Research Article

An Efficient *In Vitro* Regeneration and *Ex Vitro* Rooting of *Ceropegia thwaitesii*: An Endemic Species from Western Ghats

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ABSTRACT

The aim of this study was to optimize indirect organogenesis and plant regeneration of endemic medicinal plant *Ceropegia thwaitesii*. Internode and young leaf found to be the most suitable explant for culture establishment and further regeneration. Callus was induced from Internode explants on MS medium containing 3.0 mg/l of 2,4-D. Proliferation of callus was observed on MS + 0.3 mg/l of 2,4-D and 0.3 mg/l of NAA. The shoot regeneration was achieved when proliferated callus was transferred to MS medium supplemented with BA either alone or in combination with IBA or NAA. Maximum numbers (19.43) of shoots were regenerated on MS medium containing 1.0 mg/l BA and 0.1 mg/l NAA. Shoots were successfully rooted in *in vitro* and *ex vitro* and the rooted plantlets survived and grew normally. This protocol for *in vitro* plant regeneration provides a tool not only for vegetative propagation but also for plant genetic transformation and gene function studies of *C. thwaitesii*.

Keywords: *Ceropegia thwaitesii*, organogenesis, explants, seaweed extracts, *ex vitro* rooting.

INTRODUCTION

The genus Ceropegia L. (Apocynaceae, APG III[1]) is the largest genus of flowering family with comprises about 200 species distributed in tropical and sub-tropical regions.[2] It is represented by about 50 spp. in India of which 35 spp. exists alone in the Western Ghats.[3] Ceropegias are highly noticeable due do their edible tubers and their medicinal purposes.

The Ceropegia species are much importance in industry due to their presence of starch, sugars, albuminoids, carbohydrates and etc.[4,5] The cerpegin pyridine alkaloid isolated from root tubers of Ceropegia exhibited promising hepatoprotective, antipyretic, anti -ulcer etc.[6,7] Numerous species are described as rare and endangered due to over exploitation so recent scenario integrated programs are required to prevent and protect the current biodiversity.[8]

Regeneration via indirect organogenesis is generally preferable to direct regeneration in plant transformation efforts, as effective selection of a homogeneous transgenic plant is easily attainable. The indirect regeneration (IR) of adventitious shoots is an alternative method to somatic embryogenesis in obtaining whole plant regeneration of explants. There are some reports on adventitious regeneration from various tissues in Ceropegia.[9-11] Shoot regeneration has also achieved from Thin Cell Layers (TCL).[12] This paper presents a plant regeneration protocol via indirect organogenesis for *C. thwaitesii* which will be helpful in Ceropegia tissue culture and gene function studies.

MATERIALS AND METHODS

Establishment of explant source

To establish the experimental plants, the nodal explants were cultured on the MS medium containing KIN (3.0mg/l) + IAA (5.0mg/l), in addition to 30 g/l sucrose and 8 g/l agar. As earlier a detailed method for the optimized culture initiation from nodal explants of *Ceropegia thwaitesii* has been reported by.[13] Adventitious shoot cultures were subcultured on the fresh initiation medium every 4-5weeks. The regenerated adventitious shoot clusters from the *in vitro* cultures were maintained and proliferated on the initiation medium for about 2 months. For experimental purpose these shoot cultures were maintained in MS basal medium up to 5-6 months. Explants were collected from these stabilized mature plants.

Callus Induction and proliferation

To initiate the callus from young leaf (YL) and internode explants, *in vitro*-regenerated shoots were maintained for nearly 5-6 months on the BCM (Basic culture medium) were used as source material. Semi mature leaf and internode were excised and abaxial sides in contact with the medium. The BCM supplemented with 3% (w/v) sucrose at different concentrations of 2,4-D, TDZ and Pic at 0.1, 0.3, 0.5, 0.7, 1.0, 2.0 and 3.0mg/l were tested in the callus induction experiment in combination with auxins NAA, IBA, and IAA at 0.1, 0.3, 0.5, 0.7, 1.0, 2.0 and 3.0 mg/l. These cultures were incubated at 26±2°C in 16/8h light incubation. Callus was induced in wounded region of the fragments after culturing for 2 weeks. Callus response and callus size were determined 90days after culture. Callus response was calculated as the percentage of the number of wounded region forming callus. Each

treatment contained at least 10 explants and the experiment was repeated two times.

Adventitious bud differentiation and shoot development

Calli produced on callus induction medium (CIM) containing 2, 4-D (0.3mg/l) and NAA (0.3mg/l) with 30g/l sucrose were used to evaluate medium for inducing shoot formation. Three-month-old calli were selected randomly and cultured on shoot development medium (BSM) which was a modified BCM containing cytokinins BA, KIN and TDZ at 0.1, 0.3, 0.5, 0.7, 1.0, 2.0 and 3.0 mg/l. Calli were cultured in BSM. There was one callus per tube, 10 tubes per replicate for each treatment. Cultures were kept under the same condition as described above. After 2 months of further culture, the adventitious bud differentiation was determined as the percentage of the number of calli with buds. The total number of shoots developed per callus was recorded. In addition, for shoot multiplication optimized concentrations of cytokinins were subjected with auxins like IAA, IBA and NAA at 0.1, 0.5, 1.0 and 2.0. These shoots were later used for rooting observation.

