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Full Length Research Paper

In vitro propagation of *Ceropegia thwaitesii* Hook- an endemic species of Western Ghats of Tamil Nadu, India

S. Muthukrishnan[1], J. H. Franklin Benjamin[2], M. Muthukumar[1], N. Ahamed sherif[1], T. Senthil kumar[3], M. V. Rao[1]*

[1]Department of Plant Science, Bharathidasan University, Tiruchirappalli 620 024, Tamil Nadu, India.
[2]Botanical Survey of India, Southern Circle, Coimbatore- 641 003, Tamil Nadu, India.
[3]Department of Industry University Collaboration, Bharathidasan University, Tiruchirappalli 620 024, Tamil Nadu, India.

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***In vitro* propagation of *Ceropegia thwaitesii* Hook (Asclepiadaceae), an endemic plant species of Western Ghats, Tamil Nadu (India), was carried out. Auxiliary bud explants were cultured on Murashige and Skoog (MS) medium fortified with cytokinins (KIN), 6-benzyl adenine (BA), 2-isopentenyladenine (2-iP) and thidiazuron (TDZ) in various concentrations and in combination with auxins indole-3-butyric acid (IBA), indole-3-acetic acid (IAA) and naphthalene acetic acid (NAA). High frequency of shoot bud proliferation and multiplication was observed on KIN (13.94 µm) + IAA (28.54 µm). Rhizogenesis was observed on MS medium supplemented with IBA 2.46 µm and plantlets produced through micropropagation were hardened with the survival success of 73.33%. The efficient Micropropagation regeneration protocol developed would aid *ex situ* conservation of this endemic species.**

Key words: *Ceropegia thwaitesii, in vitro* propagation, Asclepiadaceae, plant growth regulators, micropropagation.

INTRODUCTION

India has a rich and varied heritage of biodiversity, encompassing a wide spectrum of habitats from rainforests to alpine vegetation and from temperate forests to coastal wetlands (Bapat et al., 2008). In India, about 1500 species are listed as rare and threatened belonging to both flowering and non flowering plant groups (Myers, 1988). *Ceropegia* L. (commonly known as lantern flowers) is an old world tropical genus comprising about 200 species, which are distributed from south-east Asia, India, Madagascar, Tropical Arabia, Canary Islands, Africa except Mediterranean region, New Guinea and northern Australia (Meve and Liede, 2007; Mabberly et al., 1997; Dyer 1983). *Ceropegias* are known to store starch, sugars, gum, albuminoid, fats, crude fibre and other valuable phytoconstituents which are routinely used in traditional Indian ayurvedic drugs (Kirtikar and Basu, 1935). The pharmacological importance of the genus *Ceropegia* is mainly due to the presence of a pyridine alkaloid called 'Ceropegin' (Sukumar et al., 1995).

In India, about 50 species are present and most of them are endemic to Western Ghats which is one of the centres of diversity of *Ceropegia* (Malpure et al., 2006; Yadav, 1996; Surveswaran et al., 2009; Jagtap and Singh, 1999), and most of them are enlisted under endangered category (Nayar and Sastry, 1987). Most of the endemic species of *Ceropegia*, by virtue are being restricted only to a special habitat and narrow ecological niche; they are highly vulnerable and merit special consideration in their conservation. Reasons for their decline include destruction of forests, modifications of habitats, industrialization, pollution and introduction of exotic weeds (Yadav and Mayur, 2008). *Ceropegia thwaitesii* Hook, an endemic plant species, has a very restricted distribution in the Western Ghats of Tamil

*Corresponding author. E-mail: mvrao_456@yahoo.co.in.

Abbreviations: AA, Ascorbic acid; **BA,** 6-benzyl adenine; **CH,** casein hydrolysate; **CA,** citric acid; **IAA,** indole-3-acetic acid; **IBA,** indole-3-butyric acid; **KN,** kinetin; **NAA,** naphthalene acetic acid; **TDZ,** thidiazuron; **YE,** yeast extract; **2-iP,** 2-isopentenyladenine; MS, Murashige and Skoog medium.

Nadu, India. It inhabits bare slopes of Pambar shola, and is highly vulnerable due to habitat destruction and over exploitation (Nayar and Sastry, 1988). Establishment of an efficient protocol of micropropagation is necessary to ensure large scale multiplication and preservation of this plant. However, the species is difficult to propagate by conventional methods of cuttings and seedling because of *in vivo* contamination that is difficult to overcome. These difficulties could be overcome using alternative and more effective plant tissue culture. Very few reports are available on the micropropagation of *Ceropegia* species (Patil, 1998; Beena et al., 2003; John Britto et al, 2003; Karuppusamy et al., 2009; Nikam and Savant, 2009; Chandore et al., 2010; Chavan et al., 2011a, 2011b). Though being a highly vulnerable plant, *C. thwaitesii* has never been subjected to *in vitro* studies. The present investigation, reports for the first time, the *in vitro* propagation of *C. thwaitesii* through axiliary bud prolife-ration. These results could serve as a guide for future regeneration studies of this vulnerable plant.

