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Investigating the Effects of Exogenous Factors on Growth, Photosynthetic Pigments and Bud Induction in *Gracilaria corticata* var. *cylindrica* under *In vitro* Conditions

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A B S T R A C T

Effect of kind and concentration of culture media, and plant growth regulators on *in vitro* response was studied in an economically important seaweed species *viz. Gracilaria corticata* var. *cylindrica*. Filter sterilized autoclaved artificial seawater medium at 100 % concentration (A_2) was found to be the most optimum for *in vitro* culture as the cultured explants showed superiority in terms of growth and photosynthetic pigment content, apart from inducing lateral bud formation. Incorporation of cytokinin alone or in combination with auxin (IAA) promoted growth of seaweed explants in A_2 media.A_2 medium supplemented with kinetin at different concentrations showed the highest total chlorophyll content (K_2), highest total carotenoid content (K_3) and better induction of lateral buds (K_1). Hence, considering the promising response of artificial seawater supplemented with cytokinins and auxin for *in vitro* culture of the species, the present investigation could serve as a base study for formulating future research programmes.

Introduction

In the ever expanding billion-dollar seaweed industry, production contribution is mostly from the organized culture sector, while wild harvests accounts for mere 5% of the global seaweed production (FAO, 2014). Species of *Gracilaria* are cultivated in many parts of the world (FA0, 2016) and forms an important natural source of phyco-colloid- agar. However, the conventionally used vegetative fragments from the same mother plants over generations have reported to decrease the agar yield and quality, apart from increasing their susceptibility to various diseases (Hurtado and Chenney, 2003). Micropropagation is an *in vitro* culture technique, wherein axenic explants from any part of the seaweed are used to develop clones in artificial media under controlled conditions. Utility of *in vitro* multiplication techniques has been emphasized for commercial scale seaweed culture (Bohra *et al.*, 2017). Supply of good quality seed material through *in vitro* means could improve the yield significantly through improved growth and phyco-colloid recovery,

when compared with conventional vegetative fragments (Kumar *et al.,* 2004). This technique could be employed for large scale production of superior quality seed material with uniform characteristics for seaweed farming. Despite the fact that tissue culture aspects have been attempted in 85 species (Reddy *et al.,* 2008), the repeatability of the developed protocol has not very been successful even in same species grown elsewhere.

Addition of exogenous hormones to culture media in controlled environments could enhance the culture establishment and subsequent development. Endogenous presence of plant growth regulators (PGRs) such asauxins and cytokinins have been reported in different seaweed species (Jacobs *et al.,* 1985; Bradley, 1991; Jacobs, 1993; Stirk and van Staden, 1997; Stirk *et al.,* 2003, 2004). Imelda *et al.,* (1998) suggested that use of exogenous PGRs could act synergistically with the native hormones and promote the culture growth.

Positive responses with use of PGRs in some seaweed species have been reported by earlier researchers (Kaczyna and Megnet, 1993; Yokoya *et al.,* 2004; Hayashi *et al.,* 2008; Yong *et al.,* 2014a). Report by Aguirre-Lipperheide *et al.,* (1995) also suggested that red seaweed species respond more to exogenously supplied PGRs than other seaweeds.

Addition of PGRs aids in regaining the morphology of seaweeds treated with antibiotics in laboratory to obtain axenic explants (Bradley and Chenney, 1991). The present study concerned a preliminary attempt to investigate the effects of culture media similar to the natural environment and PGRs on the micropropagation of *Gracilaria corticata* var. *cylindrica* in Andaman and Nicobar Islands, India.

Materials and Methods

Segments of seaweed species *Gracilaria corticata* var. *cylindrica* were collected during low tide from Burma Nallah region of South Andaman, Andaman and Nicobar Islands (India). Healthy segments (2- 5 cm) were collected and cleaned off with sterilized cotton and tweezers to eliminate the epiphtyes followed by rinsing in filter sterilized (0.45 µ) seawater. Such explants were first treated with antibiotic mixture A_3 (Liu and Kloareg, 1992) in autoclaved beaker for 5 d. Subsequently, fragments were washed with filter sterilized autoclaved seawater and treated with 0.1 % detergent (Charmy green) for 10 min (Kumar *et al.,* 2007) and thereafter with betadine.

The explants were finally cut into *ca*. 1 cm size using sterile blades and such explants were used for successive experiments.

