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Branch, micropropagule and tissue culture of the red algae *Eucheuma denticulatum* and *Kappaphycus alvarezii* farmed in the Philippines

Clinton J. Dawes [1] & Evamaria W. Koch [2]
[1] *Department of Biology, University of South Florida, Tampa, FL 33620-5150, USA;* [2] *Department of Marine Science, University of South Florida, St Petersburg, FL 33701, USA*

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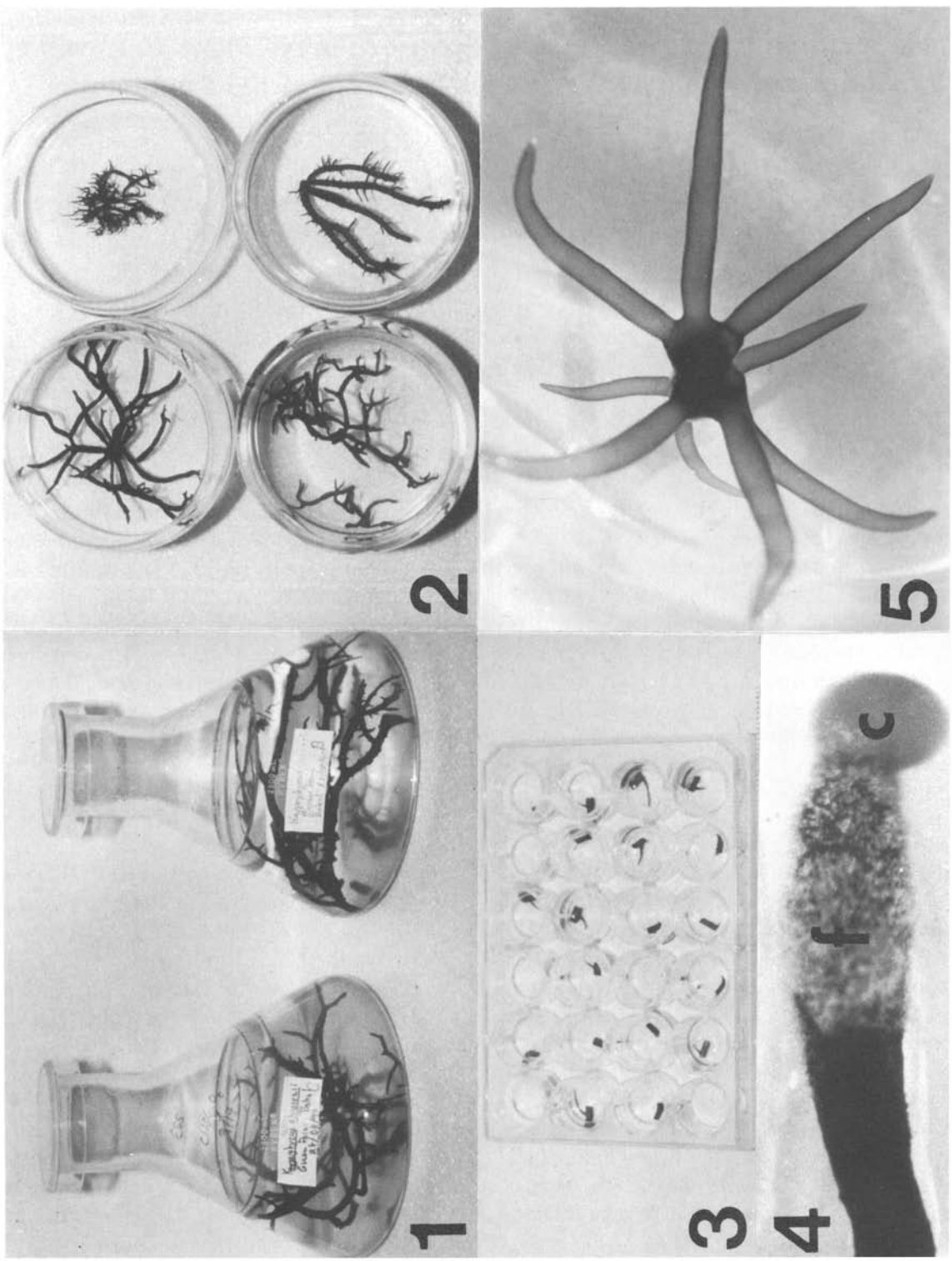
Abstract

