ResearchGate

See discussions, stats, and author profiles for this publication at: https://www.researchgate.net/publication/320422184

Micro-propagation of Kappaphycus and Eucheuma: Trends and Prospects

Chapter · October 2017

DOI: 10.1007/978-3-319-63498-2_5

CITATIONS
0

READS
419

5 authors, including:

CRK Reddy
Institute of Chemical Technology, Mumbai
145 PUBLICATIONS **2,557** CITATIONS

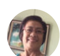
SEE PROFILE

Wilson Thau Lym Yong
Universiti Malaysia Sabah (UMS)
36 PUBLICATIONS **188** CITATIONS

SEE PROFILE

Maria Rovilla J. Luhan
6 PUBLICATIONS **35** CITATIONS

SEE PROFILE


Anicia Q. Hurtado
Integrated Services for the Development of Aquaculture and Fisheries, Inc.
66 PUBLICATIONS **1,158** CITATIONS

SEE PROFILE

Some of the authors of this publication are also working on these related projects:

A consolidated biomass process for integrated production of multiple products from fresh marine macroalgae View project

Integration of bio-processing technology with seaweed farming View project

All content following this page was uploaded by CRK Reddy on 22 February 2018.

The user has requested enhancement of the downloaded file.

5 Micro-propagation of *Kappaphycus* and *Eucheuma*: Trends and Prospects

C.R.K. Reddy, Nair S. Yokoya, Wilson Thau Lym Yong, Maria Rovilla J. Luhan, and Anicia Q. Hurtado

Abstract

Among the red algae, *Kappaphycus* and *Eucheuma* are the two most commercially important carrageenophytes farmed extensively in Southeast Asian countries; they rank top in production in terms of volume of raw material produced. The farming of carrageenophytes has emerged as a successful enterprise and provides a promising, alternative livelihood option for low-income, coastal communities in a number of countries. In 2014, carrageenophyte production worldwide, surpassed other red seaweeds and was placed at the top of the production rankings with 10.99 million MT wet weight (77% of total production of farmed red seaweed) and Indonesia being a major producer. However, over time, the productivity of the crop (carrageenophytes) has declined in some regions due to sourcing of seedlings from single, selected genetic stocks considered to have higher yield potential which resulted in strain fatigue, or loss of vigour. The incidences of disease and epiphytic infestations are on rise in recent times which have severely affected biomass production, as well as the yield and product quality (carrageenan). In order to circumvent the crop productivity issues arising from clonal propagation, the raising of planting materials from spores, derived through the red algal sexual reproductive cycle, has been initiated to support the sustainability of selected, farmed carrageenophytes. Alternatively, *in vitro* tissue culture techniques have also been explored to not only rejuvenate the vigour of seedlings (i.e. the out-planting material) but also to seek seedlings resilient to stress, disease and epiphytes to act as an invigorated mother stock. These efforts have succeeded to a great extent in the development of appropriate techniques for explant culture, callus induction, callus sub-culture and regeneration to micro-propagules with improved traits. The present chapter briefly summarizes the developments and success achieved in micro-propagation of *Kappaphycus* and *Eucheuma* and also provides pointers to both gaps and priority areas for future research required for the advancement of sustainable farming of these carrageenophytes.

C.R.K. Reddy (✉)
Division of Marine Biotechnology and Ecology,
CSIR-Central Salt and Marine Chemicals Research Institute,
Bhavnagar 364002, India
e-mail: crk@csmcri.res.in

N.S. Yokoya
Institute of Botany, Environmental Secretary of São Paulo State,
P.O. Box 68041, 04045-972 São Paulo, Brazil

W.T.L. Yong
Biotechnology Research Institute, Universiti Malaysia Sabah,
Jalan UMS, 88400 Kota Kinabalu, Sabah, Malaysia

M.R.J. Luhan
Aquaculture Department, Southeast Asian Fisheries Development Center (SEAFDEC), Tigbauan, Iloilo 5021, Philippines

A.Q. Hurtado
Integrated Services for the Development of Aquaculture and Fisheries (ISDA) Inc., McArthur Highway, Tabuc Suba, Jaro, 5000 Iloilo City, Philippines

© Springer International Publishing AG 2017
A.Q. Hurtado et al. (eds.), *Tropical Seaweed Farming Trends, Problems and Opportunities*, Developments in Applied Phycology 9, DOI 10.1007/978-3-319-63498-2_5

5.1 Introduction

Kappaphycus and *Eucheuma* are the two important carrageenophyte genera that are extensively, commercially farmed in tropical and subtropical waters. In 2014, carrageenophyte production worldwide surpassed other red seaweeds and remained at the top of the production rankings with 10.99 million MT wet weight (77% of total production of farmed red seaweed with a value over USD 1.1 billion), and Indonesia being a major producer (Nayar and Bott 2014; FAO 2016). Carrageenophyte farming has indeed developed into a successful enterprise in a number of tropical (and subtropical) developing countries and has emerged as a promising alternative livelihood option for low-income, coastal communities (Valderrama et al. 2013). The economic value of carrageenophytes mainly stems from their cell wall polysaccharides including several types of carrageenan which are used as ingredients in a number of processed food products in dairy, meat, food, cosmetics, personal care and also some applications in the pharmaceutical industry; usage continues to grows by 4–5% per annum (Bixler and Porse 2011). Another emerging application of *Kappaphycus* biomass is the production of plant growth biostimulants, as an integrated biorefinery product, manufactured as a part of kappa-carrageenan extraction, through a unique liquefaction process using fresh seaweed directly harvested from the sea (Ghosh et al. 2015; Singh et al. 2016).

Dwindling supplies of cultivated carrageenophytes, more particularly from the Philippines (for various reasons – see elsewhere in this book), coupled with the constantly growing global market demands for carrageenan, provided impetus to seek out new farming grounds in other parts of the world, with similar climatic zones (see chapter by Hayashi et al.). This initiative of crop dissemination (not unlike many terrestrial crops spreading globally over centuries) led to the introduction of *Kappaphycus* and *Eucheuma* in 32 countries, in a relatively short time-frame, in order to sustain commercial production around the world (Zemke-White and Smith 2006).

Commercial farming of carrageenophytes traditionally employs vegetative thalli as asexual propagules broken into convenient segments and termed "seedlings" by the industry (Hurtado et al. 2006). The practice of propagule selection in *Kappaphycus* and *Eucheuma* involves setting aside the fastest growing plants from one season to be utilised as "seed" for the successive season – unfortunately when practiced over and over again, this led to decline in "seedling vigour" and inevitably a decline in a crop production (Dawes et al. 1993). The persistent outbreaks of disease such as: 'ice-ice' and the over-growth of harmful epiphytes and endophytes, the successes of which have been attributed to farm management practices as well as (at least in part) to environmental stresses (Largo et al. 1995) arising from climate change, have also further seriously affected the carrageenophyte farming industry. As a consequence of the downturn and loss of income, there have been serious social and trade-related issues leading to social unrest and unethical practices (Valderrama et al. 2013). Consequently, the importance for the selection and establishment of new varieties, strains and cultivars, together with their preservation using tissue culture and genetic manipulation techniques, has been a real necessity for survival. These techniques provide an immense potential to increase not only crop production but also carrageenan yield and quality and hence value addition to the farmers. In addition, this pioneering work, as applied to seaweeds with marine agronomic value, will also provide further positive applications in marine macroalgal biotechnology as a whole.

A number of studies have dealt with the necessary adaptations and application of pre-existing (as previously used in revolutionary roles for terrestrial crop production), tissue culture techniques to that of economically important seaweeds. As a result, a great deal of success with respect to callus induction and thallus regeneration were reported for a diverse range of seaweeds (Polne-Fuller and Gibor 1987; Butler and Evans 1990; Aguirre-Lipperheide et al. 1995; Reddy et al. 2008, 2010). Although the initial studies were centred on the development of various basic techniques in seaweed tissue culture, their application for *in vitro* selection and propagation of desired strains (Dawes et al. 1993; Kirihara et al. 1997) and production of secondary metabolites (Lawlor et al. 1990; Maliakal et al. 2001; Rorrer and Cheney 2004) has also been accomplished. The present chapter briefly summarizes the developments and successes achieved in micro-propagation of *Kappaphycus* and *Eucheuma* and points to both gaps and thrust areas required for future research in order to further continue and advance sustainable, extensive and future intensive, farming of carrageenophytes.

5.2 Spore Production and Their Developmental Morphology

Trono and Ohno (1989), and Trono and Lluisma (1992) had already reported dramatic decreases in the hardiness and carrageenan quality of the then cultivated varieties of carrageenophytes. One factor which may have contributed to this phenomenon first reported nearly 30 years ago, could have been the common practice of farmers using simple, vegetative cuttings from their pre-existing stocks as further propagules for the succeeding generations of cropping. It was a type of "strain selection" from the "best looking, fastest growing" plants at the earliest sites where cultivation was initiated. Unfortunately, other than growth and colour, there were no other traits selected for such as yield and properties of the carrageenans (Hurtado and Agbayani 2000). This

continuous re-transplantation from a single parent stock is likely to have eroded some important genetic traits that resulted in the lower quality of the present stocks (though no studies using molecular markers are known to have taken place). Periodic replacement of propagules with new stocks (various strains, colour and physical morphotypes) as sources of invigorated plantlets was necessary to prevent the deteriorating agronomic traits of the seedlings used at that time. Hence, initiated by the farmers, the industry began to look for alternative approaches to vegetative cuttings in order to produce the new plantlets with vigour and value for a changing sustainable, carrageenophyte seaweed industry.

The earliest studies made extensive efforts to produce seedlings from reproductive, as well as somatic sources. For instance, mature carposporophytes of *K. alvarezii* were shown to produce viable spores *in vitro* (Azanza-Corrales et al. 1992; Azanza and Aliaza 1999; Azanza-Corrales and Ask 2003). Successful culture of micro-propagules of *K. alvarezii* was already undertaken at the laboratory scale (Dawes and Koch 1991; Dawes et al. 1993, 1994). The explants of seaweeds were used for tissue culture and achieved great success (Reddy et al. 2003; Munoz et al. 2006; Hurtado and Biter 2007; Hurtado et al. 2009). Tetrasporophyte progenies were shed (released) in the laboratory and grown outdoors (Bulboa et al. 2007, 2008; Bulboa and de Paula 2005).

The use of spores in seaweed farming has been demonstrated successfully for *Porphyra (Pyropia)*, *Gracilaria*, *Laminaria (Saccharina)* and *Undaria*. In *Kappaphycus*, the convenient use of spores is still at its earliest stage of development as the technique applied to produce plantlets for culture results in the higher production costs of propagules. Of greater importance will be the determination of the quality of seedlings raised from the spores for their growth, carrageenan yield and quality, disease susceptibility etc.. A similar concern is applicable to seedlings generated through *in vitro* tissue culture or any other means, in order to ascertain beneficial features of seedlings prior to their distribution to farmers. Such endeavours are necessary to further understanding of the species in order to achieve enhanced efficiencies of biomass production. *Kappaphycus* has a *Polysiphonia*-type triphasic life cycle (Dawes 1993). Female gametophytes have a carpogonial carpogonial branch while male gametophytes have a superficial spermatangia. In the water column, the spermatia released by the spermatangia find their way to the protruding carpogonial branches on the surface of the thallus and lead the spermatia to the carpogonium for fertilization from which carpospores will develop. The diploid carpospores are a product of syngamy of non-flagellate, spermatia and oogonia; they are contained within cystocarpic bodies and grow on the haploid, female gametophytes. The carposporophytes are attached and acquire nourishment from the female gametophytes. Mature carpospores will be shed and find a substratum up on which to settle and germinate. The germinated carposporeling (2N), remain vegetative until it develops spores which mature into tetrasporophytes. Tetrasporophytes develop tetraspororangial bodies and are the site of meiosis to release haploid tetraspores, which will develop to gametophytes (N). A further complicating factor is that the male and female gametophytes and the tetrasporophytes are isomorphic and are therefore not distinguishable at their early stages of somatic growth. This is yet another major track essential to basic and applied research endeavours that would lead to identify each genotype at the earliest stages of development necessary for improvement of cultivars.

In “wild populations”, adult *Kappaphycus,* with mature carposporophytes, are usually found in areas where cover of the seagrasses *Thallasia* and *Cymodocea* is dense. The carposporophytes are also found associated with other seaweeds growing on dead coral and rock substrata. Often they are undistinguishable due to a covering of fine silt. A very keen eye is therefore required to scrutinize seaweed patches in order to collect mature, reproductive *Kappaphycus,* cystocarpic individuals. Cystocarpic *Kappaphycus* could be present year-round, but since mature specimens are rare to find in the field, few individuals are ever collected. Hence there are relatively small amounts of biomass to work with at any one time using this method of selection and propagation. The processing of seaweed tissue in the laboratory involves the use of sterile materials. Seawater is filtered using a 0.45 μm GFC Whatman filter and other tools such as brush, plankton nets, bottle caps, forceps, Petri plates and autoclaved needles. Glassware must be heat sterilized at 100 °C in an oven. Other materials that cannot be autoclaved should be placed under a UV light inside a laminar flow bench. Directly from the point of collection, live seaweed material is placed in a container with sterile seawater (SSW). This tissue is cleaned using a soft brush and rinsed repeatedly to remove adhering debris. Portions of thalli with cystocarps are excised and placed on to a glass slide in a Petri dish with SSW (34‰) and placed at ambient temperature (28 °C) in the dark. The excised cystocarps, thus incubated in Petri plates, showed shedding of spores immediately. Carposporophytic *Kappaphycus* plants will also shed spores *in vitro* by manipulation of culture conditions. “Shocks” caused by increasing or decreasing temperature or light can induce the release of spores. Continuous exposure to light and longer incubation in the dark also induces the shedding of spores in mature carposporophytes (Azanza and Aliaza 1999).

