CHAPTER 5

Discussion

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The objectives of this study are to develop a complete system for successful tissue culture and protoplast isolation of the *Gracilaria changii* Abbot, Zhang and Xia (Rhodophyta). The success of this micropropagation system requires the establishment of an optimal plant sterilisation process. Once this is obtained, work on tissue and protoplast isolation can proceed. As with terrestrial plants, the success of callus and plants regeneration in *G. changii* required the establishment of correct media composition. The major prerequisite for cell culture was the isolation of viable protoplast using the right enzyme or combination of enzymes.

5.1 Sterilisation of *Gracilaria changii* Explants

Experiments with *G. changii* showed that the most effective method for producing axenic explants involved the use of non-bleaching agents such as cleansing with UHQ, 1% w/v povidone iodine and incubation in a combination of antibiotics coupled with mild physical treatment such as brief sonication (Table 9, Treatment 6). Using this mode of treatment, the thalli remained healthy and maintained its brownish colour and were free of microbial contamination when tested on Marine Agar 2216E (Difco). The use of bleaching agents such as hypochlorite and alcohol were unsuitable although they produced axenic explants as the thalli were severely bleached.

An almost similar sterilisation technique was employed by Kaczyna and Megnet (1993) on *Gracilaria verrucosa*. They found that treatment of the seaweed thalli in 1% PVP-iodine in 30ppt seawater for 3 minutes alone gave the best results. PVP-iodine is mild to the thalli and enables them to retain their colour. It also acts as fungicide, bactericide, virucide and protozide (Kaczyna and Megnet, 1993). On the other hand, Cheney *et al.*, (1986) managed to obtain axenic material from a few species of *Gracilaria* by subjecting the explants to antibiotic treatment alone (AM9 antibiotic mixture containing 85 mg/ml polymyxin E).

As with *G. changii*, hypochlorite and alcohol were shown to be damaging to *G. verrucosa* and resulted in bleaching and death of plants although axenicity was achieved (Kaczyna and Megnet, 1993). Treatment 5, employing non-bleaching agents such as potassium iodide and penicillin G, produced healthy thalli but at the expense of contamination. This proved that the use of KI and single antibiotic (pennicillin G) was ineffective in producing healthy and axenic explants. In contrast, when overnight incubation of thalli in seawater containing six different types of antibiotics (0.1 g/L kanamycin, 0.3 g/L Penicillin G, 0.02 g/L polymyxin B sulphate, 0.001 g/L nalidixic acid, 0.02 g/L cefotaxine and 1 g/L streptomycin sulphate) were employed in Treatment 6, bacterial growth was successfully retarded when tested in Marine Agar 2216 (Table 9).

5.2: Tissue Culture of *Gracilaria changii* Abbot, Zhang and Xia

5.2.1. Effects of the forms of Culture Medium

Three different forms of culture media were investigated. They were solid, semi-solid and liquid media. The results showed conclusively that the types and form of culture medium affected the regeneration of branches and callus. Both solid and semi-solid media mentioned in Sections 4.2.1 and 4.2.2 (MS, MS + vitamins, MS + vitamins + growth substances, PES + vitamins and PES + vitamins + growth substances) were unsuccessful in generating branches or callus. The explants cultured in these media survived up to a maximum of 42 days but failed to undergo further organogenesis. As for liquid media, only PES + vitamins + growth substances medium induced branching and callus-like-structures (CLS) regeneration when supplemented with single and combinations of growth substances respectively (Tables 10 and 11).

The effect of culture medium on callus regeneration has also been demonstrated in other species of red algae. Morphology and regeneration of different species of *Laminaria* and *Porphyra* cultures were found to be controlled by the form of media. Polne-Fuller (1986) showed that agar solidified medium induced the development of calluses in these species while liquid media allowed the development of different fronds. Studies with *Gelidium vesicolor*, *Grateloupia deryphora* and *Laurencia* sp. cultivated in PES medium showed, in contrast to the results obtained here, that an increase in agar concentration of the culture medium was positively

correlated with callus formation but agreed that liquid medium was needed for bud regeneration (Robiana *et al.*, 1990).