Three forms of the iota-producing carrageenophyte, *Eucheuma denticulatum*, and four forms of the kappa-producing carrageenophyte, *Kappaphycus alvarezii*, obtained from seaweed farms in the Philippines have been grown in the laboratory under unialgal and axenic conditions. Comparison of media indicates that seed stocks of both species can be cultured using enriched seawater media ranging from ESS and SWMD-1 to inexpensive soil extract (Erdshreiber's) or holding in sterile seawater for up to three weeks. Micropropagation has been successful with at least two forms of each species resulting in clonal propagation from axenic explants within 4 to 8 weeks. Callus development and branch regeneration has also been induced in two forms of each species. The results indicate that culture facilities in the farming areas of the Philippines could maintain high-yielding and rapidly growing seed stock for the seaweed farmers.

Introduction

A variety of morphological and pigment forms of the red seaweeds *Eucheuma denticulatum* (Burnan) Collins and *Kappaphycus alvarezii* (Doty) Doty (Solieriaceae) are extensively farmed in the Philippines, as well as in Indonesia and elsewhere in the South China Sea, for the phycocolloid carrageenan; iota and kappa respectively (Dawes, 1981; Doty, 1985; Doty & Norris, 1985). Dried *Eucheuma* and *Kappaphycus* from Philippine farms alone account for 70% of all the semi-refined carrageenan in the world (Llana, 1990). The farming of these forms began in the early 1970's and is presently estimated to be a major source of livelihood for over 10000 coastal inhabitants of the Philippines (Llana, 1990; Trono, 1987). Harvests of farmed weed declined by 22.5% in 1987 (22038 to 17086 t dry m) with only a slight (0.2%) increase in 1988 to 1989 (18336 to 18386 t dry m; Llana, 1990). Furthermore, the harvest of natural populations has declined by 80.8% from 1984 to 1987 (2533 to 488 t dry m) with this decline continuing into 1990 (Llana, 1990).

The decline in farmed seaweed appears to be due in part to the selection procedures of seed stock by the *Eucheuma* farmers in the Philippines (G.C. Trono, pers. comm.). The farmers select the best growing plants from one harvest for the next crop, thus selecting a seed stock that is not acclimated to seasonal shifts in growing condi-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tions. In addition, there no longer is a sufficiently large source of natural populations to serve as new seed stock for each growing season. This suggests a need for laboratory culture and maintenance of high-yielding and rapidly growing seed stock for each farming season. The cultured stocks could also be used in the selection of new high-yielding strains through genetic engineering (Cheney, 1986).

The overall objective of our studies with Philippine species of *Eucheuma* has been to develop procedures for branch, micropropagule and tissue culture so that seed stock can be maintained inexpensively in facilities near the farming areas of the Philippines. The plantlets generated through micropropagation and tissue culture will also be used in seed stock selection via physiological studies. This paper reports the culture procedures utilized to transport, grow branches, produce micropropagules and callus, and regenerate seed stock of *Kappaphycus alvarezii* and *E. denticulatum*.

Materials and methods

Transportation

Three forms of *Eucheuma denticulatum* (A: red, spinose; D: green, spinose; H: brown, flew blunt branchlets) and four forms of *Kappaphycus alvarezii* (B: brown, blunt branches; C: green, blunt branches; F (brown) and G (green) both with irregular recumbent branching were obtained by air freight from the Philippine farms on the Danajon Reef (Azanza-Corrales, 1990) and an experimental farm of the University of the Philippines Marine Science Institute at Bolinao on northern Luzon. Branches up to 30 cm long and 3 cm diameter were shipped in foam plastic coolers with 1 cm diameter holes in the sides (Trono, pers. communication). Either live *Sargassum* or wet paper towels were used as packing material to keep the branches moist, the branches being layered loosely between the packing material. The plants were cleaned of epiphytes and debris and rinsed in seawater prior to transportation.

