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Use of Acadian marine plant extract powder from *Ascophyllum nodosum* in tissue culture of *Kappaphycus* varieties

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Abstract Three varieties of *Kappaphycus alvarezii* (Kapilaran, KAP), Tambalang purple (PUR), Adik-adik (AA), and one variety of *Kappaphycus striatum* var. *sacol* (green sacol (GS) were used to determine the efficiency of Acadian marine plant extract powder (AMPEP) as a culture medium at different concentrations, for the regeneration of young plants of *Kappaphycus* varieties, using tissue culture techniques for the production of seed stock for nursery and outplanting purposes for the commercial cultivation of carrageenophytes. A shorter duration for shoot formation was observed when the explant was treated with AMPEP+Plant Growth Regulator (PGR=PAA+zeatin at 1 mg L^{-1}) compared to AMPEP when used singly. However, four explants responded differently to the number of days required for shoot formation. The KAP variety took 46 days to form shoots at 3–4 mg L^{-1} AMPEP+PGR; while PUR required 21 days at 3–5 mg L^{-1} AMPEP and 3–4 mg L^{-1} AMPEP+PGR. AA required 17 days at 3–5 mg L^{-1} AMPEP and AMPEP+PGR; and GS 25 days at 1 mg L^{-1} AMPEP+PGR. It was observed that among the four explants used, PUR and AA initiated shoot formation with the use of AMPEP only at higher concentrations (3–5 mg L^{-1}) after a shorter period. Only PUR responded positively to ESS/2 for shoot initiation. The use of AMPEP alone and/or in combination with PGR as a culture medium in the propagation of microplantlets using tissue culture technique is highly encouraging.

Keywords Soluble seaweed extract powder · Tissue culture · Shoot growth · *Kappaphycus*

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Introduction

Historically, seaweeds have been used as soil conditioners in improving plant growth in agricultural crops especially in areas near to coastlines (Chapman and Chapman 1980). The invention of liquid fertilizer by Milton (1952) and dry powder by Stephenson (1974) provided more access of fertilizer to users in horticulture and agriculture activities. Commercially, extracts from brown algae are good sources of fertilizer.

Ascophyllum nodosum (rockweed) is a brown seaweed known to grow abundantly in temperate countries such as Canada, France, Iceland, Ireland, Norway, and the United Kingdom. This seaweed is usually replaced or mixed with other related species such as *Fucus* sp. in the most exposed or iced scoured areas (Sharp 1986). It is sustainably harvested by hand cutter rake in The Maritimes, Canada (Ugarte and Sharp 2001; Sharp et al. 2006; Ugarte et al. 2006) with an estimated 7,500 WT in 2004. *Ascophyllum nodosum* is the most important commercial seaweed in Canada and it is the dominant perennial seaweed in the intertidal zone along the Atlantic coastline of the Maritimes

where it forms extensive beds. The extract products of *A. nodosum*, both liquid concentrate and soluble powder, are traded globally for agricultural farming purposes.

The chemical composition for product consistency and performance of *A. nodosum* has been determined quantitatively by ^{1}H NMR and Principal Component Analysis (PCA) techniques (Craigie et al. 2007). The authors found a relatively dense cluster using PCA indicating small gross variations in chemical composition suggesting that harvesting of *A. nodosum* for soluble extracts could be undertaken any time of the year and therefore its performance as a seaweed-based fertilizer is remarkably consistent. The performance of *A. nodosum* as a plant growth promoter was further tested in *Arabidopsis thaliana* as a model plant (Rayorath et al. 2007). Other investigations show that extracts from *A. nodosum*, both as soluble powder and as liquid extract, promoted root-tip elongation and shoot growth compared to controls in the model land plant. Rayorath et al. (2008) showed that organic components of *A. nodosum* extract induced amylase activity independent of GA_3 and might act in concert with GA-dependent amylase production leading to enhanced germination and seedling vigor in barley. The performance of *A. nodosum* extract was further tested in carrots. A significantly reduced incidence of foliar fungal disease in carrots was observed when sprayed with extract of *A. nodosum* at 0.2% compared to control plants sprayed with water (Jayaraj et al. 2008). Furthermore, the authors claimed that *A. nodosum* extract is more effective than salicylic acid in reducing infection.