Effect seaweeds

Preparation of seaweed extracts

Enteromorpha prolifera, Enteromorpha intestinalis and *Padina tetrastromatica*, were collected by handpicking during low tide from Hare Island in the Gulf of Mannar of Tuticorin coast (08°46'25.15" N lat., 78°11'16.05" E long.) during 2012. The seaweeds were cleaned with distilled water to remove impurities and epiphytes and then shade dried. The shade-dried algae were finely chopped and powdered. About 500g of seaweeds were boiled in sterile distilled water for 50 min. Then the extracts were initially filtered through a muslin cloth and then filtered through Whatman No1 filter paper and stored at 4°C for further experimental studies.

Seaweed liquid extracts like *E. prolifera*, *E. intestinalis* and *P. tetrastromatica*, 25, 50, 100 ml/l were added based on the response observed in previous experiments to the shoot multiplication medium (BA 1.0+ NAA 0.1 mg/l) to assess the enhancement of shoot multiplication rate, but it was fail to induce the shoot bud, so in other hand it was carried out for enhance its shoot elongation rate. For shoot elongation, each treatment was replicated twice and each replicate consisted of at least 10 explants. 12 weeks after the culturing, shoot length were recorded.

***In vitro* rooting**

In order to induce high-frequency *in vitro* rooting, healthy elongated shoots were tested in auxins (NAA, IAA and IBA) at 0.1, 0.3, 0.5, 0.7,1.0 and 3.0 mg/l and Seaweed liquid extracts like *E. prolifera*, *E. intestinalis* and *P. tetrastromatica* at 5, 25, 50, 100 ml/l also tested, MS medium without any plant growth regulators as a control. The pH of the medium was adjusted to 5.7±2 before autoclaving for 15 min at 121°C. The plantlets were maintained at 26 ± 2°C, 16 h photoperiod, 80 to 85% relative humidity and 60 μmol^{-2} sec^{-1} light intensity. The successfully rooted plantlets were transferred to acclimatization.

***Ex vitro* rooting in regenerated shoots**

To induce roots under *ex vitro* conditions, individual regenerated shoots excised from parent cultures were pulse treated with various freshly prepared auxins (IAA, NAA, and IBA-50, 100, 200 and 300 mg/l) for different time durations (1–5 min). These were transferred to sterilized 1:1:1 (w/w/w) red soil to sand and coconut coir mixture and enriched with MS liquid medium.

Acclimatization and Hardening

Plantlets developed from *in vitro* and *ex vitro* rooting were placed on the tissue paper to remove the water and to reduce the contamination during potting. These plants were planted in 3-cm paper cups containing a sterile 1:1:1 (w/w/w) red soil to sand and coconut coir mixture, enriched with MS salt solution, covered with a polythene bag to maintain the high humidity, and plantlets were maintained at 26 ± 2°C, 16 h photoperiod, 80 to 85% relative humidity and 60 μmol^{-2} sec^{-1} light intensity. The paper cups were covered with transparent polythene cover to maintain humidity until the plantlet survived. Then the paper cups were transferred to green house and the polythene covers removed. Hardened plants were transferred to pots containing mixture of red soil mixed sand and forest humus and coconut coir (1:1:1:1). The pots were watered on dry and pour method under greenhouse condition.

Experimental Design and Data Collection

All the experiments were conducted using the completely randomized block design. Comparisons between treatments were made with Duncan's new multiple range test (DMRT) (Using SPSS software Version18) at p=0.05.

RESULTS AND DISCUSSION

Callus induction and proliferation

In vitro propagation efficiency differs due to variation in parameters such as concentrations of plant growth regulators and other supplements added to culture media.[14] A number of preliminary experiments revealed that the PGRs were essential for the induction of callus from the internode and YL explants and no callus was induced by basal MS medium supplemented with IAA and IBA. A series of attempts were made to initiate the shoots from the primary callus using the individual cytokinins. It was noticed that the tested cytokinins failed to initiate the shoots from the callus and the callus explant darkened within few days. Scarcity of explant in order to obtain the alternate source of explant for shoot regeneration, axenic cultures derived by the protocol was used as explant and this is the first report on organogenesis of *C. thwaitesii*.[13] In general, the explants derived from the micropropagated shoots have an early and greater capacity for morphogenesis than the tissue

