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Regeneration Studies in *Grateloupia filicina* (J.V. Lamouroux) C. Agardh – An Important Carrageenophyte and Edible Seaweed

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Grateloupia filicina (J.V. Lamouroux) C. Agardh (Halymeniaceae, Cryptonemiales, Rhodophyta) is an edible seaweed as well as an important source of carrageenan. In the present study, attempt has been made to develop a suitable protocol for effective regeneration of the seaweed and the rapid multiplication of the desired varieties. The young upright thallus of *G. filicina* was grown in axenic culture using both solid and liquid media. The various media tested were f/2, Provasoli's Enriched Seawater (PES) and Enriched Seawater (ESW). The effect of glycerol (as a carbon source) and various plant growth regulators i.e. auxin (NAA) and cytokinins (Kinetin and BA) were tested. Although, regeneration of young thalli was observed from the cut ends in all the media, better growth was found in f/2, PES, f/2 (0.5% Glycerol), f/2 (NAA 10^{-5}_{M}) and f/2 (BA 10^{-6}_{M}). On the other hand callusing was observed only in solid media supplemented with low concentration of Glycerol (0.5%) in f/2, NAA 10^{-5}_{M} in f/2, PES and BA 10^{-5}_{M} in f/2. Young thalli were developed from the callus sub culture after 40 days of inoculation.

Key Words: callusing, carrageenophyte, *Grateloupia filicina*, regeneration

INTRODUCTION

There has been growing interest in the application of tissue and protoplast culture techniques in seaweeds for genetic manipulation and micro propagation both in order to improve the aqua cultural crops and to increase the knowledge about the process of differentiation, morphogenesis and regeneration (Yokoyo and Handro 1996). The marine algal biotechnology and the basic knowledge and technique for tissue and cell culture in this field are still in the state of development. For the last several years attempts have been made with seaweed tissue culture, following the success achieved in higher plants.

In seaweeds the first demonstration of totipotency of a piece of tissue was obtained from the work of Chen and Taylor (1978) on commercially important red alga *Chondrus crispus*. Chen (1982) induced callus like structure from *Chondrus crispus* on solid media but the regeneration was obtained in liquid media. Calluses and callus like structures have been reported from *in vitro* cultures of brown algae such as *Laminaria* spp; *Sargassum* spp; *Undaria pinnatifida*; *Ecklonia* spp and red algae such as *Agardhiella subulata*; *Eucheuma denticulatum* and *Gelidium vagum* (Polne-Foller and Gibor 1987).

Among red macroalgae, however, less is known about the effect of carbon sources and plant growth regulators on cell cultures. The ability to assimilate carbon sources has been well documented (Robaina *et al*. 1995). Glycerol acts as the exogenous carbon source to provide both carbon skeleton and energy through respiratory catabolism. Glycerol promoted morphogenesis (rapid axial regeneration) at lower concentration in *Grateloupia doryphora* being an effective organic carbon source for this alga but at higher concentration it is not relevant for normal ecological or metabolic processes (Robaina *et al*. 1990a,b). Similarly the studies on effect of plant growth regulators on the red macroalgae are very scanty.

Plant growth regulators have been studied in relation to their occurrence with in different groups (Hamana and Matasuzaki 1982; Yokoyo and Handro 1996) and their involvement in cell division (Cohen *et al*. 1984). Dawes and Koch (1991) and Dawes *et al*. (1993) investigated the effect of plant growth regulators on callus development and regeneration process. Similarly, Hurtado and Cheney (2003) through tissue culture studies on *Eucheuma denticulatum* (Burman) Collins et Harvey generated the new strains. Therefore, in the present study an attempt has been made to develop a suitable protocol for effective callusing and regeneration in *G. fil-*

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Table 1. Effect of phytoregulators on explants (f/2 liquid/solid medium)