Simple, filtered seawater is a good medium to initiate the release of spores. A substratum is needed for the spores to settle upon. Glass slides are preferred for easy handling, cleaning and replenishing the culture medium. Spores that are released early disperse the furthest distance from the cystocarps on the substrata provided and can be most easily

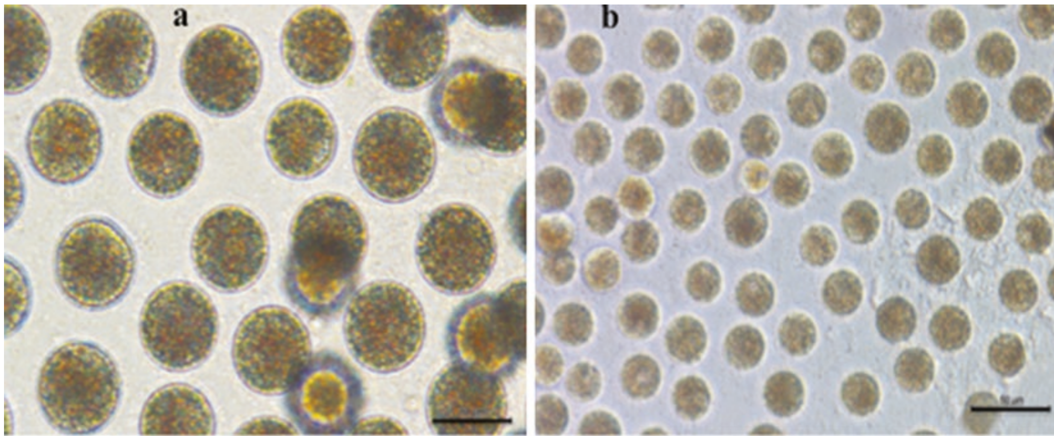


Fig. 5.1 (**a**) Diploid spores and (**b**) haploid spores (bar = 50 μm) (Photos courtesy of MRJ Luhan)

collected and/or separated. Those spores released later will be trapped in the natural mucilage of the thalli. The majority of released spores are embedded in the mucilage which does dissolve, but leaves many individuals coalesced. Some spores will be suspended in the medium but settle within 24–48 h after shedding. Spores may settle individually or two or more may coalesce and develop into a juvenile plantlet. There are approximately 200 spores released per mature cystocarp. The mean diameter of diploid and haploid spores is 44.9 ± 2.5 μm and 19.2 ± 1.9 μm, respectively. Diploid spores (Fig. 5.1a) are relatively larger than the haploid spores (Fig. 5.1b). Hyaline hairs appear 6–8 days after settlement and are visible until the sporelings develop a dome shape or shoot primordia. Contamination in culture will appear after 24 h. After 48 h the culture vessel should be changed and a fresh medium added. This process is repeated regularly in order to reduce contamination and over-growth of contaminants such as diatoms, ciliates and bacteria. Initially the growth of sporelings is very slow but can be boosted by gradually increasing the culture medium volume and supply of nutrients in pace with the growth of the sporelings (Luhan and Sollesta 2010).

The first stage of laboratory culture is to grow the new sporelings on glass slides placed onto the bottom of the Petri dish (Fig. 5.2). Following the settlement of spores and their attachment on to the glass slides, the slides are then placed inside a wide-mouth, capped bottle (50 mL capacity) filled with sterile seawater enriched with modified Grund medium (Brown et al. 1977) and kept in an incubator with a fluorescent lamp, with an irradiance of 50 μmol m^{-2} s^{-1}, 13:11 light:dark photoperiod and at 25 °C. An irradiance between 50–175 μmol m^{-2} s^{-1} should be maintained during the culture. Slides are oriented at 45°. Small volumes of medium in bottles are used during the initial culture period with tiny sporelings (<3 mm). The culture medium should be replenished on a daily basis. Sporelings are then scraped off the glass when they reach 3 mm and

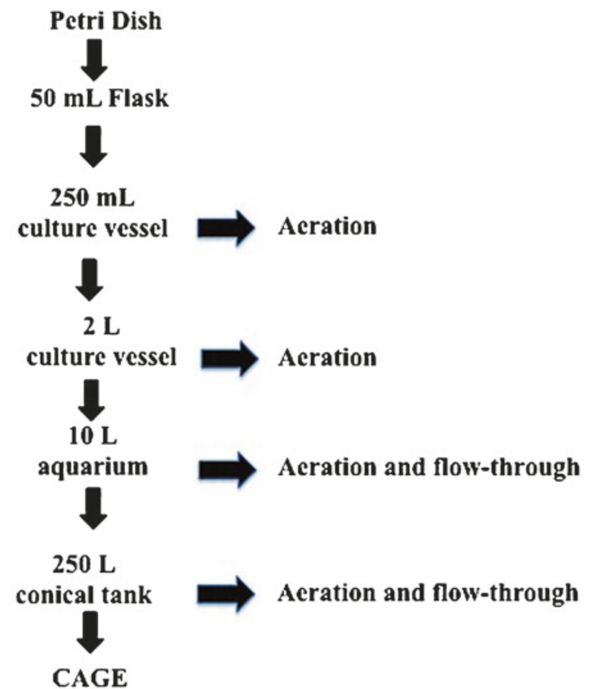


Fig. 5.2 Multi-step culture of *Kappaphycus* in the land-based nursery

are transferred to a container with 50 mL medium and cultured in suspension without aeration. As the culture progresses, larger flasks (250 mL) are used and aeration provided to tumble the water and simulate natural conditions. The use of bubblers prove to be ideal for the mixing of water in order to prevent shading and ensure the efficient distribution of nutrients for the sporelings.

Sporelings harvested from the 2L flasks are then transferred to a 10L aquarium outdoors. Filtered seawater is used to grow the plantlets in the aquarium. The water, in the aquarium is vigorously aerated with flow-through, filtered seawater. Fertilization is made twice a week by adding

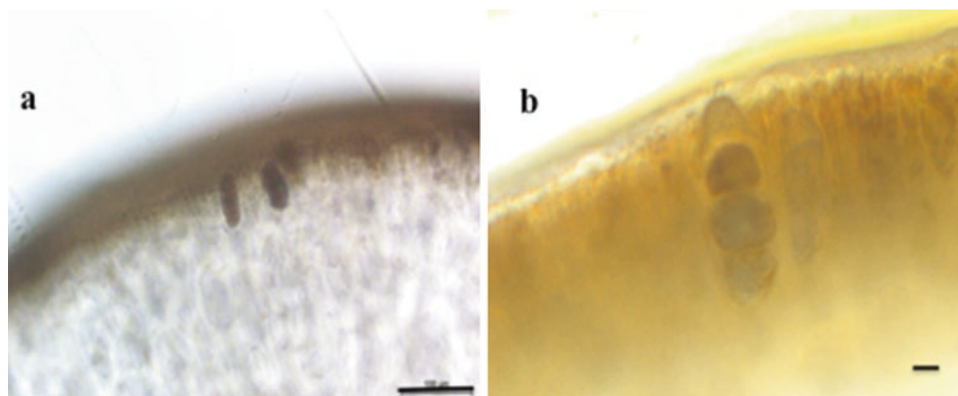


Fig. 5.3 (**a** and **b**) Cross-section of tetrasporophytes (bar = 100 μm) (Photos courtesy of MRJ Luhan)

modified Grund medium. The water supply is shut off before the application of fertilizer. The water supply is reopened after 2 h. Irradiance (172–1200 μmols m^{-2} s^{-1}), salinity (32–34 ppt) and temperature (28–30 °C) are to be maintained under ambient outdoor conditions. Plantlets are grown for 28 days in the aquaria. Plantlets are stocked at 50 individuals per aquarium. Sporelings from the 10 L aquaria are further grown in 250 L outdoor tanks with 200 L seawater in a flow-through system, with 400 individuals, at a flow rate of 1L min^{-1} with vigorous aeration. A flow-through water system brings constant water movement that further simulates natural conditions, preventing self-shading and providing additional nutrients favouring good growth. The tank culture is the last step in the land-based nursery.

Five transfers/steps are made until the sporelings reach approximately 50–60 mm in length. Generally, a marked increase in growth is noted when the sporelings are transferred from a smaller to larger culture containers. The additional extra space and nutrient availability explains the increase in growth at each change. Initially the growth of sporelings is very slow. It will take 150 days from spores before the sporelings are transferred to 250 mL flasks which is between 5 and 8 mm. The growth of sporelings in 250 mL (approx. 0.04 g), 2 L (approx. 0.6 g), aquaria (approx. 0.28 g) and tanks (approx. 15 g) are 4.9%, 9.6%, −2.7% and 4.6% day^{-1}, respectively. Negative growth was observed in the aquaria which could be due to static conditions (i.e. the absence of flow-through) in the first few days after stocking in outdoor conditions. 'Ice-ice' was observed after 5 days of culture and recovered after 12 days when the water was continuously changed or flow-through system started.

The diploid *Kappaphycus* sporelings will mature and can be transferred to produce mature tetrasporophytes *ex situ* in cages. Similarly, mature tetrasporophytes will shed their own (haploid) spores *in vitro* when environmental parameters are manipulated. Again, exposure to longer periods of light, or dark, can be used to induce the release of spores. Tetrasporangia of *Kappaphycus* are zonate bodies (Fig. 5.3). The spores can be found mostly solitary on the glass substratum.

Not all species (strains/variants and morphotypes) of *Kappaphycus* thrive well in tanks. However, a tank-culture phase is essential if a land-based nursery is to provide a continuous supply of healthy seedlings for out-planting by the farmers especially during those situations where there are shortages of seedlings arising from high mortalities at the cultivation grounds (this is not unlike the general practice for a number of aquaculture species nor indeed the concept of specialist (expert centres) which look after breeding and improvements of major terrestrial crops such as banana, rice, potato, corn, etc.). In countries where several typhoons may occur each year (see Chapter by Largo et al.), a land-based nursery is essential over a sea-based nursery, since total loss of stocks and mortalities due to typhoons can be avoided. The land-based system is a secure repository which also will be increasingly important for chain of custody assurances required by high value applications of biomass, i.e. pharmaceutical applications where there must be strict traceability back to the original germplasm. In many instances, destruction of seaweed stocks has been observed in grow-out farms after typhoons, even contributing to a decline in Philippines' production and the surge of Indonesia as the number one producer (since the coastlines of that country effectively do not suffer the devastating consequences of typhoons and storm surges). It is expensive to maintain land-based nurseries, but this may be undertaken by institutions with existing facilities or perhaps funded by the companies buying biomass for processing.

5.3 Clonal Propagation

Most of the carrageenophytes cultivars which are farmed at present, in a circumferential band around the oceans bounded by the tropics (see the maps in, Chap. 3) have been sourced from limited seed stocks with proven productivity and high yield of product. The widespread proliferation of such stocks across different geographical regions led to very large populations. However, these stocks need to be reinvigorated and improved in order to achieve overall sustainability in the carageenophyte farming industry.

All of the studies dealing with clonal selection have successfully utilised the regeneration potentials for isolating superior clones from wild stocks. The species studied include: *Chondrus* (Cheney et al. 1981), *Gigartina* (Sylvester and Waaland 1983), *Gracilaria* (Patwary and van der Meer 1982, 1983) and *Kappaphycus* (Doty and Alvarez 1973). From the very inception, the seaweed selection approach has been from an empirical perspective for a single trait but later studies have combined superior growth with superior polysaccharide yields. Unfortunately, these studies, apart from confirming the faster growth in a particular location, did not progress further to an understanding of the genetics of these traits. This has led to the development of modern molecular biology techniques in order to transform and improve the cultivars (Wang et al. 2010). Recently Handayani et al. (2014) attempted to produce transgenic *Kappaphycus* resistant to bacterial infections by cloning the lysozyme (*Lys*) gene from chickens through an *Agrobacterium*-mediated gene transfer.