excised from the field plants,[15] which is attributed to the absence of lag period between the explanting and adaptation to *in vitro* conditions. The presence of smaller yet active meristematic centers of the microplants compared to relatively larger but quiescent meristems of the shoots of mature plants might be the reason for the successful regeneration of the explants from the micropropagated shoots in the present report.[16] The internode and YL explants from the axenic shoots served as the explants for the induction of organogenic callus. The callus initiation was observed on the cut region of YL and internodes 10-15 days after the culture incubation. The induction of callus was mainly influenced by the 2, 4-D, TDZ and Pic and different concentration used (Table 1 Fig. 1a &b). With increasing concentrations of 2, 4-D the callus induction rates were increased significantly. Of the different PGRs, 2, 4-D yielded better results for callus initiation. Initially, all the calli were light transparent white in color with soft, friable and unorganized morphology. This callus was sub cultured every two weeks for 60 days on to fresh medium of the same composition after which the callus showed varied nature. 2, 4-D and BA are the most frequently used cytokinins to induce regeneration, but their efficiency depends on genotype and other factors.[17] The role of 2, 4-D in the regulation of morphogenetic potential was reviewed,[18] and stated that 2, 4-D is closely related to the metabolism and action of purine-based cytokinins and auxins. 2, 4-D has been shown to stimulate shoot organogenesis and somatic embryogenesis.[19] It has been used in regeneration of Ceropegia by several research groups. Increasing the callus amount 2, 4-D 0.3mg/l subjected to different types of auxins. Among the tested auxins, NAA 0.3mg/l gives six fold of the total weight of individual callus obtained. Most of the calli became yellow to green compact organogenic in nature after a total of 90 days (Fig. 1c & d). Only green compact callus was investigated further and reported. The mean percentage of callus induction of internode ranged from 36.0-100% while the YL ranged from 24.6 – 100% based on the concentration of auxins used (Table 2). Maximum percentage of the organogenic callus was obtained on MS medium supplemented with 2, 4-D with NAA at 0.3+0.3 mg/l. IAA and IBA had no significant effects in individual and combination with 2, 4-D in terms of increasing callus percentage. In contrast, NAA had a positive effect in combination with 2, 4-D and significantly increased the callusing rate. The results also reveal that 2, 4-D with NAA had significant effects on callus proliferation. The combined effect of 2, 4-D and NAA has been reported recently in *Achyranthes aspera*[20] and *Leptadenia reticulate.*[21] Internode explants yielded a mass of compact green callus, whereas YL segments produced white, soft callus with a smooth, wet-looking surface. Of the 2 explant types, internode produced the maximum callusing rate followed by YL segments. However, many factors such as composition of the nutrient medium, and physical growth factors like light, temperature, humidity, and endogenous supply of growth regulators are important for callus induction.[22] Several reports have been published about the effects of plant growth regulators on callus culture in different plants such as *C. santapaui, Hoya wightii* ssp. *palniensis, C. pusilla* and *C. bulbosa*.[23-25] In the present study, it was observed that 2, 4-D with NAA was the best combination for callus induction and proliferation of *C. thwaitesii.*

Effect of Cytokinins on Shoot Regeneration

Ninety days old organogenic callus obtained from the CIM was transferred to the BSM containing MS basal medium supplemented with BA, KIN and TDZ. For the Internode and YL derived callus, a highest number of responses of shoot regeneration was obtained when the culture medium was supplemented with BA 1.0 mg/l (97%) with 6.4 shoots/explant and 86.3% with 6.14 shoots/explants respectively (Table 3 and Fig. 1e & f). BA has been used most frequently for the *in vitro* shoot regeneration and multiplication studies of many plants. It showed higher amount of cytokinins activity by promoting the shoot multiplication and growth of internode and leaf of *Aconitum violaceum* and *Aesculus hippocastanum*.[26,27] This result was reflected in the present experiment also. Cytokinins play a multiple role in the control of plant development. Some are present in t-RNA molecules and it activates RNA synthesis and the activities of some enzymes. The superior result of BA over other cytokinins for shoot bud formation has been attributed to the abilities of plant synthetic growth regulators and degrades the cytokinins into zeatin endogenously. The MS medium supplemented with KIN also showed marked influence on shoot bud formation but the number of shoots formed was lower than on BA amended medium. This could be due to the fact that the KIN was less effective than the BA in the formation of multiple shoot induction. TDZ fails to induce shoot bud on both the explants used. The difference in the number of shoots formed in the internode and YL explant could be a result of difference in the regeneration potential of different explants, which was attributed by the physiological condition, age and cellular differentiation among the constituent cells.[28] Among the explants tested, the internode explant showed high morphogenic efficiency. The efficiency of the internode callus over the leaf callus might be due to the passage of some internode components from the pre-existing axillary buds that were essential to evoke the caulogenesis. Moreover, internodes contained sufficient cytokinins at the time of excision for the adventitious shoot production on a medium, and that the response is species and genotype dependent.[29]

Effect of Auxin on Shoot Multiplication

Cytokinins are very effective in promoting direct or indirect shoot initiation. A balance between auxin and cytokinin normally gives the most effective on organogenesis. Cytokinins together with auxins take part in the regulation of the cell cycle in plant cells. The effect of different auxins (IAA, IBA and NAA) was tested on

shoot multiplication in the BCM containing BA (1.0 mg/l). The MS basal medium amended with BA 1.0 mg/l and NAA 0.1 mg/l showed high percentage of response on internode and YL explants.

Table 1: Callus induction of *Ceropegia thwaitesii*, from leaf and internode explant on MS medium supplemented with different concentrations of PGRs, after 60days of culture.