Branch culture

After arrival, the branches were trimmed to 15 cm, rinsed in filtered (Whatman #2) seawater, placed in Fernbach flasks (up to four 15 cm branches) with 1800 ml 34 ppt salinity autoclaved seawater and left overnight in a growth chamber (25 °C, 10 to 12 h photoperiod, 25 μmol photon m^{-2} s^{-1}). The next day, the branches were gently damp dried with paper towels and rinsed in distilled water. If bacterial contamination was evident (turbid media), the branches were rinsed for 30 s to 2 min in an antibiotic solution (15 mg erythromycin, 10 mg gentamicin, 30 mg neomycin, 35 mg polymixin B l^{-1}) and transferred without rinsing to either Fernbach flasks (15 cm branches, Fig. 1) or 300 ml storage dishes (5 cm branches, Fig. 2). Enriched seawater media were tested using 5 cm branches in 300 ml storage dishes. Media included a modified form of ESS (Saga, 1986) which contained plant growth regulators (PGR's) and an antibiotic mixture (see Azanza-Corrales & Dawes, 1989 for modifications), VS media (Guiry & Gunningham, 1984), Grund (Von Stosch, 1963), soil extract (Erdschreiber's media; McLachlan, 1973), and a commercial media, Alga Grow (Carolina Biological Supply Co.), as well

Fig. 1. Two Fernbach flasks (2800 ml) containing form C (left) and B (right) of *Kappaphycus alvarezii* that had been transported from the Philippines to the U.S.A. (× 0.25).

Fig. 2. Four storage dishes (300 ml) containing branches of form A of *Eucheuma denticulatum* (2A), a form of *E. isiforme* from the Florida Keys (2B), and two plants regenerated from micropropagules of form C (2C, 2D) (× 0.5)

Fig. 3. 24-well (3 ml each) multiwell plate containing micropagules of form C (× 0.5).

Fig. 4. Callus developed at the cut end of a main axis (c) and crystalline filamentous growth (f) of form C over the surface of an explant cultured on 8% agar (× 5).

Fig. 5. Multibranched plant regenerated from callus (central portion) of form C (× 2).

as SWMD-1 (Chen & Taylor, 1978). To reduce handling, lower cost of culturing, and avoid contamination, various regimes for nutrient enrichment were tested ranging from culture in continuously enriched seawater media to culture in sterile seawater with limited (1–3 d wk^{-1}) periods of enrichment. Also, a range of temperatures (18 to 26 °C), irradiances (20 to 160 μmol photon $m^{-2} s^{-1}$), pH (6.5 to 9.0), and methods of cleaning (use of distilled water, brushing, ultrasonic bath) were compared. Since they are stenohaline seaweeds (Dawes, 1979), salinity was maintained at 34 to 36 ppt.

Micropropagule production

In order to determine minimum explant size, epiphyte-free main axes of four forms A, C, D, and F were cut into 1, 2, 3, and 5 mm segments ($N = 6$) using sterile procedures and growth was monitored over a 4 week period. A factorial experiment ($n = 3$ explants) tested for the phytotoxicity of antibiotics at various concentrations (15, 30 mg l^{-1} erythromycin, rifampin, and neomycin each; 300, 600 units 100 ml^{-1} polymyxin B), an antiseptic betadine (1, 10%) and a fungicide (2.5 g l^{-1} captan) by soaking the explants for 3 days. After axenic procedures for handling explants were determined, the explants were soaked in the appropriate antibiotic and transferred to 3 ml wells of Falcon Multiwell Tissue Culture Plates (Fig. 3) with 2 ml of sterilized seawater enriched with ESS (without PGRs). Factorial combinations ($N = 3$; 0, 1, 5, 10 mg l^{-1}) of auxins (PAA = phenylacetic acid; IBA = indol-3-butryic acid; IAA = indole-3-acetic acid; NAA = -naphthalenacetic acid) and cytokinins (2iP = N^6-[-2-isopentenyl]adenine; BAP = 6-benzylaminopurine; Z = zeatin; K = kinetin) were tested over a 4-week period. Axenic testing was carried out by pipetting 1–2 ml of the used culture media into autoclaved seawater containing 0.5% sucrose ($N = 10$).