Recently, Fadilah et al. (2016) employed recurrent selection methods to select high growth rate germplasm from *K. alvarezii*, from a population having a DGR >10%. Incidentally, the selected germplasm also had the favourable characteristics of higher clump weight, average daily growth rate, longer thallus potential, with a greater number of branches with shorter internodes, as compared to the control plants. Also, total sugar content and the ratio of kinetin and indole-3-acetic acid were reportedly higher in the selected germplasm than that of the control control. The growth rate of this germplasm was assessed for four generations and found that more than 90% showed aconsistent DGR of 10%. Although this approach, in general, has been hailed as successful (van der Meer and Patwary 1983; van der Meer 1986), it entails continuous, cumbersome monitoring and isolation of clones with superior quality for a given trait and finally the costly maintenance of the selected strains. In another study, Titlyanov and Titlyanova (2006) and Titlyanov et al. (2006) reported methods for the mass production of *Gelidium* for land-based cultivation, using fragments and cell aggregates of apical meristems. Furthermore, freeze-thawing of apical meristem tissues enabled the production of plantlets which produced rhizoids that could be used for cultivation in the sea. These methods effectively maximized the number of propagules per donor plant and further facilitated mass production of seed stock. However, the selection of the "elite germplasm" through clonal propagation is a continuous process and a substantial amount of harvest is required to be utilized as initial seed material from which the subsequent selections are to be made, which are then require to be validated in subsequent cultivation cycles. This largely forms part of "domestication of species" to bring wild germplasm under a farming line. Further, isolation of useful germplasm lines for cultivation through this process is less likely to be successful as genetic variant cells are small in number and are masked by more common non-variant cells (Garcia-Reina et al. 1991).

5.4 Seaweed Extracts as Sources of Bio-Stimulant Used in Generating Microplantlets of *Kappaphycus*

5.4.1 Brief Description of AMPEP (Acadian Marine Plant Extract Powder) as a Bio-Stimulant for a red Seaweed Propagation and Enhanced Vigour

AMPEP (Acadian Marine Plant Extract Powder) is an acronym proposed and popularised by Hurtado (pers comm). Although based on an existing commercial agrochemical, extract, the product is not currently promoted. It is however a brown seaweed-based bio-stimulant (bioeffector) extracted from *Ascophyllum nodosum.* This brown seaweed grows luxuriantly along coasts of the North Atlantic, notably Canada, France, Iceland, Ireland, Norway and the United Kingdom (the limits to distribution are north from New York in the US to Portugal) (Ugarte and Sharp 2012).

Ascophyllum nodosum is an important commercial seaweed in Canada (Ugarte et al. 2006). It is a dominant, perennial seaweed and forms extensive beds where conditions are suitable (Ugarte and Sharp 2001). In the past, *A. nodosum* was used as a source of biomass for the extraction of alginate and production of "kelp meal" (unspecified origin, dried and milled seaweed flakes and/or powders). Other uses of this resource include manufacture of extracts (various methods of extraction) to produce a bio-stimulant i.e. a liquid concentrate and dried seaweed powder extract for agricultural and horticultural uses. The production of this agrochemical extract is an important economic activity of the Maritime Provinces, Canada, and similarly extracts are manufactured in Ireland, Norway and US.

AMPEP is brownish-black in colour, crystalline in nature with a typical marine odour, 100% soluble in water with a pH 10.0–10.5. Its total nitrogen (N), available phosphoric acid (P_2O_5) and soluble potash (K_2O) contents are 0.8–1.5%, 1–2% and 17–22%, respectively. Its total amino acid content is 4.4% plus other elements such as sulphur, magnesium, calcium, sodium range from 0.2–5%; boron, iron, manganese, copper, zinc range from 1–250 ppm. This seaweed extract is a proven source of bio-stimulant (bioeffector) properties (Khan et al. 2009; Craigie 2011).

Earlier studies reported on the significant role of *A. nodosum* extract (ANE) on the improvement of disease tolerance in some agricultural crops, as reported by Jayaraj et al. 2008, 2011; Fan et al. 2011, to name a few. AMPEP has been used not only in the micro-propagation of *Kappaphycus* plantlets,

Table 5.1 Varieties of *Kappaphycus* treated in various concentrations of AMPEP and AMPEP + PGR produced shoot primordia at different rates (Hurtado et al. 2009)

Treatment	Concentration	Shoot primordia (days post-culture initiation)			
	Mg L^{-1}	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	–	–

Note: (−) indicates absence of cell stage

in the Philippines (Hurtado et al. 2009; Yunque et al. 2011) but also to enhance the growth rate of field cultivated *Kappaphycus* (Philippines, Hurtado et al. 2012) and to mitigate the incidence of epiphytes (in Brazil, Loureiro et al. 2010, 2012) and the harmful endophyte *Neosiphonia* (Borlongan et al. 2011), improved carrageenan yield (Loureiro et al. 2014) and the impact of epibiont settlement on *Kappaphycus* (Marriog et al. 2016; see chapter by Loureiro et al.).

The role of AMPEP as an efficient medium for direct shoot formation and development in the micro-propagation of *Kappaphycus* plantlets is briefly discussed below. These results are likely to be of great interest in the pursuit of developing plantlets at the commercial scale. AMPEP studies with *Kappaphycus* have indeed provided a lot of proof of concept information, but there is still much further research required, not least in the efficacy of various types of other extracts from the other seaweeds along with understanding the underlying mechanisms imparting the positive responses observed.

5.4.2 Use of AMPEP + Plant Growth Regualtors (PGRs)

When three varieties of *Kappaphycus alvarezii:*viz. (Kapilaran, KAP), Tambalang purple (PUR), Adik-adik (AA) and one variety of *Kappaphycus striatum* var. sacol (green sacol; GS) were tested with different concentrations of AMPEP + PGR (=Phenyl Acetic Acid (PAA) + Zeatin at 1 mg L^{-1}), each variety responded differently in the number of days taken for shoot formation, as shown in the Table 5.1. PUR and AA initiated shoot formation with the use of AMPEP only at higher concentrations (i.e. 3–5 mg L^{-1}) after a shorter period. Only PUR responded positively to ESS/2 for shoot initiation.

5.4.3 Optimization of AMPEP as a Culture Medium

The use of different low concentrations of AMPEP with, or without, PGR led to the enhanced development of shoots in six varieties of *Kappaphycus*. The earliest shoot emergence was observed to occur at low concentrations of AMPEP (e.g. 0.001–1.0 mg L^{-1}) with PGR, or at high concentrations of AMPEP (3–4 mg L^{-1}) without PGRs (Table 5.2). Only the control treatment of PUR formed shoots after 78 days of culture in ESS/2. The rate of production of new and improved *Kappaphycus* explants for a commercial nursery stock was improved through the use of AMPEP with optimized culture media pH, temperature and density conditions.

Explants of TGR cultured at all AMPEP concentrations with, or without PGR, produced the first shoots within the same time frame (i.e. 19 days post-culture). An optimum concentration of 3.0 mg L^{-1} AMPEP and 0.001 mg L^{-1} AMPEP + PGR was determined for TGR, based on the generated shoot length (Fig. 5.4). Significant differences ($P < 0.01$) were observed between the lengths of shoots under different treatments. However, no definite pattern of response to AMPEP with, or without PGR, was observed (Fig. 5.5).

Table 5.2 Summary of media composition that produced the earliest shoot primordia in the six *Kappaphycus* varieties tested, as compared to the control medium (ESS/2 + PGRs) (Yunque et al. 2011)

	Variety	First occurrence of shoot primordia (no. of days)	AMPEP concentration	ESS/2
K. alvarezii	Tambalang purple (PUR)	21	3 mg L^{-1}; 0.1 mg L^{-1} + PGR	78 days
	Kapilaran brown (KAP)	49	1 mg L^{-1} + PGR	–
	Vanguard brown (VAN)	22	0.1 mg L^{-1} + PGR	–
	Adik-Adik brown (AA)	17	3 mg L^{-1}; 0.001 mg L^{-1} + PGR	–
	Tungawan green (TGR)	19	3 mg L^{-1}; 0.001 mg L^{-1} + PGR	–
K. striatum	Sacol green (GS)	25	1 mg L^{-1} + PGR	–

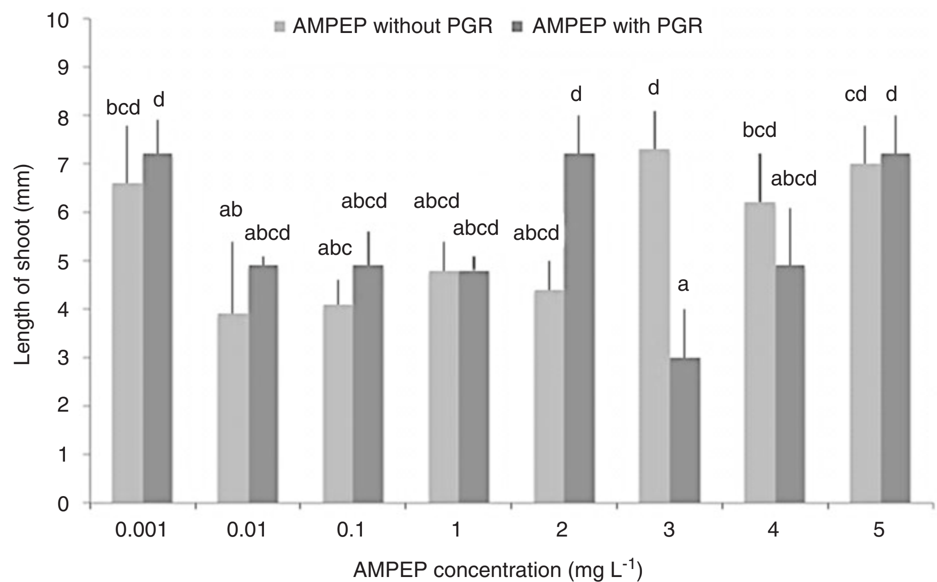


Fig. 5.4 Average length (±SD) of shoots developed from TGR grown in various concentrations of AMPEP with and without PGRs after 19 days of culture. Means of the same letter are not statistically different at the p = 0.01 level of significance (Yunque et al. 2011)

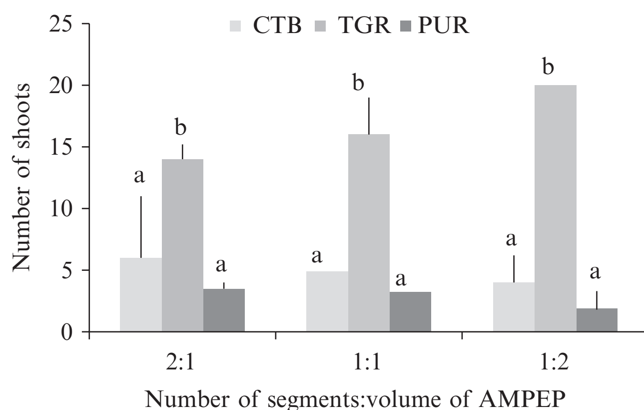


Fig. 5.5 Average density (±SD) of segments with shoots developed from CTB, TGR, and PUR. Means having the same letter are not statistically different at the p = 0.01 level of significance (CTB curly *top brown*, TGR Tambalang *Green*, PUR *Purple*) (Yunque et al. 2011)

The culture medium supplemented with optimum medium concentrations of AMPEP, in the presence of plant growth regulators (PGR), were used *vis-a-vis*: (1) pH– temperature combinations (2) explant density and volume of medium in order to improve the production of *Kappaphycus* plantlets in tissue culture.

Various pH–temperature combinations elicited shoot growth in GS, PUR and BRN. After 54 days of incubation, the highest number of shoots formed with the least mortality, were pH 8.7 and 9.7 at 25 °C for GS (11 = 46%), pH 6.7 at 25 °C for PUR (15 = 63%) and pH 6.7 and 7.7 at 20 °C for BRN (5 = 21%). All varieties responded positively to almost all pH–temperature combinations, except GS, which did not produce shoots at any pH value at 20 °C (Table 5.3).

Explant number versus the culture medium volume combination was also tested. Formation of *Kappaphycus* shoot

primordia was observed as early as day 9 for TGR and PUR and after day 21 for Tungawan CTB. The highest average number of new shoots formed was determined at 2:1 density of 1.0 mg L^{-1} AMPEP + PGR for both brown and purple color morphotypes (35% and 100%, respectively). The green morphotype produced the most number of shoots at 1:2 density of the same culture media (16.7%). Remarkably, no mortality was recorded in either brown or green morphotypes. Mortality was only observed in PUR cultures with the highest value of 53.3% at the 2:1 density. Significant differences ($P < 0.01$) were observed between the strains and volume of AMPEP:density treatments.

