

IN VITRO MULTIPLE SHOOT INDUCTION OF SELECTED CEROPEGIA SPECIES - MEDICINALLY IMPORTANT PLANTS

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Abstract

An efficient protocol has been developed for inducing multiple shoots from nodal explants of two asclepiads *Ceropegia bulbosa* Roxb and *Ceropegia juncea* Roxb, important medicinal plants. The sterilized nodal explants were inoculated in MS medium containing various concentrations (0.5 to 2.5mg/L) of cytokinins (BAP, Kn and TDZ) added separately. In *C. juncea* (6.23 shoots/explant) and *C. bulbosa* (5.66 shoots/explant) maximum shoots formed when nodal segments were cultured on full strength Murashige and Skoog's (MS) basal medium fortified with 1.0mg/L BA. In vitro Shoots were subculture on the same medium. Shoots of 3-4 cm length were transferred into half strength MS medium fortified (0.3 to 1.5mg/L) with IBA, NAA and IAA. The highest numbers of roots were observed in *C. bulbosa* (5.8roots/explant) fortified with IBA 0.6mg/L and *C. juncea* (5.37roots/explant) at IBA 0.3mg/L. The rooted plantlets were hardened and fruitfully recognized in pots at 80% achievement rate.

Keywords: *Ceropegia*; Rare medicinal plant; Nodal explants; Multiple shoots.

Introduction

Ceropegia (Asclepiadaceae) is a genus of 200 species of climbers, herbs and rarely subshrubs distributed in tropical and subtropical Asia, Africa, Australia, Malaysia and Pacific islands. Among the 28 species are endemic to peninsular India [1-4]. *Ceropegia bulbosa* and *C. juncea* were endangered and endemic to India. The overexploitation and poor seed germination of *C. bulbosa* and endemic species of *C. juncea* going to rare and endangered category in various place of India mentioned by standard articles [5-8]. The leaves and tubers of *C. bulbosa* are edible and considered to be tonic digestive and cure urinary disorders. They are also reported to be a cure for diarrhea, dysentery and appetizer [9]. *Ceropegia juncea*, which is used as a source of "Soma" a plant drug of the Ayurvedic medicine with a wide variety to cure the various ailments [10, 11]. The fleshy stem is used as a raw material for traditional and folk medicines for the treatments of stomach and gastric disorders [12]. Due to habitat encroachment and overexploitation for medicinal purposes in local healers and poor seed germination of *C.*

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juncea has become extinct in its natural habitat [6]. Both species of *C. bulbosa* and *C. juncea* are important sources of Ayurvedic drug, but the lack of proper cultivation practice, low number of seed germination, destruction of wild habitats and its removal, all lead to a progressive devastation of these species [13, 14].

The vegetative propagation through stem cuttings of Indian *Ceropegia* species is not possible as they do not have semi-hard wood [15]. However the increasing of habitat disturbances, overexploitation and poor reproduction and need of appropriate pollinator, limit the population of Asclepiadaceae family plants [16, 17]. On the other hand the *in vitro* propagation is an alternative tool for large scale production and may increase the number of propagules for cultivation as well as aid the replacement of natural populations [18, 19]. Many researchers reported that the *in vitro* studies in *Ceropegia* species, such as *Ceropegia bulbosa* Roxb. var. *bulbosa* [20], *Ceropegia bulbosa* [13], *Ceropegia spiralis* [21], *Ceropegia pusilla* [22] and other Asclepiadaceae family plants, *Hemidesmus indicus* [23], *Wattakaka volubilis* [24] and *Leptadenia reticulata* [25]. The development of an efficient method for rapid micropropagation techniques used to meet the demand and conservation of important medicinal plants. In the present study was described the *in vitro* propagation of *C. bulbosa* and *C. juncea* through the nodal explants by standard method. Similar studies were done on medicinally or endangered plants [26-29].

Material and methods

Source of plant material

C. bulbosa and *C. juncea* was collected from Madukkarai Hills of Coimbatore district, Western Ghats of Tamilnadu, India. The plants were grown in the greenhouse of Kongunadu Arts and Science College, Coimbatore, Tamilnadu, India.

Explant sterilization

Collected explants were transferred into 250mL sterile conical flask and they were washed first under running tap water (5-7 minutes) and treated with 0.1% (v/v) Tween - 20 for 5 minutes followed by repeated rinsing with distilled water. Further sterilization was done under aseptic environment in a laminar air-flow hood. Then the explants were surface sterilized with 20% (v/v) ethanol (1 minutes) and then by 0.01% (v/v) HgCl₂ (2-3 minutes). Finally, the explant was washed thoroughly (6-8 times) with sterile double distilled water. Sterilized explant was inoculated into Murashige and Skoog medium (MS medium).