Plant growth regulators	Percentage of response		Nature and colour of the callus	
	Internode	Leaf	Internode	Leaf
IAA and IBA	No Response			
2,4-D 0.1	74.33±0.66e	70.66±0.33de	LGC	LGC
0.3	82.00±0.57c	76.66±1.20c	LGC	LGC
0.5	82.66±0.33c	87.00±0.57b	GC	GC
0.7	74.00±0.57e	69.00±0.57e	GC	GC
1.0	100.00±0.00a	99.66±0.33ab	GC	GC
2.0	100.00±0.00a	98.33±0.88ab	GC	GC
3.0	100.00±0.00a	100.00±0.00a	GC	GC
TDZ 0.1	77.00±0.57de	72.00±0.57de	YC	YC
0.3	82.00±0.57c	76.33±0.88c	YC	YC
0.5	98.00±0.57ab	99.00±0.57ab	YC	YC
0.7	97.33±0.33ab	99.33±0.33ab	YC	YC
1.0	93.66±1.45b	99.66±0.33ab	YC	BF
2.0	73.66±2.18ef	78.66±0.88c	YC	BF
3.0	62.66±2.18g	72.33±0.33d	YC	BF
Pic 0.1	34.00±0.57j	24.00±0.57i	LG	BF
0.3	42.66±0.33i	26.00±0.57i	LG	BF
0.5	43.66±1.33i	34.66±0.33h	YC	BF
0.7	49.66±0.88h	32.66±3.84h	YC	BF
1.0	62.66±1.20	34.00±0.57h	YC	BF
2.0	70.33±3.28f	39.00±0.57g	RF	BF
3.0	79.00±1.52cd	55.00±0.57f	RGC	BF

BF- Brown Friable; GC- Green compact; LGC- Light Green Compact; RF- Red friable RGC- Red Green Compact; Yellow compact

Table 2: Callus proliferation and multiplication of *Ceropegia thwaitesii*, from leaf and internode explant on MS+2, 4-D (0.3mg/l) in combination with auxins, after 90days of culture

Plant growth regulators	Percentage of response		Fresh weight (g)		Nature and colour of the callus	
	Internode	Leaf	Internode	Leaf	Internode	Leaf
NAA0.1	84.00±0.57c	83.00±0.57c	2.7±0.63cd	2.67±0.52fg	GC	GC
0.3	**100.00±0.00a**	**100.00±0.00a**	**6.87±0.45a**	**6.35±0.50a**	**GC**	**GC**
0.5	98.66±1.33ab	98.33±0.88b	5.65±0.37b	5.80±0.20ab	GC	GC
0.7	99.00±1.00b	99.66±0.31ab	5.03±0.75b	5.18±0.50bc	LG	LG
1.0	99.33±0.66b	97.66±1.20bc	4.81±0.51b	4.41±0.63cd	LG	LG
2.0	89.33±0.33b	85.00±0.47bc	0.36±0.01i	0.46±0.57i	YG	YG
3.0	81.33±1.85cd	77.00±0.57cd	1.66±0.13gh	1.48±0.13hi	YG	YG
IBA0.1	79.33±0.33de	35.00±0.57k	1.21±0.40hi	1.07±0.36i	WF	WF
0.3	78.00±0.57e	58.33±0.88ef	2.67±0.30cde	2.66±0.29fg	WF	WF
0.5	99.00±0.57b	63.33±0.88de	1.75±0.24fg	1.56±0.28hi	WF	WF
0.7	87.66±0.57ab	24.66±2.40l	2.67±0.34cde	2.91±0.46ef	WF	WF
1.0	88.00±0.88ab	47.66±0.83gh	0.76±0.05hi	0.69±0.09i	WF	WF
2.0	72.00±1.15f	57.33±0.48f	0.63±0.12hi	0.90±0.17i	WF	WF
3.0	51.00±0.57g	52.00±0.37g	0.58±0.07hi	0.88±0.11i	WF	WF
IAA0.1	42.33±0.57h	81.00±7.02bc	2.21±0.25f	2.32±0.25gh	RGC	WF
0.3	87.66±0.33b	37.00±1.15jk	2.29±0.19cde	2.50±0.26gh	RGC	WF
0.5	36.00±2.84j	38.33±1.20jk	2.47±0.29cde	3.78±0.31de	RGC	WF
0.7	38.00±0.57ij	41.33±1.20ij	2.33±0.35cde	3.09±0.65ef	BF	YC
1.0	40.33±1.45hi	68.33±1.20d	2.32±0.14cde	3.06±0.29ef	YC	YC
2.0	72.00±0.33f	86.66±1.20ab	3.33±0.12c	3.40±0.15de	YC	YC
3.0	42.33±0.57h	43.66±0.15hi	1.55±0.17gh	3.13±0.60ef	YC	YC

BF- Brown Friable; GC- Green compact; LG- Light Green; RGC- Reddish Green Compact; WF- White Friable; Yellow compact

Table 3: Shoot regeneration from callus of *Ceropegia thwaitesii* on MS medium supplemented with different concentrations of cytokinins, after 90days of culture