Effect of media on seaweed cultures

Following the surface sterilization procedure, effect of culture media on establishment of seaweed cultures were determined using two different media *viz*. filter sterilized autoclaved seawater (FSAS) and filter sterilized autoclaved artificial seawater (FSAAS) at three different concentrations (50% (15 ppt), 100% (30 ppt) and 200% (60 ppt)). The optimized concentration of media was used for the subsequent experiment.

Effect of PGRs on seaweed cultures

The effect of cytokinins (6-Benzylaminopurine (B), Kinetin (K) and *meta*-topolin (M)) at different combinations with or without addition of auxin (indole-3-acetic acid (I))was studied on culture response at three concentrations i.e. 2.5 mg/L (B_1, M_1, K_1), 5 mg/L (B_2, M_2, K_2) and 7.5 mg/L (B_3, M_3, K_3)in FSAAS at 100% concentration. In

auxin supplemented treatments, a common dose of I (0.2 mg/L) was used. Both the experiments were conducted for 30 d and subculture was performed by changing the culture media after 15 d. A photoperiod of 16:8 (L:D) was maintained throughout the culture period with 20 W fluorescent tubes (Phillips, India). Weight of the explants was recorded at fortnightly interval, whereas number of buds, chlorophyll content and carotenoid content were recorded before initiation and atthe end of the experiment. Chlorophyll and carotenoid contents were estimated following Wellburn (1994). Daily growth rate (DGR) was calculated following earlier reports (Loureiro*et al.,* 2010). The statistical analysis of the data was done using Web Agri Statistical Package (WASP v. 2.0, Indian Council for Agricultural Research-Research Complex for Goa, Old Goa, India).

Results and Discussion

The results of the experiment showed that both kind and concentration of culture media had a profound influence on the growth of cultured explants ($p<0.05$, Table1-2). Natural seawater (S_2) promoted maximum increase in weight of explants with highest DGR among the three different FSAS media tested. Culture of explants initiated in A_2 established themselves with highest DGR and also gained maximum weight during subculture, while explants showed maximum weight during the first 15 d in A_1 among the different FSAAS media (Table 1 and 2). The growth rate also gradually decreased with duration of culture. Both suboptimal and supraoptimal levels of media were not conducive for culture growth as bleaching of explants was observed in these concentrations of FSAAS media. Although *Gracilaria* has been reported to grow at low saline conditions (Bird and McLachlan, 1986), reduced growth (Graham and Wilcox, 2000; Jong *et al.,* 2015) and bleaching (Jong *et al.,* 2015) have been noticed. Loss of thallus rigidity has been reported at low salinity levels (Kumar *et al.,* 2010). Salinity level has been reported to affect growth in red algae (Daugherty and Bird, 1988) and both low and high salinity levels inhibited culture growth in earlier reports (Cai, 2011). Decrease in growth rate with increase in salinity (Ding *et al.,* 2013) was also evident in present experiment. This might be due to the damage to the outer protective covering of explants due to hyper saline conditions, which in turn hindered the culture growth. Negative growth rate was observed in media at high concentration which might be due to bleaching of explants. Ding *et al.,* (2013) also observed negative growth at high salinity in *Gracilaria* species. In present experiment, the optimum salinity for growth of explants was observed to be 30 ppt in both the media (S_2& A_2), which is in line with the earlier reports (Bird and McLachlan, 1986; Ding *et al.,* 2013; Jong *et al.,* 2015). Further, A_2 media at this salinity provided better environment for culture growth, which is in agreement with Kaladharan *et al.,* (2003), who reported suitability of artificial seawater for *in vitro* culture of *Gracilaria* species.

Salinity of the culture media affected the photosynthetic pigments of the explants *i.e.* total chlorophylls and total carotenoids (Fig. 1). The concentration of chlorophylls decreased with salinity stress (low and high salinity media), when compared to the concentration prior to the treatments although carotenoid contents showed increase in all media under study except A_3. Irrespective of the culture medium, chlorophyll content reached maximum level at 30 ppt and was found to be the highest in A_2 media. These findings are supported by earlier reports in which photosynthetic rate was found to decrease in *Gracilaria verrucosa* in low salinity (Wang *et al.,* 1993), while decrease of photosynthetic pigments was observed above and below 25-30 ppt in *Hypnea cervicornis* (Ding *et al.,* 2013).