Culture media and conditions

The explant was inoculated on MS basal medium supplemented with various Plant growth regulators of auxins and cytokinins either individually, containing 3% (w/v) sucrose. The pH was adjusted to 5.8 prior to the addition of 8g/L agar and autoclaved at 121°C for 20 minutes. After that cultures were incubated at 20±20°C with 16/8 h photoperiod by cool white fluorescent tubes and 75-80% relative humidity.

Shoot induction

Nodal segments were placed on MS medium with BAP, Kn and TDZ (0.5 to 2.5mg/L) at different concentrations for multiple shoot induction and proliferation. MS medium without any growth regulator was served as a control. The percent of regeneration, number of shoots per explant and mean shoot length were recorded after 4 weeks of culture.

Rooting

Micro shoots from shoot clusters were carefully separated and axillary nodes (3–4cm long with two pairs of fully expanded leaves) were transferred to half-strength MS medium supplemented with various root inducing growth regulators. Efficacy of IBA, IAA and NAA (0.3 to 1.5mg/L) were checked for root induction. Data on percentage of root induction, average number of roots and average root length were recorded after 4 weeks of culture.

Plant acclimatization

Plantlets with well-developed shoots and roots were removed from the culture medium carefully, washed gently under running tap water and transferred to small plastic pots containing a potting mixture. Special efforts were taken during hardening process of *C. bulbosa* and *C. juncea* by using different substrates such as, sterile garden soil, river sand and coco peat either individually or in combinations. Potted plantlets were covered with transparent polythene bags to retain humidity and were watered every 3 days with ½ MS medium without sucrose for 2 weeks. These plastic pots were kept under diffuse light (16h photoperiod) conditions. Polythene bags were opened after 2 weeks in order to acclimatize plants to field conditions. After 4 weeks, acclimatized plants were transferred to pots containing normal soil, sand, and vermiculite (1:1:1). The pots were watered daily under greenhouse condition.

Experimental design and data analysis

The cultures were observed periodically and morphological changes were recorded at regular intervals. All the experiments were repeated twice with 20 replicates per treatment. The experiments were conducted in a completely randomized design and the means and the standard deviation (SD) were compared. Data were subjected to analysis of variance (ANOVA) and comparisons of means were made with the Duncan's multiple range test (DMRT).

Result and Discussion

For the induction of multiple shoots of two among the species through the nodal explants were inoculated on MS medium fortified with various concentrations of cytokinins. All the concentrations of BAP (0.5 to 2.5mg/L), Kn (0.5 to 2.5mg/L) and TDZ (0.5 to 2.5mg/L) individually facilitates the multiple shoot formation. Multiple shoot formation from the *in vitro* culture of nodal segments has proved to be an effective and low cost method for mass multiplication. Among the different concentrations of cytokinins used, BA was the most effective growth regulators for the induction of multiple shoot formation in *C. bulbosa* and *C. juncea*. Of the three cytokinins tested, the maximum number of shoots formation from single explants of *C. juncea* (6.23 ± 0.08 cm) shoots, shoot length (7.15 ± 0.10 cm) followed by *C. bulbosa* induced (5.66 ± 0.11 cm) shoots and shoot length (6.50 ± 0.40 cm) at 1.0mg/L BA in MS medium. 100% nodal explants of among the species were induced shoot formation at BA 1.0mg/L (Figs. 1 and 2). Kn and TDZ also induced shoot multiplication but the number of shoots per explant was less than BA fortified medium. However, with Kn in the medium, the number of shoots per explant *C. bulbosa* at 0.5mg/L was (4.31 ± 0.21 and 5.09 ± 0.12 cm) and followed TDZ 0.5mg/L induced number of shoot (3.79 ± 0.17 and 4.42 ± 0.21 cm) respectively. In *C. juncea* 0.5mg/L Kn induced the number of shoots and shoot length (4.12 ± 0.20 and 5.27 ± 0.29 cm) was observed and TDZ (4.27 ± 0.41 , 5.86 ± 0.03 cm) at 1.0mg/L also recorded (Table 1). The high concentration (2.5mg/L) of BAP, Kn and TDZ affected shoot formation of both species. Similar results were found in *Sophora tonkinensis* [30], *Boucerosia diffusa* [31]. Dominance of BA for shoot multiplication in Asclepiadaceae has been reported in many other species like *Tylophora indica* [32], *Hemidesmus indicus* [33], *Caralluma bhupenderiana* [34] and *Ceropegia fimbriifera* [35].

