CHAPTER 3

Materials and Methods

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3.1 *Gracilaria changii* Abbott, Zhang and Xia

(Phang and Lewmanomont, 1991; Lewmamont and Ogawa, 1995)

Gracilaria changii Abbott, Zhang and Xia (Plate 1), was previously known as *Polycavernosa changii* and *Hydropuntia changii*. It is purplish brown to dark brown with one to many branches arising from a disk-like holdfast or from a percurent axis. The thalli range from 6 - 20 cm tall. Branching may be two to four orders; irregular, alternate or secund; branches turgid, cylindrical 0.3 – 2.5 mm in diameter, abruptly constricted at the base forming a slender stipe, slightly swollen distal of stipe, tapering towards the tip. Fronds in traverse section consist of large rounded medullary cells (350 – 570 μm) with thick walls (7.5 – 20 μm), and two to three layers of small, pigmented cortical cells; transitions of cells abrupt. Tetrasporangial and gametophytic plants similar in size and branching pattern. Tetrasporangia ovoid to elongated, densely scattered over frond surface; cruciately divided. Spermatangial conceptacles verrucosa-type, oval to nearly globosed, adjacent conceptacles may coalesce forming polycavernosa-type spermatangia. Cystocarps not constricted at base; gonimoblast consisting of small cells; basal absorbing filaments few, lateral and upper; carposporangia rounded or ovoid, 18 – 32.5 μm diameter; pericarp thick consisting of two kinds of tissue; the outer five to six rows of rounded oval cells and the inner 7 – 9 rows of compressed cells.

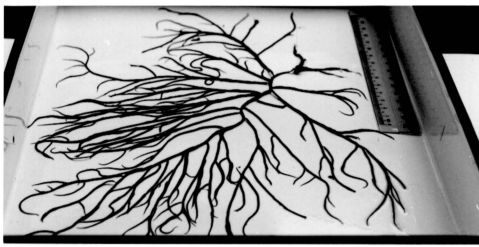

Plate 1. *Gracilaria changii* Abbott, Zhang and Xia.

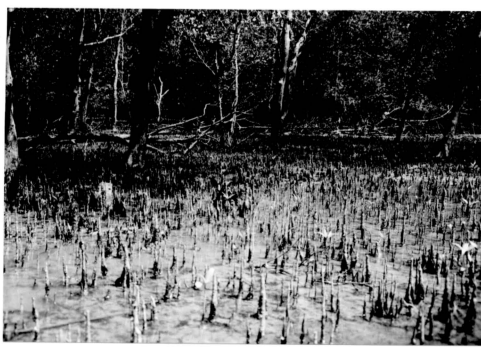

Plate 2. Collection site at the mangrove area of Pulau Carey.

3.2 Collection of *G. changii*

Vegetative plants of *Gracilaria changii* Abbott, Zhang and Xia were collected from the mangrove areas of Pulau Carey (Plate 2) and Pantai Morib, Selangor, in clean plastic bags and kept in an ice-chest to be transported back to the laboratory. The average transportation time is approximately two hours. Ideally, the whole bunch of the seaweed was collected to minimise fragmentation or breakage of the thalli.

3.2.1 Maintenance of *Gracilaria changii* in the Laboratory

Once the seaweeds arrived in the laboratory, they were cleansed of the mud and epiphytes by rinsing them in clean seawater. The individual bunches of the seaweeds were separated and cultured in tanks of clean and aerated seawater containing a marine nutrient supplement, "Norimax" (Prof. Masao Ohno, Japan), in order to maintain them for use in tissue culture and protoplast isolation. The salinity of the seawater was kept at 30 parts per thousand (ppt) as measured using the refractometer (Atago S/Mill-E). The pH of the seawater was maintained at 7.8 – 8.0 while the temperature was 25°C. The light intensity was 25 $\mu Em^{-2}s^{-1}$ with 12 hours light and dark cycles.