Callus production

Epiphyte-free main axes of forms A_2 and D (*Eucheuma denticulatum*) and forms C and F (*Kappaphycus alvarezii*) were cut into 0.5 cm sections using sterile procedures and the explants soaked in polymyxin B sulfate (3000 units l^{-1}) for up to 3 h, based on the results from micropropagule production. The segments were shaken gently every 3 to 5 min while in the antibiotic solution. Substrates used were 0.8%, 3%, and 8% agar and 0.8%, 1.6% carrageenan all dissolved in seawater and ESS enriched. The media was also supplemented with 10 mg l^{-1} NAA and 1 mg l^{-1} 2iP in addition to the base levels of those plant growth regulators in the ESS media (Azanza-Corrales & Dawes, 1989). The segments were placed on the agar or carrageenan substrate so that one of the cut surfaces was exposed to the air.

A factorial experiment ($n = 2$ explants) tested for callus development of two auxins at four concentrations (NAA, PAA, 0, 0.1, 1.0, 10.0 mg l^{-1}) and of two cytokinins at two concentrations (2iP, BAP; 0, 1 mg l^{-1}) for four weeks using 3% agar and ESS enriched seawater without standard PGRs. Plant regeneration from callus was carried out by cutting the callus into 2 mm pieces and transferring them to test tubes with sterile liquid ESS media, with growth regulator supplements as described for the agar substrate.

Results

Branches of the 7 forms remained healthy for at least 5 days during air shipment using the shipping conditions described above. The branches of 5 forms have been cultured successfully for over a year, the other 2 forms (G, H) for over 6 months.

All of the media tested except Grund and Alga Grow were effective in maintaining the branches (Table 1). A modified form of ESS was chosen because growth rates were highest (up to 1.58% d^{-1}). The soil extract was effective even without supplementation with NO_3 and PO_4 for periods up to 4 to 8 weeks and SWMD-1 could be used

Table 1. Growth rate (percent wet wt d^{-1}) of 5 cm branches of *Eucheuma denticulatum* (form A_1) and *Kappaphycus alvarezii* (form C) in enriched seawater using VS_5 and VS_{10} (Guiry and Cunningham, 1984), modified ESS (Azanza-Corrales & Dawes, 1989), Grund (Von Stosch, 1963), soil extract, (Erdschrieber Media, McLachlan, 1973) and Alga Grow (Carolina Biological Supply). $N = 3$ (± 1 standard error).

	Form A_1	Form C
ESS (52 days)	0.64 (0.78)	1.58 (1.30)
VS_5 (52 days)[1]	1.03 (0.17)	0.52 (0.19)
VS_{10} (52 days)[1]	0.53 (0.12)	0.32 0.12)
Grund (44 days)	0.77 (0.30)	plants died
Soil Extract (44 days)	0.86 (0.33)	0.33 (0.08)
Alga Grow (44 days)	0.84 (0.41)	plants died

[1] 5 and 10 ml of stock added to 1 liter seawater, respectively.

if supplemented with meso-inositol (not shown in Table 1). It was not necessary to keep branches continuously in enriched seawater media, growth rates of 0.5 to 1% d^{-1} occurred if plants were maintained in sterile seawater and pulse-enriched for 1–3 d every 1 or 2 wk with modified ESS enriched, autoclaved seawater.

The various forms of both Philippine species grew under irradiances of 25 to 160 μmol photon m^{-2} s^{-1} with optimum growth around 60 μmol photon m^{-2} s^{-1}. Branches of both forms grew at 22 to 26 °C and died at lower temperatures. The pH of the medium was critical with highest growth rates (1.5% d^{-1}) around 7.5 to 8.0; at lower pH levels the plants showed tip discoloration and dieback suggesting a limitation in CO_2 availability.

Weekly cleaning to remove epiphytes was required for the first 3 to 6 weeks. Cleaning procedures included rinsing under tap or distilled water, brushing with a soft toothbrush under running water, or using an ultrasonic bath (1 megacycle s^{-1}) with distilled water for 30 s. Reduction of bacterial contamination was best accomplished by dipping the branches for 30 s in the antibiotic solution (full strength) described for branch culture in the Materials and Methods. Germanium dioxide (1 to 3 days, 5 $\mu g\, l^{-1}$) was effective in removing diatom contamination but the Philippine forms showed tip dieback if exposed over longer periods (5 + days) or to higher concentrations.