Recently, the liquid seaweed extract Kelpak was used to promote growth in the cultivation of *Gracilaria* and *Ulva* under tank and field conditions in South Africa (Robertson-Anderson et al. 2006). The authors reported that the addition of Kelpak or a combination of Kelpak and fertilizer could be useful in commercial seaweed mariculture. Furthermore, the intermediate Kelpak concentration (1:2,500) produced the highest growth of *Ulva* in the turbot water, while the highest Kelpak concentration (1:500) inhibited *Ulva* growth.

Successful micropropagation of *Kappaphycus alvarezii* and *Eucheuma denticulatum* has been reported by Dawes and Koch (1991), Dawes et al. (1993, 1994), Hurtado and Cheney (2003) and Hurtado and Biter (2007) using different culture media. Reddy et al. (2003) reported the somatic embryogenesis and regeneration of embryos from pigmented callus of *K. alvarezii* as a potential means for clonal production of seed stock for commercial farming. All the authors reported the use of inorganic elements in the preparation of culture media.

The present study was conducted to determine the efficiency of AMPEP from *Ascophyllum nodosum,* a seaweed-based culture medium in the regeneration of *Kappaphycus* varieties using tissue culture techniques for the production of seed stock for nursery and out-planting purposes for the commercial cultivation of carrageenophytes.

Materials and methods

Three varieties of *Kappaphycus alvarezii* (Kapilaran, KAP), Tambalang purple (PUR), Adik-adik (AA) and one variety of *Kappaphycus striatum* var. *sacol* (green *sacol* (GS) were collected from a farming area in Tictauan Is., Zamboanga City, Philippines (6°54′53.06″ N and 122°10′43.33″ E) and used as explants in the study. Explants were placed in an ice chest, covered with cheesecloth previously soaked in seawater and brought to the Aquaculture Department Southeast Asian Fisheries Center (SEAFDEC), Tigbauan, Iloilo. The plants were acclimatized in concrete tanks for 5 days, provided with continuous aeration and a flow-through water system. Only healthy apical 2-cm segments were cut using a sterile blade, wiped with a paper towel, shaken three times with glass beads (1 mm size), brushed with 0.05% Povidone iodine, and finally rinsed three to four times in autoclaved seawater. Cleaned segments were incubated in 9.1% E3 antibiotic solution for 3 days at 23°C, 13:11 h L:D cycle and 10–15 μmol photons m^{-2} s^{-1} irradiance.

After 3 days, each segment was cut into 2-mm thick sections, rinsed three to four times with autoclaved seawater, placed individually in a 48-well culture plate containing AMPEP, a gift from Acadian Seaplants Limited, Nova Scotia, Canada (Table 1). Since this was a novel application of AMPEP for seaweeds, different concentrations (0.001, 0.01, 0.1, 1.0, 2.0, 3.0, 4.0, and 5.0 mg L^{-1}) of AMPEP were prepared at pH 7.8 and filtered through a 0.22-μm nylon membrane (Whatman) and used as culture media to the different varieties of *Kappaphycus*.

Another set of sections (n=8) was incubated in 48-cell well plates with the same concentrations of AMPEP but with additional 0.1% plant growth regulator (=PAA+Zeatin at 1 mg L^{-1}; Hurtado and Cheney 2003; Hurtado and Biter 2007). ESS/2 (half strength of Enriched Sterile Seawater)+ E3 (antibiotic mixture)+PGR was used as a control. The cell culture plates were sealed with Parafilm and completely covered with aluminum foil for 7 days. Incubation was done at 23°C, 13:11 h L:D cycle and the culture plates were exposed at 10–15 μmol photons m^{-2} s^{-1} irradiance after 7 days. Media were changed weekly. Release of new cells and consequent growth and development were observed periodically using an Olympus IX70 Coolsnap CF Monochrome Microscope

Sections with young 1–2-mm shoots were transferred to T25 flasks with 40 mL of the corresponding medium concentration, and incubated at 23°C, 13:11 h L:D cycle and 5–10 μmol photons m^{-2} s^{-1} irradiance. Shoots of

Table 1 Composition of Acadian marine plant extract powder 1–1–17 from *Ascophyllum nodosum*