5.4.4 Use of AMPEP + Spindle Inhibitors + PGRs

AMPEP K^+ is a bio-stimulant produced from *A. nodosum*, however, the K^+ content is higher than in the regular AMPEP (Tibubos et al. 2016). When AMPEP K^+ was used, singly or in combination with spindle inhibitors (colchicine and oryzalin) + PGR, the production of 1–4 direct axes (shoots) of *K. alvarezii* were recorded. This confirmed the observations of Hayashi et al. (2008) and Neves et al. (2015) for *K. alvarezii*. A highly significant difference ($p < 0.01$) in shoot length was observed when *K. alvarezii* was compared with those treated with AMPEP K^+ + PGR (Control B), indicating the efficacy of the PGR addition in shoot formation and elongation. No significant differences ($p > 0.05$) in shoot lengths were observed when *K. alvarezii* shoots were treated with AMPEP K^+ (Control A) vs AMPEP K^+ + colchicine + PGR at all levels of concentrations, which simply indicated that colchicine demonstrated the same effect as the Control A in shoot formation and elongation. However, highly significant differences ($p > 0.01$) were observed when *K. alvarezii* was treated with AMPEK K^+ + oryzalin + PGR vs. Control A. This indicated a strong efficacy of oryzalin to aid in shoot formation and elongation. When AMPEK K^+ + oryzalin + PGR and AMPEP K^+ + colchicine + PGR were compared in relation to their shoot elongation, no significant differences ($p > 0.05$) were observed at all concentrations, which simply indicated that either of these two types of spindle inhibitor could be useful in controlling shoot formation and elongation in *K. alvarezii* (Figs. 5.6a–e, 5.7a–e, 5.8a–o and 5.9a–o).

Table 5.3 Effect of pH and temperature combinations on number of explants with shoots developed from GS, PUR, and BRN after 54 days incubation, in 1.0 mg L^{-1} AMPEP + PGR enriched medium (n = 24) (Yunque et al. 2011)

Variety	Temperature (°C)	pH			
		6.7	7.7	8.7	9.7
Sacol green (GS)	20	0	0	0	0
	23	5	2	4	3
	25	10	8	11	11
Tambalang purple (PUR)	20	6	4	6	4
	23	8	6	10	5
	25	15	13	11	4
Tambalang reddish brown (BRN)	20	5	5	4	3
	23	2	2	0	0
	25	3	5	0	3

5.5 Tissue Culture and Micropropagule Production

Several protocols for callus induction, callus sub-culture and thallus regeneration have been reported for a wide range of seaweeds (Polne-Fuller and Gibor 1984; Aguirre-Lipperheide et al. 1995; Kumar et al. 2007; Reddy et al. 2003). The earliest tissue culture attempts for *Eucheuma* and *Kappaphycus* species were those of Polne-Fuller and Gibor (1987) followed by Dawes and Koch (1991). The methods employed in seaweed tissue culture generally involve the preparation of axenic explants; their culture in optimized, enriched growth media supplemented with plant growth regulators; callus induction; callus sub-culture and *de novo* thallus regeneration (Bradley 1991; Reddy et al. 2003). The first report for the production of callus from the cortical tissues of *Eucheuma uncinatum* (Polne-Fuller and Gibor 1987), followed by medullary tissue of *Kappaphycus alvarezii* (Dawes and Koch

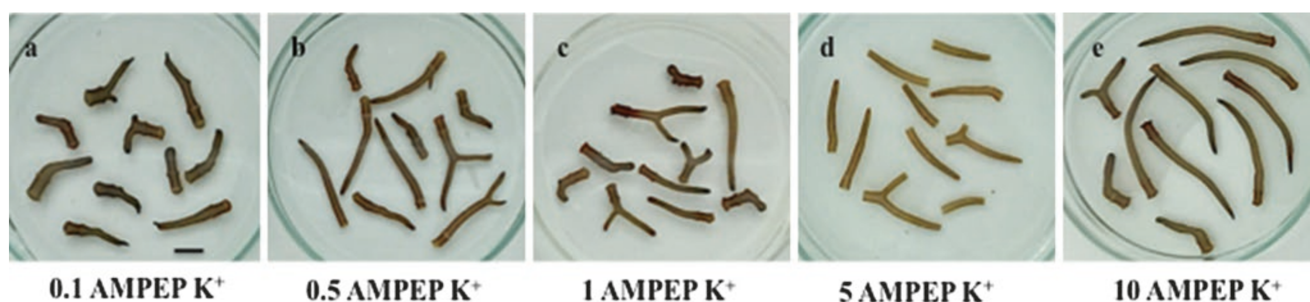


Fig. 5.6 (**a–e**) Length of shoots (mm) developed after 90 days using different concentrations (mg L^{-1}) of AMPEP K^+ (bar = 1 cm)

Fig. 5.7 (**a–e**) Length of shoots (mm) developed after 90 days using different concentrations (mg L^{-1}) of AMPEP K^+ + 1 mg L^{-1} PGR (IAA + Kinetin) (bar = 1 cm) (Photos by AQ Hurtado)

0.1 AMPEP K+ + 0.1 ORY + 1 PGR
0.1 AMPEP K+ + 0.5 ORY + 1 PGR
0.1 AMPEP K+ + 1 ORY + 1 PGR
0.5 AMPEP K+ + 0.1 ORY + 1 PGR
0.5 AMPEP K+ + 0.5 ORY + 1 PGR
0.5 AMPEP K+ + 1 ORY + 1 PGR
1 AMPEP K+ + 0.1 ORY + 1 PGR
1 AMPEP K+ + 0.5 ORY + 1 PGR
1 AMPEP K+ + 1 ORY + 1 PGR
5 AMPEP K+ + 0.1 ORY + 1 PGR
5 AMPEP K+ + 0.5 ORY + 1 PGR
5 AMPEP K+ + 1 ORY + 1 PGR
10 AMPEP K+ + 0.1 ORY + 1 PGR
10 AMPEP K+ + 0.5 ORY + 1 PGR
10 AMPEP K+ + 1 ORY + 1 PGR

Fig. 5.8 (**a–o**) Length of shoots (mm) developed after 90 days using different concentrations (mg L^{-1}) of AMPEP K^+ + Oryzalin (mg L^{-1}) + 1 mg L^{-1} PGR (IAA + Kinetin) (bar = 1 cm) (Photos by AQ Hurtado)

1991) stimulated researchers to develop further successful tissue culture protocols for *Eucheuma* and *Kappaphycus* species encouraged by their economic importance. A number of studies subsequently showed regeneration of micropropagules directly from explants of *E. serra* (Sahoo et al. 2002), *K. striatus* (Yunque et al. 2011), *K. alvarezii* (Neves et al. 2015; Yong et al. 2014b) and other red seaweeds, as reviewed by Reddy et al. (2008) and Baweja et al. (2009). Seaweed breeders used micro-propagation technology as a means of maintenance and clonal propagation of seedstock for the mariculture of economically important seaweeds including *Kappaphycus* and *Eucheuma* (Hurtado et al. 2015).

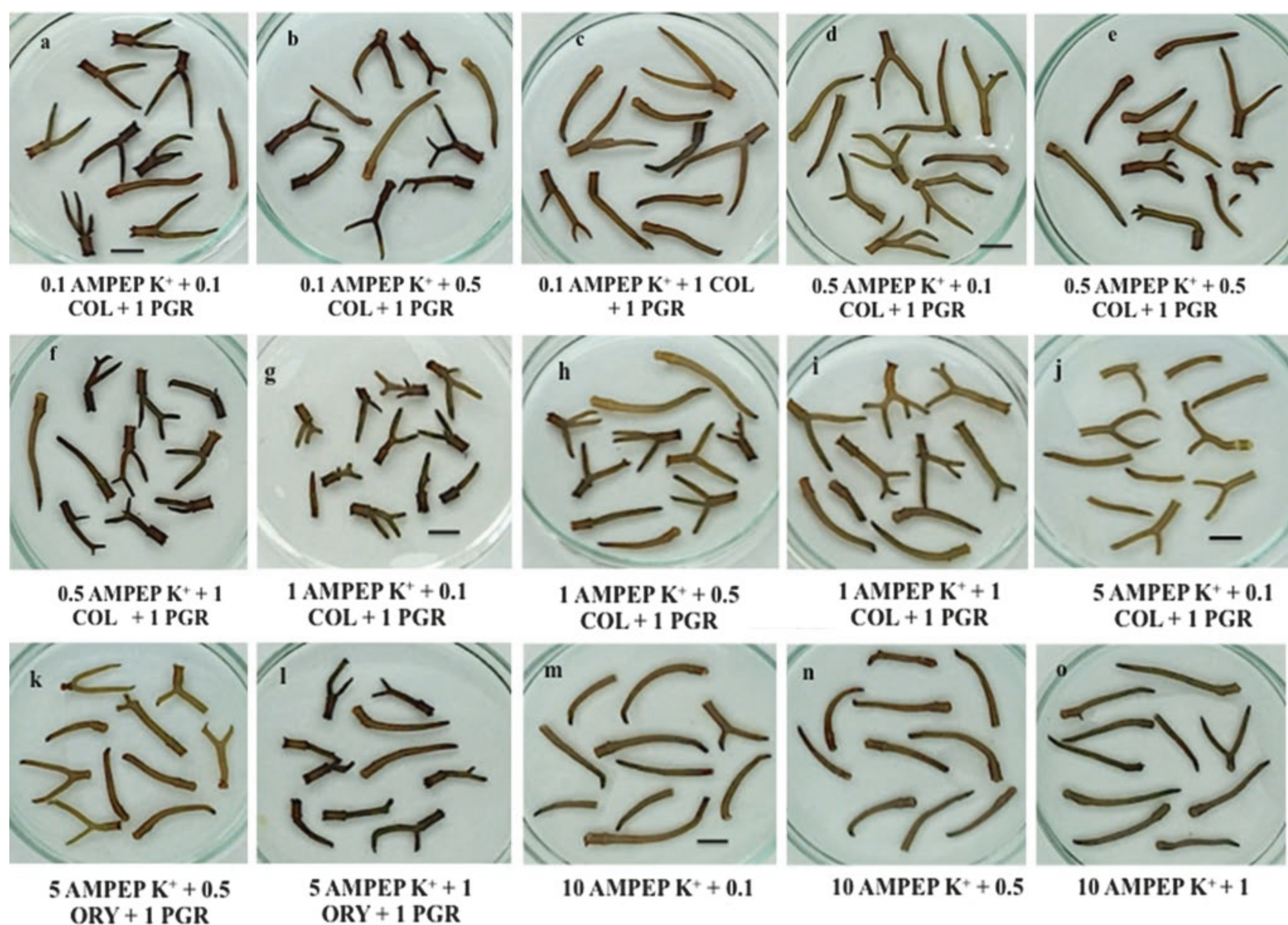


Fig. 5.9 (**a–o**) Length of shoots (mm) developed after 90 days using different concentrations (mg L^{-1}) of AMPEP K^+ + Colchicine (mg L^{-1}) + 1 mg L^{-1} PGR (IAA + Kinetin) (bar = 1 cm) (Photos by AQ Hurtado)

While tissue culture offers an exciting avenue for the selection of high quality germplasms and improvement of productivity, achievement of definite goals in seaweed tissue culture is often accompanied by the adoption of appropriate techniques and culture methods. In general, callus induction and somatic embryogenesis are used for the selection and propagation of desired strains, with economic importance, as reported by Reddy et al. (2003) and Yeong et al. (2014) in *K. alvarezii*. Somaclonal variation induced from undifferentiated callus with a high degree of genetic (including genomic, chromosome and epigenetic) variability provides a significant opportunity for the selection of improved phenotypes with desired heritable changes from regenerated cultures (Garcia-Reina et al. 1991).

While achieving rapid propagation of a superior strain, maintaining the genotype, micro-propagation via direct regeneration is an advantage over typical callus culture in order to produce “true-to-type” materials devoid of somaclonal variation which can arise from callus de-differentiation (Yong et al. 2014b). Establishment of seaweed culture through the development of adventitious rhizoids from small thallus fragments has been recommended to exploit the regeneration potential of explants for the production of uniform seedlings in high quantity, from a small number of donor plants (Garcia-Reina et al. 1991; Yunque et al. 2011). With the cutting-edge production technology, as compared to conventional vegetative propagation, tissue culture and micro-propagation have provided tools for an improved strategy to enhance the production of commercially viable *Kappaphycus* and *Eucheuma* seaweeds in order to fulfil the growing global demands of their raw materials (Hurtado et al. 2015; Yong et al. 2014b; Yunque et al. 2011). Muñoz et al. (2006) and Yong et al. (2014b) published the detailed design and application of an air-lift photobioreactor which could achieve optimal growth of *K. alvarezii* controlling all of the important abiotic growth factors such as light delivery, mixing time and supplementation of plant growth regulators. Successful implementation of bioreactors in future seaweed cultivation enterprises will be of benefit to control and intensify the biomass production while circumventing the barriers

of seasonality and environmental variability (Reddy et al. 2008; Yong et al. 2014b). Further investigations are aimed at cutting down the capital and operational costs of bioreactors. Additionally, bioprocess engineering of cell and tissue cultures created a potential platform for the controlled production of high value chemicals such as pharmaceuticals and nutraceuticals from marine seaweeds (Rorrer and Cheney 2004), including *Eucheuma* and *Kappaphycus* which could provide rich sources of valuable, bioactive compounds (Farah Diyana et al. 2015; Holdt and Kraan 2011; Kumar et al. 2008; Lau et al. 2014; Matanjun et al. 2008; Raman and Doble 2014; Reddy et al. 2008). However, the manufacturing capacity, chain of custody and security of supply are found lacking and required for safer and more efficacious biopharmaceuticals production from valuable species. A listing of *Eucheuma* and *Kappaphycus* species from which different types of cell and tissue cultures have been reported to date is provided in Table 5.4.

