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Induction of Morphogenetic Callus and Multiple Shoot Regeneration in *Ceropegia pusilla* Wight and Arn.

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Abstract: This study was undertaken to evaluate the most suitable concentration of plant growth regulators and perfect explant (node, internode and thin cell layer explants-TCLs) for callus induction and subsequent organogenesis in an endangered medicinal *Ceropegia pusilla*. The best callus induction was found on the MS medium supplemented with 6-benzylaminopurine (BAP) 13.32 μM LG^1 + 2, 4-dichlorophenoxy acetic acid (2,4-D) 0.45 μM LG^1 from TCLs. After the initiation of the callus, it was immediately transferred to MS medium supplemented with BAP along with other auxins like 2, 4-D, Indol-3-Acetic Acid (IAA), Indole-3-Butyric Acid (IBA), Naphthalene Acetic Acid (NAA). The regenerative calli were raised on the MS medium supplemented with 1.13 μM LG^1 of 2,4-D. Whereas, the organogenic calli was raised on the medium containing 22.7, 40.86, 45.4 μM LG^1 of Thidiazuron (TDZ) induced 37.54±0.29, 37.12±0.18 and 34.32±0.17 shoots, respectively. On the media containing BAP 13.32 + IBA 0.49 to 1.23 μM LG^1 micro shoots rooted best and 75% of the shoots were survived. The plantlets were established, acclimatized and thrived in green house conditions with 80%. The regeneration protocol developed in this study provides a basis for germplasm conservation and for further investigation of bio active constituents of this medicinal plant.

Key words: Callus, organogenesis, multiple shoots, *in vitro* propagation, medicinal plant

INTRODUCTION

Ceropegia L. is an old world tropical genus containing about 200 species of which 48 *Ceropegia* species found in India (Bruyns, 2003). Twenty eight species of *Ceropegia* are endemic to the Peninsular India (Ansari, 1984; Ahmedulla and Nayar, 1986). The existence of the *Ceropegia* species has become restricted to remote pockets in the Himalayas and the Western Ghats, two biodiversity hot spots. Regrettably, the *Ceropegia* genus has now been added to the list of Indian endangered plants (Botanical Survey of India, 2002). *Ceropegia pusilla* is an annual herb grown wildly in South India and is in endangered category (Nayar and Sastry, 1987; Madhav, 2004; Walter and Gillett, 1998). The root tubers contain an alkaloid called Ceropegin (Nadkarni, 1976), consumed after cooking (Mabberley, 1997). The root tubers also contain starch, sugars, gum, albuminoids, fats, crude fiber and valuable constituents in many traditional Indian Ayurvedic drug preparations that are active against many diseases especially diarrhea and dysentery. The Ceropegin was an analgesic drug, tranquilizer and known to use against ulcers, inflammation etc., (Adibatti *et al*., 1991). Optimization for this plant's flowering (*in vivo*/*in vitro*) is an important issue because of the commercial value of this beautiful flower in the market. It is important to prevent the extinction of *C. pusilla* for its taxonomic and ethanobotanical importance as well as for the fact that it can be used as root stock for propagation of *C. pusilla*, however the *in situ* conservation effort has had the limited impact on halting the decline in the population. It is therefore, necessary to establish *ex-situ* conservational methods like micropropagation as supplementary measures. The present *in vitro* propagation study was taken to develop a method for multiplication of this endangered progenitor species.

MATERIALS AND METHODS

Ceropegia pusilla Wight and Arn. (Asclepiadaceae) was collected from the Shevaroy hill ranges, Tamil nadu and the voucher specimen was deposited in the herbarium of Department of Biotechnology, Montessori Mahila Kalasala, Vijayawada Andhra Pradesh, India. Plant material was collected during November and December

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2007, due to scarcity of the plant material, the work was started only with single plant. The nodes were excised from the one month-old plant to initiate *in vitro* cultures. The nodes containing axillary buds were washed in the running tap water, followed by a fungicide and bactericide each 0.3% for 10 min and with 5% tween 20 (v/v) for 4 min. Then, with surface disinfectant 0.1% $HgCl_2$ (w/v) for 2 min, after repeated washes in double distilled water, the sterilized segments were then washed thoroughly with sterilized distilled water, cut into appropriate sizes and cultured on nutrient medium.