Table.1 Effect of media on the seaweed explants (Culture initiation)

Treatment	Mean Initial Weight (g)	Mean Final Weight (g)	%Weight Change	Paired t test	DGR (Daily growth rate)	Remarks
S_1(50%FSAS media)	0.14±0.06	0.15±0.04	+12.54	NS	+0.06	
S_2(100%FSAS media)	0.13±0.02	0.19±0.02	+42.16	*	+2.81	
S_3(200%FSAS media)	0.12±0.04	0.12±0.04	+0.93	NS	+1.32	**
A_1(50%FSAS media)	0.11±0.04	0.16±0.03	+47.04	*	+0.84	
A_2(100%FSAS media)	0.14±0.03	0.17±0.04	+19.81	*	+3.14	
A_3(200%FSAS media)	0.15±0.02	0.16±0.02	+2.17	NS	+0.14	**

Data are presented as means ± standard error (S.E.).,*- Significant; NS- Not significant; ** - Bleaching observed

Table.2 Effect of media on the seaweed explants (Sub culture)

Treatment	Mean Initial Weight (g)	Mean Final Weight (g)	%Weight Change	Paired t test	DGR (Daily growth rate)	Remarks
S_1 (50% FSAS media)	0.15±0.04	0.20±0.02	+9.48	NS	+0.63	
S_2(100% FSAS media)	0.19±0.02	0.16±0.04	+9.95	*	+0.66	
S_3(200% FSAS media)	0.12±0.04	0.12±0.04	-4.88	NS	-0.33	**
A_1(50% FSAS media)	0.16±0.03	0.13±0.03	-14.31	NS	-0.95	**
A_2(100% FSAS media)	0.17±0.04	0.19±0.04	+13.54	*	+0.90	
A_3(200% FSAS media)	0.16±0.02	0.13±0.03	-16.87	*	-1.12	**

Data are presented as means ± standard error (S.E.); *- Significant; NS- Not significant; ** - Bleaching observed

Table.3 Effect of PGRs on the proliferation of explants (Culture initiation)

Treatment	Mean InitialWeight (g)	Mean Final Weight (g)	%Weight Change	Paired t test	Daily Growth Rate (DGR)
A_2	0.14±0.005	0.17±0.015	+26.21	*	+1.75
B_1	0.09±0.016	0.17±0.015	+35.54	*	+2.37
B_1I	0.08±0.029	0.12±0.028	+39.85	*	+2.66
B_2	0.08±0.056	0.11±0.038	+33.71	*	+2.25
B_2I	0.11±0.035	0.12±0.072	+38.75	*	+2.58
B_3	0.12±0.055	0.15±0.04	+20.67	*	+1.38
B_3I	0.12±0.05	0.14±0.066	+23.93	*	+1.60
M_1	0.08±0.014	0.14±0.06	+27.07	*	+1.80
M_1I	0.11±0.037	0.11±0.018	+40.15	*	+2.68
M_2	0.10±0.011	0.14±0.045	+31.04	*	+2.07
M_2I	0.12±0.031	0.13±0.021	+31.59	*	+2.11
M_3	0.12±0.034	0.15±0.037	+23.17	*	+1.54
M_3I	0.10±0.025	0.16±0.034	+39.28	*	+2.62
K_1	0.09±0.035	0.13±0.017	+32.75	*	+2.18
K_1I	0.09±0.013	0.13±0.058	+31.13	*	+2.08
K_2	0.05±0.036	0.12±0.019	+47.75	*	+3.18
K_2I	0.09±0.016	0.10±0.033	+44.19	*	+2.95
K_3	0.12±0.018	0.13±0.022	+45.90	*	+3.06
K_3I	0.09±0.03	0.18±0.041	+46.64	*	+3.11

Data are presented as means ± standard error (S.E.); *-Significant

A_2 (control) - 100% FSAAS media; B_1, B_2 & B_3 and B_1I, B_2I & B_3I- A_2 media supplemented with 6-Benzylaminopurine and A_2 media supplemented with 6-Benzylaminopurine and IAA; M_1, M_2& M_3 andM_1I, M_2I & M_3I- A_2 media supplemented with with *meta*-topolin and A_2 media supplemented with *meta*-topolin and IAA; K_1, K_2& K_3 and K_1I, K_2I &K_3I- A_2 media supplemented with Kinetin and A_2 media supplemented with Kinetin and IAA.