5.5.1 Axenic Culture Development

The steps adopted for preparing axenic, viable explants from seaweeds are quite similar to those techniques followed for land plant tissues, and are briefly described in the following.

The development of axenic culture techniques is an indispensable feature for successful tissue culture. However, it is difficult to achieve due to the absence of a cuticle on the algal surface, which would normally protect land plant tissues from damage caused by sterilizing chemicals. Therefore, it is very important to develop a protocol that selectively eliminates the contaminating bacteria, fungi and other microorganisms without affecting the viability of the donor plant tissues.

The initial explants to establish axenic tissue culture of *Kappaphycus* species are generally taken from either apical segments or thallus sections isolated from epiphyte-free, thallus or unialgal cultures (Table 5.5). Basically, the protocol for preparing axenic explants employs two steps, i.e. surface sterilization and incubation in a culture medium supplemented with a broad spectrum antibiotics and antifungal agents. The former employs the treatment of explants - first with detergent and then povidone iodine (Reddy et al. 2003; Yunque et al. 2011; Sulistiani et al. 2012), or sodium hypochlorite (Hayashi et al. 2008; Zitta et al. 2013). The latter involves the incubation in broad spectrum antibiotics with an antifungal agent, as summarized in Table 5.5, for eliminating microbial contaminants from both epiphytic and endophytic origin. In contrast, Dawes and Koch (1991) succeeded in producing axenic explants with antibiotic treatment alone from *Eucheuma* and *Kappaphycus*, and the most effective antibiotic soak was 30 min in polymixin B sulphate (300 mg 100 mL^{-1}).

The procedure to obtain axenic material of *K. alvarezii* described by Reddy et al. (2003) was the following: apical thallus fragments of approximately 5 cm in length were manually cleaned with a brush in filtered seawater with 0.5% liquid detergent (Charmy green, Japan) in sterile seawater for 10 min. Explants were then treated with 2% povidone iodine (available iodine 0.5% w/v) in sterile seawater for 3 min. Finally the explants were incubated in sterile PES medium (Provasoli 1968) containing 3% filter-sterilized broad spectrum antibiotic mixture (Polne-Fuller and Gibor 1984) with nystatin (fungicide) for 2 days (Table 5.5).

Sulistiani et al. (2012) employed a similar protocol, as described by Reddy et al. (2003). Apical segments (4–5 cm) were soaked in Tween® solution (5 drops in 200 mL) for 10 min, after which they were rinsed twice in 0.5% povidone iodine for 3 min. After, explants were rinsed 3–4 times with sterilized seawater, dried with sterilized tissue paper, and immersed in Conwy medium (Liao et al. 1983) containing 3% antibiotic mixture of Polne-Fuller and Gibor (1984).

Yunque et al. (2011) studied seven varieties of *K. alvarezii* and one variety of *K. striatus* (var. Sacol Green), and used the following protocol: apical segments (2 cm) were brushed with 0.05% povidone iodine, cleaned by shaking three times in 50 mL centrifuge tubes with fine glass beads and sterile filtered seawater. Cleaned segments were incubated in 9.1% E3 anti-bacterial solution (Bradley et al. 1988) in sterile, filtered seawater.

Hayashi et al. (2008) incubated explants in antibiotic and anti-fungal solutions. Apical branches of *K. alvarezii* (5 cm length) were selected from unialgal cultures of tetrasporophytes (red, brown and green strains) and a gametophyte strain (named as "Edison de Paula", EP). These branches were incubated with seawater enriched with a half strength Von Stosch solution (Oliveira et al. 1995) with anti-fungal and antibiotic solution (composed of 10,000 units Penicillin G, 10 mg Streptomycin sulphate and 25 μg Amphotericin B per liter, and 0.1 μg mL^{-1} Nystatin, Sigma) for 2 days. After, explants were washed with a solution of sterile seawater with 0.05% sodium hypochlorite and 2% organic detergent (Amway, USA) for 20 s, followed by successive rinsing with sterile seawater inside a laminar flow chamber.

Zitta et al. (2013) reported on the callus ontogeny of the brown tetrasporophyte of *K. alvarezii* and used a similar protocol to obtain axenic explants, as described by Hayashi et al. (2008), with the exception that explants were washed four times in a solution of sodium hypochlorite 0.05% and four times in 2% organic Triton X-100 detergent (Sigma) for 5 s at each washing.

Table 5.4 Types of cell and tissue culture for *Eucheuma* and *Kappaphycus* species with related culture media and exogenous factors

Species	Tissue	Culture	Medium	Irradiance (μmol m^{-2} s^{-1})	Photoperiod (L:D)	Temperature (°C)	References
E. uncinatum & *E. alvarezii*	Explants	Callus formation & regeneration	PES (solid)	40–60	12:12	18	Polne-Fuller and Gibor (1987)
E. denticulatum	Branches	Direct regeneration & callus formation	ESS (solid)	20–160	12:12	18–26	Dawes and Koch (1991)
E. denticulatum	Branches	Clonal propagation & callus formation	ESS + CW (solid)	25–50	NA	23–25	Dawes et al. (1993)
E. serra	Thallus	Direct regeneration	ASW (liquid)	7–50	12:12	12–25	Sahoo et al. (2002)
E. denticulatum	Explants	Callus formation & regeneration	ESS/2 (liquid)	5–80	16:8	25	Hurtado and Cheney (2003)
K. alvarezii	Branches	Direct regeneration & callus formation	ESS (solid)	20–160	12:12	18–26	Dawes and Koch (1991)
K. alvarezii	Branches	Clonal propagation & callus formation	ESS + CW (solid)	25–50	NA	23–25	Dawes et al. (1993)
K. alvarezii	Callus	Somatic embryogenesis & regeneration	PES (liquid)	5–70	12:12	22 (±1)	Reddy et al. (2003)
K. alvarezii	Thallus	Protoplast isolation & germination	F/2 and F/4 (liquid)	20–160	12:12	20–30	Salvador and Serrano (2005)
K. alvarezii	Explants	Callus formation & regeneration	PES (solid and liquid)	5–90	12:12	26 (±1)	Muñoz et al. (2006)
K. alvarezii	Explants	Callus formation & regeneration	ESS/2 (solid and liquid)	10–15	13:11	23–25	Hurtado and biter (2007)
K. alvarezii	Explants	Callus formation & regeneration	VS 50 (solid)	40 (±10)	14:10	23 (±2)	Hayashi et al. (2008)
K. alvarezii, *K. striatum*	Explants	Shoot formation	ESS/2 (liquid)	5–45	13:11	23	Hurtado et al. (2009)
K. striatum	Spores	Sporeling development	MGM (liquid)	50	13:11	25	Luhan and Sollesta (2010)
K. alvarezii, K. striatum	Explants	Shoot formation	ESS/2 (liquid)	10–45	13:11	20–25	Yunque et al. (2011)
K. alvarezii	Thallus	Callus formation & regeneration	PES (solid)	1500 (lux)	12:12	22–25	Sulistiani et al. (2012)
K. alvarezii	Thallus	Callus formation & regeneration	PES (solid and liquid)	25	12:12	25	Yeong et al. (2014)
K. alvarezii	Thallus	Direct regeneration	PES (liquid)	100 (±10)	12:12	25 (±1)	Yong et al. (2014a, b)
K. alvarezii	Explants	Protoplast isolation & germination	PES (liquid)	1500–2000 (lux)	12:12	15–40	Zhang et al. (2014)
K. alvarezii	Thallus	Direct regeneration	VS 50 (liquid)	15–50	12:12	25 (±1)	Neves et al. 2015

PES = Provasoli enriched seawater (Provasoli 1968), ESS and ESS/2 (half strength) = Enriched sterile seawater (Saga 1986), CW = coconut water, ASW = autoclaved seawater, F/2 (half strength) and F/4 (quarter strength) = F medium (Guillard and Ryther 1962), VS 50 = 50% von Stosch's solution (Guiry and Cunningham 1984), MGM = modified Grund medium (McLachlan 1973), NA = not available

5.5.2 Callus Morphogenesis

Dawes and Koch (1991) observed a higher rate of "bubbly type" of callus in green and brown forms of *K. alvarezii*, which was formed on the cut ends which were exposed to the air. These then formed an undifferentiated brown to beige mass of cells which originated from the medullary and inner cortical cells. Additionally, crystalline filaments grew from the cortical region of the explants. On the other hand, the majority of *E. denticulatum* explants formed a filamentous type of callus. Similarly, Reddy et al. (2003) observed filamentous outgrowths originating from both medullary and cortical regions of the cut surface of *K. alvarezii* explants, which gave rise to pigmented, uniseriate-branched filaments, which after 2 months' growth became a bright whitish, cap-like structure. Callus formation on cut surfaces, is indeed common in seaweed tissue culture (e.g. Polne-Fuller and Gibor 1987; Huang and Fujita 1996; Reddy et al. 2008), and is induced by the wounding process.

Apical calluses developed from the intact apical tips of *K. alvarezii* explants which originated from the apical region as a result of wound healing (Reddy et al. 2003). Apical calluses

Table 5.5 Protocols employed for obtain axenic explants from *Kappaphycus* and *Eucheuma*

Explants	Culture medium	Antibiotic and/or antimycotic solution	Surface sterilization solution	References
Thallus section (5 mm) from epiphyte-free main axes of *K. alvarezii* and *E. denticulatum*	ESS enriched seawater (Saga 1986)	Polymyxin B sulphate (3000 units L^{-1}) for up to 3 h	–	Dawes and Koch (1991)
Apical fragments (5 cm) of *K. alvarezii*	PES medium (Provasoli 1968)	Explants were incubated in 3% filter-sterilized spectrum antibiotic mixture (Polne-Fuller and Gibor 1984) for 2 days	Firstly, thallus was brushed in filtered seawater and then in 0.5% liquid detergent in sterile seawater for 10 min, and treated with 2% povidone iodine (available iodine 0.5% w/v) in sterilized seawater for 3 min	Reddy et al. (2003)
Apical branches (5 cm) of brown, red and green tetrasporophytes, and gametophyte ("Edison de Paula" strain) of *K. alvarezii*	Von Stosch (half strength, Oliveira et al. 1995)	Firstly, explants were incubated in antibiotic and antifungal solution (composed by 10,000 units Penicillin G, 10 mg Streptomycin sulphate, and 25 µg Amphotericin B per liter, and 0.1 µg mL^{-1} Nystatin) for 2 days	Explants were washed with sterilized seawater with 0.05% sodium hypochlorite and 2% organic detergent for 20 s	Hayashi et al. (2008)
Apical segments (2 cm) of seven varieties of *K. alvarezii* and one variety of *K. striatum* (var. sacol green)	Sterile filtered seawater	Cleaned segments were incubated in 9.1% E3 anti-bacterial solution (Bradley et al. 1988) in sterilized filtered seawater.	Firstly, apical segments were brushed with 0.05% povidone iodine, cleaned by shaking three times in 50 mL centrifuge tubes with fine glass beads in sterilized filtered seawater	Yunque et al. (2011)
Apical segments (4–5 cm) of *K. alvarezii*	Conwy medium (Liao et al. 1983)	Explants were immersed in medium with 3% antibiotic mixture (1 g Penicillin G, 2 g Streptomycin sulphate, 1 g Kanamycin, 25 mg Nystatin, 200 mg Neomycin in 100 mL distilled water)	Firstly, explants were soaked in Tween solution (5 drops in 200 mL) for 10 min, rinsed twice in 0.5% povidone iodine for 3 min, and then washed 3–4 times with sterilized seawater	Sulistiani et al. (2012)
Apical branches (5 cm) of brown tetrasporophytes of *K. alvarezii*	Von Stosch (half strength, Oliveira et al. 1995)	Firstly, explants were incubated in 2 mL L^{-1} antibiotic and anti-fungal solution (composed of 10,000 units mL^{-1} Penicillin G, 10 mg mL^{-1} Streptomycin sulphate and 25 µg mL^{-1} Amphotericin B, Sigma) and 0.1 µg mL^{-1} nystatin (Sigma) for 2 days	Explants were washed four times in solution of sodium hypochylorite 0.05% and four times in 2% organic Triton X-100 detergent (Sigma) for 5 s each rinsing	Zitta et al. (2013)

were also formed in *Solieria filiformis* (Robledo and Garcia-Reina 1993), *Meristotheca papulosa* (Huang and Fujita 1997), *Gracilariopsis tenuifrons* (Yokoya 2000), *Hypnea musciformis* (Yokoya et al. 2003), *Gracilaria perplexa* and *G. tenuistipitata* (Yokoya et al. 2004) and *G. domingensis* (Ramlov et al. 2013).