Medium	Liquid			Solid	
	No. of Explants	% of Regeneration	Colour of regenerated filaments	% of Callusing	Nature & Colour of Callus
f/2 Control	15	50%	Pink	30%	Friable, pink
f/2 Glycerol (0.5%)	15	40%	Pink	70%	Crystaline and pink callus which subsequently gave rise to young filaments
f/2 Glycerol (1%)	15	5%	Green	10%	Crystalline, white
f/2 Glycerol (1.5%)	15	5%	Green	No callusing	-
f/2 NAA (10^{-5}_{M})	15	60%	Pinkish	40%	Friable, pinkish
f/2 NAA (10^{-6}_{M})	15	No regeneration	-	25%	Crystalline
f/2 NAA (10^{-7}_{M})	15	No regeneration	-	No callusing	-
f/2 BA (10^{-5}_{M})	15	10%	Green	No callusing	-
f/2 BA (10^{-6}_{M})	15	60%	Pinkish	30%	Friable, pinkish
f/2 BA (10^{-7}_{M})	15	No regeneration	-	No callusing	-
f/2 Kinetin (10^{-5}_{M})	15	10%	Pinkish	No callusing	-
f/2 Kinetin (10^{-6}_{M})	15	10%	Pinkish	No callusing	-
f/2 Kinetin (10^{-7}_{M})	15	No regeneration	-	No callusing	-

icina (Lamouroux) C. Agardh from Indian Coast.

MATERIALS AND METHODS

The plants of *Grateloupia filicina* (Fig. 1) were collected from Baga beach, Goa in the month of September. Healthy thalli were selected, washed several times in seawater in the field, wrapped with absorbent cotton and transported to the laboratory in an air-conditioned compartment between 20-22°C. Healthy plants were selected and visible epiphytes were removed with the help of a brush under a Bausch & Laumb binocular microscope. Subsequently, the cleaned thalli were treated with IKI solution for one minute to eliminate the surface microbes (2 g KI and 1 g I_2 dissolved in 300 ml of distilled water. Out of this 1 ml of IKI solution was added to 250 ml of sterilized seawater). Further sterilization was done by using broad-spectrum antibiotic mixture and 1% GeO_2 for 2 days to prevent the growth of bacteria and diatoms (Polne-Fuller and Gibor 1984).

The sterilized healthy thalli were kept in 500 ml of autoclaved seawater in a flat-bottom, Florence flask and aerated with compressed air at 20°C temperature and 12:12 light:dark photoperiod as stock culture. The salinity was maintained at 30 ppt throughout the experiment.

Before inoculation thalli were thoroughly cleaned 3-4 times in autoclaved seawater and excised into 3-5 mm in length. Each explant (taken from the central part of thallus)was then wiped gently with sterile filter paper (Whatman no. 1, Maidstone, U.K.) to remove moisture and mucilage exuded from the cut ends.

Three different marine media such as f/2 (Guillard and Rhyther 1962); PES (Provasoli 1968) and ESW (Freshwater and Kapraun 1986) were used, both in solid and liquid form and the pH was adjusted to 8.2. Besides, these media were further supplemented with a number of phytoregulators such as Glycerol (0.5%; 1% &1.5%); Napthalene Acetic Acid (NAA), Benzylaminopurine (BA) and Kinetin all at the concentration of 10^{-5}_{M}, 10^{-6}_{M}, 10^{-7}_{M}. The phytoregulators were added in both solid and liquid media. A control was set for each experiment using autoclaved seawater. Each treatment consisted of three replicates with five explants in each. The explants were cultured in petridish with 20 ml of solid or liquid medium. The medium was solidified using 1.5% agar. Cultures were maintained at 20°C with cool white fluorescent light in 12:12 light:dark photoperiod (Philips fluorescent tubes 36 W/54, 6500 K: 15 μmol m^{-2} s^{-1}). The results were recorded after every 3 days.

RESULTS

Grateloupia filicina (Lamouroux) C. Agardh is an important carrageenophyte, which inhabits intertidal tropical and subtropical marine environments. It tolerates a wide range of temperature and salinities (Baweja and Sahoo 2002). The plants of *G. filicina* were 5-6 cm in height having deep reddish brown color (Fig. 1).