Other authors have reported the formation of callus and callus-like structures induced by semi-solid (agarised) state of the medium but not in liquid medium (Fries, 1980; Saga *et al.*, 1982; Saga and Sakai, 1983; Polne-Fuller and Gibor, 1984 and Garcia-Reina *et al.*, 1988). It was also reported that there is a relationship between the form of media and growth substances in controlling plant organogenesis in seaweeds. The process of callus-like structures (CLS) regeneration observed on *Grateloupia dichotoma* was shown to be stimulated significantly by treatment with a high concentration of benzyladenine (BA), a cytokinin, or IAA in semi-solid and liquid media. In contrast, the growth of upright axes was stimulated by treatment with 2,4-D in semi-solid medium and IAA + BA in liquid medium (Yokoya *et al.*, 1996).

Research conducted by Jin *et al.*, (1997) showed that the types of gelling agent used affected callus and blade formation in the seaweed, *Hizikia fisuformis*. The author reported that agar is not physiologically inert as it contains a variety of growth inhibiting and growth stimulating contaminants. As highly purified agar (Sigma) was used in this experiment, it is beyond doubt that the failure of solid and semi-solid to produce branches and callus was not due to contamination of the agar, the solidifying agent.

5.2.2 Branching or Shoot Formation

Many seaweeds were found to abscise fragments of branches as a regular part of the life cycle (Buggeln, 1981; Norton *et al.*, 1981 and Norton *et al.*, 1983). Incidence of branching or shoot formation from cut cross-sections of thalli cultured in liquid PES medium supplemented with vitamins and a single growth substance was recorded (Table 10). The low concentrations of auxins, NAA and IAA, in concentrations of 0.001 and 0.01 $mg.L^{-1}$ respectively generated the highest incidence of branching (20%) compared to the cytokinin, kinetin (13.33% for 0.001 $mg.L^{-1}$ and 6.67% for 0.01 $mg.L^{-1}$). The auxin, 2,4-D, did not generate branching within the range of concentrations tested. The effect of growth hormones on regenerative fragmentation or branching in red algae has rarely been reported. Thus it remains unclear if growth hormones have significant roles in branching. Fragments of red algae are known to be highly regenerative. Field farming of *Gracilaria* in Chile is mainly based on the regeneration capacity of thallus fragments as a result of intercalary growth (Santelices and Verela, 1995).

Studies with another species of red algae, *Gelidium*, showed that finely chopped branches were able to regenerate into young plants and later attach to plastic ropes by rhiziods which were induced as a result of contact with solid surfaces. All this took place without the addition of any growth hormone (Polne-Fuller, 1988). Thus, it can be concluded from this study that either cytokinin or auxins, except 2,4-D, tested alone in PES medium was necessary for branching but when applied in high concentrations, may be inhibitory (Table 10). This conclusion is drawn as no

incidence of branching was recorded when the concentrations of the auxins and kinetin tested were above 0.1 $mg.L^{-1}$ (Table 10) and when PES medium without growth substances (the experimental control) was used (Section 4.2.3). It is also suggested that branching in *G. changii*, a form of organogenesis, bypassed the callus stage as shoot formation arose directly from the cut-section of the explants. Further experiments should be aimed at separating the new shoots from the explants and culture them separately to observe their development into matured plants.

5.2.3 Regeneration of Callus-like Structures (CLS)

Work with terrestrial plants gave evidence that growth hormones usually act in combination (Hughes, 1981) and interact with other compound that have protective, stimulative or inhibitive properties (Kafeli and Dashek, 1984). While 2,4-D failed to give rise to shoot formation when used alone (Section 5.2.2), friable, ball-like callus-like structures (CLS) together with young shoots were developed from cross-sections of thallus cultured in liquid PES medium supplemented with combinations of 2,4-D (an auxin) and kinetin (cytokinin) at different concentrations within 35 to 42 days of culture (Table 11, Plate 10). Microscopic examination showed that the phenomenon for shoot formation was independent from CLS regeneration. The former consisted of lateral branches regenerated from cut surfaces of the explants while the latter arose from the cortical and epidermal cells (Plate 10). This long period for CLS regeneration is expected of red algae as studies with agarophytes showed that the time for callus formation or development required from a few months to a year (Chen and Taylor, 1978).

