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IN VITRO PROPAGATION OF CEROPEGIA FIMBRIIFERA BEDD., AN ENDANGERED, ENDEMIC PLANT OF SOUTH INDIA

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ABSTRACT

A successful and efficient in vitro propagation protocol for C. fimbriifera, a rare and threatened species from South India, has been developed. Nodal explants were cultured on Murashige and Skoog (MS) medium fortified with different concentrations of cytokinines BAP (6-benzylaminopurine), Kinetine (Kn), TDZ (Thidiazuron) for multiple shoot formation along with Auxin IAA (Indole-3-Acetic Acid), IBA (Indole-3-Buyric acid) and NAA (Napthalein-3-Acetic Acid) for root induction. Among the four cytokinines tested BAP 3 mg/L showed higher number of multiple shoots (7.5±0.3) with length 11.2±0.8 cm. Induced multiple shoots were excised and rooted best (5.1±0.4) on half Strength MS medium with IAA 2mg/L. The well grown plantlets were subsequently transferred to greenhouse and acclimatized further for field transfer.

KEYWORDS: Ceropegia Fibriifera, Micropropagation, Acclimatization

INTRODUCTION

The genus Ceropegia L. (1) with more than 220 species is distributed in tropical and sub-tropical regions of the old world (2). Presently there are a total of 67 taxa (ca. 33% of total diversity of the genus in the world) (60 species, 2 subspecies and 5 varieties) of Ceropegia in India (3). Out of 67 taxa 42 species and 5 varieties are distributed in the Western Ghats of peninsular India, which is the centre for diversity of the genus in India (4). Many Ceropegia species have been domesticated as ornamental house plants and some are commercially available in Europe and United States (5, 6). The tuberous roots of many Ceropegia species are edible (7). The root tubers contain starch, sugar, gum, albuminoids, fats and crude fibre and are valuable constituents in many traditional medicinal systems in India (8). Active principle of tuberous roots contains an alkaloid ceropegin which is active against diarrhea and dysentery (9).

Ceropegia fimbriifera Bedd., a perennial erect tuberous herb possesses attractive flower which is hairy at the base of corolla lobes (Fig.1 a,b). The species has scanty distribution in the edges of dry deciduous forests of Karnataka, Tamil Nadu and evergreen forests of Kerala. According to the IUCN criteria the species falls under the Critically Endangered CR B1ab (i, ii) (IUCN 2010) category (10). 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 (11).Vegetative propagation by root tubers and stem cuttings is very arduous. To overcome all these difficulties large scale micropropagation system has been developed. Only few reports are available on the micropropagation of Ceropegia species (12-18).Therefore multiple shoot induction through plant tissue culture may prove to be pivotal for conservation of this rare endangered threatened species.

MATERIALS AND METHODS

A single plant was collected from the view of conservation and due to the scarcity of the plant from the Silent valley National Park, Palakkad District, Kerala. For periodic harvest the tuber of plant was maintained in Botany Department, Shivaji University, Kolhapur, Maharashtra.

The plant material was thoroughly washed under running tap water for 30 minutes to remove dust particles and other superficial contaminants. For the inception of culture establishment the nodal segments were cut and decontamination was achieved by using diluted liquid disinfectant Savlon for 10 minutes followed by treatment with Bavistine (0.4% w/v) for 3 min. These explants were treated with surface sterilant 0.1% $HgCl_2$ for 4 minutes and subsequently thrice washings of sterile distilled water were given. The exposed area of explant was cut and then explant inoculated on culture medium (Fig. 1 c) under the laminar air flow chamber.

MS basal salts (19) with 3% sucrose, 0.2% gelrite along with various concentrations of plant growth regulators were used. The pH of the medium was adjusted to 5.8 ±0.1 using 0.1N NaOH or 0.1N HCl prior to autoclaving at 121^0C for 15 min. All the in vitro cultures were maintained in a 25 ± 2^0C with a 16 h photoperiod with 33µmole $m^{-2}s^{-1}$ light intensity from cool fluorescent tubes followed by 8 h dark period with 60% relative humidity. All the cultures were sub cultured on the fresh medium after every 4 weeks.