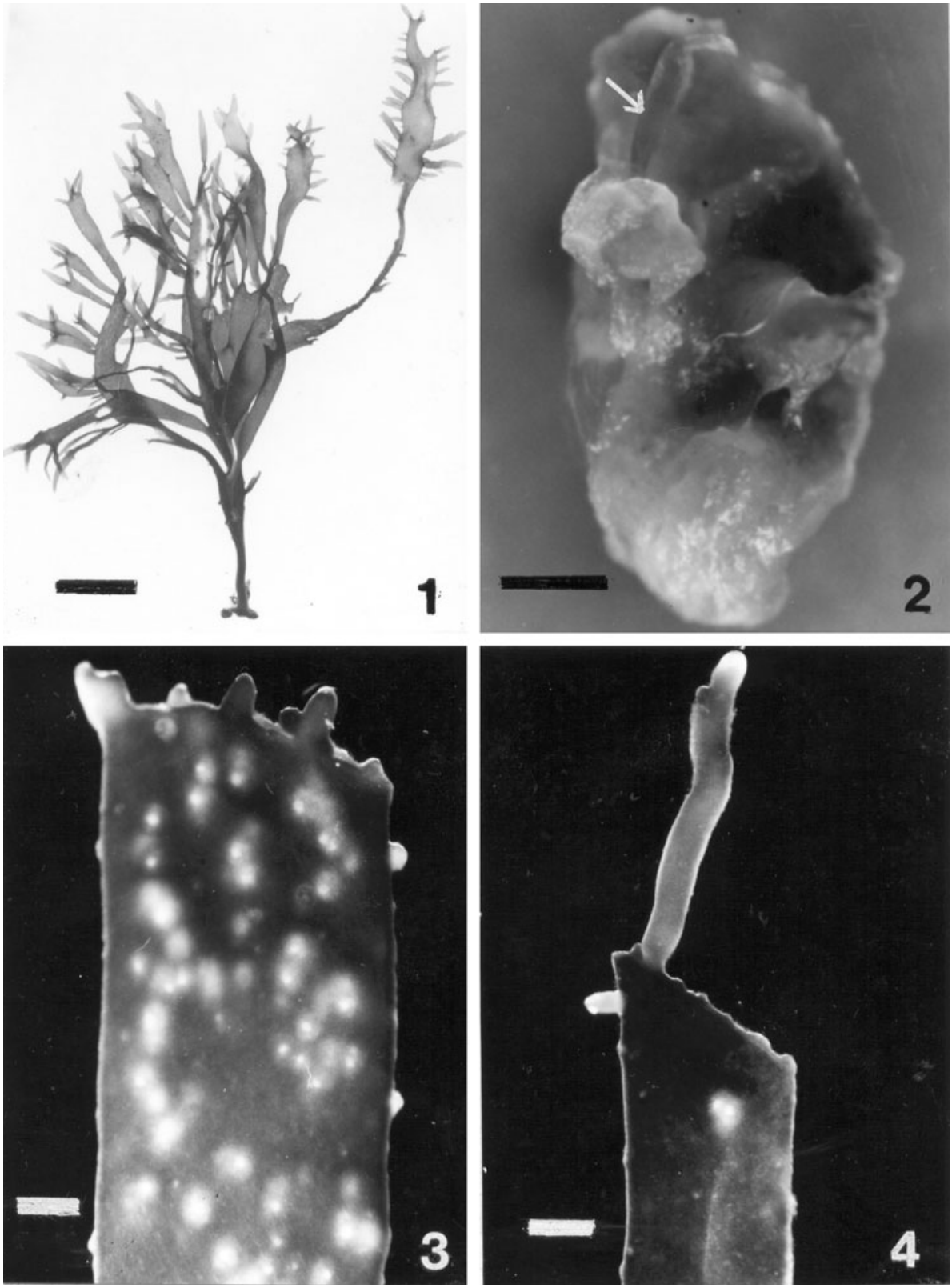

Fig. 1. Habit of *Grateloupia filicina* from Baga Beach (Goa). (scale bar = 0.25 cm).