The induction of CLS and shoots formation in *G. changii* was shown to relate positively to higher concentrations of 2,4-D and lower concentrations of kinetin (Table 11). The combination of 10 $mg.L^{-1}$ 2,4-D and 0.001 $mg.L^{-1}$ kinetin resulted in formation of CLS and shoots development in all the explants. The phenomenon observed in this study is in agreement with studies of regeneration from caprosporelings of another red algae, *Grateloupia doryphora*, which showed that

higher 2,4-D concentration (0.001 M) induced callus while the same concentration of kinetin was inhibitory (Garcia-Jimenez, *et al.*, 1998).

Microscopic examination of the CLS cross-sections showed that the callus arose from both the epidermal and cortical cells and ranged between 10-50 μm in diameter and pigmented red. Gusev *et al.*, (1987) showed that the callus of agarophytes are long-living and can be maintained for up to 30 months through subculturing into new medium. The combination of 2,4-D and a cytokinin is known to induce CLS in some red seaweeds. Yokoya *et al.*, (1996) reported that the development of CLS from apical segments of *Grateloupia dichotoma* was significantly greater in treatments with the auxin, 2,4-D (1 $mg.L^{-1}$), and the cytokinin, BA (5 $mg.L^{-1}$), compared to other auxins such as IAA.

Although it has been reported in many papers that a combination of auxin and cytokinin is required for callus formation the knowledge about the function of growth substances in algae is poorly understood. Cytokinins in seaweeds have been detected by bioassay and through the use of high-performance liquid chromatography. Endogeneous applications of cytokinins to algal explants indicated that these hormones play a role in algal growth and development, including the processess of cell division and elongation and organogenesis (Mooney and Staden, 1984). Except for Combination No.4 (Table 10), the percentage of CLS formation in explants is positively related to the increase of concentration of 2,4-D and inversely related to the increase in kinetin concentration. The exception of Combination No.4 may be due to the levels of endogeneous cytokinin activity already existed in the explants. This phenomenon is common among seaweeds. Studies with *Sargassum heterophyllum*

(Mooney, 1983) and *Ecklonia maxima* (Featonby-Smith, 1984) indicated that endogeneous cytokinin activity was correlated with seasonal vegetative growth, stages of development and the time of the lunar cycle at which harvesting of the seaweeds is carried out. Thus although the percentage of CLS formation in combination No.4 should be expected to be in the range of 93 to 100%, it is suggested that the deviation might be due to the endogeneous level of natural cytokinin already present. In fact it has been shown that kinetin alone could stimulate callus formation in *Gracilaria verrucosa* (Gusev *et al.*, 1987).

Bradley and Cheney (1990) examined the effects of auxin and cytokinin, either singly or in combination, on the stimulation of cell division in the tissue culture of the red seaweed, *Agardhiella subulata*. They showed among other combinations, IAA and Kinetin combination resulted in filamentous and CLS growth that resulted from cross-sectional discs cut from young branches. They also showed that, in agreement with the results in Table 10, high concentrations of cytokinins (10 $mg.L^{-1}$) had inhibitory effects on the regeneration of plants in some of the cultures. Auxins and cytokins also resulted in cell division and callus formation in cell cultures of *Enteromorpha*, *Phyllophora nervosa*, *Eucheuma denticulatum* and *Kappaphycus alvarezii* (Fries and Aberg, 1978; Gusev *et al.*, 1987 and Cheney *et al.*, 1991).

