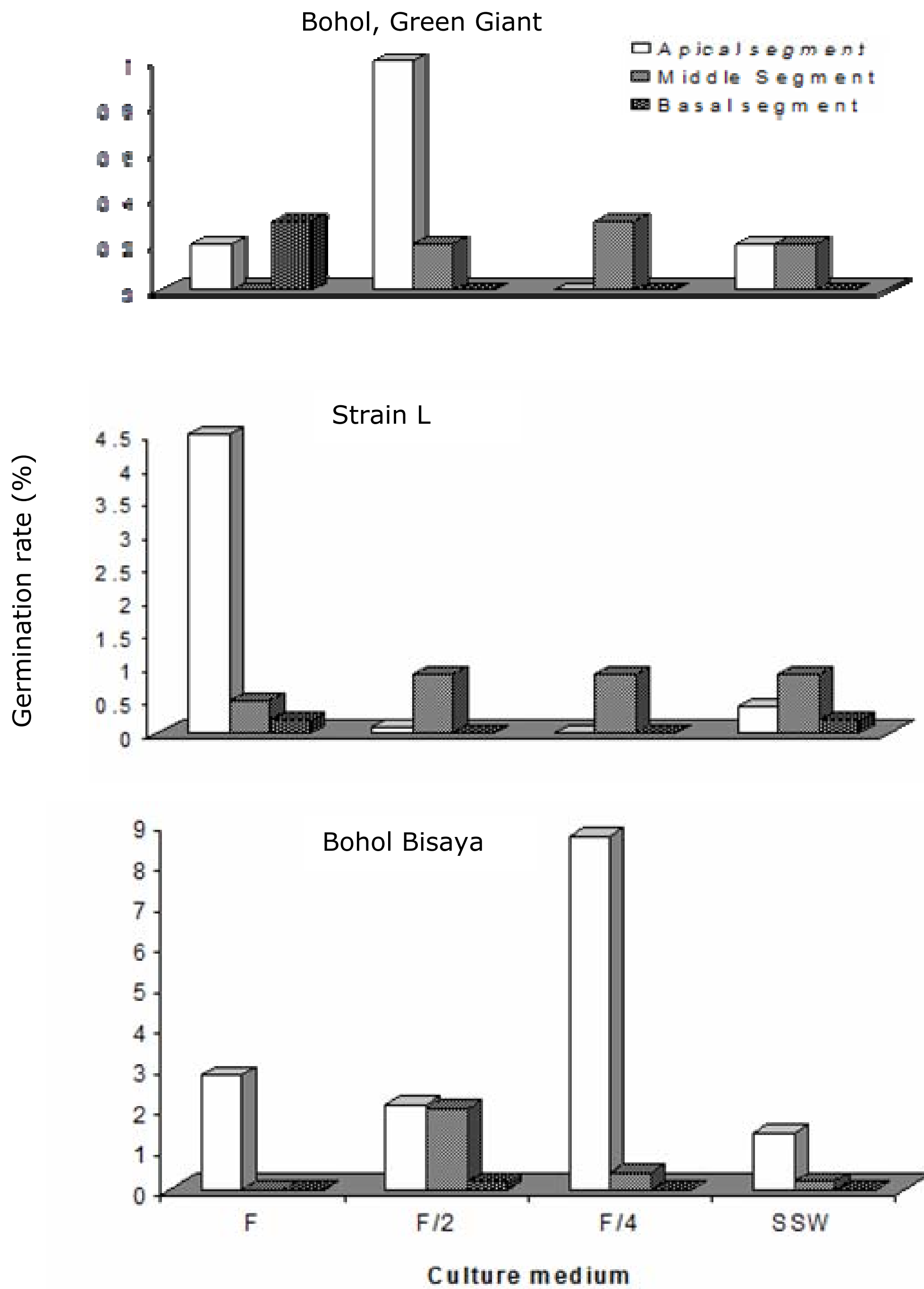

Figure 3. Germination rate (%) of vegetative cells from different thallus segments of three *Kappaphycus alvarezii* cultivars cultured in different media for 18 – 20 days.