Fig. 2. Regeneration of a young filament (arrow) from the subcultured callus in f/2 solid medium supplemented with 0.5% glycerol. (scale bar = 0.05 mm).

Fig. 3. Explant showing regeneration in f/2 liquid medium after 3 days of inoculation. (scale bar = 0.6 mm).

Fig. 4. Explant in PES medium showing regeneration after 7 days of inoculation. (scale bar = 1 mm).

Table 2. Effect of phytoregulators on explants (PES liquid/Solid medium)

Medium	Liquid			Solid	
	No. of Explants	% of Regeneration	Colour of regenerated filaments	% of Callusing	Nature & Colour of Callusing
PES Control	15	40%	Reddish-brown	20%	Friable, pink
PES Glycerol (0.5%)	15	30%	Pink	25%	Crystaline, pink
PES Glycerol (1%)	15	No regeneration	-	10%	Crystalline,Pinkish
PES Glycerol (1.5%)	15	No regeneration	-	10%	Friable, white
PES NAA (10^{-5}_{M})	15	10%	Pinkish	25%	Friable, white
PES NAA (10^{-6}_{M})	15	10%	Pinkish	10%	Friable, white
PES NAA (10^{-7}_{M})	15	No regeneration	-	No callusing	-
PES BA (10^{-5}_{M})	15	15%	Pinkish	No callusing	-
PES BA (10^{-6}_{M})	15	15%	Pinkish	No callusing	-
PES BA (10^{-7}_{M})	15	No regeneration	-	No callusing	-
PES Kinetin (10^{-5}_{M})	15	15%	Reddish Brown	5%	Friable, Pinkish
PES Kinetin (10^{-6}_{M})	15	No regeneration	-	No callusing	-
PES Kinetin (10^{-7}_{M})	15	No regeneration	-	No callusing	-

Table 3. Effect of phytoregulators on explants (ESW liquid/Solid medium)

Medium	Liquid			Solid	
	No. of Explants	% of Regeneration	Colour of regenerated filaments	% of Callusing	Nature & Colour of Callusing
ESW Control	15	30%	Reddish-brown (++)	10%	Friable, White
ESW Glycerol (0.5%)	15	No Regeneration	-	No callusing	-
ESW Glycerol (1%)	15	No Regeneration	-	No callusing	-
ESW Glycerol (1.5%)	15	No Regeneration	-	No callusing	-
ESW NAA (10^{-5}_{M})	15	20%	Pinkish (+)	No callusing	-
ESW NAA (10^{-6}_{M})	15	20%	Pinkish (+)	No callusing	-
ESW NAA (10^{-7}_{M})	15	No Regeneration	-	No callusing	-
ESW BA (10^{-5}_{M})	15	5%	Reddish Brown (+)	No callusing	-
PES BA (10^{-6}_{M})	15	No Regeneration	-	No callusing	-
ESW BA (10^{-7}_{M})	15	No Regeneration	-	No callusing	-
ESW Kinetin (10^{-5}_{M})	15	-	No regeneration	No callusing	-
ESW Kinetin (10^{-6}_{M})	15	5%	Reddish Brown (+)	No callusing	-
ESW Kinetin (10^{-7}_{M})	15	No Regeneration	-	No callusing	-

Regeneration of small protrubences was observed from the cut ends of the explants which were inoculated in f/2, PES & ESW liquid media after 3 days of inoculation. Better regeneration of thallus was obtained in f/2 liquid medium (Fig. 3) and PES liquid medium (Fig. 4) after 3 and 7 days of inoculation respectively. No callusing was observed in the explants cultured in the liquid media (Tables 1, 2 and 3).