Plant growth regulators	% of explants forming shoots		No. of shoots/explants		Shoot length (cm)	
	Internode	Leaf	Internode	Leaf	Internode	Leaf
TDZ	No response					
BA						
0.1	34.00±0.47i	32.33±1.45f	1.71±0.28i	2.00±0.21f	0.22±0.02f	0.40±0.04g
0.3	36.66±0.31i	33.00±0.52f	1.81±0.26h	2.28±0.18e	0.44±0.12e	0.62±0.11f
0.5	47.00±0.57g	43.33±0.83e	2.28±0.18g	2.28±0.18	0.54±0.05de	1.04±0.16cd
0.7	81.33±2.02c	74.66±1.20b	4.28±0.35b	4.28±0.39b	0.50±0.04de	0.80±0.10
1.0	**97.00±1.52a**	**86.33±0.76a**	**6.42±0.40a**	**6.14±0.29a**	**2.15±0.22a**	**2.81±0.25a**
2.0	75.33±1.02c	64.33±0.31c	3.42±0.42cd	3.71±0.28bc	1.92±0.11ab	2.37±0.31ab
3.0	68.00±0.31de	56.66±0.88dc	3.00±0.30de	3.14±0.34cd	1.44±0.06c	1.51±0.13c
KIN						
0.1	42.00±0.47h	43.66±0.66e	1.85±0.26h	2.28±0.28de	0.50±0.04de	0.75±0.11e
0.3	64.66±0.20e	64.33±1.76c	2.57±0.29efg	2.71±0.28de	0.51±0.06de	0.70±0.12e
0.5	53.00±0.20f	52.33±1.85d	2.42±0.36fg	2.85±0.26de	0.65±0.07d	0.85±0.07de
0.7	72.33±0.33cd	73.66±2.10b	3.57±0.36cd	3.85±0.34bc	0.60±0.06de	0.87±0.07de
1.0	84.00±1.00b	74.66±0.02b	4.00±0.53bc	4.71±0.35b	1.92±0.21ab	2.17±0.29b
2.0	81.66±0.40b	79.66±5.48b	5.71±0.18a	5.58±0.35a	1.74±0.19cd	2.40±0.29ab
3.0	63.33±0.88e	66.00±0.57c	2.57±0.36ef	3.85±0.63bc	1.58±0.13cd	2.21±0.21b

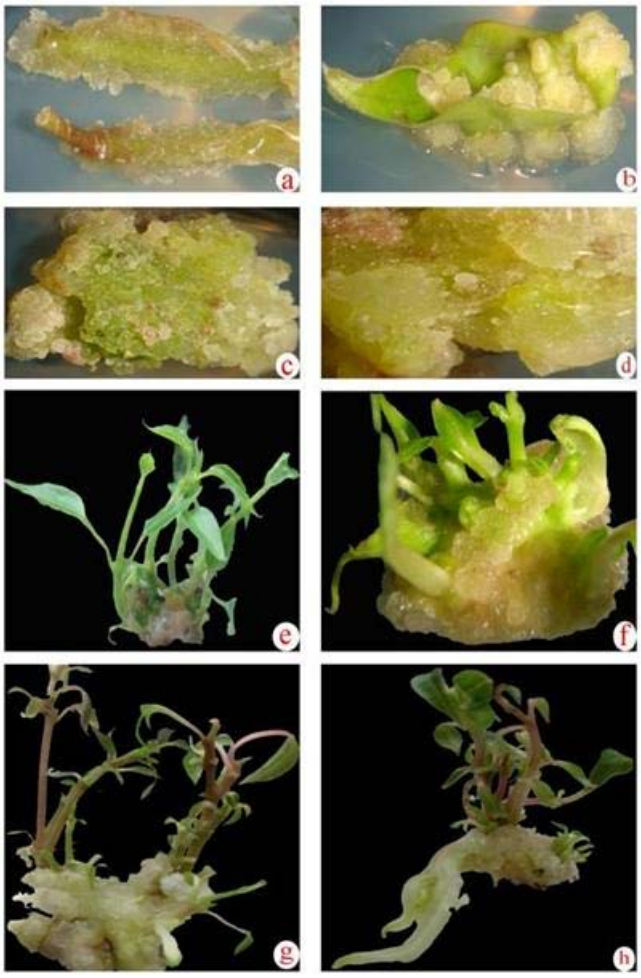

Figure 1: Indirect organogenesis of *Ceropegia thwaitesii*

(a & b), internode & leaf explant; (c &d), callus proliferation (2,4-D+NAA 0.3+0.3mg/l); (e & f), shoot induction (BA 1.0mg/l); (g & h), shoot multiplication (BA+NAA 1.0+0.1mg/l).

A superior response for the number of shoots 19.4 and 17.1 from the internode and YL explants was observed on the MS medium incorporated with BA 1.0 mg/l + NAA (0.1 mg/l) (Table 4 Fig. 1g & h). The superiority of the NAA over IAA and IBA on shoot regeneration through organogenesis has also been reported in some species, including *Eclipta alba*; *Digitalis lamarckii*; *Begonia rex*; *Acacia mearnsii* and *Rehmannia elata*.[30-34] Among the explants tested, Internode explants showed high response in the BCM supplemented with BA with NAA showed a better shoot induction response as compared to IBA and IAA. Explants treated with NAA showed the best response in shoot induction. A total of 19.4 shoots/explants of shoot induction were observed in the BSM containing 0.1 mg/l of NAA in internode explants, whereas YL explant showed 17.2 shoots/explants. Using different explants for organogenesis, stem explants only shows high response in shoot induction of *Labisia pumila*.[35] IBA showed minimum numbers and also the quality of shoots was not desirable. The IAA supplementation on MS+BA 1.0 mg/l was failed to induce shoot multiplication. IAA are stable and persist, in the media IAA is less stable and is especially sensitive to the light and to oxidants, probably due to its instability, it is usually less effective than other auxins.[36]