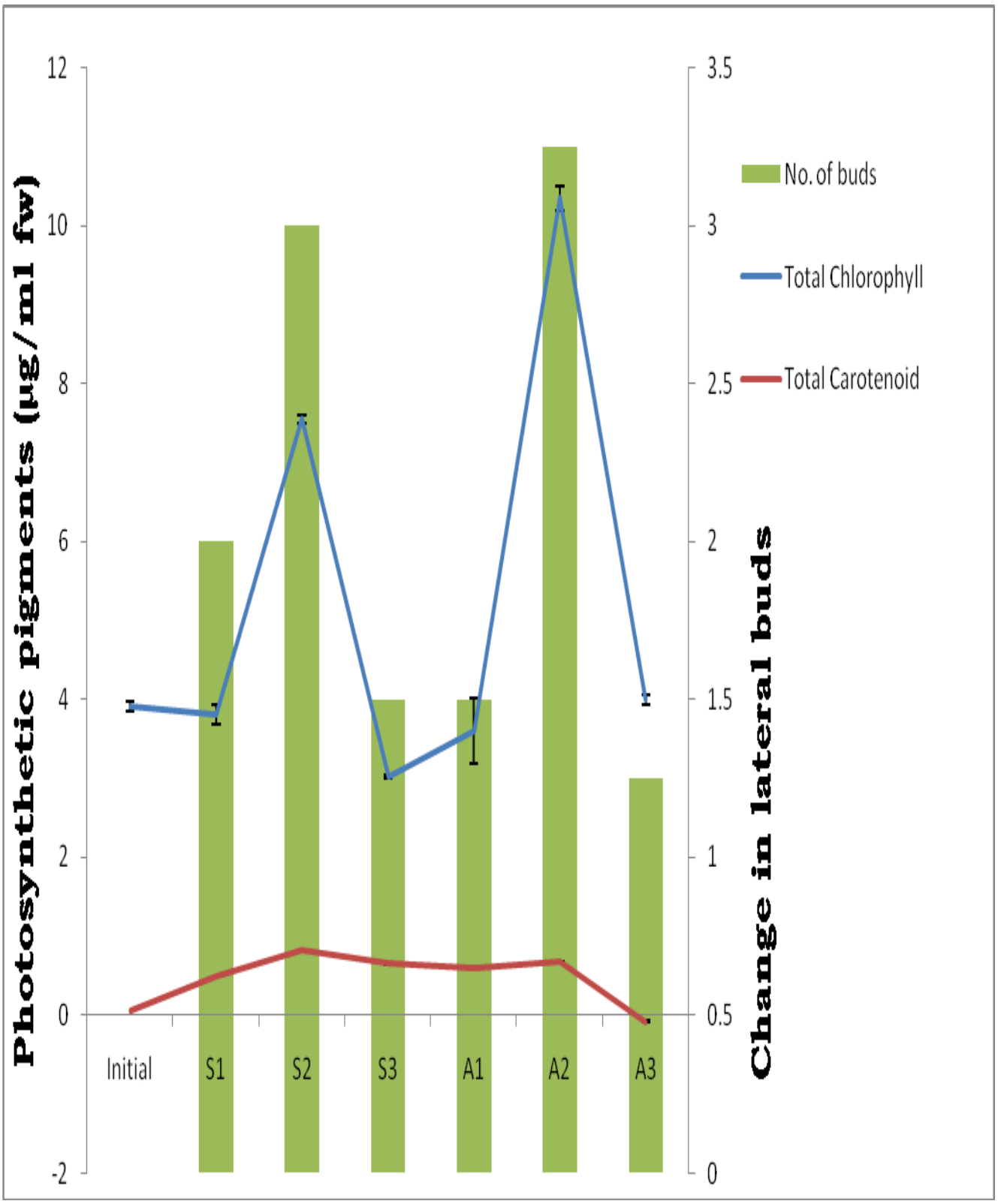
Table.4 Effect of PGR's on the proliferation of explants (Subculture)

Treatment	Mean Initial Weight (g)	Mean Final Weight (g)	%Weight Change	Paired t test	Daily Growth Rate (DGR)
A2	0.17±0.015	0.19±0.023	+12.75	*	+0.85
B1	0.12±0.028	0.13±0.034	+7.70	NS	+0.51
B1I	0.11±0.038	0.12±0.044	+7.48	NS	+0.50
B2	0.12±0.072	0.07±0.053	+4.56	*	+0.30
B2I	0.15±0.04	0.15±0.038	-37.63	*	-2.51
B3	0.14±0.066	0.16±0.085	-5.99	NS	-0.40
B3I	0.14±0.06	0.14±0.067	+7.40	NS	+0.49
M1	0.11±0.018	0.12±0.015	+12.55	*	+0.84
M1I	0.14±0.045	0.16±0.048	+13.59	*	+0.91
M2	0.13±0.021	0.14±0.019	+5.94	*	+0.40
M2I	0.15±0.037	0.16±0.036	+7.54	*	+0.50
M3	0.16±0.034	0.17±0.042	+6.67	*	+0.44
M3I	0.13±0.017	0.13±0.019	+9.38	*	+0.63
K1	0.13±0.058	0.14±0.062	+16.23	*	+1.08
K1I	0.12±0.019	0.14±0.026	+15.05	*	+1.00
K2	0.10±0.033	0.11±0.035	+11.03	*	+0.74
K2I	0.13±0.022	0.14±0.029	+12.16	*	+0.81
K3	0.18±0.041	0.20±0.042	+11.76	*	+0.78
K3I	0.13±0.052	0.14±0.051	+8.43	*	+0.56