Reports of similar CLS formation in other species of seaweeds include *Gracilaria textorii*, *G. filicina* (Huang and Fujita, 1997) *Porphyra lanceolata* and *P. perforata* (Polne-Fuller and Gibor, 1987). Both *G. textorii* and *G. filicina* developed callus with filamentous, oval and spherical cell chains when cultured in ASP12 NTA solid medium supplemented with IAA and 6-benzylaminopurine (BAP). The callus

was induced from the outer and cortical tissue of the explants after three weeks in culture. As for *Eucheuma* sp., calluses developed from filaments, which grew from cortical cells when cultured in liquid PES and ASP12 NTA medium. The callus was made up of thick filamentous ball-like growth, resembling conchosporangial branches much like the CLS of *G. changii* obtained in this experiment (Plate 10).

Studies with *Gracilaria verrucosa* showed that in addition to growth substances, glycerol (0.217 M) and diphenylurea (5 $mg.L^{-1}$) in ASP5F2 medium were required for callus induction on the whole surface of the explants (Kaczyna and Megnet, 1993).

The culture conditions dictating callus formation in seaweeds are diverse as evidenced from the number of literatures available. Much intensive study is still needed to optimise cultural conditions, which support efficient organogenesis and embryogenesis in tissue culture of important seaweeds such as *G. changii*.

5.3 Optimal Mannitol Concentration

Osmolarity of the enzyme solution for protoplast isolation affects protoplast viability. Ideally, the osmolarity of the osmoticum should not be any greater than is necessary to induce plasmolysis; too high an osmotic pressure can impair metabolism and cell wall synthesis (Shepard and Totten, 1977). Table 12, Plate 12 and Figure 1 show that 0.8 M mannitol in seawater (30 ppt salinity) was required for plasmolysis to occur. This mannitol concentration agreed with that reported by Cheney *et al.*, (1986) on *Gracilaria tikvahiae* (0.7 M) and Araki and Morishita (1998) on *G. verrucosa* (0.7 M). The percentage of plasmolysed cortical cells increased with mannitol concentration and a maximum was achieved at 1.2 M (71%). However, the number of plasmolysed epidermal (or outer cortical) cells declined in 1.2 M of mannitol. This decline might be due to the use of cross-section from thallus that are more resistant to external osmotic pressure.

Generally, the percentage of plasmolysed cortical cells was higher than the percentage of plasmolysed epidermal cells for each concentration of mannitol tested. From this experiment, mannitol in concentrations of 1 and 1.1 M was shown to induce sufficiently high percentage of plasmolysed cortical (48%) and epidermal (16%) cells without any indication of over-plasmolysed (Plate 12). Mannitol concentration of 1 M was considered as the optimal for ease and accuracy of calculation during the formulation of enzyme isolating solution. Although 1.2 M produced higher number of plasmolysed cells, a large percentage of the cortical cells were over plasmolysed and the number of plasmolysed epidermal cells were lower (14%).

5.4 Protoplast Isolation Using Single and Combination of Enzymes.

Protoplasts are most useful materials for manipulation of cells. The removal of cell walls while simultaneously conserving the cytoplasmic and nuclear constituents of the cells necessary for subsequent cell wall deposition and division, leaves the plasma membrane fully exposed as the only barrier between the external environment and the interior of the totipotent cell (Burbidge *et al.*, 1993 and Mantell *et al.*, 1985).

This experiment showed conclusively that incubation of cut cross-sections of *G. changii* in enzyme solution containing 1% w/v abalone acetone powder for two hours produced the highest number of viable protoplasts (5.0×10^4 protoplast g^{-1} fresh weight) compared to all the other enzymes tested (Figure 2 and Table 13). Polne-Fuller (1986) reported that abalone-gut acetone powder was effective in isolating protoplasts from red and brown seaweeds. The author reported that the rates of tissue dissociation and success of regeneration of the isolated protoplasts were dependent on the types of tissues from which the cells were isolated.