Effect of seaweed extracts

For the shoot multiplication, the regenerated shoots were transferred to different seaweed extracts at different concentration in shoot regeneration medium (MS+BA 1.0 mg/l +NAA 0.1 mg/l). But, the seaweed extracts fails to increase the shoot number. So in other hand regenerated shoots were tested for shoot elongation. The MS medium supplemented with 50ml/l of *E. intestinalis* extract recorded a (14.26 and 12.82 cm) shoot length were

observed in internode and YL explant respectively and *E. prolifera* and *P. tetrastromatica* also response in shoot elongation (Fig. 2). Recently reported seaweed extracts act as biostimulant and increase the shoot length on *Lycopersicon esculentum.*[37] The beneficial effect of the seaweeds extracts on growth and elongation of plants may be due to the presence of plant growth promoting hormones like cytokinins and auxins in the seaweeds.[38,39] Recently various cytokinins were identified in seaweed extracts.[40] In the present study we found that various seaweed extracts have a different effectiveness towards the shoot elongation.

In vitro Rhizogenesis

Different explants responded differently to the PGRs. Among the explants used in the present study, internode explant was found to be the suitable explant in adventitious root induction. This was demonstrated in terms of higher percentage of rooting, average number of roots and the average length of root formed on MS medium supplemented with auxins and seaweed extracts at various concentrations. Though roots were successfully induced from the shoots regenerated from YL explants, the frequency and amount of roots induced were relatively low when compared with the internode explants. It was observed that, root generation always took place near the cut exterior of the basal ends of the leaf and stem explants and roots grew into the air column above the medium. This might be due to the strong polarity in the regeneration of roots.[41] Among the different types of auxins and seaweed extracts used, seaweed extracts were successful in inducing rooting response (Table 5).

Table 4: Shoot regeneration from callus of *Ceropegia thwaitesii* on MS medium supplemented MS+BA (1.0mg/l) in combination with auxins, after 90days of culture

Plant growth regulators	No. of shoots/explants		Shoot length (cm)	
	Internode	Leaf	Internode	Leaf
IAA	No response			
NAA				
0.1	19.43±0.84a	17.14±0.70a	2.99±0.14a	3.94±0.21a
0.5	13.43±0.97bc	13.86±0.45b	1.77±0.46b	2.40±0.93b
1.0	12.14±0.14cd	11.57±0.20d	1.54±0.09bc	1.27±0.05cd
2.0	11.86±0.14d	11.43±0.20e	1.39±0.06cd	0.77±0.10e
IBA				
0.1	11.71±0.18e	11.57±0.20c	1.14±0.22d	1.09±0.19d
0.5	11.29±0.18f	11.57±0.20c	0.61±0.19f	0.69±0.07f
1.0	12.00±0.00cd	12.14±0.14c	0.91±0.05e	1.86±0.02c
2.0	13.86±0.50b	13.71±0.39b	1.14±0.08cd	1.03±0.28d

Table 5: Rhizogensis of *Ceropegia thwaitesii* on MS medium supplemented with different concentrations of auxins, after 60days

Plant growth regulators	Days for root induction	No. of roots		Root length(cm)	
		Internode	Leaf	Internode	leaf
IAA					
0.1	33-35	0.57±0.29gh	0.57±0.20g	0.60±0.28e	0.70±0.25ef
0.3	32-34	0.43±0.20g	0.42±0.20h	0.52±0.24e	0.52±0.24f
0.5	29-32	0.86±0.40fg	1.14±0.20f	0.65±0.31de	0.78±0.28de
0.7	27-30	0.57±0.20h	1.42±0.33ef	0.68±0.24cde	0.97±0.25d
1.0	25-27	1.14±0.40fg	1.14±0.40ef	0.71±0.26cde	0.45±0.16gh
3.0	25-27	1.71±0.28ef	1.42±0.36ef	0.60±0.10de	0.47±0.12fg
IBA					
0.1	25-27	1.86±0.34ef	2.00±0.37de	0.74±0.14cde	0.95±0.19de
0.3	23-25	4.89±0.47b	4.25±0.40b	3.01±0.21b	2.78±0.36a
0.5	21-23	**5.53±0.29a**	**5.27±0.42a**	**3.31±0.32a**	**2.87±0.22a**
0.7	23-25	3.86±0.45c	3.28±0.35c	1.88±0.14ab	1.65±0.03ab
1.0	23-25	3.86±0.45c	3.85±0.45c	2.17±0.21ab	1.42±0.09bab
3.0	25-27	0.57±0.29	0.85±0.34fg	0.34±0.14	0.32±013
NAA					
0.1	27-29	0.43±0.20gh	0.42±0.20h	0.45±0.06	1.25±0.81cd
0.3	28-30	2.57±0.36de	2.00±0.37de	1.27±0.57c	0.52±0.14e
0.5	30-33	3.00±0.37cd	2.28±0.28d	1.25±0.05cd	0.65±0.12e
0.7	32-33	1.86±0.14ef	2.28±0.28d	0.74±0.05cd	0.60±0.08e
1.0	33-35	1.86±0.14ef	1.71±0.18ef	0.75.06cd	0.47±0.07fg
3.0	33-35	0.57±0.20g	0.57±0.20g	0.48±0.17f	0.44±0.16h