Physical data	
Appearance	Brownish-black crystals
Odor	Marine color
Solubility in water	100%
pH	10.0–10.5
Typical analysis	
Maximum moisture	6.5%
Organic matter	45–55%
Ash (Minerals)	45–55%
Total nitrogen (N)	0.8–1.5%
Available phosphoric acid (P_2O_5)	1–2%
Soluble potash (K_2O)	17–22%
Sulfur (S)	1–2%
Magnesium (Mg)	0.2–0.5%
Calcium (Ca)	0.3–0.6%
Sodium (Na)	3–5%
Boron (B)	75–150 ppm
Iron (Fe)	75–250 ppm
Manganese (Mn)	5–20 ppm
Copper (Cu)	1–5 ppm
Zinc (Zn)	25–50 ppm
Carbohydrates	Alginic acid, mannitol, laminarin
Amino acids (total 4.4%)	
Alanine	0.32%
Arginine	0.04%
Aspartic acid	0.62%
Cystine	0.01%
Glutamic acid	0.93%
Glycine	0.29%
Histidine	0.08%
Isoleucine	0.26%
Leucine	0.41%
Lysine	0.16%
Methionine	0.11%
Phenylalanine	0.25%
Proline	0.28%
Serine	0.08%
Threonine	0.04%
Tyrosine	0.17%
Valine	0.28%
Tryptophan	0.07%

2–5 mm were placed in 200-mL culture medium in 250-mL culture flasks and incubated at 23°C, 13:11 h L:D cycle and 30–45 μmol photons m^{-2} s^{-1} irradiance with gentle aeration.

Regenerated microplantlets (>5 mm) were placed in perforated plastic containers (50 mL capacity) and transferred to a 1-tonne capacity tank at ambient temperature and irradiance. Salinity was lowered from 39 ppt by approximately 3 ppt every week to acclimatize the thallus to ambient seawater (≈30 ppt). Dipping of each microplantlet in twice its previous medium concentration for 45 min was performed every 3 days. Containers and microplantlets were cleaned with a soft toothbrush before every dipping.

Results

New cells were released at the cut surfaces of the different varieties after 4–7 days of culture in increasing concentrations of AMPEP with and without PGR in the dark at 23°C (Fig. 1). Incubation at 23°C with 13:11 h L:D cycle and 10–15 μmol photons m^{-2} s^{-1} irradiance led to the differentiation of the cells into meristimatic or filamentous cells (Figs. 2 and 3). Filamentous cell growth from the medullary and cortical layers was observed to occur earlier in all varieties and concentrations (1–3 weeks) while meristimatic cell growth generally emerged at a much later period (1–8 weeks) within lower concentrations of AMPEP with and without PGR. These cell types aggregated at the medullary layer to form a dome (Fig. 4) and consequently led to the formation of shoot primordia (Fig. 5a). Earliest emergence of shoot primordia varied in concentration and day of emergence amongst the *Kappaphycus* varieties.

Further growth of the shoot primordia led to the development of 1.0-mm young shoots (Fig. 5b). The young shoots were transferred to T25 plastic culture flasks with 40 mL of their corresponding medium and incubated in 23°C, 13:11 h L:D cycle and 10–15 μmol photons m^{-2} s^{-1} irradiance in a rotary shaker at 80 cycles/min. Elongation of the shoots to 2–3 mm was observed after one week (Fig. 5c). Incubation in 250 mL culture flasks with 200 mL of their corresponding culture media and in 23°C,

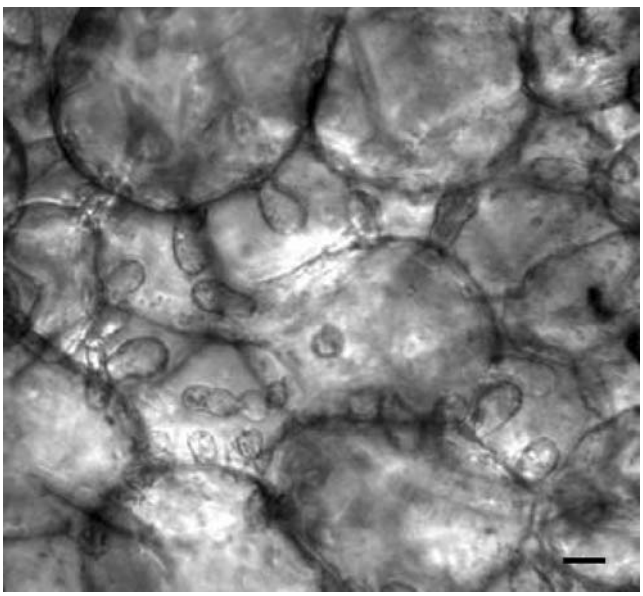

Fig. 1 Newly released cells (*bar* 20 μm)