Before placing onto MS medium (Murashige and Skoog, 1962) solidified with agar 0.9% (w/v) HiMedia Laboratories Pvt. Ltd. Mumbai and different growth regulators (BAP, Kn, TDZ, 2,4-D, IAA, IBA and NAA) at different concentrations either alone or in combinations were added to the medium. In the present study, all the media were autoclaved at 121°C and 15 lbs pressure for 20 min after adjustment of the pH to 5.7±2 with 1 N NaOH and 1 N HCl. To study the callogenesis, about 6-7 TCLs (3 from each side) were taken from either side of the *in vitro* grown plant nodes. All the *in vitro* cultures were maintained at 24±2°C and illuminated for 16 h with fluorescent light (18-24 µmol/m/sec) followed by 8 h dark period and the relative humidity was about 60-80% within the 250 mL bottles and 25×150 mm culture tubes covered with the aluminum foil. When the hormones failed to induce a specific response (callus, somatic embryos and direct organogenesis) at the end of the first cycle, it was marked as inappropriate combination. Twenty cultures were raised for each treatment and all experiments were repeated thrice. Cytokinins were tested individually to estimate the callogeny, caullogeny and organogenesis of the nodal/TCL explants.

In vitro regenerated shoots were inoculated for rooting on full strength medium supplemented with IAA, IBA and NAA in combination with BAP and sucrose. The rooted shoots were washed with distilled water to remove the traces of the medium. The *in vitro* rooted plantlets were transferred to vessels containing autoclaved vermiculite and sand in 1:1 ratio. Those vessels were covered initially with polythene bags to maintain humidity and placed in a mist chamber. After every alternative day, quarter strength MS medium salt solution was supplied to the plantlets. After two weeks of growth, the complete plants were established, acclimatized and thrived in green house conditions.

Statistical analysis Experiments were set in the Completely Randomized Block Design (CRD). The 25×1.5 cm test tubes formed a replicon, 20 such replicants were provided for every culture. Similarly 20 replicates were provided during the trials on shoot organogenesis from callus derived axillary shoot and root induction was from microshoots. However, media in these cases were dispensed in Erlenmayors' flasks of 250 mL capacity, 30 mL of media was poured in each flask. The parameters studied were percentage of explant that under went callusing average number of shoots regenerated from each callus, average number of shoots developed for axillary bud, percentage of shoots from which roots developed, average number of shoots that developed per shoot and mean root length, data were subjected to Analysis of Variance (ANOVA) and comparison among mean of treatments were made by Tukey's HSD test with p#0.05 was considered to be statistical significant, using statistical software Graphpad instat.

RESULTS AND DISCUSSION

Effect of growth regulators on the callus formation: The combination of BAP and NAA induced an excellent amount of callus from the nodes of *Ceropegia pusilla* and the morphology of the callus was friable, cream to yellow colored and nodular in its nature (Table 1). It was observed that the 2, 4-D at any concentration will stimulate the TCLs callus proliferation together with BAP. The medium supplemented with 2, 4-D in the range of 0.45 to 4.52 µM LG^1 together with BAP 13.32 µM LG^1 had the ability to produce the excellent embryogenic callus (Fig. 1A). While the explants cultured on the medium supplemented with 2, 4-D 1.13 µM LG^1 + BAP 13.32 µM LG^1 had optimum effect on organogenesis. Since, 2, 4-D is said to show unorganized growth regulation and is supportive for the organogenesis together with the cytokinins as in the findings of Jager and van Staden (1996). In this study, all the media were supplemented with cytokinin (constant), the necessity of the cytokinin for shoot initiation was well established as in the findings of Beck and Componetti (1983).

Effect of auxins and BAP on efficiency of callus formation: Induction of callus from the Thin Cell Layer (TCL) explants of *Ceropegia pusilla* were initiated on the medium supplemented with various concentrations of

Table 1: Callus induction from stem explants of *Ceropegia pusilla* cultured on MS medium supplemented with various hormonal concentrations

Concentrations of PGR	Morphology of the callus	Response±SD
BAP 4.44 + NAA 2.68 µM	YC, N, F	99±3.47
BAP 8.88 + NAA 2.68 µM	YC, N, F	99±3.50
BAP 13.32 + NAA 2.68 µM	YC, N, F	98±0.46
BAP 17.76 + NAA 2.68 µM	YC, N, F	97±0.48
BAP 22.2 + NAA 2.68 µM	YC, N, F	99±3.49

YC: Yellowish cream; N: Nodular; F: Friable

Table 2: Induction of callus from the TCLs on the medium containing different concentrations of auxins in combination with BAP