Micropropagation was successful for all four forms tested. Explant size was critical with survival rates of 17% (1 mm), 50% (2 mm), and 100% (3 and 5 mm) after four weeks ($N = 6$). The most effective antibiotic soak for producing axenic explants was 30 min in polymixin B sulfate (300 mg 100 ml^{-1}) which reduced contamination rates (<25%). No bacterial contamination was evident in all 10 samplings of media in which explants grew indicating axenic culture conditions had been established. Other antibiotics used at similar concentrations have caused tip damage on branches (neomycin, rifampin), discoloration (erythromycin, captan) or were ineffective at nontoxic concentrations to the plant (1 or 10% Betadine). To insure axenic culture, 1 ml of the stock antibiotic of polymixin B was added to each litre of ESS enriched seawater. Based on the results from branch culture, the multiwell plates were incubated at 25 °C, 25 μmol photons m^{-2} s^{-1} and 12:12 h light:dark photoperiod.

The factorial experiment ($N = 3$) testing effects of 4 auxins and 4 cytokinins using 0, 1, 5, and 10 mg l^{-1} concentrations showed different responses by each of the 4 forms (Tables 2A, 2B, 2C, 2D). Form A_2 of *Eucheuma denticulatum* showed growth in all explants with 5 and 10 mg l^{-1} IBA and all concentrations of K, 2iP, BAP (Table 2A). The green form, D, of *E. denticulatum* also showed strong responses to 1 or 5 mg l^{-1} IAA with Z, 2iP, and BAP (Table 2B). Forms B and F of *Kappaphycus alvarezii* showed positive growth in all concentrations of IBA, IAA, and NAA with all four cytokinins while PAA caused variable responses (Tables 2C, 2D).

During the first week, 1 to 2 mm of growth occurred in the form of dark pigmented cells at the medullary region (center of cut area) in the explants of the 6 forms of both species as well as by elongation of the spines of *Eucheuma denticulatum* (forms A, D). Growth was rapid and most explants produced 5 to 10 spines from a 0.5 cm segment. Within 3 to 4 weeks the explants had outgrown the 3 ml wells and were transferred either into 10 ml multiwell plates or 300 ml storage

Table 2A. Micropropagation of 5 mm explants of *Eucheuma denticulatum* (form A_2) using 16 combinations of four auxins (phenylacetic acid = PAA, indole-3-butryic acid = IBA, indole-3-acetic acid = IAA, α-naphthalenacetic acid = NAA) and four cytokinins (zeatin = Z, kinetin = K, N^6-(2-isopentenyl) adenine = 2iP, 6-benzylaminopurine = BAP). All factorial experiments used 0, 1, 5, 10 mg l^{-1} concentrations of each plant growth regulator in modified ESS media (Saga, 1986) without PGR's. New growth (= +) was measured at the end of 4 weeks (1, 2, 3 = healthy segments out of 3 replicates).

		PAA				IBA				IAA				NAA			
		0	1	5	10	0	1	5	10	0	1	5	10	0	1	5	10
Z	0	3	3	2	2	3	2	3	3‡	3	3	3	3	3+	3	2	2
	1	3	3‡	2+	2	2	3	2	2‡	3+	3	3+	3	3+	3+	3	2
	5	3	3+		1	3	3‡	2‡	3‡	1+				3+	3		1
	10	3	3+			2	2‡	2‡	2‡	1				3‡	3+	3	3
K	0	3	3‡			3+	3‡	3‡	3‡	3	3	3‡	2‡	3	3	3	3
	1	3				3+	3‡	3‡	3‡	3	3+	3+	3‡	3	3	3	3
	5	3	3+			3+	3‡	3‡	3‡	3	3	3‡	3‡	3	3	3	3
	10	3	3+			3+	3‡	3‡	3‡	3	3‡	3‡	3‡	3	3	3	3
2iP	0	3	3			3	3	3‡	3‡	3	3	1	1	3	3	3	3
	1	3	3‡			3	3	3‡	3‡	3	3		1	3	3	3	3
	5	3	3+			3	3	3‡	3‡	3	3			3	3	3	3
	10	3	3+			3	3	3‡	3‡	3	3		1	3	3	3	3
BAP	0	3	3+			3	3	3‡	3‡	3	3	1		3	3	3	3
	1	3	3+			3	3	3‡	3‡	3	3			3	3	3	3
	5	3+	3			3	3	3‡	3‡	3	3			3	3	3	3
	10	3	3			3	2	3‡	3‡	3	3	1		3	3	2	2

dishes with modified ESS media for continued growth. After 4 months in culture micropropagules of both species reached 3 to 4 g wet weight in the 300 ml storage dishes and were again dissected for micropropagation or callus production.