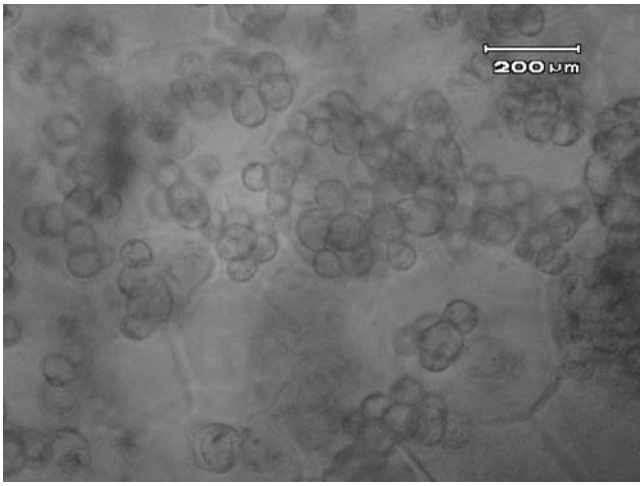

Fig. 2 Growth and aggregation of meristimatic cells

13:11 h L:D cycle and 30–45 µmol photons m^{-2} s^{-1} irradiance with gentle aeration resulted in further elongation of the shoot to 5 mm. Acclimatization of the microplantlets to ambient salinity was done in a 1-tonne capacity tank with ambient light and 24 h aeration. Increase in shoot length was observed after continuous weekly fertilization of the microplantlets with their corresponding media (Fig. 5d). After 3 months, the young plants were hung from a bamboo slat to promote branching and shoot growth. Sections cultured in ESS/2+PGR+E3 shrank and died after 4 weeks.

Growth of the different varieties of *Kappaphycus* used in the present study is shown in Table 2. Kapilaran produced shoot primordia after 49 days of culture at 1.0 mg L^{-1} AMPEP+PGR, Purple after 21 days at 3.0 mg L^{-1} AMPEP+PGR, Adik-adik at 17 days in 3.0 mg L^{-1} AMPEP and Green *sacol* after 25 days in 1.0 mg L^{-1} AMPEP+PGR.

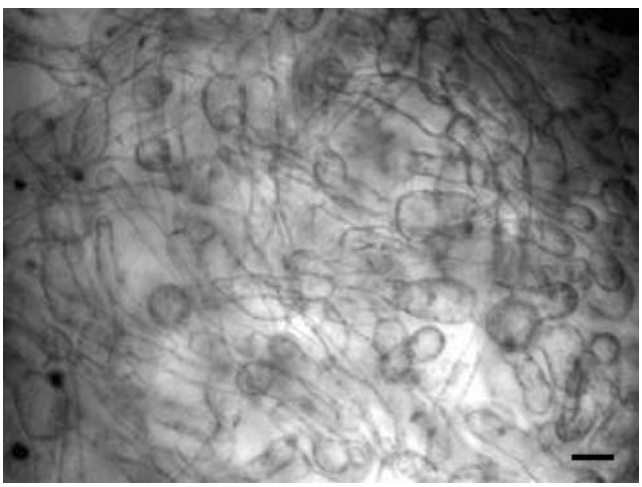

Fig. 3 Cell surface covered with two-celled filamentous cells (*bar* 20 µm)

Discussion

Several reports have shown the positive effect of seaweed extract on land-based crops for growth, yield and quality, pest and disease resistance, and environmental stress tolerance (Featonby-Smith and van Staden 1987; Crouch 1990; Rayorath et al. 2007; Rayorath et al. 2008; Jayaraj et al. 2008). Earlier reports were made by Fries (1984) in *Fucus* and Dawes et al. (1994) in *Kappaphycus* on the use of seaweed extract for tissue culture. However, the use of AMPEP with known N–P and other macronutrients composition is used for the first time in tissue culture technique in the present study to germinate shoots and regenerate microplantlets.

