Original Research Article

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

A. Anuraj*1, Ajit Arun Waman2, Chandra Prakash3, S. Dam Roy4, M. Viji1, Manoj Baidya1 and N.K. Chadha3

1Division of Fisheries Science, 2Division of Horticulture and Forestry, ICAR-Central Island Agricultural Research Institute, Port Blair, India
3Division of Aquaculture, ICAR-Central Institute of Fisheries Education, Mumbai, India
4Director, ICAR-Central Island Agricultural Research Institute, Port Blair, India

*Corresponding author

**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 (A2) 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 A2 media. A2 medium supplemented with kinetin at different concentrations showed the highest total chlorophyll content (K2), highest total carotenoid content (K3) and better induction of lateral buds (K1). 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.

**Keywords** Rhodophyta, Seaweed, Media, PGR, Explants.

**Article Info**

Accepted: 28 August 2017
Available Online: 10 September 2017

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 (FAO, 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 as auxins 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 epiphytes followed by rinsing in filter sterilized (0.45 µ) seawater. Such explants were first treated with antibiotic mixture A3 (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 (B1, M1, K1), 5 mg/L (B2, M2, K2) and 7.5 mg/L (B3, M3, K3) 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 at the 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, Table 1-2). Natural
seawater (S2) promoted maximum increase in
weight of explants with highest DGR among
the three different FSAS media tested. Culture
of explants initiated in A2 established
themselves with highest DGR and also gained
maximum weight during subculture, while
explants showed maximum weight during the
first 15 d in A1 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.

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 A3. Irrespective of
the culture medium, chlorophyll content
reached maximum level at 30 ppt and was
found to be the highest in A2 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 |
|----------------------------|-------------------------|-----------------------|-----------------|---------------|-------------------------|---------|
| S1 (50% FSAS media)        | 0.14±0.06               | 0.15±0.04             | +12.54          | NS            | +0.06                   |         |
| S2 (100% FSAS media)       | 0.13±0.02               | 0.19±0.02             | +42.16          | *             | +2.81                   |         |
| S3 (200% FSAS media)       | 0.12±0.04               | 0.12±0.04             | +0.93           | NS            | +1.32                   | **      |
| A1 (50% FSAS media)        | 0.11±0.04               | 0.16±0.03             | +47.04          | *             | +0.84                   |         |
| A2 (100% FSAS media)       | 0.14±0.03               | 0.17±0.04             | +19.81          | *             | +3.14                   |         |
| A3 (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 |
|----------------------------|-------------------------|-----------------------|-----------------|---------------|-------------------------|---------|
| S1 (50% FSAS media)        | 0.15±0.04               | 0.20±0.02             | +9.48           | NS            | +0.63                   |         |
| S2 (100% FSAS media)       | 0.19±0.02               | 0.16±0.04             | +9.95           | *             | +0.66                   |         |
| S3 (200% FSAS media)       | 0.12±0.04               | 0.12±0.04             | -4.88           | NS            | -0.33                   | **      |
| A1 (50% FSAS media)        | 0.16±0.03               | 0.13±0.03             | -14.31          | NS            | -0.95                   | **      |
| A2 (100% FSAS media)       | 0.17±0.04               | 0.19±0.04             | +13.54          | *             | +0.90                   |         |
| A3 (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 Initial Weight (g) | Mean Final Weight (g) | % Weight Change | Paired t test | Daily Growth Rate (DGR) |
|-----------|------------------------|-----------------------|-----------------|--------------|------------------------|
| A2        | 0.14±0.005             | 0.17±0.015            | +26.21          | *            | +1.75                  |
| B1        | 0.09±0.016             | 0.17±0.015            | +35.54          | *            | +2.37                  |
| B1I       | 0.08±0.029             | 0.12±0.028            | +39.85          | *            | +2.66                  |
| B2        | 0.08±0.056             | 0.11±0.038            | +33.71          | *            | +2.25                  |
| B2I       | 0.11±0.035             | 0.12±0.072            | +38.75          | *            | +2.58                  |
| B3        | 0.12±0.055             | 0.15±0.04             | +20.67          | *            | +1.38                  |
| B3I       | 0.12±0.05              | 0.14±0.066            | +23.93          | *            | +1.60                  |
| M1        | 0.08±0.014             | 0.14±0.06             | +27.07          | *            | +1.80                  |
| M1I       | 0.11±0.037             | 0.11±0.018            | +40.15          | *            | +2.68                  |
| M2        | 0.10±0.011             | 0.14±0.045            | +31.04          | *            | +2.07                  |
| M2I       | 0.12±0.031             | 0.13±0.021            | +31.59          | *            | +2.11                  |
| M3        | 0.12±0.034             | 0.15±0.037            | +23.17          | *            | +1.54                  |
| M3I       | 0.10±0.025             | 0.16±0.034            | +39.28          | *            | +2.62                  |
| K1        | 0.09±0.035             | 0.13±0.017            | +32.75          | *            | +2.18                  |
| K1I       | 0.09±0.013             | 0.13±0.058            | +31.31          | *            | +2.08                  |
| K2        | 0.05±0.036             | 0.12±0.019            | +47.75          | *            | +3.18                  |
| K2I       | 0.09±0.016             | 0.10±0.033            | +44.19          | *            | +2.95                  |
| K3        | 0.12±0.018             | 0.13±0.022            | +45.90          | *            | +3.06                  |
| K3I       | 0.09±0.03              | 0.18±0.041            | +46.64          | *            | +3.11                  |