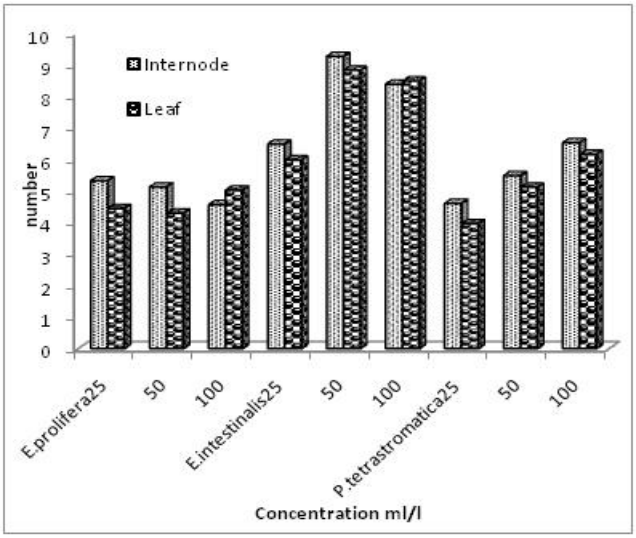

Figure 2: Effect of seaweeds on shoot elongation of *in vitro* regenerated shoots

Based on the results obtained, the highest number (9.0 and 7.4) of roots/shoots resulted from internode and YL explants in the medium supplemented with *E. intestinalis* at 50 ml/l. Whereas in the media supplemented with IBA 0.5mg/L roots/explants were 6.53 and 5.27 roots/shoots respectively internode and YL explants (Fig. 3a & b). The percentage and number of roots were declined in the media contain IAA and *E. phaprolifera.* Data also showed that low concentration of IAA and high concentration of NAA inhibited the growth of roots. Likewise in the media contain *E. phaprolifera* and *P. tetrastromatica* also too. Roots formed from internode explants with *E. intestinalis* appeared as whitish, strong, and healthy. The use of internode explants in tissue culture has the advantage of being a system where phytochromes can be easily manipulated to direct pluripotent cells to a particular cell fate.[42]

The ability of producing its endogenous auxins might also contribute to the more effective adventitious root formation from the internode than that of YL explants which lack this ability. [43] Among the different types of PGRs and seaweed extracts tested, only *E. intestinalis* and IBA were successful in showing a positive rooting response. Among four different concentrations of *E. intestinalis* tested, the highest percentage of roots was induced from internode explants in the medium supplemented with 50ml/l (Table 6).

Figure 3: Rooting and Hardening of indirect organogenesis: (a), *in vitro* rooting IBA 0.5mg/l; (b), effect of seaweed *E. intestinalis* 50ml/l; (c), *ex vitro* rooting IBA 200mg/l; (d), enlarge portion; (e), Hardening

Table 6: Rhizogensis of *Ceropegia thwaitesii* on MS medium supplemented with different concentrations of seaweeds, after 60days

PGRs (ml/l)	No. of roots/explants		Root length (cm)	
	Internode	Leaf	Internode	Leaf
Enteromorpha prolifera				
5	1.60±0.74bc	0.80±0.20e	1.56±0.88cd	0.18±f
25	1.20±1.20e	2.00±0.70cd	0.86±0.86f	0.28±e
50	1.20±0.48e	1.60±0.50de	2.48±0.82b	0.50±cd
100	1.60±0.60bc	2.40±0.67cd	2.02±0.52c	0.48±d
Enteromorpha intestinalis				
5	2.40±1.16bc	2.60±0.50c	1.34±0.54d	1.04±b
25	4.00±0.89b	1.60±0.24de	1.38±0.08d	1.00±bc
50	**7.00±0.70a**	**6.40±0.50a**	**2.68±0.06a**	**2.72±a**
100	3.00±0.70bc	4.00±0.44b	2.00±0.08c	1.16±b
Padina tetrastromatica				
5	1.40±0.50c	1.60±0.40de	1.82±0.33cd	0.62±cd
25	1.20±0.37d	1.60±0.04de	1.04±0.08e	1.02±bc
50	3.20±0.48bc	2.20±0.37cd	2.56±0.80ab	0.96±bc
100	1.80±1.11bc	2.00±0.54cd	0.68±0.41f	0.42±d

Apart from *E. intestinalis* and IBA, internode and YL explants in the MS medium containing other seaweed extracts and IAA, NAA also showed rooting response. The percentage of root formation from the YL explants declined as the concentration of IAA increased from 1 to 3 mg/l. Besides that, higher concentrations (1 and 3 mg/l) of NAA inhibited the growth of roots from the both explants. This was evidenced by[44] who mentioned that endogenous IAA was detected in root explants on media supplemented with NAA. Furthermore, more energy may be needed by the explants to convert the absorbed synthetic NAA from the medium to a natural form of IAA before being used by the explants. This condition could likely explain the low efficiency in root induction on explants placed in medium added with NAA. Whitish, strong and healthy roots were formed from the shoots regenerated from internode and YL explants in the medium with *E. intestinalis* 50ml/l and IBA 0.5mg/l. Studies by[11] found that internode explants from *in vitro* culture could induce root formation when treated with IBA. Similar report has been reported in some plant species such as *Jatropha curcas*; *Simmondsia chinensis* and *Aerva lanata*.[45-47] The higher concentration of NAA and IAA declined the rooting. This could be explained by the fact that auxins at high concentration may possess herbicidal properties which inhibit the adventitious root induction.[48] IBA was believed to be the most suitable PGR in inducing roots from explants. Thus, results of this study showed that IBA was more effective than NAA in adventitious root induction from *C.thwaitesii*. Although internode explants are capable of inducing adventitious roots. It was found that *E. intestinalis* was a much better than PGR used in inducing roots. In general biostimulants are capable of raising the root volume of plant system and endorse the root initiation and growth development of plants.[49] In our reports clearly indicate that the seaweed extracts have growth promoting activities than compared to synthetic auxins tested. Based on report and our knowledge this is the first report on establishment of *in vitro* regeneration system for this species using different seaweed extracts.