Cortical cells germinated better than medullar cells in all treatments (Figure 4). At 20 days of culture, filament lengths of germlings from cortical cells cultured at 20°C were longer (mean 1.62 mm).

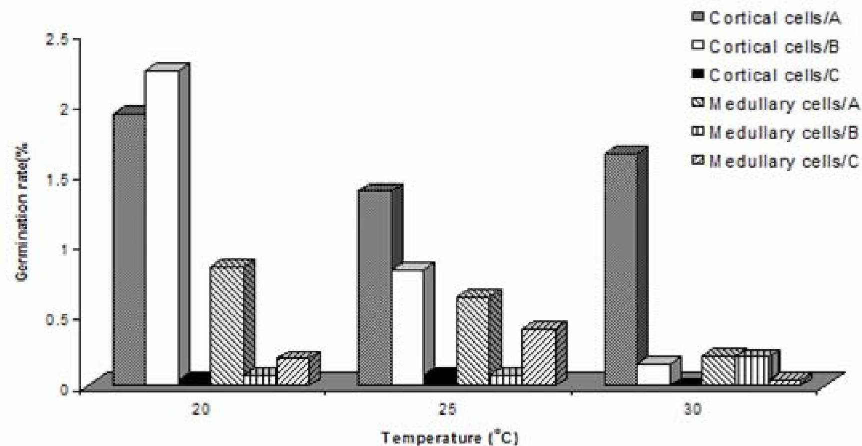

Figure 4. Germination rates (%) of cortical and medullar cells isolated from the apical segment of *Kappaphycus alvarezii* using three methods and cultured at different temperature for 15 days. Methods of cell isolation: A= cells dissociated from softened thalli, B= cells dissociated from fresh thalli, C= cells dissociated by heating.

Discussion. Germination of *Kappaphycus alvarezii* cells appears to be monopolar-forming a single protruberance or bud in one pole from which filaments arise. Formation of tiny buds, referred to as somatic embryos, from callus cells of *Kappaphycus alvarezii* has also been reported by Reddy et al (2003). This germination pattern is known to occur among spores of many red seaweeds such as the zygotospores of some *Porphyra* species (Guiry 1990), but differs from polarity of regeneration reported for isolated vegetative cells of higher Florideophycidae by Waaland (1990) and Guiry (1990). Liu & Gordon (1987) have also observed formation of buds from cortical layer of *Pterocladia* segments within 4 days of culture in PES medium. In the present work, some of the protuberances or buds appeared similar to the cellular extensions reported by Azanza-Corrales & Dawes (1989) which developed from medullar and cortical cells of wounded *Eucheuma alvarezii* var. *tambalang* tissues. The buds later became a mass of brown cells, serving as initial prostrate basal system, from which one or more thalli later developed. Similar germination pattern, which favored secure attachment prior to development of erect thallus, was also observed in spores of *Gracilaria cornea* (Orduña-Rojas & Robledo 1999).

In the present work, the germination process in *Kappaphycus alvarezii* cells was similar to spore development in most red seaweeds, thus providing evidence of the totipotency of the isolated vegetative cells. However, the possibility that the single cells may have undergone modification into reproductive cells could not be discounted. Modification of single cells into reproductive cells that discharge many spores (which germinate parthenogenetically) has been observed in three species of *Ulva* (Reddy et al 1989).

In the present study, the filaments radiating from *Kappaphycus alvarezii* cells which developed in 18 to 20 days were composed of elongated cells. These were morphologically similar to the outward-growing uniseriate filaments arising from the outer cortex of cross-

sectional discs of *Agardhiella subulata* (Cheney et al 1987). Erect shoot formation from spores occurred within 21 days at 28°C in *Gracillaria cornea* (Orduña-Rojas & Robledo 1999) and 28 days in *Gracilariopsis bailinae* (Rabanal et al 1997).