Commercial seaweed extracts remain so ill-characterized despite their long history. Chemical analyses of extracts generally have focused on those components known to act as plant growth regulators such as auxins, betaines, cytokinins, and gibberellins, either in the commercial products or in the native seaweeds (Senn and Kingman 1978; Jameson 1993; Craft et al. 2007; Hiltz et al. 2007). The work of Patier et al. (1993) on bioactive β-1,3-oligoglucans and humic acid in brown seaweeds by Radwan et al. (1997) has broadened the work on bioactive compounds. Knowledge of the composition of the parent seaweed is useful but insufficient, since the use of alkali is common in the liquefaction process and can greatly increase the complexity of the resulting chemical matrix (Niemela and Sjostrom 1985).

Different culture media with and without plant growth regulators have been used successfully to micropropagate *Kappaphycus alvarezii* (Dawes and Koch 1991; Dawes et al. 1994; Reddy et al. 2003; Hurtado and Biter 2007; Hayashi et al. 2007). One of the culture media used by Dawes et al. (1994) was Algafer, a liquid fertilizer from a

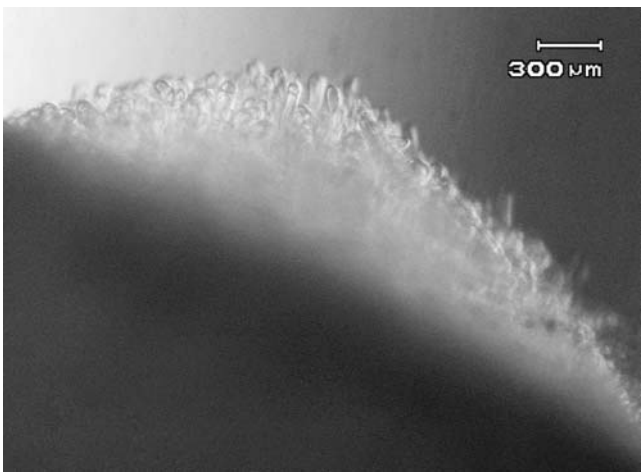

Fig. 4 Dome formation of filamentous cells

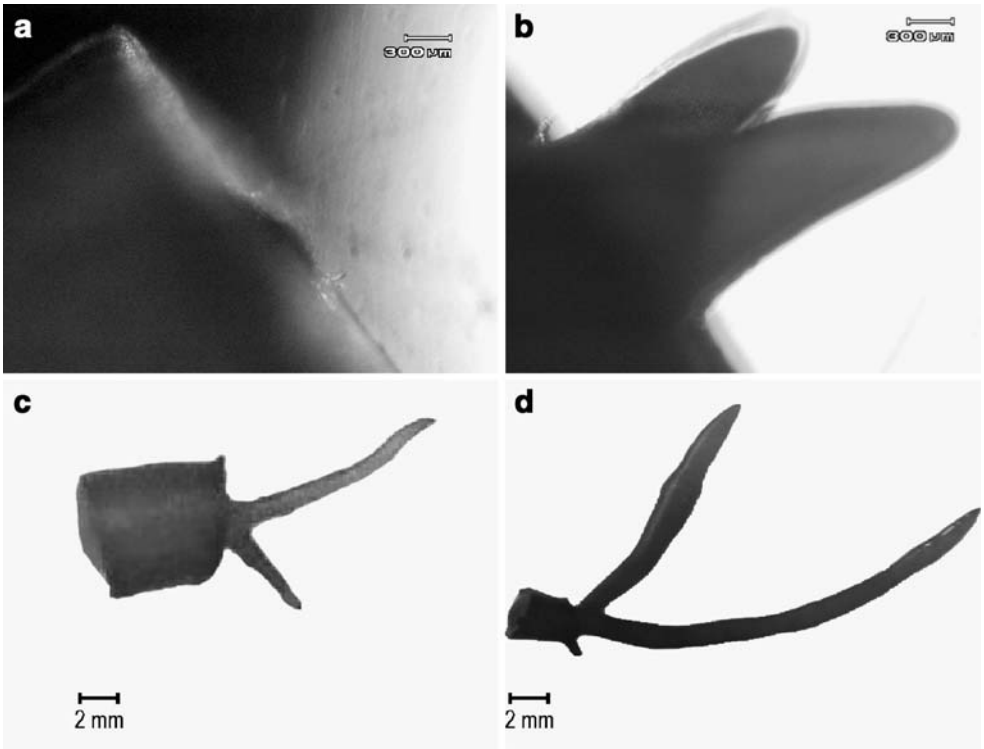

Fig. 5 Increase in shoot length was significantly observed in *tambalang* (purple) sections treated with 3.0 mg L^{-1} ASL+ PGR. Young shoots of a *tambalang* (purple) section lengthened from **a** newly formed dome, **b** 2 mm at day 34, **c** 5 mm at day 87, and **d** 22 mm at day 195