Data are presented as means ± standard error (S.E.); *Significant
A2 (control) - 100% FSAAS media; B1, B2 & B3 and B1I, B2I & B3I- A2 media supplemented with 6-Benzylaminopurine and A2 media supplemented with 6-Benzylaminopurine and IAA; M1, M2 & M3 and M1I, M2I & M3I- A2 media supplemented with with meta-topolin and A2 media supplemented with meta-topolin and IAA; K1, K2 & K3 and K1I, K2I & K3I- A2 media supplemented with Kinetin and A2 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
A2 (control) - 100% FSAAS media; B1, B2 & B3 and B1I, B2I & B3I- A2 media supplemented with 6-Benzylaminopurine and A2 media supplemented with 6-Benzylaminopurine and IAA; M1, M2 & M3 and M1I, M2I & M3I- A2 media supplemented with with meta-topolin and A2 media supplemented with meta-topolin and IAA; K1, K2 & K3 and K1I, K2I & K3I- A2 media supplemented with Kinetin and A2 media supplemented with Kinetin and IAA.

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

Data are presented as means ± standard error (S.E.); S₁, S₂ and S₃ - 50%, 100% and 200% FSAS media respectively. A₁, A₂ and A₃ - 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$_1$I, B$_2$I & B$_3$I- A$_2$ media supplemented with 6-Benzylationminopurine and A$_2$ media supplemented with 6-Benzylaminopurine and IAA; M$_1$, M$_2$& M$_3$ and M$_1$I, M$_2$I & M$_3$I- 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$_1$I, K$_2$I & K$_3$I- 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 (S2 and A2). Lateral bud formation was more at lower salinities when compared with higher salinities in both the media (FSAS and FSAAS). Use of artificial media (A2) promoted more lateral bud formation, when compared with the natural (S2) media.

All the PGRs, either alone or in combination with IAA, promoted the growth of seaweed explants in A2 media except B3, B3I and M3 during the initial period and positive effect was shown throughout the entire period in the media supplemented with M1I, K1 and K1I (p<0.05) (Table 3,4). Positive response with the addition of cytokinins has been reported earlier (Hayashi et al., 2008; Yong et al., 2014a). Cytokinin, 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. M1 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 A2 media, both total chlorophyll and total carotenoid pigments of explants increased only with the addition of cytokinins such as B3I, M2, M3I, K1 and K3 when compared with either initial content or control A2 (Fig.2). The total chlorophyll pigment was found to be highest with the addition of K2 while total carotenoid was highest in media supplemented with K3. In relation to formation of lateral buds, A2 media supplemented with K1 regenerated 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 suggeste 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.

Acknowledgements

Authors are thankful to the Indian Council of Agricultural Research, New Delhi for providing financial assistance for carrying out this work. First author also acknowledge helpful suggestions and research facilities provided by Dr. Pooja Bohra, Scientist, Division of Horticulture and Forestry, ICAR-CIARI, Port Blair.

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How to cite this article:

Anuraj, A., Ajit Arun Waman, Chandra Prakash, S. Dam Roy, M. Viji, Manoj Baidya and Chadha, N.K. 2017. Investigating the Effects of Exogenous Factors on Growth, Photosynthetic Pigments and Bud Induction in Gracilaria corticata var. cylindrica under In vitro Conditions. Int.J.Curr.Microbiol.App.Sci. 6(9): 3235-3246. doi: https://doi.org/10.20546/ijemas.2017.609.398