MATERIALS AND METHODS

The plants of *C. thwaitesii* were collected from Pambar Shola in the Western Ghats of Tamil Nadu, India and maintained in earthen pots in the glass house of Bharathidasan University, Tiruchirappalli under controlled conditions (Temp, 26 ± 2°C and relative humidity (RH), 70%). Explants were collected from these plants after stabilization.

Surface sterilization

Axillary bud explants (2 cm length) were washed under running tap water for 20 to 30 min and surface sterilized with 70% ethanol for 30 s, rinsed with sterile distilled water and treated with 0.1% $HgCl_2$ for 3 min duration. To remove every trace of sterilant, the shoot material was then washed with sterile distilled water at least three to five times. The shoot segments containing nodes (1 to 2 cm) were prepared from the surface sterilized shoots and were used as explants. These sterilized explants were inoculated on culture medium. This whole process was carried out under the laminar air flow chamber.

Culture conditions

Murashige and Skoog **(**1962) medium was fortified with 30 g/L sucrose (Himedia, India) and gelled with 0.8%(w/v) agar (Himedia, India), and the pH of the medium was adjusted to 5.7 ± 0.2 with 0.1 N NaOH or 0.1 N HCl after addition of the growth regulators. The medium was autoclaved at 121°C, for 30 min. All the cultures were maintained in sterilized culture room at 26 ± 2°C, under 16/8 h light regime provided by cool white fluorescent light (60 μmol^{-2} sec^{-1} light intensity) and with 55 to 60% relative humidity. All the cultures were sub cultured on the fresh medium after 15 to 20 days.

Shoot bud induction and multiplication

After surface sterilization, the nodal segments were placed on MS medium containing different concentrations of cytokinins individually 6-benzyl adenine (BA, 0.44 to 22.19 µm), cytokinin (KIN, 0.46 to 23.23 µm), thidiazuron (TDZ, 0.45 to 22.50 µm), 2- isopen-tenyladenine (2-iP, 0.49 to 24.61 µm) for shoot bud proliferation. Six weeks later, the shoot regeneration frequency was observed. Effect of the optimal cytokinin was tested for more shoot regeneration frequency was observed. Effect of the optimal cytokinin in combination with different range of auxins naphthalene acetic acid (NAA, 0.54 to 26.86 µm), indole-3-acetic acid (IAA, 0.57 to 28.54 µm), 2,4-dichlorophenoxyacetic acid (2,4-D, 0.45 to 22.62 µm), indole-3-butyric acid (IBA) (0.49 to 24.60 µm) concentrations was tested for shoot multiplication. MS medium without any growth regulator was served as a control.

Effect of supplements

Organic additives like casein hydrolysate and yeast extract, antioxidants namely citric acid and ascorbic acid: 50, 100 and 150 mg/L (w/v) were supplemented to the shoot multiplication medium (cytokinin-auxin optimal combination) to assess the enhancement of shoot multiplication rate.

Acclimatization and transplantation of plantlets

Microshoots from shoot clusters were carefully separated and axillary nodes (3 to 5 cm long fully expanded leaves) were transferred to MS medium supplemented with various root inducing growth regulators. Efficacy of IBA (0.049 to 2.46 µm), IAA (0.057 to 2.854 µm), NAA (0.054 to 2.685 µm), were checked for root induction. The rooted plantlets were removed from the culture tubes and washed with tap water to remove trace of agar. Then they were planted onto paper cups (2.5 cm diameter) containing a mixture of red soil mixed with sand and coconut coir (1:1:1) 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. MS major salts solution was poured with four days intervals up to four weeks. 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 daily under green house condition.

Experimental design and data collection

Observations of the culture were made every week and data measured after 35 and 45 days for shoot multiplication and rooting respectively. All the experiments were conducted as a randomized complete design. For each experiment, a minimum of seven replicates were taken and repeated three times. Data related to shoot multiplication (frequency of response, number of shoots, number of node, number of leaves and shoot length). Comparisons between treatments were made with Duncan's new multiple range test (DMRT) (Duncan, 1955).