The concentration of the plant growth regulator hormone tested in the present study was within the range of auxin levels that produce healthy segments and growth in 5 mm explants of *Eucheuma denticulatum* and *Kappaphycus alvarezii* (Dawes & Koch 1991, Dawes et al 1994). However, except for differences in filaments width and color, there was no marked effect of IBA levels on germination and growth under the range of experimental conditions studied. This finding was similar to the results obtained by Liu & Gordon (1987) on the effects of the growth hormone naphthalene-acetic acid (NAA) on *Pterocladia* and *Porphyra* species, wherein growth is inhibited at higher concentration. The addition of the growth hormone zeatin at 1 mg L^{-1} and NAA at 0.01 mg L^{-1} in the culture medium is routine in the germination of cross-sectional discs of *Agardhiella subulata* (Cheney et al 1987), but in *Kappaphycus alvarezii* in the present study, IBA appeared to be required only by cells cultured in SSW but not by those cultured in rich medium. Supplementation of NAA and BAP individually or in combination did not also result in increases in callus induction rate or callus growth in *Kappaphycus alvarezii* (Reddy et al 2003). *Kappaphycus alvarezii* cells require low light intensity for growth and this finding was consistent with the generalization that red algae grow better in a relatively low irradiance level (Gantt 1990). For instance, specific growth rates of *G. cornea* carposporelings were also highest at low irradiance level (10 μmol photon m^{-2} s^{-1}) (Orduña-Rojas & Robledo 1999).

The SGR values obtained in the present study was comparable to the growth rate value (3.6% day^{-1}) considered optimum for commercial production of *Eucheuma* (Parker 1974), but was lower than the values (5.8 – 7.2 % day^{-1}) reported by Hurtado & Cheney (2003) for outplanted propagules of *Eucheuma denticulatum* produced through tissue culture. Compared with many *Gracilaria* species, the specific growth rates of *K. alvarezii* germlings were low (max. 3.38% day^{-1}). Growth rate of *G. cornea* was 5.2% day^{-1} while those of other *Gracilaria* species ranged from 6.0-25% day^{-1} (Orduña-Rojas & Robledo 1999).

Cells from the different thallus segments of *Kappaphycus alvarezii* germinated and grew under a broad range of conditions (i.e. different culture media and temperature levels of 20-30°C), but highest germination rate and growth were from cortical cells of the apical segments at the lowest irradiance level (20 μmol photon m^{-2} s^{-1}) and at the lowest temperature level (20°C) tested, at relatively longer duration of light exposure (14L: 10D photoperiod). In the natural environment however, high light intensity (1000 – 2750 μE m-2 s-1) and high temperature levels have been found necessary for better growth of farmed *K. alvarezii* (Trono & Valdestamon 1994). These findings indicated that the growth requirements of single cells differed from relatively large *K. alvarezii* tissues. However, it was also possible that the three cultivars studied were adapted to lower irradiance levels. Based on photosynthetic-irradiance responses, Dawes (1992) found sun-type responses (adaptation to high irradiance levels) and shade-type responses (adaptation to low irradiance levels) in Philippine cultivars of *Eucheuma denticulatum*.

The relatively high germination and growth rates of apical cells (young cells) compared to cells from basal segments justify the use of apical parts of the thallus as seedstocks in commercial farming, and the use of cortical cells from the apical segments in the production of germlings as precursors of *Kappaphycus alvarezii* seedstocks. This finding also confirmed the occurrence of an apico-basal gradient in thallus age in *Kappaphycus alvarezii*, which has been reported by Waaland (1990) to occur among advanced red seaweeds (Florideophyceae) exhibiting pseudoparenchymatous growth type.

Conclusions. The series of conducted studies demonstrated the feasibility of producing *Kappaphycus alvarezii* germlings from single cells or clump of cells. However, the possible effects of growth hormones other than IBA on germination of *Kappaphycus alvarezii* cells

and growth of germlings need to be investigated. The conditions for growth of germlings to seedstock size (ca. 50 g), such as duration of light exposure, frequency of nutrient enrichment and cell density, should be optimized, and experiments comparing growth of seedstocks produced from cells with cuttings from previous cropping season under field conditions should also be conducted.