Callus has been obtained from explants of *Kappaphycus alvarezii* (forms C and F) and to a much smaller degree by *Eucheuma denticulatum* (forms A and D) depending on the combination of auxin and cytokinin (Table 3). The highest NAA concentration tested (10 mg l^{-1}) killed all explants of forms A_2 and D of *E. denticulatum* (Table 3). Additionally, most of the callus formed on these explants was of the filamentous type while explants of *K. alvarezii* had a higher rate of 'bubbly' type of callus from which new plants would regenerate.

Rates of contamination were lowest (< 10%) when the explants were taken from micropropagules. Callus formed on 75% of the explants originated from older branches. Fewer (10%) and smaller callus growths occurred on young branches or spines. The callus was formed on the cut ends exposed to the air and consisted of an

Table 2B. Micropropagation of 5 mm explants of *Eucheuma denticulatum* (form D). See Table 2A for details.

		PAA				IBA				IAA				NAA			
		0	1	5	10	0	1	5	10	0	1	5	10	0	1	5	10
Z	0	3	3			3	3	3	3	3+	3‡	3+	3+	3	3	3	3
	1	3	3+	1	1	3	3	3	3	3	3‡‡	3‡	3+	3	3+	3	3
	5	3	2	1		3	3+	3	3	3‡	3‡‡	2+	2+	3	3+	3	3+
	10	2	1			3+	3‡	3+	3	3‡	3‡	1		3	3	3	3
K	0	3	3‡			3	3	3‡	3+	3	3	3		3	3	3	3
	1	3	3‡‡			3	3+	3‡‡	3‡	3	3	3		3	3	3	3
	5	3‡	2			3	3+	3‡‡	3‡	3	3	2+		3	3	3	3
	10	3‡	1+			3	3+	3‡	3‡	3	3	1+	2	3	3	3	3
2iP	0	3	3‡			3	3	3‡‡	3‡‡	3	3	3‡‡	3‡‡	not done			
	1	3‡	3‡‡			3	3	3‡	3‡‡	3	3‡‡	3‡‡	3‡‡				
	5	3+	2‡			3	3	3‡	3‡‡	3	3‡‡	2‡					
	10	3‡	3+			3	3	3‡‡	3‡‡	3	3‡‡						
BAP	0	3+	3‡			3	3	3‡	3‡‡	3	3	2‡	3‡‡	not done			
	1	3	3‡‡			3	3	3‡	3‡‡	3	3	3‡‡	1+				
	5	3	3‡			3	3	3‡	3‡‡	3	3	3‡‡	1+				
	10	3	1			3	3	3+	3‡‡	3	3	1+					

undifferentiated brown to beige mass (forms C and F) or white to pink masses (forms A and D) of tissue. The cell masses originated from the medullary and inner cortical cells at the cut surface. They reached 1 to 5 mm in height within 4 weeks, with a bubbly appearance ('c' of Table 3, Fig. 4). In addition, white crystalline, filamentous cells grew along the epidermis of the explant ('f' of Table 3), originating from the epidermal cells at the cut edge (Fig. 4).

Callus and crystalline filamentous cells were produced only in media containing 3 or 8% agar and not on either concentration of carrageenan. The use of agar in modified ESS enriched seawater supplemented with 1 mg l^{-1} NAA or 0.1 and 1 mg l^{-1} of PAA and 1 mg l^{-1} 2iP or BAP induced the largest callus in *Kappaphycus alvarezii* within 4 weeks (Table 3). Explants placed in 0.8 or 1.6% carrageenan produced side branches that grew into the media but could not be induced to form callus.