mix of red seaweed extract. The performance of Algafer as culture medium at 0.01% was demonstrated on the growth rate of brown (1.7–6.1% day^{-1}) and green (0.6–3.3% day^{-1}) *K. alvarezii*. However, the authors failed to describe the performance of this liquid fertilizer on the growth and development of callus cells and regeneration of young plants which is the focus of the present study using AMPEP as the main component of the culture media.

AMPEP is derived from fresh *Ascophyllum nodosum* sustainably harvested from the North Atlantic coastal waters of Nova Scotia, Canada. It is a source of naturally occurring major and minor nutrients, carbohydrates, amino

Table 2 Varieties of *Kappaphycus* sections treated in various concentrations of AMPEP and AMPEP+PGR produced shoot primordia at different rates (note: (−) indicates absence of cell stage)

Treatment	Concentration (mg/L)	Shoot primordium (days post culture initiation)			
		Kapilaran (KAP)	*tambalang* (PUR)	Adik-adik (AA)	Green *sacol* (GS)
AMPEP	0.001	–	–	25	–
	0.01	–	–	39	–
	0.1	72	–	39	–
	1.0	–	–	25	–
	2.0	–	–	19	–
	3.0	–	21	17	–
	4.0	60	21	17	–
	5.0	60	21	17	–
AMPEP+PGR	0.001	–	41	17	–
	0.01	–	22	–	–
	0.1	–	22	17	–
	1.0	49	22	21	25
	2.0	49	22	19	–
	3.0	46	21	17	–
	4.0	46	21	17	–
	5.0	–	31	17	–
ESS/2 (Control)		–	78	–	–

acids, and plant growth promoting substances, which enhance crop health, nutrition and quality. AMPEP is known to contain the following major macronutrients: total nitrogen (N)=08–1.5%, available phosphoric acid (P_2O_5)=1–2% and soluble potash (K_2O)=17–22% which are necessary for growth and development of callus cells as evidenced from the initiation of shoots as early as 17 days in adik-adik. The use of AMPEP alone at higher concentrations (1.0–5.0 mg L^{-1}) and AMPEP+PGR at lower concentrations (0.001–0.1 mg L^{-1}) resulted in the initiation of shoots in adik-adik at a faster rate than the other varieties used. The results suggest that initiation of shoots of each variety is dependent on the concentration of AMPEP alone and or in combination with PGR. In contrast, the use of ESS/2 in the present study resulted in negative growth of callus cells compared to an earlier report of Hurtado and Biter (2007) which may be attributed to the quality and age of the explant and preparation of the culture media.

Growth and development of callus cells of the different varieties of *Kappaphycus* in response to the different concentrations of AMPEP were not the same. Likewise, the initiation of shoot and their further growth differed from one variety to another. The results simply indicated that the performance of AMPEP on the regeneration of micro-plantlets from the different varieties used in the present study was variety specific.

AMPEP promoted root and shoot growth of *Arabidopsis thaliana* as reported by Rayorath et al. (2007). They provided evidence using DR5:GUS that components of the commercial *A. nodosum* extracts modulates the concentration and localization of auxins which could account, at least in part, for the enhanced plant growth. The organic components of *A. nodosum* extract induce amylase activity independent of GA_3 and might act in concert with GA-independent amylase production leading to enhanced germination and seedling vigor in barley (Rayorath et al. 2008). The findings of Rayorath et al. (2007) and Rayorath et al. (2008) could be related to the early emergence of shoots from the *Kappaphycus* varieties tested in the present study.

The use of AMPEP alone and/or in combination with PGR as a culture medium in the propagation of micro-plantlets using this tissue culture technique is highly encouraging. The major and minor nutrients needed for growth and development of the *Kappaphycus* varieties used in the study were present naturally in the seaweed extract. Further studies are required to test the economic feasibility of this seaweed extract for larger scale micropropagation especially in tropical countries where the cultivation of *Kappaphycus* is practiced.

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