		Organogenesis				
Treatment BAP 13.32 µM LG^{1}	Callus formation (%)	No. of shoots	No. of roots	No. of Shoots±SE	Embryogeny	Texture and color of the callus
2,4-D 0.45	98.67[a]	-	-	2	EC	YC, N, F
2,4-D 1.13	97.34[a]	4	-	0	EC	YC, N, F
2,4-D 2.26	97.34[a]	-	-	0	EC	YC, W, F
2,4-D 4.52	94.45[a]	-	-	0	EC	YC, C
IAA 0.57	59.47[a]	-	-	3	NE	G, NP
IAA 1.44	59.47[a]	-	-	2	NE	G, NP
IAA 2.88	59.47[a]	-	-	0	NE	G, NP
IAA 5.77	59.46[a]	-	-	0	NE	G, NP
IBA 0.49	49.47[b]	3	-	4	NE	SC
IBA 1.23	49.47[b]	-	-	4	NE	SC
IBA 2.46	49.48[b]	-	-	3	NE	SC
IBA 4.92	49.47[b]	-	-	3	NE	SC
NAA 0.53	97.34[a]	-	2	1	EC	SRC
NAA 1.34	96.37[a]	-	2	0	EC	SRC
NAA 2.68	98.66[a]	-	2	2	EC	SRC
NAA 5.37	97.37[a]	4	3	2	EC	MRC

Values with same letters was not significantly different. E: Embryogenic; NE: Non embryogenic; YC: Yellowish cream; N: Nodular; C: Compact; F: Friable; G: Greenish; W: Whitish; NP: Non proliferating; SC: Slight callus; SRC: Slight rhizoidal callus; MRC: Moderate rhizoidal callus

auxins and cytokinins. Callus was observed from TCL explants on MS medium containing combinations of BAP, 2, 4-D, IAA, IBA and NAA. The callusing response of most of these PGRs has been studied while, multiplication of *Gymnema sylvestre* (Reddy *et al*., 1998); *Hemidesmus indicus* (Siddique *et al*., 2003). The callus was induced at the periphery of the explants by culturing the TCLs for 2-3 weeks on almost all the media tested (Fig. 1A). It was observed that the tTCLs are immediately ready to produce an extensive callus, the variation in the callusing of different tissue layers was observed clearly. However, the degree of the callus formation varied with the treatments.

The young stem derived callus is highly viable, whereas the callus derived from the leaf bits was soft and could not be maintained beyond a second or third sub cultures similar observations also found in *Tylophora indica* (Rao and Narayanaswamy, 1972); *Ceropegia jainii*, *C. bulbosa* var. *bulbosa* and *C. bulbosa* var. *lushii* (Patil, 1998). On the contrary, in *Ceropegia candelabrum* (Beena and Martin, 2003); *Decalepis hamiltonii* (Giridhar *et al*., 2004) produced callus from the leaf and internodal explants. Later, same callus has produced somatic embryos too. Whereas, Neetha *et al*. (2005) obtained his embryogenic callus from roots and leaves of *Hemidesmus indicus*. The optimum callusing was observed on the MS medium fortified with BAP 13.32 µM LG^{1} + 2,4-D 0.45 µM LG^{1}, as in case of *Ceropegia sahyadrica* (Nikam and Savant, 2007). The 2, 4-D is the principle auxin to induce the callus, it was best observed in case of other taxa too, *Ceropegia* species viz., *C. jainii*, *C. bulbosa* var *bulbosa* and *C. bulbosa var lushii* (Patil, 1998); *Gymnema sylvestris* (Gopi and Vatsala, 2006; Roy *et al*., 2008). Whereas, the other auxin also used to induce the callus in *Tylophora indicia* (Faisal, 2003); 2, 4-D + 2iP were used to induce the callus in *Pergularia daemia* (Kiranmai *et al*., 2008).

The callus produced on the medium containing BAP 4.44 µM LG^{1} + 2, 4-D 0.45 µM LG^{1} + NAA 2.26 µM LG^{1}. The 2, 4-D 0.45 µM LG^{1} had the ability to produce hairy roots (99%). The texture of the callus depends on the concentration and type of growth regulators. The callus formed on the media augmented with IAA and NAA quite often showed fine hairy mass on the surface of calli while the calli induced on the 2,4-D were soft, pale yellow and slight morphogenetic, similar findings were noticed in *Ceropegia sahyadrica* (Nikam and Savant, 2007).