Within 2 days after transfer to ESS enriched seawater, growth on the pieces of callus became visible and within 5 days small green branch buds were evident in forms C and F. Branch buds on callus of forms A and D were slower to develop, taking over 4 weeks to produce 1 cm-long branches. Green pigmented branches developed from the callus tissue of Form C within a week and reached 3 to 4 cm in length within 4 weeks of transferring the branches to 300 ml storage dishes in ESS enriched seawater (Fig. 5). The crystalline cells that developed from all forms did not regenerate.

Table 2C. Micropropagation of 5 mm explants of *Kappaphycus alvarezii* (form B). See Table 2A for details.

		PAA				IBA				IAA				NAA			
		0	1	5	10	0	1	5	10	0	1	5	10	0	1	5	10
Z	0	2	3‡	1+	2‡	All 3+++				3‡	3‡	3‡	3‡	All 3+++			
	1	2	1+	2‡	2‡					3‡	3‡	3‡	3‡				
	5	2	1+	2‡	3‡					2	3‡	2‡	1+				
	10		2‡							3‡	3‡	3‡	3‡				
K	0	2‡	1			3+	3+	3‡	3‡	3+	3+	3‡	3‡	All 3+++			
	1	3‡	2‡			3	3+	3‡	3‡	3+	3‡	3‡	3‡				
	5	2‡	1			3‡	3+	3‡	3‡	3+	3‡	3‡	3‡				
	10	2‡	3‡			3‡	3‡	2‡	3‡	3‡	3‡	3‡	3‡				
2iP	0	3‡	3‡	2‡	2‡	3+	3‡	3‡	3‡	3‡	3‡	3‡	3‡				
	1	3‡	3‡	2‡	2‡	3‡	3‡	3‡	3‡	3‡	3‡	3‡	3‡				
	5	3‡	2‡	1+	1+	3‡	3‡	3‡	3‡	3‡	3‡	3‡	3‡	Not done			
	10		1+			3‡	3‡	3‡	2+	3‡	3‡	3‡	1+				
BAP	0	3‡	1+			3+	3	3‡	3‡	3‡	3‡	3‡	3‡	Not done			
	1	3‡	3‡			3	3	3‡	3‡	3‡	3‡	3‡	3‡				
	5					2	2+	2‡	2+	3‡	3‡	3‡	2‡				
	10					1+	1+			1+	2‡	3‡	1+				

Discussion

The present study has demonstrated techniques for the successful culture of branches of 3 forms of *Eucheuma denticulatum* and 4 forms of *Kappaphycus alvarezii* obtained from seaweed farms in the Philippines. The highest growth rates and healthiest plants were obtained using a modified form of ESS (Saga, 1986), which contains meso-inositol; a compound that increases the rate of wound healing in all forms of *Eucheuma* and *Kappaphycus* (Azana-Corrales & Dawes, 1989). The use of the soil extract enrichment for culturing, and the holding of branches up to 4 weeks in filtered seawater without enrichment offer inexpensive alternatives for maintenance of seed stock in Philippine culture facilities.

Cloning of branch segments to produce micropropagules was successful for all 6 forms tested. This procedure, which resulted in production of a large number of new plants, is simple, rapid, and less expensive than propagation from callus. The multiwell plates can be reused after cleaning, soaking in 10% bleach overnight, rinsing in distilled water and microwave irradiation (3 x 2 min at full power). In terms of producing seedstock for *Eucheuma* farms, the micropropagation method appears to have great potential.

The classic paper of Chen & Taylor (1978) was the first to describe callus production in a carrageenophyte, *Chondrus crispus*, and the regeneration of the callus into new plants. More recently Polne-Fuller & Gibor (1987) reported successful callus development and regeneration of new

Table 2D. Micropropagation of 5 mm explants of *Kappaphycus alvarezii* (form F). See Table 2A for details.