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References

Azanza-Corrales R., Dawes C.J. 1989 Wound healing in cultured *Eucheuma alvarezii* var. *tambalang* Doty. Bot Mar 32: 229-234.

Azanza R.V., Aliaza T.T. 1999 *In vitro* carpospore release and germination in *Kappaphycus alvarezii* (Doty) Doty from Tawi-tawi, Philippines. Bot Mar 42: 281-284.

Cheney D.P., Luistro A.H., Bradley P.M. 1987 Carrageenan analysis of tissue cultures and whole plants of *Agardhiella subulata*. Hydrobiologia 151/152: 161-166.

Cheney D.P., 1999 Strain improvement of seaweeds through genetic manipulation: current status. World Aquaculture 30: 66-67.

Dawes C.J., Koch E.W., 1991 Branch, micropropagules and tissue culture of the red algae *Eucheuma denticulatum* and *Kappaphycus alvarezii* farmed in the Philippines. J Appl Phycol 3: 247-257.

Dawes C.J., 1992 Irradiance acclimation of the cultured Philippine seaweeds, *Kappaphycus alvarezii* and *Eucheuma denticulatum*. Bot Mar 35: 189-195.

Dawes C.J., Lluisma A.O., Trono G.C., 1994 Laboratory and field growth studies of commercial strains of *Eucheuma denticulatum* and *Kappaphycus alvarezii* in the Philippines. J Appl Phycol 6: 21-24.

Gantt E., 1990 Pigmentation and photoacclimation in Cole K.M., Sheath R.G., eds. Biology of the Red Algae, Cambridge Univ. Press, Cambridge, UK, 508 pp.

Guiry M.D. 1990 Sporangia and spores in Cole K.M., Sheath R.G., eds. Biology of the Red algae. Cambridge Univ. Press, Cambridge, UK, 508 pp.

Hurtado A., Cheney D.P., 2003 Propagule production of *Eucheuma denticulatum* (Burman) Collins et Harvey by tissue culture. Bot Mar 46: Fasc. 4.

Liu X.W., Gordon M.E., 1987 Tissue and cell culture of New Zealand Pterocladia and Porphyra species. Hydrobiologia 151/152: 147-154.

Orduña-Rojas J., Robledo D., 1999 Effects of irradiance and temperature on the release and growth of carpospores from *Gracilaria cornea* J. Agardh (Gracilariales, Rhodophyta). Bot Mar 42: 315-319.

Parker H.S., 1974 The culture of the red algal genus *Eucheuma* in the Philippines. Aquaculture 3: 425-439.

Polne-Fuller M., Gibor A., 1987 Calluses and callus-like growth in seaweeds: induction and culture. Hydrobiologia 151/152: 131-138.

Rabanal S.F., Azanza R., Hurtado-Ponce A., 1997 Laboratory manipulation of *Gracilariopsis bailinae* Zhang et Xia (Gracilariales, Rhodophyta). Bot Mar 40: 547-556.

Reddy C.R.K., Migita S., Fujita Y., 1989 Protoplast isolation and regeneration of three species of *Ulva* in axenic culture. Bot Mar 32: 483-490.

Reddy C.R.K., Raja Krishna Kumar G., Siddhanta A.K., Tewari A., 2003 *In vitro* somatic embryogenesis and regeneration of somatic embryos from pigmented callus of *Kappaphycus alvarezii* (Doty) Doty (Rhodophyta, Gigartinales). J Phycol 39: 610-616.

Tait M.I., Milne A.M., Grant D., Somers J.A., Staples J., Long W.F., Williamson F.B., Wilson S.B., 1990 *Porphyra* cell cultures: isolation, growth and polysaccharide production. J Appl Phycol 2: 63-70.
Trono G.C., Valdestamon R.G. 1994 New aspects in the ecology and culture of *Kappaphycus* and *Eucheuma*. Kor J Phycol 9: 205-216.
Waaland S., 1990 Development. Cambridge Univ. Press, Cambridge, UK, p.508.

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