However, callusing was observed after 15 days of inoculation mostly in f/2 & PES solidified media supplemented with various concentrations of phytoregulators (Tables 1 and 2). In the present study no callusing was observed in ESW solid medium supplemented with phytoregulators (Table 3). When the f/2 medium was supplemented with low concentration of glycerol (0.5%) 70% callusing was observed but when the concentration of glycerol was increased the percentage of callusing declined and no callusing was observed when the medium was supplemented with 1.5% glycerol. Similarly lower concentration of NAA 10^{-5}_{M} and BA 10^{-5}_{M} produces better callusing compared to the higher concentration of the phytoregulators. The calli were mostly crystalline, friable and pinkish in colour depending on the media (Tables 1 and 2). When the calli were subcultured

regeneration of new filaments were observed only in the callus which was cultured in f/2 medium supplemented with 0.5% glycerol after 25 days of subculturing of the callus (Fig. 2) when this regenerated filaments were excised and transferred to f/2 liquid medium new branching was observed after 20 days of inoculation. Extensive branching was subsequently observed and the plants resembled in morphology to the wild plants after 30 days in culture. Thus the whole regeneration with in 90 days from the date of inoculation of explants was completed.

DISCUSSION

Regeneration is a wide spread phenomenon in seaweeds which demonstrates totipotency of algal cells, provides a mean for studying morphogenetic stages of adventive embryony, and produces correlation growth effects which may be useful for hypothesizing mechanism of regulation of morphogenesis (Buggeln 1981; Baweja *et al*. 2009). The development of *in vitro* vegetative propagation permits the rapid multiplication of desired varieties. There have been several reports on seaweed cultivation based on regeneration from fragments of upright thalli (Goldstein 1973; Bula-Mayer 1989; Hurtado-Ponce 1990; Kain 1991).

The main objective of the present study was to develop and demonstrate tissue culture methodology in tropical marine algae. In the present study direct regeneration of new plantlets were observed in *G. filicina* without any callusing in liquid media whereas callusing was observed only in the solid media. The highest percentage of direct regeneration was observed in f/2 and PES liquid media, but regeneration from callus was observed only in the f/2 solid medium supplemented with 0.5% glycerol. The callusing may be due to the presence of exogenous carbon source, and plant growth regulators present in the culture media which induces callus development. In general plant growth regulators (Glycerol, Auxins and Cytokinins) are not essential for *in vitro* callus growth in algae, but they may enhance callus induction and growth in red seaweeds (Aguirre-Lipperheide *et al*. 1995; Huang and Fujita 1997). This is substantiated in the present study as regeneration and callus like structures were also observed in cultures whether they were supplemented with phytoregulators or not. In *G. filicina* lower percentage of callusing was observed in the control f/2 solid medium, but the percentage of callusing was enhanced when the media were supplemented with glycerol and auxins at lower concentration. Robaina *et al*. (1990a, b) observed that the presence of glycerol, although not essential but can increase the percentage of calli formation in *Grateloupia doryphora*. Yokoyo *et al*. (1993) observed development of callus like structure and regeneration from it in *G. filiformis*. Yokoyo and Handro (1996) also observed callus like structures from the basal pole of both apical & intercalary segments in *Grateloupia dichotoma*. Migita (1988) found that cut fragments of basal crusts of *Grateloupia filicina* can easily and rapidly regenerate new crust. According to Polne-Fuller & Gibor (1987) the development of this callus like structures both in presence and absence of plant growth regulators is due to the wound healing process in seaweeds. Plant growth regulators also promoted callus growth in *Kappaphycus alvarezii* (Dawes and Koch 1991; Dawes *et al*. 1993). Sahoo and Singh (2006) studied thallus regeneration in *Cystoseira indica*. Athukorala *et al*. (2003) reported antioxidant activity of *G. filicina*. Since the plants are edible but found in less quantity in nature, micro propagation of suitable variety through tissue culture could be one possible way for mass cultivation of this alga. In conclusion, the present study indicates that *G. filicina* provides a good system for micro propagation.

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