RESULTS AND DISCUSSION

Effect of cytokinins on shoot multiplication

The percentage of response to culture varied according to type and concentration of cytokinins. All concentrations of cytokinins used facilitated response from axillary bud induction with respect to number of shoots obtained per

Table 1. Influence of cytokinins on shoot multiplication of *Ceropegia thwaitesii*[1]

Plant growth regulator (µm)[2]		No. of shoots	No. of nodes	No. of leaves	Shoot length (cm)	Percentage of response
BA	0.44	0.85 ± 0.14^{ef}	0.71 ± 0.18^{de}	1.71 ± 0.42^{def}	1.41 ± 0.43^{abcd}	71.42
	1.33	0.42 ± 0.20^{def}	1.42 ± 0.48^{cd}	1.14 ± 0.45^{def}	0.78 ± 0.38^{bcd}	57.14
	2.22	0.42 ± 0.20^{def}	1.00 ± 0.43^{abcd}	1.42 ± 0.71^{def}	1.55 ± 0.77^{abcd}	28.57
	3.11	1.00 ± 0.37^{cd}	1.00 ± 0.48^{abcd}	1.28 ± 0.71^{cdef}	1.41 ± 0.80^{abcd}	57.14
	4.44	1.57 ± 0.29^{ab}	1.14 ± 0.14^{abc}	2.85 ± 0.50^{ab}	1.60 ± 0.23^{abc}	66.66
	13.32	2.14 ± 0.26^{cd}	1.14 ± 0.26^{abc}	2.42 ± 0.75^{abcd}	1.22 ± 0.35^{bcd}	85.14
	22.19	1.00 ± 0.30^{cd}	1.00 ± 0.30^{abcd}	2.28 ± 0.64^{cde}	1.52 ± 0.40^{abcd}	57.14
KIN	0.46	0.71 ± 0.18^{def}	0.71 ± 0.18^{cde}	1.42 ± 0.42^{def}	1.08 ± 0.30^{bcd}	33.33
	1.39	0.85 ± 0.34^{cdef}	1.00 ± 0.43^{abcd}	1.85 ± 0.76^{def}	0.80 ± 0.28^{bcd}	42.85
	2.32	1.28 ± 0.42^{abc}	1.41 ± 0.42^{bcd}	2.00 ± 0.57^{ef}	1.41 ± 0.42^{abcd}	66.66
	3.25	1.28 ± 0.42^{abc}	1.00 ± 0.30^{abcd}	2.42 ± 0.86^{abcd}	1.17 ± 0.36^{bcd}	57.14
	4.65	1.14 ± 0.14^{ab}	2.00 ± 0.37^{ab}	2.71 ± 0.77^{abc}	1.62 ± 0.62^{abc}	85.14
	13.94	3.28 ± 0.28^{a}	2.57 ± 0.36^{a}	3.42 ± 0.71^{a}	2.78 ± 0.37^{a}	100.0
	23.23	1.71 ± 0.42^{b}	1.71 ± 0.42^{abc}	2.00 ± 0.57^{ef}	1.85 ± 0.51^{ab}	57.14
2ip	0.49	0.00 ± 0.00^{f}	0.00 ± 0.00^{e}	0.00 ± 0.00^{f}	0.00 ± 0.00^{d}	57.14
	1.48	0.42 ± 0.20^{def}	0.57 ± 0.29^{cde}	0.85 ± 0.55^{bcd}	0.54 ± 0.25^{bcd}	57.14
	2.46	0.42 ± 0.20^{def}	0.57 ± 0.29^{cde}	1.42 ± 0.84^{def}	0.74 ± 0.44^{bcd}	33.33
	3.44	0.71 ± 0.18^{def}	1.00 ± 0.43^{abcd}	1.42 ± 0.71^{def}	1.17 ± 0.62^{bcd}	42.85
	4.92	0.42 ± 0.20^{def}	0.57 ± 0.29^{cde}	1.00 ± 0.48^{bcde}	0.42 ± 0.21^{bcd}	60.00
	14.78	0.57 ± 0.20^{def}	1.14 ± 0.40^{abcd}	1.71 ± 0.60^{abcd}	1.12 ± 00.54^{bcd}	57.14
	24.61	0.28 ± 0.18^{ef}	0.42 ± 0.29^{de}	0.71 ± 0.47^{cdef}	0.62 ± 0.46^{bcd}	57.14
TDZ	0.45	0.28 ± 0.18^{ef}	0.28 ± 0.18^{de}	0.28 ± 0.18^{ef}	0.20 ± 0.13^{cd}	14.28
	1.35	0.28 ± 0.18^{ef}	0.28 ± 0.18^{de}	0.42 ± 0.29^{def}	0.20 ± 0.13^{cd}	42.14
	2.25	0.57 ± 0.29^{def}	0.57 ± 0.29^{cde}	1.14 ± 0.59^{bcdef}	0.60 ± 0.28^{bcd}	42.85
	3.15	0.42 ± 0.20^{def}	0.28 ± 0.18^{de}	0.57 ± 0.36^{def}	0.71 ± 0.46^{bcd}	14.28
	4.54	0.28 ± 0.18^{ef}	0.57 ± 0.36^{cde}	1.00 ± 0.65^{cdef}	0.51 ± 0.33^{bcd}	28.57
	13.42	0.85 ± 0.14^{def}	1.42 ± 0.42^{bcd}	2.42 ± 0.57^{abcd}	1.92 ± 0.83^{ab}	57.14
	22.50	0.28 ± 0.18^{ef}	0.14 ± 0.14^{e}	0.57 ± 0.36^{def}	0.17 ± 0.17^{cd}	28.57