Data are presented as means ± standard error (S.E.); * Significant

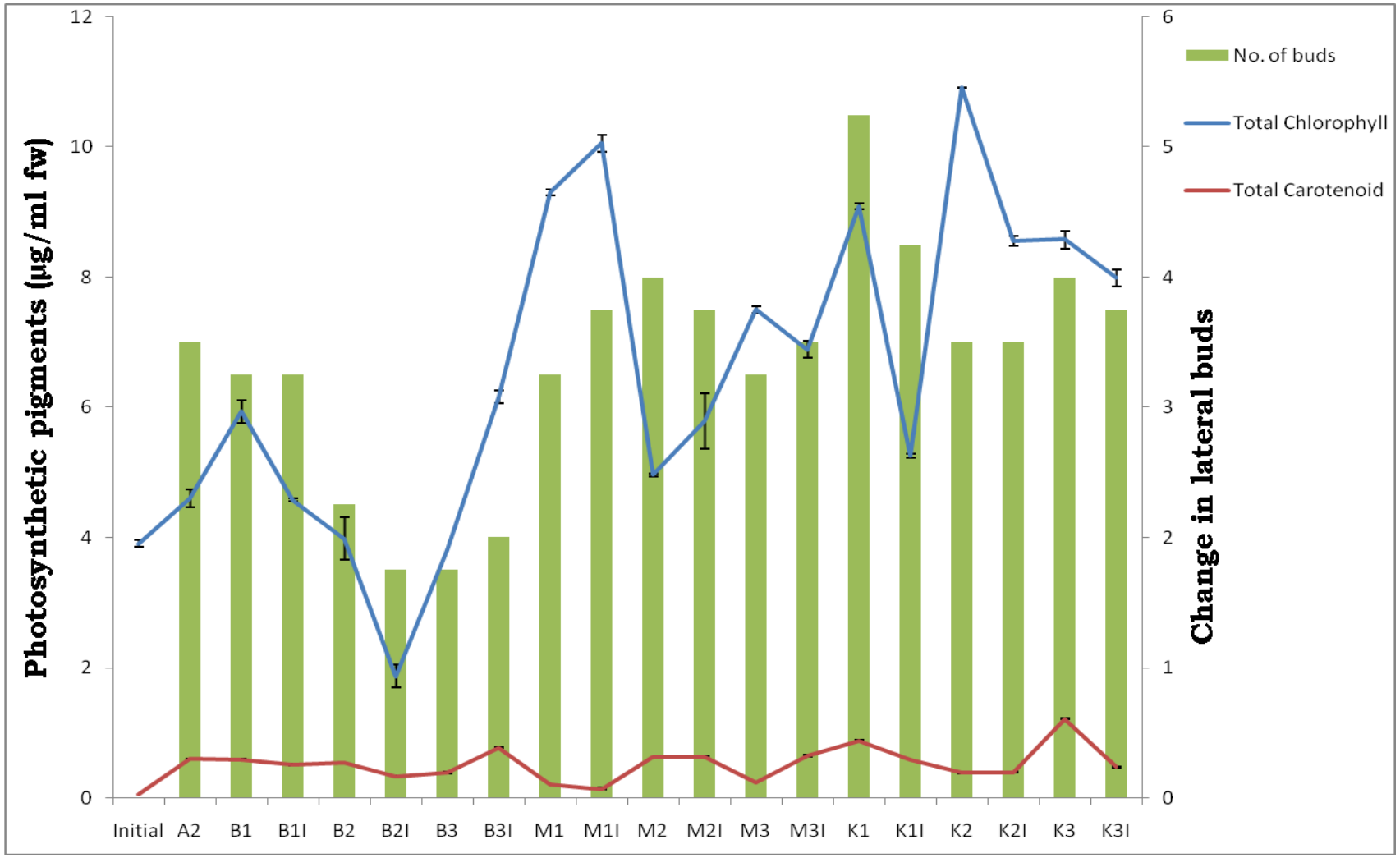
A_2 (control) - 100% FSAAS media; B_1, B_2 & B_3 and B_1I, B_2I & B_3I- A_2 media supplemented with 6-Benzylaminopurine and A_2 media supplemented with 6-Benzylaminopurine and IAA; M_1, M_2& M_3 andM_1I, M_2I & M_3I- A_2 media supplemented with with *meta*-topolin and A_2 media supplemented with *meta*-topolin and IAA; K_1, K_2& K_3 and K_1I, K_2I &K_3I- A_2 media supplemented with Kinetin and A_2 media supplemented with Kinetin and IAA.