Desirable shoots were excised and inoculated into half strength MS medium containing different concentration of auxins (IBA, IAA and NAA). Shoots grown on half-strength MS liquid medium supplemented with 0.6mg/L IBA and 3% sucrose formed roots at the highest number in *C. bulbosa* (5.8 ± 0.17 cm) roots, root length (3.52 ± 0.24 cm) and *C. juncea* (5.37 ± 0.43 cm) roots, root length (3.67 ± 0.30 cm) at 0.3mg/L IBA (Table 2; Figs. 1e and 2e). The high concentration of auxins induced swelling and callus formation at base of the shoot. In the present study poor performance was observed in high concentration of IBA, IAA and NAA regarding reduction in root number and length. Among the three growth regulators tested, IBA found to be the most effective for root induction than that of IAA and NAA. Among these

auxins higher concentrations induced callus formation at the basal end of microshoots, which inhibited the growth and formation of roots in *Wattakaka volubilis* [36], *Ceropegia juncea* [37]. Similarly, IBA has been used successfully to obtain the highest rooting frequency in *Ceropegia odorata* and *C. maccannii* [38], *Ceropegia fantastica* [39], *Wattakaka volubilis* [40], *Ceropegia thwaitesii* [41].

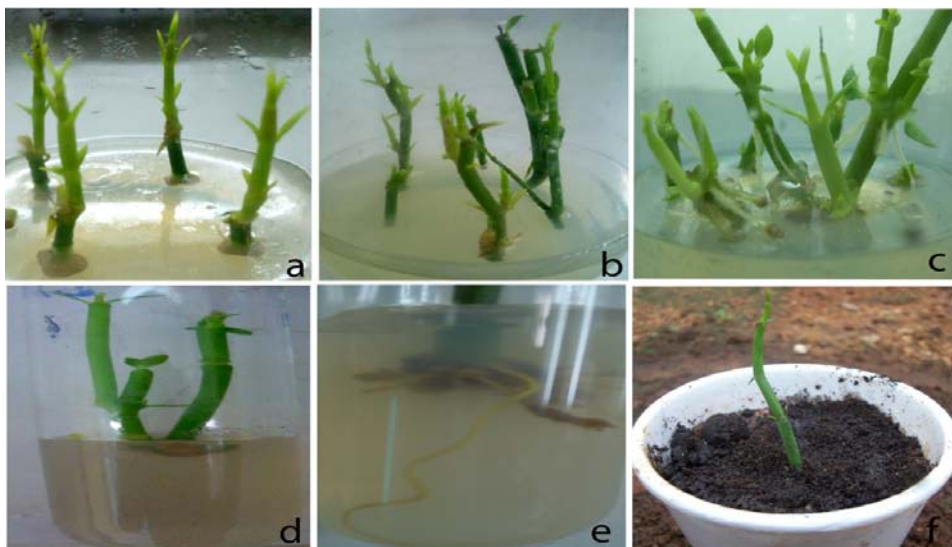


Fig. 1. *In vitro* propagation of *Ceropegia juncea* Roxb: a. Multiple shoots from nodal explants cultured on MS medium supplemented with BAP 1.0mg/L; b. Multiple shoots induction; c. Multiple shoots form nodal explants; d. Root initiation; e. *In vitro* rooting on half strength MS medium supplemented with IBA 0.3mg/L; f. Hardening.