1Data were scored after 45 days of culture.
2Cytokinins were supplemented to MS medium. For all the parameters mean values followed by the same letter are not significantly different at P = 0.05 (Duncan's New Multiple Range Test

explant and their shoot length, leaf number and node number (Table 1) on medium supplemented with KIN (13.94 µm). Whereas, TDZ resulted in a significantly lower shoot multiplication (16.66%) (Figure 1a). The axillary nodes remained green and fresh but failed to sprout on MS media without cytokinins. The medium supplemented with high concentrations of KIN (13.94 µm) produced the maximum number of shoots (3.28 ± 0.28) per explant with 100% response (Table 1 and Figure 1c) among the cytokinins, while increasing or decreasing their concentration reduced the frequency and number of shoots produced. The shoot multiplication rate was higher in KIN (13.94 µm) when compared to all other tested cytokinins. KIN induced multiple adventitious shoots as was reported in other Asclepiadaceae members as such as *Ceropegia juncea* (Nikam and Savant, 2009), the rare *Hoya wightii* spp. *palniensis* (Lakshmi et al., 2010), *Gymnema sylvestre* (Komalavalli and Rao, 2000), and in the Plantaginaceae *Isoplexis canariensis* (Arrebola et al., 1997).

Thus the degree of shoot multiplication varied considerably with the KIN concentration. In the present study, 13.94 µm of KIN supplemented MS medium promoted maximum shoot multiplication frequency, whereas BA did not improve the shoot number significantly. Similar response was also observed in the shoot multiplication of *Tylophora indica* (Faisal et al., 2005), and *Ceropegia attenuata* (Chavan et al., 2011a).