After surface sterilization, the sterilized explants were inoculated on MS medium containing different concentrations of cytokinins viz; BAP (0.5 to 3.5 mg), Kn (0.5 to 3.5 mg), TDZ (0.5 to 3.5 mg), Zeatin (0.5 to 3.5 mg) for multiple shoot induction. Subculture was done and shoot induction frequency was measured.

Regenerated shoots with 5 to 6 cm in height were inoculated on half strength MS medium supplemented with various concentrations of auxins viz; IAA, IBA, NAA for root induction. The cultures were incubated under 16 hour photoperiod for 30 days until rhizogenesis occurs and then rooting frequency is measured.

The plantlets with well developed shoots and roots were removed from the culture medium carefully, washed gently under running tap water to remove the traces of the medium. The in vitro rooted plantlets were transferred to small plastic pots containing autoclaved soil, sand and coco pit (5:3:2). These pots were initially covered with zip lock bags to maintain the humidity and placed in a mist chamber. After every alternate day, quarter strength of MS medium salt solution was supplied to the plantlets. After two weeks of growth, the proper water irrigation was provided .The rooted plants were established, acclimatized and transferred in green house condition.

Statistical Analysis:

All of the experiments were conducted with a minimum of 30 replicates per treatment. The experiments were conducted in a completely randomized design. The means and the standard error (SE) were compared. Data were subjected to analysis of variance (ANOVA) and comparisons of means were made with the Dunnett multiple comparison test.

RESULT AND DISCUSSION

Effect of Cytokinines on shoot multiplication

The main purpose of this study was to establish a rapid micropropagation system for C. fimbriifera through axillary shoot multiplication. Nodal segments containing axillary buds have quiescent or active meristem depending upon the physiological stage of plant. These buds have the potential to develop into complete plantlets. The conventional method used for the vegetative propagation of stem cuttings relies on the axillary bud taking over the function of the main shoots in the absence of a terminal bud. In nature, these buds remain dormant for a specific period depending on the growth pattern of the plant (20).

For the induction of multiple shoots explants were inoculated on MS medium fortified with various concentrations of cytokinines, as nodal explants cultured on medium without cytokinine remained green but failed to sprout. All concentrations of tested cytokinines

facilitated axillary bud induction with different frequencies of response. All the concentrations of BAP (1 to 3.5mg/L), Kn (1 to 3.5mg/L), TDZ (1 to 3.5mg/L), Zeatine (1 to 3.5 mg/L) individually facilitates the multiple shoot formation.

Among the various concentrations of cytokinines used, BAP was the most efficient cytokinine for induction of axillary bud and subsequently for multiple shoot formation. BAP alone at 3 mg/L induced maximum number of 7.5±0.3 shoots per explants with 11.2±0.9 cm shoot length with 96.66 % response (Table 1, Fig.1 d), Whereas 5.8±0.3 shoots/explants were obtained with BAP 3.5 mg/L with 90% response. Thus as BAP concentration increased further, shoot number decreased significantly. In vitro propagated plants belonging to Asclepiadaceae has been shown to have optimum overall growth in MS medium containing BAP[21]. The successful results obtained using BAP in few other species of Ceropegia viz; C.jainii(12), C.bulbosa (12,22), C. intermedia (14) have also shown similar findings. TDZ succeeded to give maximum 86.66% response with 2.3±0.3 shoots with 5.3±0.7 at 1.5mg/L concentration. Similar results were also found in C. thwaitesii (18). In present study Kinetin showed poor performance i.e. (2.4±0.2) shoots with 9.1±0.6 cm shoot length with 96.66% response at 2mg/L which is in agreement with some other instances like C. candelabrum (13). When compared to all other tested cytokinines the shoot multiplication rate was lower in zeatin as it did not enhance shoot induction significantly and produced maximum of 2.2±0.3 shoots per explants with 3.0±0.3 shoot length at 1mg/L concentration and showed 93.33% response. Unsuccessful results of Zeatine has been demonstrated in other Ceropegias viz; C. elegans (20), C. intermedia (14)

Subculture and shoot multiplication

Due to the scarcity of the plant, single plant was selected and best cytokinine was achieved for shoot bud induction. Successive subcultures of axillary buds were achieved on fresh medium of identical composition in order to assess the consistency of results in terms of appearance and quality of the multiple shoots. For subcultures, new shoots were excised and transferred on MS medium fortified with BAP 3 mg/L. In subsequent subcultures the number of shoots increased for 2nd and 3rd subculture and reduced thereafter. The third subculture during which the best multiplication i.e. 8.0±0.4 shoots with 9.2±0.5 shoot length was achieved (Table 2). Thus it can be stated that as the successive subcultures are done the rate of forming multiple shoots get reduced after third subculture. Hence it is possible to obtain significant number of shoots within 3 months through limited subcultures. There are some

other reports of Asclepiadaceae for an enhanced pace by subsequent subcultures followed by reduction in shoot numbers viz; Holostema ada-kodien (23), Hemidesmus indicus (24).