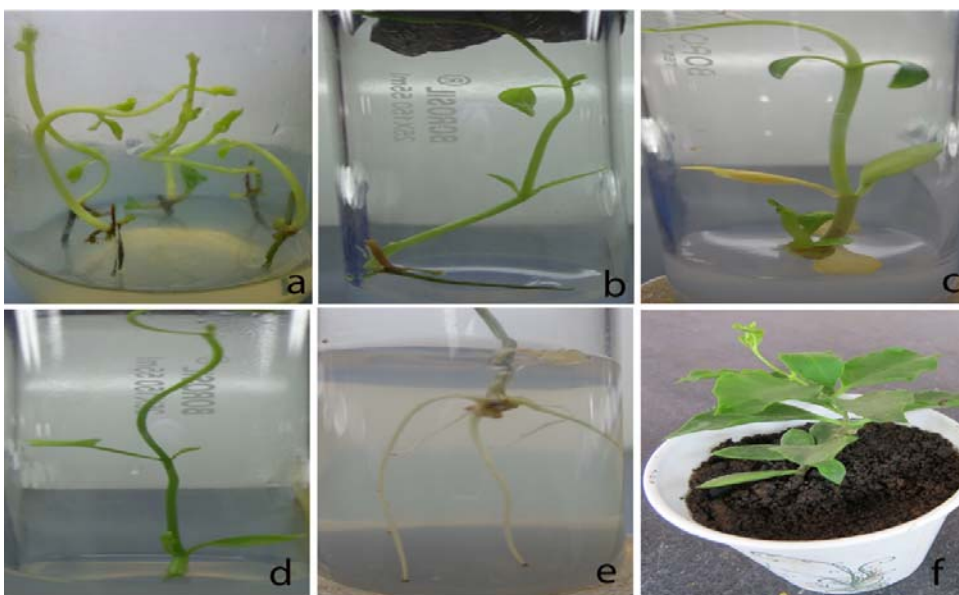


Fig. 2. *In vitro* propagation of *Ceropegia bulbosa* Roxb: a. Multiple shoots from nodal explants cultured on MS medium supplemented with BAP 1.0mg/L; b. Multiple shoots induction; c. Multiple shoots form nodal explants; d. Root initiation; e. *In vitro* rooting on half strength MS medium supplemented with IBA 0.6mg/L; f. Hardening.

MULTIPLE SHOOT INDUCTION OF CEROPEGIA BULBOSA ROXB AND CEROPEGIA JUNCEA ROXB

Table 1. The effect of plant growth regulators of cytokinins alone for shoot multiplication in *C. bulbosa* and *C. juncea*.

Samples	Growth regulators			<i>C. bulbosa</i>		<i>C. juncea</i>	
	BAP	Kn	TDZ	Number of Shoot/Explant	Shoot length (cm)	Number of Shoot/Explant	Shoot length (cm)
1	0.5	0.0	0.0	2.11±0.04 ^{ef}	3.73±0.13 ^e	4.03±0.09 ^b	5.06±0.12 ^c
2	1.0	00	00	5.66±0.11 ^a	6.50±0.40 ^a	6.23±0.08 ^a	7.15±0.10 ^a
3	1.5	0.0	0.0	3.19±0.05 ^d	4.72±0.16 ^{cd}	2.18±0.05 ^{de}	3.72±0.16 ^f
4	2.0	0.0	0.0	1.50±0.04 ^h	2.06±0.08 ⁱ	1.82±0.11 ^{ef}	2.26±0.23 ^h
5	2.5	0.0	0.0	1.11±0.10 ⁱ	1.5±0.24 ^j	1.03±0.14 ^g	1.26±0.04 ^j
6	0.0	0.5	0.0	4.31±0.21 ^b	5.09±0.12 ^c	4.12±0.20 ^b	5.27±0.29 ^c
7	00	1.0	00	3.17±0.24 ^d	3.25±0.21 ^{fg}	3.13±0.104 ^c	4.18±0.28 ^e
8	0.0	1.5	0.0	1.99±0.15 ^{fg}	2.84±0.28 ^{gh}	2.12±0.09 ^{de}	3.21±0.20 ^g
9	0.0	2.0	0.0	1.59±0.21 ^h	1.27±0.06 ^j	1.84±0.07 ^{ef}	2.08±0.12 ^h
10	0.0	2.5	0.0	1.00±0.14 ⁱ	1.22±0.12 ^j	1.481±0.17 ^f	1.62±0.10 ⁱ
11	0.0	0.0	0.5	3.79±0.17 ^c	4.42±0.21 ^d	2.44±0.29 ^d	4.63±0.17 ^d
12	00	00	1.0	2.34±0.12 ^e	5.66±0.07 ^b	4.27±0.41 ^b	5.86±0.03 ^b
13	0.0	0.0	1.5	1.75±0.15 ^{gh}	3.4±0.34 ^{ef}	2.09±0.10 ^{de}	3.4±0.12 ^g
14	0.0	0.0	2.0	1.51±0.25 ^h	2.58±0.29 ^h	1.55±0.07 ^f	2.34±0.12 ^h
15	0.0	0.0	2.5	1.0±0.16 ⁱ	1.22±0.10 ^j	1.11±0.08 ^g	1.17±0.07 ^j

Data expressed as Mean±SD. The statistical analysis of all the observations was carried out using one-way ANOVA (P < 0.05) followed by Duncan's multiple range test (DMRT). 20 replicates were used per treatment experiments and each practical was repeated at least thrice.