The combination of BAP with NAA and 2, 4-D had the organogenic ability for certain extent, The TCLs are cultured on the medium supplemented with BAP and NAA and produced an excellent callus, the callus is very competent and friable in its nature. The regeneration of shoot primordia on the callus were observed clearly (Table 3) on the medium containing BAP13.32 µM LG^{1} along with 2,4-D 1.13 µM LG^{1}, IBA 0.49 µM LG^{1}, NAA 5.37 µM LG^{1}. Dissimilar observations were noticed in case of *Gymnema sylvestris* internodes while inducing the callus (Roy *et al*., 2008). It was observed that the media supplemented with IBA and IAA had less callusing ability when compared to 2, 4-D and is non embryogenic too. But, the ability of these media to produce the considerable number of shoots (4) was recorded (Table 2).

Whereas, on the medium containing NAA along with BAP was found to produce good amount of callus with rhizoids like structures. The concentration of NAA and the rooting ability are directly proportional to one another up to 0.53 to 5.37 µM LG^{1} along with BAP 13.32 µM LG^{1} (constant). The embryogenic nature of the callus clumps was quite well, considerable organogenesis was observed on the media supplemented with 2, 4-D 1.13 µM LG^{1} along with BAP13.32 µM LG^{1} (Fig. 1B).

Table 3: Effect of cytokinins on the regeneration of *Ceropegia pusilla*

PGRs	PGR in µM	Shoot no Mean±SD
BAP	0.04	3.62±0.06[c]
	0.22	2.10±0.07[c]
	0.44	4.19±0.13[c]
	0.88	3.17±0.09[c]
	1.33	3.10±0.09[c]
	1.77	3.31±0.07[c]
	2.22	5.40±0.19[c]
	2.66	5.24±0.13[c]
	3.10	4.60±0.05[c]
	3.55	3.69±0.15[c]
Kn	0.04	2.03±0.08[c]
	0.22	1.03±0.08[c]
	0.45	1.18±0.11[c]
	0.91	1.46±0.09[c]
	1.36	1.38±0.10[c]
	1.82	1.25±0.01[c]
	2.28	1.25±0.01[c]
	2.73	2.19±0.11[c]
	3.19	2.28±0.15[c]
	3.64	1.20±0.05[c]
TDZ	4.54	11.69±0.16[c]
	9.08	11.88±0.17[c]
	13.62	8.12±0.01[c]
	18.16	17.34±0.16[bc]
	22.70	37.54±0.29[a]
	27.24	32.64±0.25[a]
	31.78	10.94±0.34[c]
	36.32	10.09±0.16[c]
	40.86	37.12±0.18[a]
	45.40	34.32±0.17[a]

Data indicate Mean±SD of the mean following by the same letter was not significantly different by the Tukey-Kramer multiple comparisons test at 0.05% probability. Twenty replicates were used per treatment experiments were repeated thrice

Effect of TDZ on induction of organogenesis from the nodes: The aim of the present study was to study the activity of TDZ in *in vitro* regeneration of *Ceropegia pusilla*. Even, TDZ is very useful to induce the callus. TDZ at 22.7, 40.86 and 45.4 µM LG^{1} had the maximum ability to induce organogenesis in the nodes, almost all the concentrations of TDZ resulted in the organogenesis, some of the concentration had the tendency to support the growth of the tubers, the protocol we generated is a reproducible protocol for the commercial companies.

All the media initially have a propensity to produce callus, later on it was noticed that only the 2, 4-D had ability to produce friable and light green colored callus. TCLs are quite active in callogenesis on the MS medium supplemented with BAP in combination with different auxins to induce callus, organogenesis and somatic embryos.

Effect of BAP, KN and TDZ on shoot regeneration: Among these three hormones, studied, first two are not much effective in induction of the shoots or for the organogenesis. The tested BAP concentrations 2.22, 2.66 and 3.10 µM LG^{1} supports to induce multiple shoots, i.e., 5.40±0.19, 5.24±0.12 and 4.60±0.05 shoots, respectively, more or less similar number of shoots were reported in *Ceropegia hirsute* (Nikam *et al*., 2008).