		PAA				IBA				IAA				NAA			
		0	1	5	10	0	1	5	10	0	1	5	10	0	1	5	10
Z	0	3‡	3‡	1+	3‡	All 3+ + +				All 3+ + + +				All 3+ + +			
	1	3‡	3‡	2‡	2‡												
	5	3‡	3‡	3‡	1+												
	10	3‡	2‡		1+												
K	0	3	3‡	1		All 3+ + +				All 3+ + +				All 3+ + +			
	1	3‡	3‡	2	1+												
	5	3‡	3‡	3‡	2+												
	10	3‡	3‡		1+												
2iP	0	3‡	3‡			All 3+ + +				All 3+ + +				All 3+ + +			
	1	3‡	3‡	1	1+												
	5	3‡	3‡														
	10		3‡														
BAP	0	3‡	3‡			All 3+ + +				All 3+ + +				All 3+ + +			
	1	2‡	3‡														
	5	2+	2‡														
	10	3	2‡														

plants in *Eucheuma uncinatum* and *E. alvarezii*. Bradley and Cheney (1990) using factorial experiments also showed that the PGRs used in this study would result in callus formation in *Agardhiella subulata*. Success in callus production of agarophytes has been more wide spread and at least 11 species have been studied (Gusev *et al.*, 1987; Garcia-Reina *et al.*, 1988; Liu & Gordon, 1987; Liu *et al.*, 1990).

In this study, callus was produced from medullary tissue in both *Eucheuma denticulatum* (forms A and D) and *Kappaphycus alvarezii* (forms C and F). Callus also developed from the medullary tissue of *Agardhiella subulata* and regenerated into new plants (Cheney *et al.*, 1987). In contrast, Polne-Fuller & Gibor (1987) reported that callus from *E. uncinatum* and *E. alvarezii* developed from the cortical tissue or from filiaments that grew out from sections placed on agar or carrageenan. The difference may be due to their use of 0.3 to 1.5% agar while this study used 3 and 8% agar. The procedures described here extend the earlier studies on *Eucheuma uncinatum* (Polne *et al.*, 1981) and confirm the techniques for callus production (Polne-Fuller & Gibor, 1987). These tissue culture procedures should prove useful in the bioengineering of plants having higher yields and quality carrageenan (Cheney, 1986).

These culture procedures and clonal production could be a source of seed stock for the *Eucheuma* farms in the Philippines. Strain selection should also be possible; for example, the 7 forms in culture are being compared with regard to pigment content and photosynthetic responses under varying irradiances. The testing of cloned plants in the field must still be carried out.

Table 3. Callus (c) and crystalline filament (f) formation in *Eucheuma denticulatum* (forms A_2, D) and *Kappaphycus alvarezii* (forms C, F) using factorial combinations of auxins (PAA and NAA, 0, 0.1, 1.0, 10 mg l^{-1}) and cytokinins (2iP and BAP, 0 and 1 mg l^{-1}). All responses are after 4 weeks in culture using 3% agar with ESS enriched seawater without standard PGRs. $N = 2$. (–) = no response, (d) = dead.

Auxin Conc:			0	0.1	1.0	10.0
Eucheuma denticulatum (form A_2)						
NAA	2iP	0	2f	1f, 1c	1f, 1 –	2d
		1	2f	1f, 1 –	1f, 1 –	2d
	BAP	1	1f, 1c	2f	1f, 1c	2d
PAA	2iP	0		2f	1f, 1 –	2f
		1		2f	1f, 1 –	2f
	BAP	1		1f, 1c	1f, 1c	2f
Euchema denticulatum (form D)						
NAA	2iP	0	2f	1f, 1 –	1f, 1 –	2d
		1	1f, 1 –	1c, 1 –	1f, 1 –	2d
	BAP	1	1c, 1 –	2 –	2 –	2d
PAA	2iP	0		2 –	2f	2 –
		1		2 –	1f, 1c	1f, 1 –
	BAP	1		1f, 1d	1c, 1 –	2f
Kappaphycus alvarezii (form C)						
NAA	2iP	0	2f	2f	2f	2 –
		1	1f, 1c	1f, 1c	2c*	2 –
	BAP	1	2f	1f, 1 –	2c*	1c, 1 –
PAA	2iP	0		2f	2c	1c, 1f
		1		2c	1c, 1f	2c
	BAP	1		1c, 1f	2f	2f
Kappaphycus alvarezii (form F)						
NAA	2iP	0	2f	1c*, 1f	1c, 1f	2f
		1	2f	1c*, 1f	2c*	1f, 1 –
	BAP	1	2c	2f	2f	1c*, 1 –
PAA	2iP	0		2f	2f	1c, 1f
		1		2c	2c*	2f
	BAP	1		2f	2f	2f

* Callus larger than 1 mm diameter.

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