3.3 Sterilisation Techniques

Thalli of *Gracilaria changii* were sterilised before being used either for tissue culture or for protoplast isolation. Six different sterilisation techniques were tested for their suitability in generating healthy axenic explants. The thalli were removed of their epiphytes and rinsed with clean seawater before subjected to the sterilization treatments. The six sterilisation techniques are as follows.

3.3.1 Treatment 1

Cleaned thalli of about three to eight cm long were soaked in seawater containing 1.5% w/v KI for 10 minutes and later rinsed in 70% v/v ethanol for 10 seconds.

3.3.2 Treatment 2

Cleaned thalli of about three to eight cm long were soaked in seawater containing 1.5% w/v KI for 20 minutes and later rinsed in 70% v/v ethanol for two seconds.

3.3.3 Treatment 3

Cleaned thalli of about three to eight cm long were soaked in seawater containing 1.5% w/v KI for 30 minutes, rinsed in 70% v/v ethanol for two seconds and finally rinsed in 1% v/v hypochlorite solution (Chlorox™) for two seconds.

3.3.4 Treatment 4

Cleaned thalli of about three to eight cm long were rinsed in 70% v/v ethanol for two seconds and later rinsed in seawater containing 1% v/v hypochlorite solution (Chlorox™) for two seconds.

3.3.5 Treatment 5

Cleaned thalli of about three to eight cm long were rinsed in 1.5% w/v KI in seawater for 20 seconds and later soaked in steriled seawater containing 1mg/ml Penicillin G (Sigma) for 30 minutes.

3.3.6 Treatment 6

Cleaned thalli of about three to eight cm long were soaked in ultra high quality water (UHQ) for one hour and sonicated for three to four minutes. The thalli were then soaked in seawater containing 1% w/v povidone-iodine for 30 seconds before incubated in seawater for 24 hours containing 0.1 g/L kanamycin (Sigma), 0.3 g/L penicillin G (Sigma), 0.02 g/L polymyxin B sulphate (Sigma), 0.001 g/L nalidixic acid (Sigma), 0.02 g/L cefotaxine (Sigma) and 1 g/L streptomycin sulphate (Sigma).

3.3.7 Sterility Test

After treating the thalli with the treatments mentioned above, the sterility (axenicity) of the treated thalli was determined by streaking them on Marine Agar 2216E (Difco) in petri dishes, to isolate the contaminating bacteria. Marine Agar 2216E is a nutrient-rich medium that includes the major mineral composition of seawater essential for the cultivation of heterotrophic marine bacteria. Petri dishes containing the Marine Agar 2216E were kept in the dark incubator at 25°C. Observations for bacterial growth were done after three days. A treatment method was deemed suitable if it was able to produce sterile and healthy explants.

3.4 Methods of Tissue Culture

Once the thalli were sterilised, healthy explants measuring about two to three millimeter in diameter were cut into sections of four to five mm in length before being inoculated onto the solid media, semi-solid media and into the liquid medium. Five cut sections were cultured in a 90 cm diameter petri dish (Plate 3) for solid media and in 250 ml volume culture vessels (Plate 4) in the case of semi-solid and liquid media.

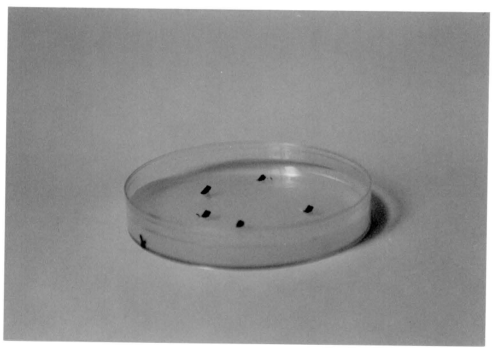

Plate 3. Culture of explants on solid media in petri dish.

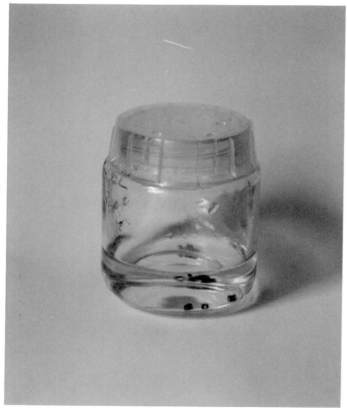

Plate 4. "Baby food jar" culture vessel used for culture of explants in semi-solid and liquid media.

