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Mechanism and Engineering of Polyamines on Growth, Regeneration and Callus Formation in *Gracilaria corticata* and *G. verrucosa* (Rhodophycea)

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Abstract: The role of Polyamines (PAs) has an important role on growth, regeneration and callus formation in *Gracilaria corticata* and *G. verrucosa*. The influence of different concentration of polyamines such as spermine, putrescine and spermidine enriched culture media was used during *in vitro* culture. Amongst different concentration of polyamines enriched media, spermine 10^{-6}M enriched f/2 medium influences maximum number of vegetative branches and a callus (disorganized cell mass that arose from the organized tissue of the explant) formation. Although regeneration was observed in putrescine and spermidine supplemented enriched media but the speed of regeneration and growth was slow in compared to spermine. The simultaneous formation of filamentous outgrowth was observed at the cut ends of explants. These filamentous outgrowths later developed several vegetative branches cultured in different concentration of polyamines enriched media. The explants taken from the basal portion of the thallus showed maximum number of regeneration of vegetative branches compared to the apical portion. In *G. corticata*, some embryoids like structure (cystocarps) were observed in spermine 10^{-6}M enriched PES medium. However, growth of cystocarps in *G. verrucosa* was observed on the surface of explants cultured in spermine 10^{-6}M supplemented f/2 medium after 21 days of inoculation. Complete plantlet of *G. corticata* and *G. verrucosa* along with several branches was grown successfully in spermine supplemented enriched f/2 medium after 2 months of culture. The color of plantlets was bushy and quite similar to that of field material in appearance.
***Keywords*:** *Gracilaria*, Polyamines (PAs), Spermine (Spm), Putrescine (Put), Spermidine (Spd), Provasoli's enriched medium (PES), f/2, enriched seawater (ESW), Regeneration, Cystocarps.

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Introduction

Polyamines (PAs) have been admitted in the category of a new class of growth substances (Galston and Kaur-Sawhney, 1990; Lee and chu, 1992; Tassoni et al., 2000). Several researcher reveals the mechanism polamines in physiological and stabilization of cell membranes (Schubert et al., 1983; Roberts et al., 1986; Kaur-Sawhney and Applewhite, 1993); stress response (Flores, 1990; Aurisiano et al., 1993; Galston et al., 1997; Kakkar et al., 2000) and senescence (Slocum et al., 1984; Rey et al., 1994; Del Duca et al., 2000). The occurrence of polyamines (PAs) have been studied within different algal groups (Hamana and Matsuzaki, 1982) and their involvement in cell division by several workers (Cohen et al., 1984). The supplementation of exogenous polyamines during the final stage of carposporophyte development in *Gracilaria cornea* promoted carpospores release, cell division and morphogenesis (Guzman-Uriostegui et al., 2002). In *Grateloupia doryphora*, carposporelings cultured in the presence of putrescine and spermidine grew and transferred into small morphogenic cell masses at both concentrations 10^{-3}M and 10^{-6}M supplemented polyamines (Garcia-Jimenez et al., 1998). Hommersand and Fredericq (1995) reported that endogenous level of polyamines in the reproductive tissue of *Gracilaria cornea*. Thus, The effect of polyamines on the growth and regeneration has been studied in different species of algae.

The objectives of this work was to study the productive role of polyamines (spermine, spermidine and putrescine) on the growth and regeneration of some economically important seaweed like *G. corticata* and *G. verrucosa*, an important source of Agarophyte in India. All these findings show great achievements in seaweed tissue culture work in our laboratory in order to select regenerate and propagate possible clones as seed stock for the commercial purpose.

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Materials and Methods

1) Survey and Collection

A regular survey was done in coastal area before collection of specimen, The thallus of *Gracilaria corticata* were collected from the coast of Manadapam in the month of July, 2010 (Figure 1). However, plants of *G. verrucosa* were collected from the coast of Chilika Lake, Orrisa in the month of December 2012 (Figure2).

a) Mandapam coast :

During collection of material, the tide of (North Latitude 78º11' to 79º15' East Longitude 8º49' to 9º15') coast was low. A few kilometer distances apart, there is Pamban Bridge connected to Rameshwaram (Figure 1)

Figure 1 : Map Showing Collection of *Gracilaria corticata* in Mandapam.

b) Chilika is Asia's largest brackish-water lagoon with an estuarine character, dotted with islands. It is situated $19^0$28' and $19^0$54' north latitude and $85^0$05' east longitude, spread over Ganjam districts of Odisha state on the east coast of India, flowing into the Bay of Bengal, covering an area of over 1,100 km (Figure 2). It is the largest coastal lagoon in India and the second largest brackish water lagoon in the world.