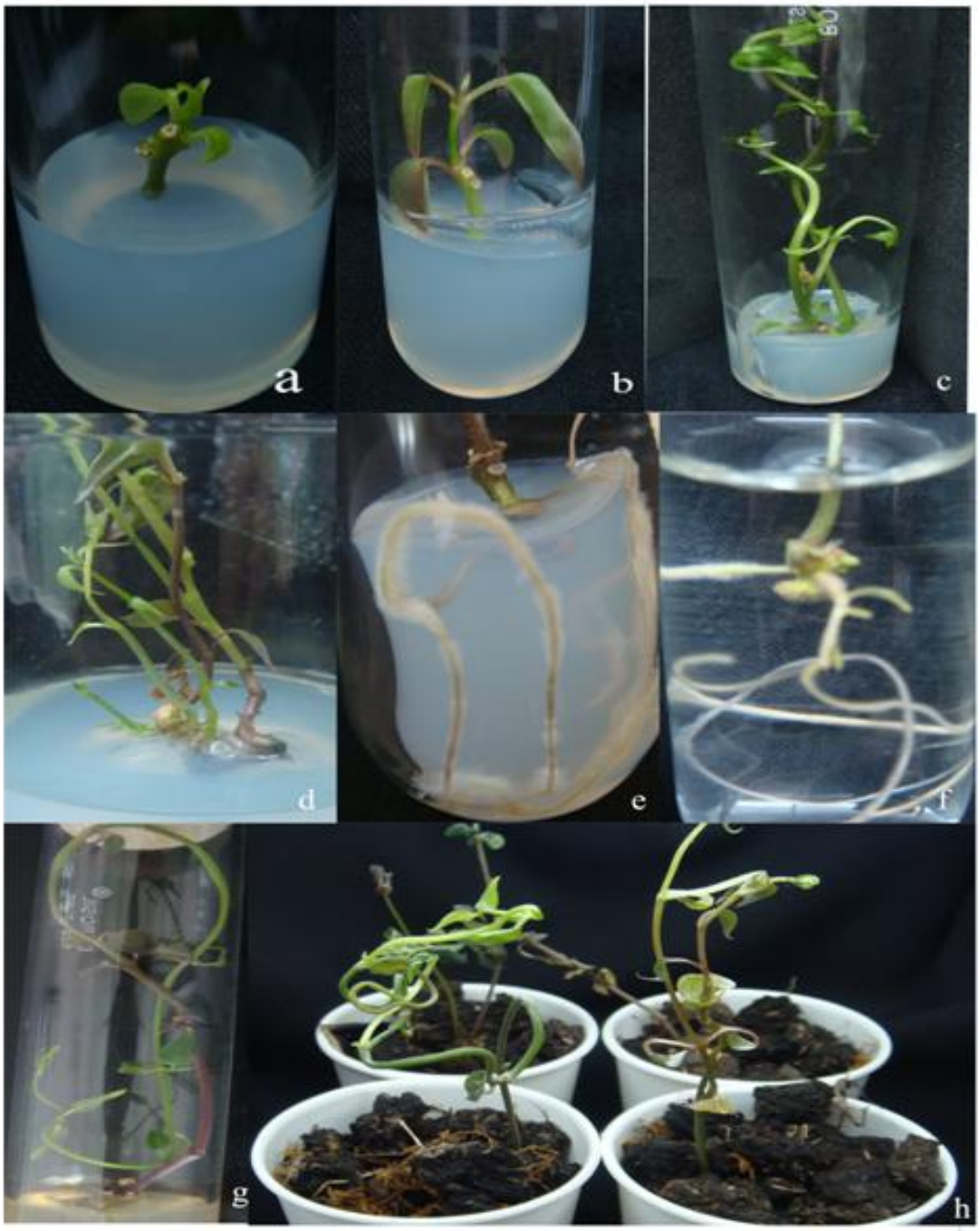

Figure 1. Micropropagation of *Ceropegia thwaitesii.* (a) Effect of TDZ on shoot induction. (b) Effect of KIN on shoot induction. (c) Shoot multiplication on MS + KN (13.94 µm). (d) Shoot multiplication on MS + KN (13.94 µm) + IAA (28.54 µm). (e, f) Effect of auxin in rooting. (g) Effect of supplement. (h) Hardening.

All the concentration of TDZ containing cultures induced a vitrified shoots invariably, though TDZ is known to induce cytokinin like effects in woody plants (Barrueto et al., 1999; Datta et al., 2007) and herbaceous crop species as well (Huetteman and Preece, 1993). High concentration of TDZ resulted in formation of stunted and vitrified shoots. It might be due to the high concentration of TDZ as it modifies the endogenous cytokinin metabolism (Capelle et al., 1983; Hare and Van Staden, 1994; Murthy et al., 1995; Hutchinson and Saxena, 1996). Alternatively, it has been suggested that TDZ may mimic an auxin response (Visser et al., 1992) or modifies endogenous auxin metabolism (Murthy et al., 1995; Hutchinson and Saxena, 1996). Similar response was

Table 2. Influence of different concentrations of auxin with KIN (13.94 µm) on shoot multiplication of *Ceropegia thwaitesii*[1]

Plant growth regulator[2] (µm)		No. of shoots	No. of nodes	No. of leaves	Shoot length (cm)	Frequency percentage	Percentage of basal callusing
	0.54	0.71 ± 0.47^{efgh}	1.00 ± 0.72^{ghij}	2.28 ± 1.5^{ij}	1.02 ± 0.75^{bc}	28.57	-
	1.61	0.71 ± 0.28^{defg}	1.85 ± 0.79^{hij}	3.71 ± 1.5^{de}	2.51 ± 1.1^{bc}	42.85	-
	2.69	0.85 ± 0.14^{efg}	2.00 ± 0.78^{ij}	4.00 ± 1.5^{ghi}	3.54 ± 1.7^{bc}	28.57	-
NAA	3.76	0.42 ± 0.20^{gh}	0.85 ± 0.45^{hij}	1.71 ± 0.91^{hij}	2.11 ± 1.0^{bc}	42.85	-
	5.37	0.85 ± 0.14^{efg}	1.85 ± 0.55^{hij}	3.71 ± 1.1^{de}	3.18 ± 1.3^{bc}	14.28	85.17
	16.11	0.00 ± 0.00^{h}	0.00 ± 0.00^{j}	0.00 ± 0.00^{j}	0.00 ± 0.00^{c}	0	100
	26.85	0.00 ± 0.00^{h}	0.00 ± 0.00^{j}	0.00 ± 0.00^{j}	0.00 ± 0.00^{c}	0	-
	0.57	0.71 ± 0.18^{fgh}	1.42 ± 0.42^{hij}	2.85 ± 0.85^{e}	3.17 ± 1.2^{bc}	57.14	-
	1.71	1.28 ± 0.52^{cde}	1.42 ± 0.61^{fhij}	3.00 ± 1.2^{hij}	3.67 ± 1.6^{bc}	28.57	-
	2.85	2.14 ± 0.34^{b}	4.28 ± 0.77^{ab}	8.28 ± 1.3^{ab}	3.98 ± 0.61^{bc}	71.42	-
IAA	3.99	1.28 ± 0.18^{cde}	3.28 ± 0.60^{bcde}	6.42 ± 1.2^{bcde}	4.45 ± 1.5^{bc}	28.57	-
	5.71	0.85 ± 0.45^{efg}	1.00 ± 0.57^{hij}	2.00 ± 1.1^{ghij}	0.85 ± 0.63^{bc}	66.66	-
	17.12	2.42 ± 0.20^{cde}	2.85 ± 0.67^{def}	5.71 ± 1.3^{bcd}	3.70 ± 1.2^{bc}	42.85	-
	28.54	6.42 ± 0.26^{a}	5.42 ± 0.57^{a}	10.85 ± 1.1^{a}	6.21 ± 0.6^{b}	100	-
	0.49	1.28 ± 0.35^{cde}	3.57 ± 1.6^{bcde}	7.14 ± 2.1^{bcd}	3.65 ± 1.2^{bc}	33.33	-
	1.48	1.57 ± 0.36^{bcd}	3.85 ± 0.96^{abc}	7.14 ± 1.7^{bcd}	3.54 ± 0.94^{bc}	66.66	-
	2.46	1.14 ± 0.26^{def}	3.57 ± 0.75^{bcde}	7.14 ± 1.5^{bcd}	10.37 ± 7.8^{a}	33.33	-
IBA	3.44	1.71 ± 0.18^{bc}	4.00 ± 0.43^{abc}	8.42 ± 0.99^{ab}	6.15 ± 0.1.2^{bc}	71.42	-
	4.90	1.28 ± 0.18^{cde}	2.71 ± 0.52^{bcd}	5.42 ± 1.0^{efg}	2.77 ± 0.78^{bc}	33.33	-
	14.8	1.57 ± 0.29^{bcd}	3.42 ± 0.36^{cde}	7.14 ± 0.73^{bcd}	3.07 ± 0.67^{bc}	33.33	-
	24.60	1.28 ± 0.18^{cde}	3.71 ± 0.28^{abc}	7.42 ± 0.57^{bc}	3.44 ± 0.81^{bc}	42.85	-