Ex vitro rooting

Ex vitro rooting method, however, resulted in favorable root development and shoot development without producing the callus and the roots observed under *in vitro* condition. *Ex vitro* root formation in microshoots was affected by auxin types, their concentrations and time duration of pulse treatment. Microshoots were optimally rooted *ex vitro*, when shoot bases were pretreated with IBA (200 mg/l) for 3-5 min. All the shoots produced roots after 6 weeks of treatment with root number (10.40±0.08 and 9.60±0.07) and average root length (6.00±0.64cm and 4.88±0.23cm) respectively internode and YL explants (Fig. 3c&d). IBA has been known as a synthetic auxin for a long time and is the major auxin used commercially for the induction of adventitious roots.[50] As different concentrations of IBA were applied, different results in rooting efficiency were obtained. It showed that a specific concentration was vital in inducing rooting. In this study, the MS medium supplemented with 200mg/l IBA demonstrated a better response with the highest percentage of rooting as well as the longest root as compared to the other concentrations of IBA and other auxins tested (Fig. 4).

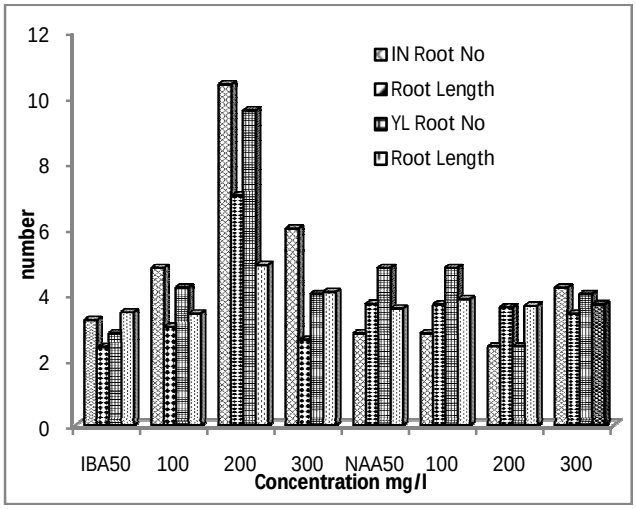

Figure 4: Effect of auxins on *ex vitro* rooting of *in vitro* regenerated shoots (after 8 weeks)

Meanwhile, as the concentration of IBA decreased lower rooting efficiency was observed. Other auxins namely IAA and NAA produced roots, but rooting response (root number and length) was inferior to IBA. In both the explants of IBA 200mg/l used in the present exhibited variable root development depending on environmental factor. For example internode explants exhibited the lowest root development under *in vitro* condition, whereas it displayed the highest rooting response under *ex vitro* condition. In recent study observed that 200mg/l produced approximately 85% *ex vitro* rooting of regenerated shoots.[21] Study the anatomy of *Camellia japonica* roots in agar and soil and found that soil grown roots had more tracheary elements compared to agar grown roots, seemingly due to thickness of cell walls and high accumulation of phenolic compounds.[51] The results of present study reveal the beneficial role of IBA in *ex vitro* rooting and this is in accordance with many other reports of some species like *Leptadenia pyrotechnica*; *C.bulbosa*.[52,25]

Acclimatization and Hardening

The rooted plants with fully matured leaves and well developed roots were transferred to the pots containing red sterile 1:1:1 (w/w/w) red soil to sand and coconut coir mixture. Growth of the potted plants was under observed after two month of transfer by both methods (*in vitro* and *ex vitro*). After two months, they were transferred to larger pots containing the mixture of red soil mixed sand and forest humus and coconut coir (1:1:1:1) and moved to a greenhouse was acclimatized successfully under the greenhouse conditions with about 80% survival rates (Fig. 3 e). In order to expose plantlets to environmental conditions, paper cups were subsequently moved from

high humidity to low humidity. The paper cups containing hardened plantlets survived in the soil conditions. Acclimatization of *in vitro* raised plantlets has been focus on researchers and it is essential to ensure appropriate hardening before transplanting to *in situ* conditions.[53] The regenerated plants did not show noticeable varieties in the morphological characteristics when compared to the mother plants.

CONCLUSION

An efficient regeneration protocol via indirect shoot organogenesis has been developed for *C. thwaitesii*, a endemic species from Western Ghats of India. Higher frequency of regeneration depends on explant type, collection of explants, combination and concentration of PGRs, culture conditions and additives in the medium. Apart from the synthetic auxins and cytokinins used in this study, our results show that seaweed extracts play a vital role in shoot elongation and rooting of regenerated plantlets. By using the naturally available marine resources, our protocol gave a better result than synthetic hormones tested. This protocol imparts a successful and rapid technique that can be utilized for the commercial propagation and *ex situ* conservation of this species.

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