Figure 2 : Map Showing Collection of *Gracilaria verrucosa* in Chilika Lake.

2) Cleaning of Thallus and Preparation of explants

Selected thalli were cleaned with seawater in the field for several times, wrapped in absorbent cotton moistened with seawater and brought to the laboratory for *in vitro* culture studies. Healthy thalli were separated and cleaned several times in filtered seawater to remove the surface contaminants and other epiphytes. Further, thalli were treated with 1ml of 1% KI-I_2 (2gm of KI and 1gm of I_2 dissolved in 300ml distilled water) in 250ml seawater. Subsequently, the thalli were treated with 1ml of antibiotic mixture (Streptomycin sulphate: Penicillin-G, 1gm each in 100ml of distilled water) in 250ml seawater to avoid the bacterial growth. This was followed by the treatment with 1ml of GeO_2 (4gm of NaOH dissolved in 100ml of distilled water and 250mg of GeO_2 in boiling condition) in 250ml seawater to check the growth of diatoms. These thalli were kept in 250ml flat-bottom flask aerated with compressed air at 20^0 C and 12 : 12 light : dark photoperiod as stock culture. The salinity was maintained at 20ppt and pH was adjusted to 7.8.

For solid culture, media were solidified with the addition of 0 .8 % agar. To examine the effect of polyamines, three different types culture media namely Provasoli's Enriched Seawater (PES) medium (Provasoli, 1968), f/2 medium (Guillard & Ryther, 1962) and Enriched Seawater (ESW) medium (Freshwater & Kapraun, 1986) supplemented with different concentration (10^{-3}M, 10^{-6}M and10^{-8}M) of polyamines (spermine, spermidine and putrescine) were used. Healthy

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thalli were selected from the stock culture and cut into small pieces (5mm) under the aseptic condition. These explants were inoculated into both liquid and solid PES, f/2 and ESW media. The experiments were carried out in three replicates with five explants. 1-2 drops of GeO_2 added to each petridishes to prevent the growth of diatoms. These petridish were kept in a thermostatically controlled culture room at 20 $\pm$ 2^0C under 12 : 12 light : dark cycle. Culture media in the petridishes were changed after 7 days and observations were made after a period of 3 days of inoculation.

Result and Discussion

Several workers reported the mechanism of polyamines on the growth, regeneration and callus formation has been studied in different species of algae (Sacramento et al., 2004; Guzman-Uriostegui et al., 2002; Baldini et al., 1994, Polne-Fuller et al. 1987). The standardization of regenerated explants shown in Table 1. In the mean time, the graphical analysis of regenerated branches measuring excellent growth in spermine supplemented marine media measuring 6.5cm length of healthy thallus (Figure 4 & 5). This luxuriant growth of *Gracilaria verrucosa* shown in Figure 3a.

In experimental start up, the first regeneration at the cut end of explants showed several outgrowth and cellular deposition, which later developed into apical cell is tetrahedral and has 3-sided cutting faces undergo multiplication to form a primary axis (Figure 3d). The repeated division of apical and sub apical cell undergo division to form several vegetative branches. Regenerated branches were irregular and sub-dichotomous to unilateral.

In *Grateloupia doryphora*, the culture of carposporeling in the presence of putrescine and spermidine supplemented marine media, grew and transferred into small morphogenic cell masses at both concentrations 10^{-3}M and 10^{-6}M (Garcia-Jimenez et al., 1998). In *G. corticata* growth and formation of embryoid like structures (cystocarps) was observed only in spermine 10^{-8}M supplemented PES medium (Figure 3 b & 3e). The size of cystocarps measuring 0.4mm in length (Present Study). Subsequentially, Spermine10^{-6}M supplemented f_2 medium initiated five

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vegetative branches in *G. corticata*, the maximum length of branches was measuring 2.0cm in length (Figure 3c & e).

In the study of solid culture using all polyamines plus marine media a white callus was observed only in spermine10^{-6}M supplemented f/2 medium respectively. The nature of callus was filamentous and white yellowish in color, measuring 2.5 m in length (Figs. 3f).

Conclusion

The overall experiment shown that although spermidine and putrescene was used along with marine media, but spermine showed its tremendous and significant effect on growth and regeneration of explants. Anyway, the output of this research concluded that this work develop a complete plantlets of *G. corticata* and *G. verrucosa* successfully grown alongwith several branches. The color and nature of these plantlets grown in culture condition were similar to that of the field material in appearance.