KIN had very low percentage of response, even high levels also not able to induce multiple shoots 3.19, 2.19 µM LG^{1} KIN had some response to induce 2.28±0.15, 2.19±0.11 shoots, respectively. Among the cytokinins tested, KIN was less effective than BAP or TDZ for multiple shoot induction. The present results are in agreement with previous reports on *C. bulbosa* and *C. jainii* revealed that the BAP alone can induce axillary shoot multiplication from nodal segments (Patil, 1998). On the other hand, a synergistic effect of a range of growth regulators in combination with BAP for shoot regeneration was well documented for members of Asclepiadaceae viz., *C. candelabrum* (Beena *et al*., 2003), *Holostemma ada kodien* (Martin, 2002) and *Hemidesmus indica* (Sreekumar *et al*., 2000). *Holostemma annulare* (Sudha *et al*., 1998; Martin, 2002) and *Leptadenia reticulate* (Arya *et al*., 2003). In other cases, the superior activity of BAP compared to other cytokinins was reported in many members of this family, i.e., *Gymnema sylvestre* (Komalavalli and Rao, 2000) and *Anisomelus indica* (John *et al*., 2001). The response on the media containing BAP/KIN was considered as very low response when compared to the TDZ concentrations. The stimulatory effect of TDZ on bud breaks and shoot regeneration has been reported earlier by Singha and Bhatia (1988). In this investigation, all the shoots regenerated on TDZ supplemented media are very small and can not be counted as it is because of their compact origin format. Although, high concentrations of TDZ induced more number of shoot buds, but failed to elongate. The formation of stunted shoots on TDZ supplemented medium has been reported earlier by Preece and Imel (1991), which could be a result of the phenyl group in TDZ.

Among the three cytokinins tested, it was TDZ which shown an excellent outstanding response showing its superiority over the other two cytokinins i.e., BAP and KIN. The TDZ combination is well suitable for the shoot regeneration and organogenesis. The optimum number 37.54±0.29 shoots were induced on the medium containing 22.7 µM LG^{1} of TDZ (Fig. 1C, D). Where as the medium containing 40.86 µM LG^{1} TDZ had the second highest number of shoots 37.12±0.18 and at 45.4 µM LG^{1} TDZ induced considerable number (34.32±0.17) of shoots. Superior activity of TDZ on the BAP was observed in the findings of Hussain *et al*. (2008). The explants cultured on either TDZ or BA differentiated multiple shoots, even though the highest number of shoots per explant was recorded on TDZ (Hussain *et al*., 2007, 2008; Huetteeman and Preece, 1993; Murthy *et al*., 1998; Thomas, 2007;

Fig. 1: Morphogenesis of *Ceropegia pusilla*. (A) Induction of callus from the nodes of *Ceropegia pusilla* on MS media containing 2, 4-D 0.45 µM L^{-1} along with BAP 13.32 µM L^{-1}. (B) Organogenesis from the callus of *C. pusilla* on the media containing 2, 4-D 1.13 µM L^{-1} along with BAP13.32 µM L^{-1}. (C) Morphogenesis of the callus to shoots on the media supplemented with TDZ 22.7 µM L^{-1}. (D) Induction of multiple shoots from the nodes of *C. pusilla* on the media supplemented with TDZ 22.7 µM L^{-1}. (E) Induction of roots from the shoots on MS medium containing BAP 13.32 + IBA 1.23 µM L^{-1}. (F) Healthy transplanted plantlet in soil after 15 days

Preece and Imel, 1991; Pradhan *et al*., 1998). Fiola *et al*. (1990) and Malik and Saxena (1992) had an opinion that the TDZ has been shown to promote shoot regeneration with efficiency comparable to or greater than that of cytokinins. On the contrary, among the cytokinins tested, BAP was found more effective than others in including shoot development and multiple shoot induction in *Andrographis paniculata* (Purkayastha *et al*., 2008). The ability of TDZ to induce high shoot regeneration efficiency in plant tissue has been reported for a number of species (Thomas and Philip, 2005; Landi and Mezzetti, 2006).

The combinations of media which was responsible for the organogenesis possessed stunted shoots were transferred to the medium containing 9.12 µM LG^1 KIN and 8.88 µM LG^1 BAP. All the plants showed considerable variation in the length in accordance with Samuel *et al*. (2009). Many combinations of auxins along with BAP 13.32 µM LG^1 and will not induce the rooting. But on the medium containing BAP 13.32 µM LG^1 + IBA 0.49 µM LG^1 to 1.23 µM LG^1 micro shoots rooted best (Fig. 1E).

Micro shoots with well-developed root system were directly transferred to small pots containing sterile vermiculite and coco peat in (1:1) ratio rejuvenated growth within 20 days. Survival rate of the plantlets is 80% and plantlets successfully established in the field exhibited morphology similar to that of mother plants (Fig.1F). About two weeks, the pots were placed in a mist chamber, where gradual decrease in the humidity was taken place.

CONCLUSION

In conclusion, the present study reported successful micropropagation protocol that can be employed in the propagation of endemic taxa *Ceropegia pusilla* and helps in conservation and domestication. There by minimizing the pressure on wild populations of the valuable flora of the forest. The TCLs here employed to reduce the negative effect of the latex on explant - medium contact. Creating genetically modified variety will also be possible from this callus (Gamborg and Phillips, 1995). This *in vitro* study will help future workers on developing related manipulations

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