1Data were scored after 45days of culture.
2Combinations of KIN and auxins were supplemented to MS medium. For all the parameters mean values followed by the same letter are not significantly different at P=0.05 (Duncan's New Multiple Range Test).

also observed in the shoot multiplication of *Ceropegia* species (Beena et al., 2003; Karuppusamy et al., 2009).

Effect of kinetin and auxin on multiple shoot formation

The effect of cytokinin on tissue or organ culture varies according to the particular compound used, the type of culture and age of explants (juvenile or mature) (Messeuger and Mele, 1987). One of the advantages of adding auxin at low concentration on the culture medium is to nullify the effect of the high concentrations of cytokinin on axillary shoot elongation (Hu and Wang, 1983). Hence, after determining the optimum level for best shoot bud multiplication with KIN (13.94 µm), screening for the effect of auxins like IAA, NAA and IBA at different concentrations was done. A combination of KIN (13.94 µm) and IAA (28.54 µm) was found effective for a bud proliferation, which developed an average of 6.42 shoots per nodal explants and the developed shoots were healthy, strong and elongated. A synergistic effect of cytokinin combination with an auxin has been demonstrated in *Ceropegia* species (Beena et al., 2003; Chavan et al., 2011b).

High shoot formation observed in the concentration of KIN, 13.94 µm and high concentration of IAA, 28.54 µm can be attributed to the synergistic effect of the combination (Table 2 and Figure 1d). Combinations of other growth regulators such as NAA, IBA were also effective in axillary bud proliferation, but the high concentration of 2,4-D gave basal callusing (100%) (data not given). The supply of exogenous growth regulators in the medium is essential for differentiation which otherwise did not occur. Improved shoot regeneration with cytokinin and auxin combination has been proved in several other Asclepiadaceae species: *Ceropegia fantastica* Sedgw (Chandore et al., 2010) and *Hoya wightii* ssp*. palniensis (*Lakshmi et al., 2010), *C. attenuata* (Chavan et al*.,* 2011a), *C. juncea* (Nikam and Savant 2009). Shoot number, shoot length, node number and leaf number increased with combination of auxin (KIN 13.94 µm + IAA 28.54 µm), more over the mean number of leaf (7.64) and shoot length were observed in high concentration of IBA (24.60 µm). In accordance with this, our study also exemplifies the positive effect of high concentration of auxin in combination with a cytokinin in shoot multiplication efficacy.

Table 3. Effect of supplements in shoot multiplication with optimal concentration of KIN (13.94 µm) + IAA (28.54 µm)[1].