Rooting

For the induction of roots elongated multiple shoots of 4 to 6 cm were excised and inoculated on half strength MS rooting medium with different concentrations of auxin as the shoots failed to induce roots on medium without hormone. The tested auxin concentrations viz; IAA (0.5 to 3 mg/L), IBA (0.5 to 3 mg/L) and NAA (0.5 to 3 mg/L) showed varied response to root induction (Table 3). Rhizogenesis was observed from the excised microshoots within 15 days. Maximum number of 5.1±0.4 roots with 0.8±0.1 cm root length was obtained with IAA 2mg/L with 100% response (Table 3, Fig.1 e). IBA showed maximum of 4.9±0.8 roots with 0.6±0.06 cm root length at 1mg/L concentration. In vitro flowering was also observed at IBA 1.5mg/L (Fig.1 f). NAA 3mg/L showed maximum number of 1.1±0.2 roots with 80% response with 0.3±0.04 cm root length. In present study poor performance was observed with NAA regarding reduction in root number and length. Increase in concentration of NAA resulted in callus induction at the base of explant (Table 3). This may be due to the residual cytokinine in shoots (25). The reduced survival at higher concentrations of auxin treatment may be due to poor vascular connection of the root with the stem because of the intervention of callus (20). Among the three growth regulator tested, IAA found to be the most effective for root induction than that of IBA and NAA. In present study better results were obtained from IAA which is similar to observations found in some other species like C. hirsute (26), C.bulbosa along with cytokinine BA (27) and Decalepis arayalpathra (28). The roots formed at IAA concentration were thick and yellowish green in colour.

These rooted plantlets were transferred to small pots with autoclaved Sand:Soil:Cocopit (5:3:2). Initially the ziplock bags were covered on these pots to maintain the relative humidity. (Fig. 1 g, h) Subsequently these bags were removed to reduce moisture and proper water irrigation was provided. These plants were transferred to greenhouse and subsequently to its natural habitats.

Conclusion

Simple and rapid micropropagation system has been established for production of large number of plantlets in shorter span as compared to conventional method. It is actually the breakthrough inception in conservation and restoration of this endemic and critically endangered species. The best multiple shoot formation (7.5±0.3) achieved on MS medium

fortified with BAP 3mg/L as well as best rooting (5.1±0.4) obtained on half strength MS medium containing IAA 2mg/L.

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Table-1: Effect of different plant growth regulators on shoot induction and multiplication from nodal segments of Ceropegia fimbriifera on MS medium.

Growth Regulators (mgL^{-1})				%Response	Shoot/explant (mean ±SE)	Length (cm)of shoot (mean ± SE)
BA	TDZ	KN	Zeatin			
Growth regulator free				00	00	00
1				93.33	1.2±0.1**	7.7±0.3**
1.5				93.33	2.3±0.2**	8.5±0.5**
2				90	4.3±0.3**	8.1±0.5**
2.5				100	5.2±0.2**	9.3±0.3*
3				96.66	7.5±0.3**	11.2±0.9**
3.5				90	5.8±0.3**	$5.5±0.6^{ns}$
	1			93.33	2.3±0.2**	$8.7±0.7^{ns}$
	1.5			86.66	2.2±0.2*	5.0±0.6**
	2			96.66	2.4±0.2*	$9.1±0.6^{ns}$
	2.5			83.33	2.0±0.2**	$7.6±0.9^{ns}$
	3			86.66	1.7±0.2**	$7.4±0.7^{ns}$
	3.5			73.33	1.0±0.1**	$3.2±0.5^{ns}$
		1		83.33	$2.1±0.2^{ns}$	$7.2±0.9^{ns}$
		1.5		86.66	$2.3±0.3^{ns}$	$5.3±0.7^{ns}$
		2		93.33	1.4±0.2**	$7.7±0.9^{ns}$
		2.5		96.66	1.4±0.1**	$6.8±0.6^{ns}$
		3		96.66	1.9±0.1**	$9.6±0.6^{ns}$
		3.5		90	1.2±0.1**	$7.4±0.8^{ns}$
			1	93.33	2.2±0.2**	$3.0±0.3^{ns}$
			1.5	80	1.1±0.1**	$3.0±0.4^{ns}$
			2	83.33	1.3±0.1**	3.8±0.6**
			2.5	66.66	0.7±0.1**	3.1±0.5**
			3	80	0.9±0.1**	$5.0±0.7^{ns}$
			3.5	86.66	1.0±0.09**	$4.1±0.5^{ns}$