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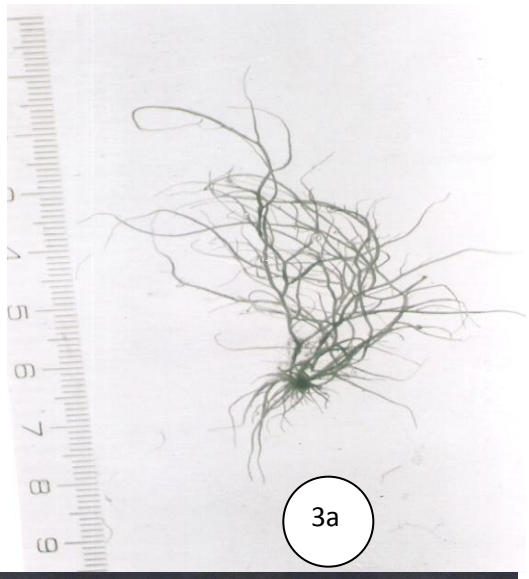

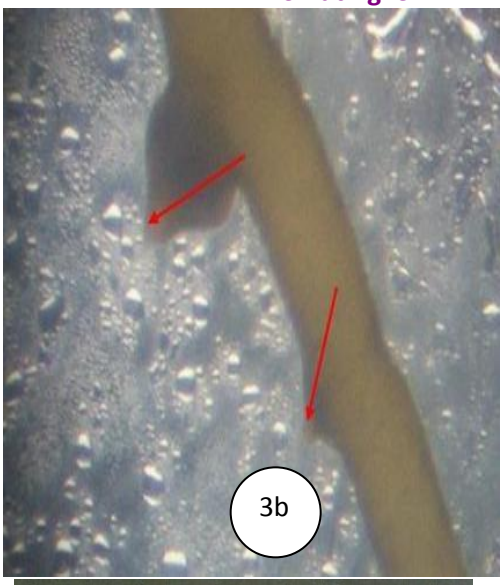

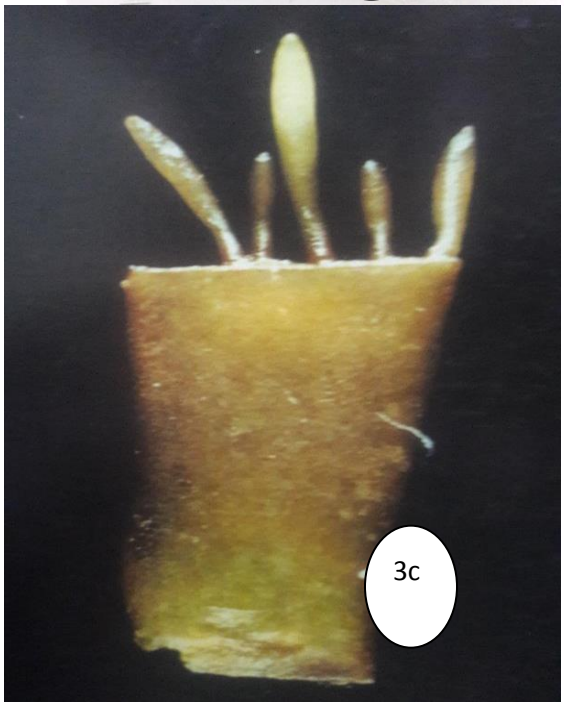

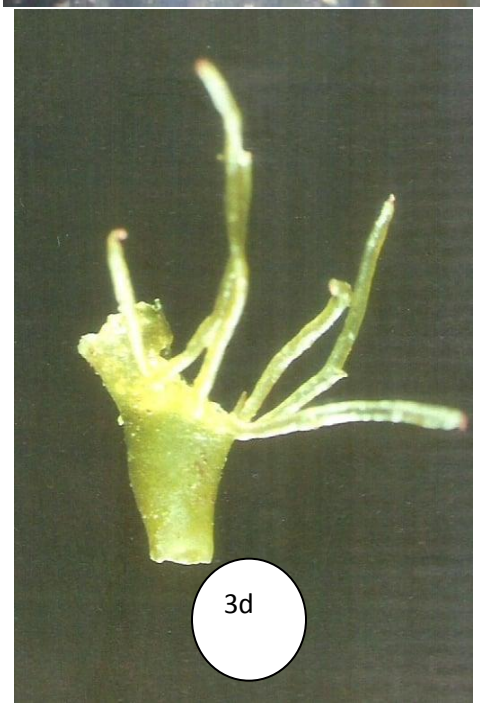

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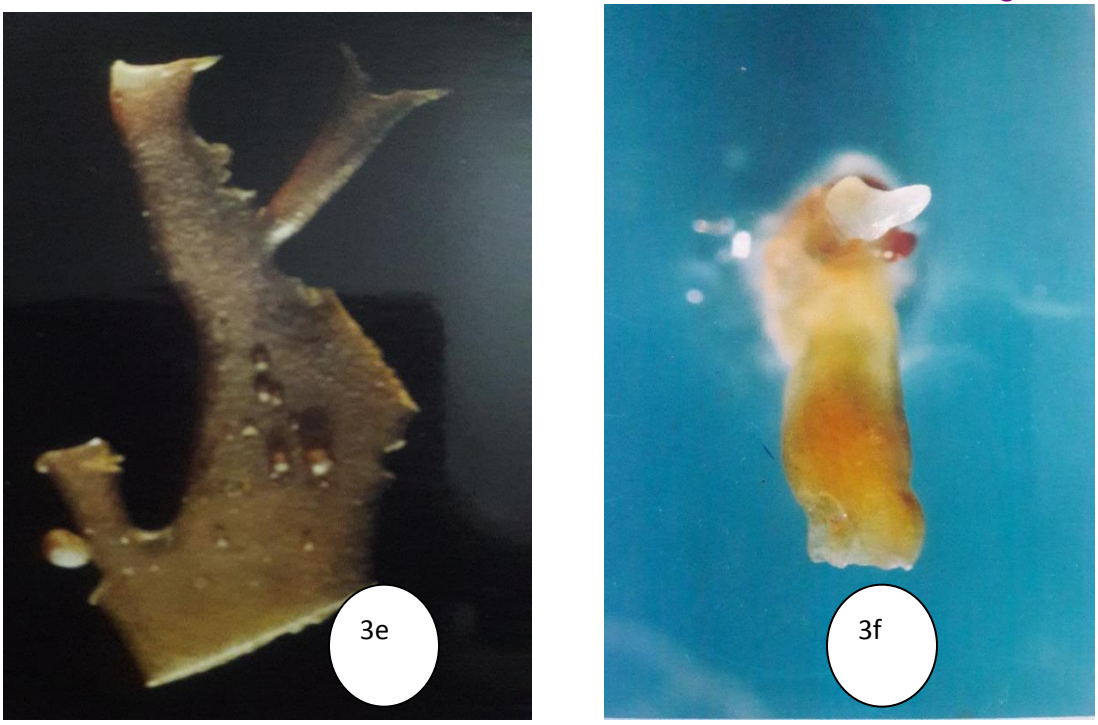

Figure 3a: A luxriant thallus of *G. verrucosa* developed in 10^{-6}M spermine supplemented f_2 Marine Media after 21 days of inoculation.
Figure 3b: The mature carpospores was observed in 10^{-6}M spermine supplemented f_2 Marine Media after 5 days of inoculation.
Figure 3c & d: Several healthy outgrowths developed from the cut end of explants.
Figure 3e : Some embroied structure shown in spermine 10^{-8}M supplemented PES marine media.
Figure 3f): A white heart shaped callus observed in 10^{-6}M spermine supplemented f_2 Marine Media.