3.4.1 Source and Preparation of Sterile Seawater

Filter-steriled natural seawater with salinity level of 30 ppt was used as the base for all media preparation. The seawater was collected from a prawn hatchery in Jeram, the coast of Kuala Selangor, and transported back into the laboratory to be kept in the aerated seawater reservoir. The seawater was passed through a coarse filtration with GFC glass fibre filter paper (Whatman®) before being used for media preparation. The coarse filtrate was then filtered using microfiltration membrane with pore size of 0.22μm. The sterilised seawater was then used for media preparation.

3.4.2 Tissue Culture on Solid MS and PES Media

Solid media were prepared using Murashige and Skoog (MS) and Provasoli's Enriched Seawater Medium (PES) in 100% seawater with salinity of 30ppt as base. The effect of these media on callus regeneration was tested in two stages. In the first stage, the effects of the media in their basic forms and the effect of the basic media with added vitamins were investigated. The test for the effect of vitamins was only investigated for MS medium as the commercially obtained PES medium already had vitamins contained in it. In the second stage, the effects of the basic media with vitamins and growth substances were investigated. Thus, three different forms of MS media were prepared for use – the basic MS media, MS + vitamins medium and MS + vitamins medium with added growth substances. Two different forms of PES media were prepared for use – the PES + vitamins medium and PES + vitamins medium with added growth substances. Observations for callus formation were done every seven days from the day of inoculation.

Solid media were prepared using 0.8% w/v of highly purified agar (Sigma). The media were autoclaved and poured into steriled 90cm petri dishes and kept for one day before being used. Axenic explants were inoculated onto the solid media aseptically in a Class 2, laminar flow cabinet. The explants were cut into sections of 5 mm each and placed onto the solid surface of the solid-media. The explants were stuck into the media at an angle of approximately 30° to the media surface so that one part of the explant was in the medium and a small part exposed to the air. Five sections of explants were placed onto a petri dish and a triplicate was done for a single

test. The petri dishes containing the explants were incubated in a chamber with the temperature set at 25°C and light intensity of 25 $\mu Em^{-2}s^{-1}$ with 12 hours light and dark cycles. Observation on the explants was made at every seven-day interval up to the 56^{th} day. The percentage of explants forming callus or giving rise to new branching and the state of the explants were recorded. The cultures were replaced with similar fresh medium every week throughout the culture period.

3.4.2.1 Murashige and Skoog Basal Salt Medium (MS)

Murashige and Skoog basal salt medium (Sigma) was prepared according to the manufacturer's prescription. A 4.4 g of the stock medium in powder form was added to one liter of seawater to achieve the macro and micro nutrients concentrations as follows.

Component	**$mg.L^{-1}$**
Ammonium nitrate	1650.0
Boric acid	6.2
Calcium chloride anhydrous	332.2
Cobalt chloride hexahydrate	0.025
Cupric sulfate pentahydrate	0.025
Disodium EDTA dihydrate	37.26
Ferrous sulfate heptahydrate	27.80
Magnesium sulphate anhydrous	180.7
Manganese sulphate monohydrate	16.9
Potassium iodide	0.83
Potassium nitrate	1900.0
Potassium phosphate monobasic	170.0
Sodium molybdate dihydrate	0.25
Zinc sulphate heptahydrate	8.6

3.4.2.2 MS basal Medium with Added Vitamins

This second form of MS basal salt medium contained MS vitamins mixtures (Sigma). One ml from the vitamin stock solution was added to one liter of MS medium to achieve the final vitamins' concentrations as follows.