Supplement (mg/L)[2]		No. of shoots	No. of nodes	No. of leaves	Shoot length (cm)	Shoot multiplication frequency (%)
	50	1.0000 ± 0.30^{b}	2.5714 ± 0.71^{cde}	5.1429 ± 1.4^{cd}	2.0714 ± 0.68^{d}	40
YE	100	1.1429 ± 0.26^{ab}	5.0000 ± 0.87^{ab}	10.7143 ± 2.1^{ab}	7.0143 ± 1.3^{bcd}	33.33
	150	1.2857 ± 0.18^{ab}	4.7143 ± 0.80^{abc}	9.1429 ± 1.3^{abcd}	6.6571 ± 2.1^{bcd}	28.57
	50	0.8571 ± 0.34^{b}	2.4286 ± 0.97^{cde}	5.4286 ± 2.2^{bcd}	2.7714 ± 1.4^{cd}	50
CA	100	0.5714 ± 0.29^{b}	2.0000 ± 0.95^{e}	4.2857 ± 2.0^{d}	3.9857 ± 2.1^{cd}	33.33
	150	0.7143 ± 0.28^{b}	2.1429 ± 0.96^{de}	4.0000 ± 1.9^{d}	3.0571 ± 1.7^{cd}	25
	50	1.8571 ± 0.26^{a}	6.1429 ± 0.40^{a}	12.8571 ± 0.88^{a}	13.0143 ± 0.91^{a}	71.4
AA	100	1.0000 ± 0.00^{b}	3.0000 ± 0.30^{bcde}	5.8571 ± 0.55^{bcd}	7.9286 ± 0.74^{bc}	0
	150	0.8571 ± 0.14^{b}	3.7143 ± 0.77^{bcde}	7.2857 ± 1.5^{bcd}	6.3286 ± 2.1^{bcd}	0
	50	1.1429 ± 0.14^{ab}	4.4286 ± 0.68^{abcd}	9.8571 ± 2.0^{abc}	7.2571 ± 1.8^{bc}	14
CH	100	1.2857 ± 0.18^{ab}	4.5714 ± 0.42^{abc}	9.1429 ± 0.7^{abcd}	10.3000 ± 1.4^{ab}	28.57
	150	1.1429 ± 0.14^{ab}	3.5714 ± 0.52^{bcde}	7.1429 ± 1.2^{bcd}	6.5571 ± 1.1^{bcd}	14

1Data were scored after 45days of culture.
2Cytokinins were supplemented to MS medium. For all the parameters mean values followed by the same letter are not significantly different at P = 0.05 (Duncan's New Multiple Range Test

Effect of supplements

After selecting the optimum concentrations and combination of cytokinin-auxin for shoot multiplication, organic supplements, casein hydrolysate and yeast extract were used to improve the shoot multiplication efficiency, casein hydrolysate is a complex source of reduced nitrogen (Adachi et al., 1991; Silvestre et al., 1994), while yeast extract is a culture media supplement because of the high concentration and quality of vitamin B present in it. Hence, their potential on shoot enhancement was analyzed. The efficiency of KIN (13.94 µm) + IAA (28.54 µm) for shoot multiplication decreased when it was supplemented with casein hydrolysate (CH) and yeast extract (YE). The addition of CH and YE did not significantly improve the shoot multiplication frequency, their number or length and node number or leaf number. Among the two organic supplements, YE showed low shoot multiplication frequency than CH. Contrary to our observation; YE and CH have been successfully used in several perennial species for shoot multiplication (Ahmad and Anis, 2005; Hussain et al., 2008; Roy et al., 1998).

Citric acid and ascorbic acid control browning of culture medium and also play a significant role in the multiplication of shoots in many plants (Sharma and Chandel, 1992; Neelam and Chandel, 1992; Faisal et al., 2005; Lakshmi et al., 2010). Hence, their efficiency on shoot multiplication was examined. Citric acid (CA) in combination with KIN (13.94 µm) and IAA (28.54 µm) enhanced shoot multiplication frequency, number of shoot, node number and number of leaf produced per explant was lower when compared to that of ascorbic acid (AA). IAA also failed to induce shoot multiplication range but it induces shoot length when compared to other supplements (Table 3 and Figure 1g). Similarly, Sanjaya and Ravishankar, (2005) observed at *Seudoxytenanthera stocksii* (Poaceae) effectiveness of CA on enhancing the shoot multiplication frequency and number of shoots per explant. In our study, ascorbic acid showed affirmative effects on shoot elongation (13.43 ± 0.9) and number of leaf (12.85 ± 0.8) than the cytokinin-auxin combination. The addition of exogenous ascorbic acid to plant tissue is said to increase their metabolic activity and accelerate the release of sugars for better growth and development (George, 1993). Similarly a simulative effect of ascorbic acid has also been reported (Neelam and Chandel, 1992; Shekhawat et al., 1993).