Hayashi et al. (2008) observed two types of callus in four colour strains of *K. alvarezii*: (a) filamentous callus developed on the explants surface exposed to the air which originated from divisions of the cortical and medullary cells, and (b) compact callus, growing on the explant surface which was in direct contact with the culture medium, also formed by the cortical and medullary cells. The "Edison de Paula" (EP) strain produced filamentous cells growing from the surface of the explant in contact with the culture medium, similarly of those described by Dawes and Koch (1991). In another instance, Sulistiani et al. (2012) observed three types of callus in explants as cultured in PES liquid medium supplemented with PGRs: (a) white compact callus, (b) white filamentous callus and (c) greenish/brownish callus. Compact (as opposed to diffuse) calluses have also been reported in other red algae, e.g. *Gracilaria domingensis* (Ramlov et al. 2013).

The ontogeny of callus development in the brown tetrasporophyte of *K. alvarezii* was analysed using light and transmission electron microscopy (TEM) by Zitta et al. (2013). Under the light microscope, callus filaments were observed to have elongated and clavate-shaped cells. Filamentous calluses composed of elongated filaments were also observed in *Agardhiella subulata* (Bradley and Cheney 1990), *Grateloupia dichotoma* (Yokoya and Handro 1996), *Solieria filiformis* (Yokoya and Handro 2002) and *Hypnea*

musciformis (Kumar et al. 2007). Under TEM analysis, the most notable feature was the thickening of the cell wall during the de-differentiation process of initially of apical cells and later, in all cells of the callus (Zitta et al. 2013). The increased thickness of the cell wall could be a defensive response against desiccation since callus was growing on a solid culture medium and exposed to the air. In addition, it was observed that the cell walls had two different types of distribution of their microfibrils, a phenomenon also reported for callus cells originating from *Gracilariopsis tenuifrons* (Bouzon et al. 2010). Other ultrastructural changes in callus cells were seen to be the proliferation of convoluted membranes, increased numbers of mitochondria and modifications to normal chloroplast ultrastructure, these observations were taken to indicate cell de-differentiation. The cytoplasm showed a large amount of starch grains, and the chloroplasts had an altered state with plasto-globules and disorganized thylakoids, as well as the presence of several convoluted membranes and vacuoles (Zitta et al. 2013).

5.5.3 Factors Affecting Callus Induction and Growth

Different types and concentrations of auxins and cytokinins have been added to culture media for callus induction and growth in *Kappaphycus* and *Eucheuma* species (see Table 5.6 for a summary). However, exogenous applications of NAA and BAP did not increase callus induction or the growth in *K. alvarezii* (Reddy et al. 2003). Similar results were reported by Sulistiani et al. (2012) using combinations of BAP + NAA and BAP + IAA. Dawes and Koch (1991) observed that the largest calluses (up to 5 mm in height) in *K. alvarezii* explants were induced by treatments with NAA + 2iP, NAA+ BAP, and PAA + 2iP at concentrations of (1 + 1) mg L^{-1}.

The type of culture medium used was also reported to affect callus formation in explants of *K. alvarezii* and *E. denticulatum*, and ESS-enriched seawater was the suitable medium for both species (Dawes and Koch 1991). Reddy et al. (2003) observed a percentage callus formation greater than 80% in explants cultured in PES medium with 1.5% of bacto-agar, without the addition of PGRs. Hayashi et al. (2008) observed the highest percentage of callus formation (100%) in the brown strain of *K. alvarezii* cultured in F/2 50 medium (seawater enriched with 50% of Guillard and Ryther solution), while the "Edison de Paula" strain showed higher levels of callus induction in explants cultured in VS 50 (i.e. seawater enriched with 50% of von Stosch solution) and F/2 50 medium. However, explants cultured in PES medium showed relatively faster callus induction after 18 days, while in CW medium callus formation was observed after 22 days (Sulistiani et al. 2012).

5.5.4 Factors Affecting Regeneration and Micro-propagation

Micro-propagation, or *in vitro* clonal propagation, allows for the production of a large number of individuals within a short period and also selection of higher yielding strains that could be used as future seedlings for seaweed cultivation (Yokoya and Yoneshigue-Valentin 2011). Micro-propagation of *Kappaphycus* and *Eucheuma* species can be achieved either indirectly from regeneration of thalli from callus cells or by directly regenerating thalli from the explant tissue by passing the callus-induction phase. Polne-Fuller and Gibor (1987) reported the successful regeneration of new thalli in *Eucheuma uncinatum* and *K. alvarezii*. Micro-propagation of *E. denticulatum* and *K. alvarezii* were stimulated by the addition of several combinations of auxins and cytokinins (see Table 5.6, Dawes and Koch 1991). Similar results were reported by Muñoz et al. (2006) and PGRs stimulated regeneration from callus in *K. alvarezii*. On the other hand, Hayashi et al. (2008) observed that explants from brown, red and green tetrasporophytes and the "Edison de Paula" strain were able to be directly regenerated; indirect regeneration was very rare (only 1%), but when it occurred, that particular explant produced approximately 90 micro-propagules.

A novel method for clonal propagation using somatic embryos from uniseriate, pigmented, filamentous callus of *K. alvarezii* was described by Reddy et al. (2003); here micropropagule production was improved by the addition of NAA and a mixture of NAA and BAP in to the culture medium**.** Also, the propagules obtained from pigmented callus of *Kappaphycus* showed an enhanced growth rate of as much as 1.5–1.8 times the rate of farmed plants which were propagated through vegetative (splitting) means, without compromising on the yield of the carrageenan after cultivation in the sea. This method could be very useful as a potential tool for mass clonal propagation of desired seed stock of *Kappaphycus*, and *Eucheuma* species for commercial farming.

5.5.5 Endogenous Concentrations of PGRs in *Kappaphycus alvarezii*

Endogenous PGRs (auxins, cytokinins, abscisic acid, gibberellins, oxylipins (jasmonic acid), polyamines are reported as detected in a number of algae belonging to different evolutionary lineages (Stirk et al. 2003, 2009; Tarakhosvskaya et al. 2007; Yokoya et al. 2010; Mikani et al. 2015). Auxin and cytokinin-like activities have been reported in a number of different commercial seaweed extracts (e.g. Stirk and van Staden 1997), which are useful as biostimulants in agriculture and horticulture (e.g. Stirk and van Staden 2006; Khan

Table 5.6 Plant growth regulators (PGRs) used for callus induction and growth, regeneration and micro-propagation of *Kappaphycus* and *Eucheuma* species

PGR type	Full name/abbreviation	Concentrations (mg L^{-1})	Culture medium	References
Auxins	Indole 3-acetic acid (IAA)	1, 5, 10	ESS	Dawes and Koch (1991)
		0.5, 1.0, 5.0	VS50	Hayashi et al. (2008)
		2.5, 5.0	PES & CW	Sulistiani et al. (2012)
	1-Naphthalene acetic acid (NAA)	0.1, 1.0	PES	Reddy et al. (2003)
		0.1–10	ESS	Dawes and Koch (1991)
		0.5, 1.0	PES & CW	Sulistiani et al. (2012)
	Indole 3-butyric acid (IBA)	1, 5, 10	ESS	Dawes and Koch (1991)
	Phenyl acetic acid (PAA)	0.1–10	ESS	Dawes and Koch (1991)
		1	ESS/2	Hurtado and Cheney (2003)
		1	AMPEP	Hurtado et al. (2009)
		1	ESS/2	Hurtado et al. (2009)
		1	AMPEP	Yunque et al. (2011)
	2,4-Dichlorophenoxy-acetic acid (2,4-D)	0.5, 5.0	VS50	Hayashi et al. (2008)
Aromatic cytokinins	6-benzylaminopurine (BAP)	0.1, 1.0	PES	Reddy et al. (2003)
		0.1–10.0	ESS	Dawes and Koch (1991)
		0.5, 1.0, 5.0	VS50	Hayashi et al. (2008)
		0.5, 1.0	PES & CW	Sulistiani et al. (2012)
	Kinetin (K)	1, 5, 10	ESS	Dawes and Koch (1991)
Isoprenoid cytokinins	Zeatin (Z)	1, 5, 10	ESS	Dawes and Koch (1991)
		1	ESS/2	Hurtado and Cheney (2003)
		1	AMPEP	Hurtado et al. (2009)
		1	AMPEP, ESS/2	Yunque et al. (2011)
	N^6-[-2-isopentenyl] adenine (2iP)	1, 5, 10	ESS	Dawes and Koch (1991)

CW – Conwy medium (Liao et al. 1983), ESS – ESS enriched seawater (Saga 1986), PES – Seawater enriched with Provasoli solution (Provasoli 1968), VS50 – Seawater with half-strength of von Stosch solution (Oliveira et al. 1995)

et al. 2009). Similarly, the sap from freshly harvested *K. alvarezii* has been reported to be a potently active, foliar spray, which could be related to the presence of the cytokinins (kinetin and zeatin), the auxin (indole 3-acetic acid), and the gibberellic acid, GA_3 reported to be present (Prasad et al. 2010). These PGRs were quantified by tandem mass spectrometry of various organic extracts, and the endogenous concentration of IAA in the sap was estimated to be 23.36 ± 0.15 ppm, while concentrations of kinetin and zeatin were 7.94 ± 0.30 and 23.97 ± 0.47 ppm, respectively; concentrations of GA_3 was 27.87 ± 0.14 ppm (Prasad et al. 2010). It is important to highlight that the first report on the occurrence of endogenous kinetin in algae was for *K. alvarezii* (Prasad et al. 2010).

5.5.6 Quality Assessment of Tissue Culture Progeny

In order to achieve the goals of cell and tissue culture in supporting the growth and value addition to the seaweed farming industry, continuous quality assessment of tissue culture progeny is mandatory to ensure the production of high quality seedlings. Stepwise acclimatization of tissue-cultured seedlings from *in vitro* conditions to partial *in vitro* tank culture, prior to transfer to an *ex situ* nursery or glasshouse is strongly recommended in order to improve the growth performance and survival capacity of the seedlings (Yong et al. 2015a). Tissue-cultured *K. alvarezii* thalli have been reported to exhibit higher growth rates and resistance to epiphytes and 'ice-ice' disease, as compared to same age, farm-propagated seedlings after out-planting to the open sea (Reddy et al. 2003; Yong et al. 2015a). Hurtado et al. (2015) reported on the production of new and improved *Kappaphycus* thalli grown under optimized growth conditions with the addition of a biostimulant to maintain the high-yielding and rapidly growing seed-stock for a commercial nursery. Moreover, comparison of the tissue culture vs farm-propagated thalli of *K. alvarezii* revealed economically important quality benefits: e.g. carrageenan yield and quality (Reddy et al. 2003; Yong et al. 2014a), higher nutritional value (Yong et al. 2015b) and less heavy metal contamination (Yong et al. 2017). This suggested that the tissue culture strains were a superior quality to serve as planting materials for the future of sustainable seaweed farming and use of the biomass for higher value applications.

5.6 Conclusions

To strengthen and indeed advance the *Kappaphycus* farming industry, it is fundamentally important to achieve success in the development of *in vitro* tissue culture technology for the furtherance of sustainable, commercial farming. The foregoing account has clearly demonstrated not only the success achieved in development of basic techniques for routine tissue culture of *Kappaphycus* and *Eucheuma,* but also underlined the benefits derived from the application of these techniques in the production of clones, their propagation and maintenance. In parallel, these efforts have also created a wide array of choices for methods, approaches and strategies for the consistent production of propagules directly from explants thereby bypassing callus induction. The propagules differentiated from callus were also shown to have improved growth characteristics suggesting the possibility of generating improved variants. Such variants will be of great benefit, especially for those countries where carrageenophytes are farmed from a single, exotic genotype. There has also been significant advancement in establishing commercial nurseries which are now considered as very important for maintaining and further boosting the activity of commercial carrageenophyte farming. The rate of production of new and improved *Kappaphycus* explants for a commercial nursery stock has been substantially improved through the novel use of AMPEP with optimized culture media, pH, temperature and density of thalli. The use of AMPEP alone and/or in combination with PGR in culture media for propagation of micro-plantlets using tissue culture techniques has been highly successful in the production of seedstock for nursery and out-planting purposes. Combinations of AMPEP K^+ with PGR + colchicine or oryzalin provided better formulations for robust seedlings and shoot production in *K. alvarezii*. Likewise, AMPEP K^+ + PGR could be used to induce direct, erect shoots for the micro-propagation of *K. alvarezii* plantlets under laboratory conditions.

It is also crucial to establish homozygous lines of economically important seaweeds for agronomically important traits such as growth, phycocolloid, disease and epiphyte resistance, stress tolerance etc. Establishment of such lines is of fundamental importance, and can form an invaluable resource for attempting breeding aimed at developing robust hybrid varieties for commercial farming of carrageenophytes, more particularly in those countries where *Kappaphycus* grows naturally. Nevertheless, the benefits of cell suspension culture have not been realized largely due to absence of friable callus formation in seaweeds, a phenomenon which is quite common for terrestrial plants. This could be possible, if the role of biostimulants in cell division and development at the molecular level is better understood.

Acknowledgment The authors would like to profoundly thank their respective heads of the organization for extending their support, encouragement and facilities while preparing this article.