Component	$mg.L^{-1}$
Glycine (Free base)	2.0
Myo-inositol	100.0
Nicotinic acid (Free acid)	0.5
Pyridoxine HCl	0.5
Thiamine HCl	0.1

3.4.2.3 Provasoli's Enriched Seawater Medium (PES)

PES medium (Sigma) was prepared according to the manufacturer's prescription and already had vitamins such Thiamine HCl and Vitamin B_{12} incorporated into it. A 20ml aliquot of the concentrated stock medium in liquid form was added to one liter of seawater to achieve the macro and micronutrients concentrations as follows.

Component	$mg.L^{-1}$
Biotin	0.001
Boric acid	5.720
Cobalt chloride hexahydrate	0.02018
EDTA disodium dihydrate	8.332
Ferric chloride hexahydrate	2.662
Manganese chloride tetrahydrate	0.7205
Sodium glycerophosphate pentahydrate	10.0
Sodium nitrate	70.0
Thiamine HCl	0.1
Vitamin B_{12}	0.002
Zinc chloride	0.05212

3.4.3 Effect of Solid MS and PES media with Growth Substances

Auxins and cytokinin were added to the vitamins-containing MS and PES media described above. Three different auxins and one cytokinin were tested individually and in combination over different concentrations for their effect on callus formation. The auxins used were 2,4-dichlorophenoxy acetic acid (2,4-D), Indole-3-acetic acid (IAA) and α-nafthalene acetic acid (NAA) (Sigma). The cytokinin used was kinetin (Sigma).

All the four different growth substances were tested individually in concentrations of 0.001, 0.01, 0.1, 1.0 and 10 $mg.L^{-1}$ in MS medium with added vitamins and PES medium as mentioned in Section 3.4.2.

A combination of auxins and cytokinin of different concentrations were carried out as follows.

Auxin ($mg.L^{-1}$)	Cytokinin ($mg.L^{-1}$)
10	0.001
1	0.01
0.1	0.1
0.01	1.0
0.001	10

3.4.4 Tissue Culture in Semi-solid MS and PES Media

Semi-solid form of the different types of media as described in Section 3.4.2 were prepared by the addition of 0.2% w/v of the highly purified agar. The semi-solid media were autoclaved and then dispensed into 'Baby Food Jar' culture vessels (Plate 4) and kept for one day before axenic cut-sections of explants were inoculated. As in Section 3.4.2, five cut sections were inoculated into each vessel and a triplicate was done for each test. The culture conditions and basis of observation were the same as described in Section 3.4.2. Observation on the explants was made at every seven-day interval up to the 56th day. The percentage of explants forming callus or giving rise to new branching and the state of the explants were recorded. The cultures were replaced with similar fresh medium every week throughout the culture period.

3.4.4.1 Semi-solid Media with Growth Substances

The method for this section is as mentioned in Section 3.4.3, except the media used here were semi-solid media. Observation on the explants was made at every seven-day interval up to the 56th day. The percentage of explants forming callus or giving rise to new branching and the state of the explants were recorded.

3.4.5 Liquid MS and PES Media

The three types of media as described in Section 3.4.2 in liquid form were prepared without the addition of agar, the solidifying agent. The liquid media were autoclaved and then poured into 250ml 'Baby Food Jar' culture vessels (Plate 4) and kept for one day before axenic cut-sections of explants were inoculated. As in Section 3.4.2, five cut sections were inoculated into each vessel and a triplicate was done for each test. The culture conditions and the basis of observation were the same as described in Section 3.4.2. Observation on the explants was made at every seven-day interval up to the 56^{th} day. The percentage of explants forming callus or giving rise to new branching and the state of the explants were recorded. The cultures were replaced with similar fresh medium every week throughout the culture period.

3.4.5.1 Liquid Media with Growth Substances

The method for this section is as mentioned in section 3.4.3, except the media used here were liquid media (no solidifying agent was added), prepared as mentioned in Section 3.4.3.

3.5 Methods of Protoplast Culture

Healthy thalli of *G. changii* collected from Pulau Carey and Pantai Morib were sterilised as described in Section 3.3 using Treatment 6. They were later used for protoplast isolation using a modification of the technique as described by Cheney *et al.*, 1986. Mannitol was used as the osmoticum for enzyme solution. Suitable concentration of mannitol was assessed prior to the formulation of enzyme solution.