Table 2. The effect of plant growth regulators of axins alone for root formation in *C. bulbosa* and *C. juncea*.

Samples	Growth regulators			<i>C. bulbosa</i>		<i>C. juncea</i>	
	IBA	IAA	NAA	Number of Root/explant	Length of root (cm)	Number of Root/explant	Length of root (cm)
1	0.3	0.0	0.0	3.44±0.22 ^c	2.69±0.12 ^c	5.37±0.43 ^a	3.67±0.30 ^a
2	0.6	00	00	5.8±0.17 ^a	3.52±0.24 ^a	3.50±0.19 ^c	2.72±0.09 ^c
3	0.9	0.0	0.0	2.3±0.33 ^{de}	1.85±0.09 ^{de}	2.39±0.34 ^e	1.86±0.10 ^{de}
4	1.2	0.0	0.0	1.24±0.10 ^h	1.57±0.34 ^{ef}	1.24±0.10 ^{gh}	1.25±0.08 ^{fg}
5	1.5	0.0	0.0	1.04±1.26 ^h	1.03±0.16 ^{gh}	0.87±0.019 ^h	1.05±0.06 ^{gh}
6	0.0	0.3	0.0	2.83±0.28 ^d	2.65±0.21 ^c	4.2±0.07 ^b	3.22±0.16 ^b
7	00	0.6	00	4.3±0.39 ^b	3.17±0.26 ^{ab}	2.88±0.23 ^d	2.98±0.45 ^{bc}
8	0.0	0.9	0.0	1.8±0.08 ^{fg}	1.52±0.24 ^{efg}	1.78±0.12 ^f	1.52±0.25 ^{ef}
9	0.0	1.2	0.0	1.41±0.31 ^{gh}	1.06±0.17 ^{gh}	1.20±0.14 ^h	1.06±0.18 ^{gh}
10	0.0	1.5	0.0	1.05±0.08 ^h	0.96±0.09 ^h	1.06±0.08 ^h	0.70±0.04 ^h
11	0.0	0.0	0.3	2.24±0.22 ^{ef}	2.2±0.33 ^d	2.00±0.14 ^{ef}	2.06±0.11 ^d
12	00	00	0.6	3.89±0.28 ^c	2.99±0.03 ^{bc}	3.8±0.18 ^{bc}	3.0±0.09 ^{bc}
13	0.0	0.0	0.9	1.79±0.19 ^{fg}	1.16±0.14 ^{fgh}	1.64±0.35 ^{fg}	1.19±0.06 ^{fg}
14	0.0	0.0	1.2	1.28±0.19 ^h	1.10±0.31 ^{fgh}	1.07±0.03 ^h	1.04±0.05 ^{gh}
15	0.0	0.0	1.5	1.14±0.08 ^h	0.84±0.10 ^h	1.02±0.09 ^h	0.78±0.13 ^h

Data expressed as Mean±SD. The statistical analysis of all the observations was carried out using one-way ANOVA (P<0.05) followed by Duncan's multiple range test (DMRT). 20 replicates were used per treatment experiments and each practical was repeated at least thrice.

The rooted plantlets were transferred into plastic cups containing sterilized sand:soil:vermiculite in the ratio of 1:1:1 for hardening. The pots were watered daily under greenhouse condition for acclimatization. The plantlets were successfully established in soil with 80% survival rate in both *Ceropegia* species. The present study revealed that the cytokinins effect of both plants to induce *in vitro* plantlet production was achieved on MS medium supplemented with BA 1.0mg/L and 3% sucrose. Roots induced on half strength MS medium fortified with IBA on *C. bulbosa* (0.6mg/L) and *C. juncea* (0.3mg/L) showed better field survival frequency.

Conclusion

In conclusion, the *in vitro* regeneration protocol established from the present study can be employed for the propagation of *C. bulbosa* and *C. juncea* in large scale which may not only help in addressing the urgent local needs for effective conservation but also meet the high pharmaceutical requirements of the country. Further, studies on genetic diversity will contribute to a better understanding of the genetic profile that can be used to develop strategies for future research on the population and evolutionary genetics of these species.

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