References

Aguirre-Lipperheide M, Estrada-Rodríguez FJ, Evans LV (1995) Facts, problems and needs in seaweed tissue culture: an appraisal. J Phycol 31:677–688

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

Azanza-Corrales R, Ask E (2003) *Kappaphycus alvarezii* (Doty) Doty carposporeling germination growth and development in the laboratory. In: Chapman A, Andersen AJ, Vreeeland V, Davison I (eds) Proc Int Seaweed Symp 17:95–99

Azanza-Corrales R, Mamauag SS, Alfiler E, Orolfo MJ (1992) Reproduction in *Eucheuma denticulatum* (Burman) Collins and Hervey and *Kappaphycus alvarezii* (Doty) Doty farmed in Danajon Reef, Philippines. Aquaculture 103:29–34

Baweja P, Sahoo D, García-Jiménez P, Robaina RR (2009) Seaweed tissue culture as applied to biotechnology: problems, achievements and prospects. Phycol Res 57:45–58

Bixler HJ, Porse H (2011) A decade of change in seaweed hydrocolloids industry. J Appl Phycol 23:321–335

Borlongan IA, Tibubos KR, Yunque DAT, Hurtado AQ, Critchley AT (2011) Impact of AMPEP on the growth and occurrence of epiphytic *Neosiphonia* infestations on two varieties of commercially cultivated *Kappaphycus alvarezii*, grown at different depths in the Philippines. J Appl Phycol 23:615–621

Bouzon ZL, Schmidt EC, Almeida AC, Yokoya NS, Oliveira MC, Chow F (2010) Cytochemical characterization and ultrastructural organization in calluses of the agarophyte *Gracilariopsis tenuifrons* (Gracilariales, Rhodophyta). Micron 42:80–86

Bradley P (1991) Plant hormones do have a role in controlling growth and development of algae. J Phycol 27:317–321

Bradley PM, Cheney DP (1990) Some effects of plant regulators on tissue cultures of the marine red alga *Agardhiella subulata* (Gigartinales, Rhodophyta). Hydrobiologia 204(205):353–360

Bradley PM, Cheney DP, Saga N (1988) One step antibiotic disk method for obtaining axenic cultures of multicellular marine algae. Plant Cell Tissue Organ Cult 12:55–60

Brown V, Ducker SC, Rowan KS (1977) The effect of orthophosphate concentration on the growth of articulated coralline algae (Rhodophyta). Phycologia 16:125–131

Bulboa CR, de Paula EJ (2005) Introduction of non-native species of *Kappaphycus* (Rhodophyta, Gigartinales) in subtropical waters: comparative analysis of growth rates of *Kappaphycus alvarezii* and *Kappaphycus striatum in vitro* and in the sea in south-eastern Brazil. Phycol Res 53:183–188

Bulboa C, de Paula E, Chow F (2007) Laboratory germination and sea out-planting of tetraspore progeny from *Kappaphycus striatum* (Rhodophyta) in subtropical waters of Brazil. J Appl Phycol 19:357–363

Bulboa C, de Paula EJ, Chow F (2008) Germination and survival of tetraspores of *Kappaphycus alvarezii* var. *alvarezii* (Solieriaceae, Rhodophyta) introduced in subtropical waters of Brazil. Phycol Res 56:39–45

Butler DM, Evans LV (1990) Cell and tissue culture of macroalgae. In: Akatsuka I (ed) Introduction to applied phycology. SPB Academic Publishing, The Hague, pp 629–645

Cheney DP, Mathiesen A, Schubert D (1981) The application of genetic improvement techniques to seaweed cultivation: I. Strain selection in the carrageenophyte *Chondrus crispus*. Proc Int Seaweed Symp 10:559–567

Craigie JS (2011) Seaweed extracts stimuli in plant science and agriculture. J Appl Phycol 23:371–393

Dawes CJ (1993) Marine botany. Wiley Interscience Publication. John Wiley & Sons, New York, p 478

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

Dawes CJ, Trono GC Jr, Lluisma AO (1993) Clonal propagation of *Eucheuma denticulatum* and *Kappaphycus alvarezii* for Philippine seaweed farms. Hydrobiologia 260(261):379–383

Dawes CJ, Lluisma AO, Trono GC (1994) Laboratory and field growth studies of commercial strains of *Eucheuma denticulatum* and *Kappaphycus alvarezii* in the Philippines. J Appl Phycol 6:21–24

Doty MS, Alvarez V (1973) Seaweed farms: a new approach for U.S. industry. Mar Technol Soc Annu Conf Pap 9:701–708

Fadilah S, Alimuddin A, Masak PR, Santoso J, Parenrengi A (2016) Growth, morphology and growth related hormone level in *Kappaphycus* produced by mass selection in Gorontalo waters, Indonesia. Hayati J Biosci. http://dx.doi.org/10.1016/j.hjb.2015.09.004

Fan D, Hodges DM, Zhang JZ, Kirby CW, Ji XH, SLocke SJ, Critchley AT, Prithiviraj B (2011) Commercial extract of the brown seaweed *Ascophyllum nodosum* enhances phenolic antioxidant content of spinach (*Spinacia aloracea* L.) which protects *Caenorhabditis elegans* against oxidative and thermal stress. Food Chem 124:195–120

FAO (2016) The state of world fisheries and aquaculture 2016. Contributing to food security and nutrition for all. FAO, Rome, 200 pp

Farah Diyana A, Abdullah A, Shahrul Hisham ZA, Chan KM (2015) Antioxidant activity of red algae *Kappaphycus alvarezii* and *Kappaphycus striatum*. Food Res Int 22:1977–1984

Garcia-Reina G, Gómez-Pinchetti JL, Robledo DR, Sosa P (1991) Actual, potential and speculative applications of seaweed cellular biotechnology: some specific comments on *Gelidium*. Hydrobiologia 221:181–194

Ghosh A, Vijay Anand KG, Seth A (2015) Life cycle impact assessment of seaweed based biostimulant production from onshore cultivated *Kappaphycus alvarezii* (Doty) Doty ex Silva—is it environmentally sustainable? Algal Res 12:513–521

Guillard RR, Ryther JH (1962) Studies of marine planktonic diatoms: I. *Cyclotella nana* Hustedt, and *Detonula confervacea* (Cleve) Gran. Can J Microbiol 8:229–239

Guiry MD, Cunningham EM (1984) Photoperiodic and temperature responses in the reproduction of north-eastern Atlantic *Gigartina acicularis* (Rhodophyta: Gigartinales). Phycologia 23:357–367

Handayani T, Alimuddin WU, Suryati E, Parenrengi A (2014) Binary vector construction and *Agrobacterium tumefaciens*-mediated transformation of lysozyme gene in seaweed *Kappaphycus alvarezii*. Biotropia 21:80–90

Hayashi L, Yokoya NS, Kikuchi DM, Oliveira EC (2008) Callus induction and micro-propagation improved by colchicine and phytoregulators in *Kappaphycus alvarezii* (Rhodophyta, Solieriaceae). J Appl Phycol 20:653–659

Holdt SL, Kraan S (2011) Bioactive compounds in seaweed: functional food applications and legislation. J Appl Phycol 23:543–597

Huang W, Fujita Y (1996) Callus induction and thallus regeneration in some species of red algae. Phycol Res 45:105–111

Huang W, Fujita Y (1997) Callus induction and thallus regeneration of the red alga *Meristotheca papulosa* (Rhodophyta, Gigartinales). Bot Mar 40:55–61

Hurtado AQ, Agbayani R (2000) The farming of seaweed *Kappaphycus*. SEAFDEC AQD, Tigbauan, Iloilo, Philippines, p 25

Hurtado AQ, Biter A (2007) Plantlet regeneration of *Kappaphycus alvarezii* var. adik-adik by tissue culture. J Appl Phycol 19:783–786

Hurtado AQ, Cheney DP (2003) Propagule production of *Eucheuma denticulatum* (Burman) Collins et Harvey by tissue culture. Bot Mar 46:338–341

Hurtado AQ, Critchley A, Trespoey A, L'honneur-Bleicher G (2006) Occurrence of *Polysiphonia* epiphytes in *Kappaphycus* farms at Calaguas Is., Camarines Norte, Phillippines. J Appl Phycol 18:301–306

Hurtado AQ, Yunque DA, Tibubos K, Critchley AT (2009) Use of Acadian marine plant extract powder from *Ascophyllum nodosum* in tissue culture of *Kappaphycus* varieties. J Appl Phycol 21:633–639

Hurtado AQ, Joe M, Sanares RC, Fan PB, Critchley AT (2012) Investigation of the application of Acadian Marine Plant Extract Powder (AMPEP) to enhance the growth, phenolic content, free radical scavenging, and iron chelating activities of *Kappaphycus* Doty (Solieriaceae, Gigartinales, Rhodophyta). J Appl Phycol 24:601–611

Hurtado AQ, Neish IC, Critchley AT (2015) Developments in production technology of *Kappaphycus* in the Philippines: more than four decades of farming. J Appl Phycol 27:1945–1961

Jayaraj JA, Wan M, Rahman PZK (2008) Seaweed extract reduces foliar fungal disease on carrot. Crop Protect 10:1360–1366

Jayaraj J, Norrie J, Punja ZK (2011) Commercial extract from brown seaweed *Ascophyllum nodosum* reduces fungal disease in greenhouse cucumber. J Appl Phycol 23:353–361

Khan W, Rayirath UP, Subramanian S, Jithesh MN, Rayorath P, Hodges DM, Critchley AT, Craigie JS, Norrie J, Prithiviraj B (2009) Seaweed extracts as biostimulants of plant growth and development. J Plant Growth Regul 28:386–399

Kirihara S, Fujikawa Y, Notoya M (1997) Axenic tissue culture of *Sargassum confusum* C. Agardh (Phaeophyta) as a source of seeds for artificial marine forests. J Mar Biotech 5:142–146

Kumar GR, Reddy CRK, Jha B (2007) Callus induction and thallus regeneration from callus of phycocolloid yielding seaweeds from the Indian coast. J Appl Phycol 19:15–25

Kumar KS, Ganesan K, Rao PVS (2008) Antioxidant potential of solvent extracts of *Kappaphycus alvarezii* (Doty) Doty – an edible seaweed. Food Chem 107:289–295

Largo DB, Fukami K, Nishijima T (1995) Occasional pathogenic bacteria promoting 'ice-ice' disease in the carrageenan-producing red algae *Kappaphycus alvarezii* and *Eucheuma denticulatum* (Solieriaceae, Gigartinales, Rhodophyta). J Appl Phycol 7:545–554

Lau TY, Vittal DF, Chew CSY, Yong WTL (2014) Antiproliferative potential of extracts from *Kappaphycus* seaweeds on HeLa cancer cell lines. Sains Malaysiana 43:1895–1900

Lawlor HJ, Borowitzka MA, McComb JA (1990) Tissue culture of brown seaweeds. Aust J Biotechnol 4:260–264

Liao IC, Su HM, Lin JH (1983) Larval foods for penaeid prawn. In: McVey JP (ed) CRC handbook of Mariculture volume 1: crustacean aquaculture. CRC Press Inc., Boca Raton, pp 29–60

Loureiro RR, Reis RP, Critchley AT (2010) *In vitro* cultivation of three *Kappaphycus alvarezii* (Rhodophyta, Areschougiaceae) variants (green, red and brown) exposed to a commercial extract of the brown alga *Ascophyllum nodosum* (Fucaceae, Ochrophyta). J Appl Phycol 22:101–104

Loureiro RR, Reis RP, Berrogain FD, Critchley AT (2012) Extract powder from the brown alga *Ascophyllum nodosum* (Linnaeus) Le Jolis (AMPEP): a "vaccine-like" effect on *Kappaphycus alvarezii* (Doty) Doty ex P.C. Silva. J Appl Phycol 24:427–432

Loureiro RR, Reis RP, Berrogain FD, Critchley AT (2014) Effects of a commercial extract of the brown alga *Ascophyllum nodosum* on the biomass production of *Kappaphycus alvarezii* (Doty) Doty ex P. C Silva and its carrageenan yield and gel quality cultivated in Brazil. J Appl Phycol 26:763–768