3.5.1 Determination of Mannitol (osmoticum) Concentration

Correct concentration of osmoticum such as mannitol is essential in achieving sufficient plasmolysis of the cells during enzymatic degradation of cell wall in generating viable protoplasts. The concentration of an osmoticum should be optimal in that it results in the cell membrane sufficiently pulled away from the cell wall but not over-plasmolysed which can result in cell death (Burbidge *et al.*, 1994).

The correct concentration of mannitol was assessed by soaking three thin cross-sections of the thallus in seawater (30 ppt salinity) with mannitol concentrations of 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1 and 1.2M for 30 minutes. The state of cellular plasmolysis in both the cortical and epidermal cells was assessed at the end of 30 minutes under light-microscope after staining with Neutral Red (1%). The percentage of plasmolysed cells were calculated based on the number of plasmolysed cell per sample of 10 cortical or epidermal cells in each cross-section soaked in seawater with

the respective mannitol concentration. Five samples each of cortical and epidermal cells were taken for each cross-section.

3.5.2 Protoplast Isolation

Thalli of healthy vegetative sections of *G. changii* were sterilised using Treatment 6 as described in Section 3.3, after being carefully cleaned of epiphytes. The entire process for protoplast isolation was carried out aseptically inside the Class 2 laminar flow cabinet. Thalli weighing about 0.5 g were chopped into small pieces with razor blades. The finely chopped thalli were rinsed three times with the rinsing medium consisting of steriled seawater (30 ppt) containing 0.3 M mannitol, 5 mM $CaCl_2$ at pH 5.8.

3.5.3 Determination of the Effect of Individual Enzyme Concentration and Incubation Time on the amount of Protoplast Isolated.

After the finely chopped thalli were rinsed with the rinsing medium as mentioned in Section 3.5.2, they were incubated in 5 ml protoplast isolating solution consisting of a single test enzyme, 1 M mannitol, 5 mM $CaCl_2$ and seawater with salinity of 30 ppt as base. The pH of the isolating solution was adjusted to 5.8. The enzymes tested individually in the isolating solution were agarase, abalone acetone powder, cellulase, pectinase and pectolyase (Sigma) in concentrations of 1, 2 and 3% w/v. The chopped thalli were incubated for 30 minutes, 1, 1.5 and 2 hours in

each isolating solution containing different enzyme concentrations and swirled gently in the dark in a water bath at 26°C. The optimal enzyme concentration and incubation time were determined based on the amount of protoplasts isolated.

The enzyme isolating solution containing 0.5 g of chopped thalli were filtered through a 70 μm nylon mesh to remove the coarse residue and the filtrate containing the isolated protoplasts was diluted one fold with the culture medium and centrifuged at 100 g for five minutes. The culture medium contained seawater (30 ppt), 0.6 M mannitol, 5 mM and $CaCl_2$ at pH 8.0. After centrifuging, the supernatant was discarded and the white protoplast pellets accumulated at the bottom of the centrifuge tube were resuspended with fresh culture medium and this protoplast-washing process was repeated twice.

The final pellet was resuspended in 1.0 ml of culture medium from which samples were removed for haemocytometer count to determine the yield of viable protoplasts. The depth of the haemocytometer was 0.01 mm. Protoplast viability was assessed by staining with Evans Blue, which only stained the membrane of dead protoplasts. Membrane of viable protoplasts was able to exclude the stain and thus appear colourless (Cheney *et al.*, 1986).

3.5.4 Effect of a Combination of Enzymes on the Amount of Protoplast Isolated at Various Times of Incubation.

The effect of a combination of the five enzymes – abalone acetone powder, cellulase, pectinase, pectolyase and agarase were carried out as mentioned above. Since, the objective was to develop the cheapest method for isolating large amount of protoplasts, a protoplast isolating solution containing 1% w/v of each enzyme was used in combination for protoplasts isolation. Viable protoplast yield was assessed as mentioned in Section 3.5.3.