Rooting

Rhizogenesis, the formation of roots is a crucial step in micropropagation for the formation of complete plantlets. Auxins induce root differentiation and their role in root development is well documented (Scott, 1972). During the study, elongated shoots (5 to 7 cm) were excised from regenerating cultures and were transferred to MS medium with varied concentration of auxins (NAA (0.54 to 26.86 µm), IAA (0.57 to 28.54 µm), IBA (0.49 to 24.60 µm) The rooting percentage, number of roots per shoot and length of roots were affected significantly among the treatments (Table 4 and Figures 1e to f). Among the three growth regulator tested, IBA was found to be most effective for root induction. IAA fails to induce the roots in

Table 4. Effect of auxin on *in vitro* rooting of *Ceropegia thwaitesii*[1].

GR	Concentration	Days for root induction	No. of roots	Root length(cm)	Basal callusing%	Frequency of responses
IAA	No response					
IBA	0.04	11 to 14	1.5714 ± 0.29^{cd}	1.9143±0.30^{b}	14.28	71.42
	0.24	11 to 14	1.2857 ± 0.18^{d}	0.7000 ± 0.11^{b}	-	85.71
	0.49	11 to 14	1.4286 ± 0.20^{d}	0.9714 ± 0.15^{b}	-	100.0
	1.48	11 to 14	1.2857 ± 0.18^{d}	0.8857 ± 0.10^{b}	-	57.14
	2.46	9 to 11	4.4286 ± 0.52^{a}	2.3714 ± 0.18^{b}	-	100.0
	3.44	0	0.0000 ± 0.00^{e}	0.0000 ± 0.00^{b}	-	0
	4.90	-	-	-	-	-
	14.8	-	-	-	-	-
	24.60	-	-	-	-	-
NAA	0.05	-	-	-	-	-
	0.27	-	-	-	-	-
	0.54	-	-	-	-	-
	1.61	0	0.0000 ± 0.00^{e}	0.0000 ± 0.00^{b}	-	0
	2.69	8 to 10	1.1429 ± 0.14^{d}	4.6714 ± 2.6^{a}	-	33.33
	3.76	8 to 10	1.2857 ± 0.18^{d}	0.9286 ± 0.12^{b}	42.85	14.28
	5.37	8 to 10	1.5714 ± 0.29^{cd}	0.8143 ± 0.15^{b}	57.14	57.14
	16.11	8 to 10	2.4286 ± 0.36^{bc}	0.9000 ± 0.11^{b}	42.85	71.42
	26.85	8 to 10	3.1429 ± 0.55^{b}	1.8000 ± 0.26^{b}	85.14	85.71

1Data were scored after 75 days of culture.
2Auxins were supplemented to MS medium. For all the parameters mean values followed by the same letter are not significantly different at P=0.05 (Duncan's New Multiple Range Test).

the microshoots (data not given). After 15 to 20 days, microshoots produced roots of various sizes and length. The highest number of roots per shoot (4.21 cm) and high frequency was obtained in IBA (2.46 µm). Similarly, IBA has been used successfully to obtain the highest rooting frequency in *Wattakaka volubilis* (Arulanandam et al., 2011), *Hoya wightii* ssp*. palniensis* (Lakshmi et al., 2010), *T. indica* (Faisal et al., 2005; Harmanjit Kaur et al., 2011) and *Leptadenia reticulata* (Shekhawat et al., 2003).

Intermittent callus formation was noticed on MS medium supplemented with NAA (26.85 µm). This may be due to the residual cytokinin in shoots (Nemeth, 1979). The highest mean of root length (4.67 cm) were obtained in NAA (2.69). Similarly, NAA has been shown to give the highest root length frequency in *Ceropegia intermedia* (Karuppusamy et al., 2009). The roots formed at the higher concentrations of NAA on MS medium were thick, stumpy and green in colour. These roots easily detach from the microshoots during transplantation and need further investigation. The rooted shoots were transferred to paper cups containing sand, red soil and coconut coir in the ratio of 1:1:1 (Figure 1h). Initially each pot was covered with a polythene bag to maintain high moisture. Subsequently, the moisture was reduced by the removal of polythene bags to harden the plants from which about 73.33% survived. These plants were transferred to pots containing mixture of red soil mixed sand, forest humus and coconut coir (1:1:1:1) to the green house. The pots were watered daily under green house condition till maturity indicating the potential use of this system for *C. thwaitesii in vitro* regeneration.

Conclusion

In conclusion, the successful micropropagation procedure described here is a simple and cost effective protocol for the rapid multiplication and conservation of the endemic plant *C. thwaitesii* from nodal explant. The species has a great regenerating potential as it exhibited a high efficiency of shoot bud formation and plant regeneration from vegetative parts taken from mature glass house grown plants. *De novo* adventitious shoot formation was observed on KIN (13.94 µm) + IAA (28.54 µm) after six to

eight weeks of culturing. This regeneration protocol will help in restoration and conservation of other rare and endemic plants. Reintroduction of hardened plants of *C. thwaitesii* will be done in their native habitat of Pambar Shola in the Western Ghats of Tamil Nadu.

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