Luhan MRJ, Sollesta H (2010) Growing the reproductive cells (carpospores) of the seaweed, *Kappaphycus striatum*, in the laboratory

until outplanting in the field and maturation to tetrasporophyte. J Appl Phycol 22:579–585
Maliakal S, Cheney DP, Rorrer GL (2001) Halogenated monoterpene production in regenerated plantlet cultures of *Ochtodes secundiramea* (Rhodophyta, Cryptonemiales). J Phycol 37:1010–1019
Marriog RG, Loureiro RR, Reis RP (2016) The effect of *Ascophyllum nodosum* (Ochrophyta) extract powder on the epibiosis of *Kappaphycus alvarezii* (Rhodophyta) commercially cultivated on floating rafts. J Appl Phycol 28:2471–2477
Matanjun P, Mohamed S, Mustapha NM, Muhammad K, Ming CH (2008) Antioxidant activities and phenolics content of eight species of seaweeds from north Borneo. J Appl Phycol 20:367–373
McLachlan J (1973) Growth media – marine. In: Stein JR (ed) Handbook of phycological methods. Culture methods and growth measurements. Cambridge University Press, Cambridge, pp 25–51
Mikani K, Mori IC, Matsuura T, Ikeda Y, Kojima M, Sakakibara H, Hirayama T (2015) Comprehensive quantification and genome survey reveal the presence of novel phytohormone action modes in red seaweeds. J Appl Phycol 28:2539–2548
Muñoz J, Cahue-López AC, Patiño R, Robledo D (2006) Use of plant growth regulators in micro-propagation of *Kappaphycus alvarezii* (Doty) in airlift bioreactors. J Appl Phycol 18:209–218
Nayar S, Bott K (2014) Current status of global cultivated seaweed production and markets. World Aq 45:32–37
Neves FAS, Simioni C, Bouzon ZL, Hayashi L (2015) Effects of spindle inhibitors and phytoregulators on the micro-propagation of *Kappaphycus alvarezii* (Rhodophyta, Gigartinales). J Appl Phycol 27:437–445
Oliveira EC, Paula EJ, Plastino EM, Petti R (1995) Metodologias para o cultivo no axênico de macroalgas marinas *in vitro*. In: Alveal K, Ferrario ME, Oliveira EC, Sar E (eds) Manual de Métodos Ficológicos. Universidad de Concepción, Concepción, pp 429–455
Patwary MU, van der Meer JP (1982) Genetics of *Gracilaria tikvahiae*. Phenotypic and genetic characterization of some selected morphological mutants. Can J Bot 60:2556–2564
Patwary MU, van der Meer JP (1983) Growth experiments on morphological mutants of *Gracilaria tikvahiae* (Rhodophyceae). Can J Bot 6:1654–1659
Polne-Fuller M, Gibor A (1984) Developmental studies in *Porphyra*. I. Blade differentiation in *Porphyra perforata* as expressed by morphology, enzymatic digestion and protoplast regeneration. J Phycol 20:609–616
Polne-Fuller M, Gibor A (1987) Calluses and callus-like growth in seaweeds: induction and culture. Hydrobiologia 151(152):131–138
Prasad K, Das AK, Oza MD, Brahmbhatt H, Siddhanta AK, Meena R, Eswaran K, Rajyaguru MR, Ghosh PK (2010) Detection and quantification of some plant growth regulators in a seaweed-based foliar spray employing a mass spectrometric technique sans chromatographic separation. J Agric Food Chem 58:4594–4601
Provasoli L (1968) Media and prospects for the cultivation of marine algae. In: Watanabe A, Hattore A (eds) Culture and collection of algae. Japanese Society of Plant Physiologists, Tokyo, pp 63–75
Raman M, Doble M (2014) Physicochemical and structural characterisation of marine algae *Kappaphycus alvarezii* and the ability of its dietary fibres to bind mutagenic amines. J Appl Phycol 26:2183–2191
Ramlov F, Plastino EM, Yokoya NS (2013) Growth, callus formation and plant regeneration in color morphs of *Gracilaria domingensis* (Gracilariales, Rhodophyta) cultured under different irradiance and plant growth regulators. Phycologia 52:508–516
Reddy CRK, Kumar GRK, Siddhanta AK, 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
Reddy CRK, Jha B, Fuhita Y, Ohno M (2008) Seaweed micropropagation techniques and their potentials: an overview. J Appl Phycol 20:609–617
Reddy CRK, Gupta V, Jha B (2010) Developments in biotechnology of red algae. In: Seckbach J, Chapman DJ (eds) Red algae in the genomic age, cellular origin, life in extreme habitats and astrobiology 13. Springer, Dordrecht, pp 307–341
Robledo DR, Garcia-Reina G (1993) Apical callus formation in *Solieria filiformis* (Gigartinales, Rhodophyta) cultured in tanks. Hydrobiologia 260(261):401–406
Rorrer GL, Cheney DP (2004) Bioprocess engineering of cell and tissue cultures for marine seaweeds. Aquac Eng 32:11–41
Saga N (1986) Pure culture of algae. In: Yamada Y, Okada Y (eds) Plant biotechnology. Tokyo Kaymkes Dojin, Tokyo, pp 55–69. (in Japanese)
Sahoo D, Ohno M, Hiraoka M (2002) Laboratory, field and deep seawater culture of *Eucheuma serra* – a high lectin yielding red alga. Algae 17:127–133
Salvador RC, Serrano AE (2005) Isolation of protoplasts from tissue fragments of Philippine cultivars of *Kappaphycus alvarezii* (Solieriaceae, Rhodophyta). J Appl Phycol 17:15–22
Singh S, Singh MK, Pal SK, Trivedi K, Yesuraj D, Singh CS, Vijay Anand KG, Chandramohan M, Patidar R, Kubavat D, Zodape ST, Ghosh A (2016) Sustainable enhancement in yield and quality of rain-fed maize through *Gracilaria edulis* and *Kappaphycus alvarezii* seaweed sap. J Appl Phycol 28:2099–2112
Stirk WA, van Staden J (1997) Screening of some South African seaweeds for cytocinin-like activity. S Afr J Bot 63:161–164
Stirk WA, van Staden J (2006) Seaweed products as biostimulants in agriculture. In: Critchley AT, Ohno M, Largo DB (eds) World seaweed resources. DVD-ROM multimedia interactive software – UNESCO Publishing/ETI Bioinformatics, University of Amsterdam, ISBN 90 75000 80 4
Stirk WA, Novák O, Strnad M, Van Staden J (2003) Cytokinins in macroalgae. J Plant Growth Regul 41:13–24
Stirk WA, Novák O, Hradecká V, Pěnčík A, Rolčík J, Strnad M, Van Staden J (2009) Endogenous cytokinins, auxins and abscisic acid in *Ulva fasciata* (Chlorophyta) and *Dictyota humifusa* (Phaeophyta): towards understanding their biosynthesis and homeostasis. Eur J Phycol 44:231–240
Sulistiani E, Soelistyowati DT, Alimuddin YSA (2012) Callus induction and filaments regeneration from callus of cottonii seaweed *Kappaphycus alvarezii* (Doty) collected from Natuna Islands, Riau Islands Province. Biotropia 19:103–114
Sylvester AW, Waaland R (1983) Cloning the red alga *Gigartina exasperata* for culture on artificial substrates. Aquaculture 31:305–318
Tarakhosvskaya ER, Maslov YI, Shishova MF (2007) Phytohormones in algae. Russian J Plant Physiol 54:163–170
Tibubos K, Hurtado AQ, Chritchley AT (2016) Direct formation of axes in new plantlets of *Kappaphycus alvarezii* (Doty) Doty, as influenced by the use of AMPEP K+, spindle inhibitors and plant growth hormones. J Appl Phycol. doi:10.1007/s10811-016-0988-z
Titlyanov E, Titlyanova T (2006) Production of plantlets of the red alga of *Gelidium* genus (Rhodophyta) from thallus fragments. Russ J Mar Biol 32:284–288
Titlyanov EA, Titlyanova TV, Kadel P, Lüning K (2006) Obtaining plantlets from apical meristem of the red alga *Gelidium* sp. J Appl Phycol 18:167–174
Trono GC Jr, Lluisma A (1992) Differences in biomass production and carrageenan yields among four strains of farmed carrageenophytes in Northern Bohol, Philippines. Hydrobiologia 247:223–227
Trono GC, Ohno M (1989) Seasonality in the biomass production of *Eucheuma* strains in northern Bohol, Philippines. In: Umezaki I (ed) Scientific survey of marine algae and their resources in the Philippine Islands. Monbusho International Scientific Research Program, Japan, pp 71–80
Ugarte RA, Sharp GJ (2001) A new approach to seaweed management in eastern Canada: the case of *Ascophyllum nodosum*. Cah Biol Mar 42:63–70

Ugarte RA, Sharp GJ (2012) Management and production of the brown algae *Ascophyllum nodosum* in the Canadian Maritimes. J Appl Phycol 24:409–416

Ugarte RG, Sharp G, Moore B (2006) Changes in the brown seaweed *Ascophyllum nodosum* (L.) Le Jol. plant morphology and biomass produced by cutter rake harvests in southern New Brunswick, Canada. J Appl Phycol 18:351–359

Valderrama D, Cai J, Hishamunda N, Ridler N (2013) Social and economic dimensions of carrageenan seaweed farming. FAO Fisheries and Aquaculture Tech Paper 580. Food and Agriculture Organization of the United Nations, Rome

van der Meer JP (1986) Genetics of *Gracilaria tikvahiae* (Rhodophyceae). XI. Further characterization of a bisexual mutant. J Phycol 22:151–158

van der Meer JP, Patwary MU (1983) Genetic modification of *Gracilaria tikvahiae* (Rhodophyceae). The production and evaluation of polyploids. Aquaculture 33:311–316

Wang J, Jiang P, Cui Y, Deng X, Li F, Liu J, Qin S (2010) Genetic transformation in *Kappaphycus alvarezii* using micro-particle bombardment: a potential strategy for germplasm improvement. Aquac Int 18:1027–1034

Yeong HY, Phang SM, Reddy CRK, Khalid N (2014) Production of clonal planting materials from *Gracilaria changii* and *Kappaphycus alvarezii* through tissue culture and airlift photobioreactors. J Appl Phycol 26:729–746

Yokoya NS (2000) Apical callus formation and plant regeneration controlled by plant growth regulators on axenic culture of the red alga *Gracilariopsis tenuifrons* (Gracilariales, Rhodophyta). Phycol Res 48:133–142

Yokoya NS, Handro W (1996) Effects of auxins and cytokinin on tissue culture of *Grateloupia dichotoma* (Gigartinales, Rhodophyta). Hydrobiologia 326(327):393–400

Yokoya NS, Handro W (2002) Effects on plant growth regulators and culture medium on morphogenesis of *Solieria filiformis* (Rhodophyta) cultured *in vitro*. J Appl Phycol 14:97–102

Yokoya NS, Yoneshigue-Valentin Y (2011) Micro-propagation as a tool for sustainable utilization and conservation of populations of Rhodophyta. Braz J Pharmacogn 21:334–339

Yokoya NS, Plastino EM, Artel R (2003) Physiological responses and pigment characterization of two colour strains of the carrageenophyte *Hypnea musciformis* (Rhodophyta). In: Chapman ARO, Anderson RJ, Vreeland VJ, Davison JR (eds) Proceedings of 17th international seaweed symposium. Oxford University Press, New York, pp 425–433

Yokoya NS, West JA, Luchi AE (2004) Effects of plants growth regulators on callus formation, growth and regeneration in axenic tissue cultures of *Gracilaria tenuistipitata* and *Gracilaria perplexa* (Gracilariales, Rhodophyta). Phycol Res 52:244–254

Yokoya NS, Stirk WA, Van Staden J, Novák O, Turecková V, Pénčík A, Strnad M (2010) Endogenous cyotikinins, auxins, and abscisic acid in red algae from Brazil. J Phycol 46:1198–1205

Yong WTL, Chin JYY, Thien VY, Yasir S (2014a) Evaluation of growth rate and semi-refined carrageenan properties of tissue-cultured *Kappaphycus alvarezii* (Rhodophyta, Gigartinales). Phycol Res 62:316–321

Yong WTL, Ting SH, Yong YS, Thien VY, Wong SH, Chin WL, Rodrigues KF, Anton A (2014b) Optimization of culture conditions for the direct regeneration of *Kappaphycus alvarezii* (Rhodophyta, Solieriaceae). J Appl Phycol 26:1597–1606

Yong YS, Yong WTL, Thien VY, Ng SE, Anton A, Yassir S (2015a) Acclimatization of micropropagated *Kappaphycus alvarezii* (Doty) Doty ex Silva (Rhodophyta, Solieriaceae) in outdoor nursery system. J Appl Phycol 27:413–419

Yong YS, Yong WTL, Ng SE, Anton A, Yassir S (2015b) Chemical composition of farmed and micropropagated *Kappaphycus alvarezii* (Rhodophyta, Gigartinales), a commercially important seaweed in Malaysia. J Appl Phycol 27:1271–1275

Yong WTL, Chin JYY, Thien VY, Yasir S (2017) Heavy metal accumulation in field cultured and tissue cultured *Kappaphycus alvarezii* and *Gracilaria changii.* Int Food Res J 24:970–975

Yunque DA, Tibubos KR, Hurtado AQ, Critchley AT (2011) Optimization of culture conditions for tissue culture production of young plantlets of carrageenophyte *Kappaphycus*. J Appl Phycol 23:433–438

Zemke-White WL, Smith JE (2006) Environmental impacts of seaweed farming in the tropics CD-ROM World Seaweed Resources – an authoritative reference system Version:1

Zhang S, Liu C, Jin Y, Chi S, Tang X, Chen F, Fang X, Liu T (2014) Studies on the isolation and culture of protoplasts from *Kappaphycus alvarezii*. Acta Oceanol Sin 33:114–123

Zitta CS, Rover T, Hayashi L, Bouzon ZL (2013) Callus ontogeny of the *Kappaphycus alvarezii* (Rhodophyta, Gigartinales) brown tetrasporophyte strain. J Appl Phycol 25:615–629

View publication stats