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Table 1. Standardization of cultured media with different concentration of polyamines (SPM, PUT and SPD) in *Gracilaria sp.*

Medium	concentration	Polyamines	Parts of explant used Basal	Apical
PES	(N)		+	-
	10^{-3}M	SPM	+	+
	10^{-3}M	PUT	+	-
	10^{-3}M	SPD	+	-
	10^{-6}M	SPM	++	-
	10^{-6}M	PUT	+	-
	10^{-6}M	SPD	+	-
	10^{-8}M	SPM	+	+
	10^{-8}M	PUT	+	-
	10^{-8}M	SPD	+	-
f/2	(N)		+	-
	10^{-3}M	SPM	+	+
	10^{-3}M	PUT	+	-
	10^{-3}M	SPD	+	-
	10^{-6}M	SPM	++	+
	10^{-6}M	PUT	+	-
	10^{-6}M	SPD	+	-
	10^{-8}M	SPM	++	+
	10^{-8}M	PUT	+	-
	10^{-8}M	SPD	+	-
ESW	(N)		+	-
	10^{-3}M	SPM	+	+
	10^{-3}M	PUT	+	-
	10^{-3}M	SPD	+	-
	10^{-6}M	SPM	+	+
	10^{-6}M	PUT	+	-
	10^{-6}M	SPD	+	-
	10^{-8}M	SPM	+	+
	10^{-8}M	PUT	+	-
	10^{-8}M	SPD	+	-