Fig.1 Media effect on photosynthetic pigments and lateral bud development



Data are presented as means ± standard error (S.E.); S_1, S_2 and S_3 - 50%, 100% and 200% FSAS media respectively
A_1, A_2 and A_3 - 50%, 100% and 200% FSAAS media respectively

Fig.2 PGR's effect on photosynthetic pigments and lateral bud development



Data are presented as means ± standard error (S.E.); A_2 (control)- 100% FSAAS media; B_1, B_2&B_3 and B_1I, B_2I & B_3I- A_2 media supplemented with 6-Benzylaminopurine and A_2 media supplemented with 6-Benzylaminopurine and IAA; M_1,M_2& M_3 andM_1I, M_2I &M_3I- A_2 media supplemented with with *meta*-topolin and A_2 media supplemented with *meta*-topolin and IAA; K_1,K_2& K_3 andK_1I, K_2I &K_3I- A_2 media supplemented with Kinetin and A_2 media supplemented with Kinetin and IAA.

Salinity stress also reduced the lateral buds formation, when compared to the normal salinity media (S_2 and A_2). Lateral bud formation was more at lower salinities when compared with higher salinities in both the media (FSAS and FSAAS). Use of artificial media (A_2) promoted more lateral bud formation, when compared with the natural (S_2) media.

All the PGRs, either alone or in combination with IAA, promoted the growth of seaweed explants in A_2 media except B_3, B_3I and M_3 during the initial period and positive effect was shown throughout the entire period in the media supplemented with M_1I, K_1 and K_1I ($p<0.05$) (Table 3,4). Positive response with the addition of phytoregulators has been reported earlier (Hayashi *et al.,* 2008; Yong *et al.,* 2014a). Phtyoregulators, auxins and cytokinins affect growth in red seaweeds (Jennings, 1971; Fries, 1974; Fries and Iwasaki, 1976). All the PGRs used in the experiment showed effect at their lowest concentration suggesting that continuous stimulatory effect are possible at this concentration. M_1 in combination with IAA showed stimulatory effect for longer period. Combinations of PGRs enhanced growth in axenic cultures (Fries and Aberg, 1978, Bradley, 1990).Growth promotion of Kinetin on seaweed has been documented (Yokoya *et al.,* 2004). The results of the experiment showed Kinetin at 2.5 mg/L when added to artificial seawater media promote growth of explants under *in vitro* condition. Among the different combinations of PGRs used in A_2 media, both total chlorophyll and total carotenoid pigments of explants increased only with the addition of phytoregulators such as B_3I, M_2, M_2I, M_3I, K_1 and K_3 when compared with either initial content or control A_2 (Fig.2). The total chlorophyll pigment was found to be highest with the addition of K_2 while total carotenoid was highest in media supplemented with K_3. In relation to formation of lateral buds, A_2 media supplemented with K_1regenerated the highest no of buds. Our results were not corresponding to the results of Yokoya (2000) and Hayashi *et al.,* (2007), where direct regeneration was promoted by combinations of IAA and BAP. These effects may be due to the difference in the media or species of seaweed used in the study coupled with the combination of various PGRs synergizing with endogenous hormones in a different way.

The present study illustrated that axenic explants could be maintained in artificial seawater media for *in vitro* culture. The preliminary results also suggestuse of kinetin as a potential PGR for growth and regeneration in *in vitro* culture of *Gracilaria corticata* var. *cylindrica*. The combination of auxin and cytokinin was also found promising. Further studies need to be focused on the mechanism of action of PGRs for understanding their role in culture proliferation in seaweed species.

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