This experiment had shown that other enzymes such as cellulase, pectinase, pectolyase and agarase were also effective in producing viable protoplasts although to a lesser extent (Table 12). Cellulase has been used by Fujita and Migita (1985) and Saga (1984) to produce protoplasts from several green seaweeds but was less successful with the red and brown seaweeds such as *Laminaria japonica*, *Undaria pinnatifida and Porphyra yezeonsis*. Cheney *et al.* (1986) suggested from their study

with *Gracilaria tikvahiae* that agarase and cellulase were essential for cell wall removal and that pectinase and pectolyase contributed by improving yields.

It is common that a combination of different enzymes is employed for protoplast isolation. Figure 7 shows that a combination of 1% w/v each of the five enzymes listed in Table 12 failed to produce the maximum achieved by using abalone acetone powder alone. In contrast, using similar combination of enzymes (3% w/v cellulase, 3% w/v pectinase, 1% w/v agarase and 0.5% w/v pectolyase), Cheney *et al.* (1986) in an experiment with *Gracilaria tikvahiae* showed greatest degree of cell wall digestion and highest of protoplasts yield ($3 - 10 \times 10^4$ protoplasts g^{-1} fresh weight). They further showed that lower concentrations of these enzymes produced substantially reduced yields without improving protoplast viability. Yan and Wang (1993) showed that protoplasts were effectively isolated from vegetative tissues of *G. asiatica* using a combination of 4% w/v cellulase and 1% w/v sea-snail enzymes and crude agarase. Yield of $2 - 4 \times 10^5$ protoplasts g^{-1} fresh weight was obtained after three hours incubation in the enzyme combination mentioned. This protoplast concentration is comparable to that obtained in this study with *G. changii* using abalone acetone powder alone (Table 12). Araki and Morishita (1998) reported high protoplast yield (1.03×10^8 protoplasts g^{-1} fresh weight) from *G. verrucosa* using an enzyme mixture consisting of four units of agarase extracted from the marine bacterium, *Vibrio* sp. PO-303, 4% cellulase and 2% pectinase obtained commercially. To date, this was the highest protoplast yield ever reported for red algae.

It is difficult to compare the effectiveness of the same enzymes used for isolating protoplast from different species of seaweeds. This is because protoplast viability was also found to be highly variable and is dependent upon the strain (genotype) and age of the parent plant material (Cheney *et al.*, 1986). The reduction in the number of viable protoplasts using a combination of enzymes compared to using only abalone acetone powder suggests that the former might had affected cell viability by digesting the cell membrane as well (Burbidge *et al.*, 1993).

It is of paramount importance that a high enough protoplast density is obtained for further investigation in any cell culture system. The maximum protoplast density (5.0×10^4 protoplasts g^{-1} fresh weight) obtained in this study using abalone acetone powder alone is the minimal density useful for further investigations such as protoplast culture (Mantell *et al.*, 1985). Perhaps a method proposed by Packer, (1994) and used successfully for generating high protoplast yield from *Porphyra* should be tested for *G. changii*. This method involves the mechanical breakdown of the tissue to osmotically stable individual cells and groups of cells in pairs, followed by a 20 minutes treatment with a mixture of crude enzyme extracts from *Haliotis iris* (abalone), *Pseudomonas elongata* (a marine bacterium) and *Aspergillus nidulans* strain 051 biA1, (a terrestrial fungi). The advantage of this method over the method employed in this study with *G. changii*, is that the algal cells were exposed to the enzyme for a shorter time (20 minutes compared to 2 hours), which guarantees a higher proportion of viable protoplasts. The amount of debris in tissue digest was also significantly reduced because much of the intercellular cell wall polysaccharides were removed in the mechanical breakdown of the tissues and the grinding of the tissues that have been treated in enzymes was no longer required.

Future investigation should include the following.

1. Improve yield of protoplast, by optimising the protoplast isolating conditions not investigated in this study such as temperature and pH of the protoplast isolating solution and the combination of mechanical and enzymic methods for protoplast isolation. High protoplast yield is important for cell culture and protoplast fusion.

2. Investigate the effect of growth substances on protoplast division not done in this preliminary study.

3. Isolate protoplast from other sources such as the spores that promised higher number of viable totipotent cells (Cheney *et al.*, 1986).