Values represent mean ±SE of 30 replicates per treatment; all the experiments were repeated twice. The values are significantly different * P<0.05 and **P<0.01 level when compared by Dunnett multiple comparisons test using one way ANOVA, ns-Non significant

Table-2 Effect of subculture on average number of shoot production of Ceropegia fimbriifera cultured on MS medium supplemented with BA 3 mg L^{-1}

Subculture	% Response	Shoot/explant (mean	Length (cm) of shoots (mean ± SE)
1st	96.66	7.5±0.3**	11.2±0.9**
2nd	96.66	7.6±0.3**	9.2±0.7**
3rd	93.33	8.0±0.4**	$9.2±0.5^{ns}$
4th	86.66	6.0±0.5*	$7.2±0.7^{ns}$
5th	90	$4.3±0.4^{ns}$	8.4±0.7**

Values represent mean ±SE of 30 replicates per treatment; all the experiments were repeated twice. The values are significantly different * P<0.05 and **P<0.01 level when compared by Dunnett multiple comparisons test using one way ANOVA, ns-Non significant

Table-3. Effect of various concentrations of auxins on in vitro root induction from micro shoots of Ceropegia fimbriifera on half strength MS medium.

Growth regulators mg L^{-1}			%response	Roots/shoot (mean ± SE)	Root length (cm) (mean ± SE)	Basal callus induction
IAA	IBA	NAA				
½ ms basal medium			00	0.0	0.0	-
0.5			90.	$3.3±o.4^{ns}$	$0.3±0.03^{ns}$	-
1			93.33	3.1±0.4*	$0.3±0.03^{ns}$	-
1.5			93.33	3.2±0.3*	0.5±0.03**	-
2			100	5.1±0.4*	0.8±0.1**	-
2.5			96.66	4.2±0.6**	$0.4±0.03^{ns}$	-
3			96.66	4.0±0.8**	0.7±0.2**	-
	0.5		90	$2.8±0.3^{ns}$	0.4±0.03**	-
	1		90	4.9±08*	$0.6±0.06^{ns}$	-
	1.5		93.33	$4.4±0.5^{ns}$	$0.6±0.06^{ns}$	-
	2		86.66	2.4±0.4**	0.5±0.05**	-
	2.5		93.33	2.2±0.4**	0.5±0.04*	-
	3		90	1.6±0.3**	0.4±0.04*	-
		0.5	70	0.9±0.1**	0.2±0.04**	-
		1	80	1.1±0.2**	$0.3±0.04^{ns}$	+
		1.5	80	0.8±0.08**	$0.3±0.04^{ns}$	+
		2	73.33	0.7±0.08**	0.2±0.02**	+
		2.5	76.66	0.7±0.07**	0.3±0.04*	+
		3	66	0.6±0.08**	0.2±0.03**	+

Values represent mean ±SE of 30 replicates per treatment; all the experiments were repeated twice. The values are significantly different * P<0.05 and **P<0.01 level when compared by Dunnett multiple comparisons test using one way ANOVA, ns - Non significant, + indicates basal callusing.

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Legends:

Fig 1 Ceropegia fimbriifera Beddome: **a**-Habit, **b**-Flower, **c**-Nodal explants inoculated on MS medium, **d**-Multiple shoot induction (MS+BAP 3mg/L), **e**-In vitro Flowering (IBA 1.5mg/L), **f**-Rooting (IAA 2mg/L), **g** and **h**-Hardened plants .