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AN EFFICIENT INDIRECT REGENERATION AND MULTIPLE SHOOTS FORMATION FROM NODAL EXPLANT OF *CEROPEGIA JUNCEA* ROXB.

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Abstract

An effective protocol has been developed for indirect shoots regeneration from nodal explant of C. juncea. Explants were cultured on MS medium inclusion with alone 2,4-dichlorophenoxyacetic acid (2, 4-D), Thidiazuron (TDZ) or in combined with concentrations of 6-benzylaminopurine (BA) and Kinetin (KN) for callus induction. The best response (73.5%) was observed from nodal explants on MS medium 0.5mg/L 2,4-D in combination with 0.05mg/L BA.Thecalli derived from nodal explants were subcultured on MS medium supplemented with BA in combination with NAA or IAA for shoot induction. The more number of shoots (5.37) and shoot length (6.13cm) was observed on MS medium supplemented with 1.0 mg/L BA with 0.10mg/L NAA of shoot regeneration in nodal derived callus. Nodal callus derived microshoots gave the highest rooting percentage (77.9%), root numbers (9.75) and length of roots (5.32 cm) were observed on half strength MS basal medium Inclusion of 0.5 mg/L IBA. Regenerated plantlets with well-developed shoots and roots successfully transferred to soil. This protocol could be useful for conservation and cultivation of C. juncea.

Keywords: *Ceropegia; Node; Callus initiation; 2, 4-dichlorophenoxyacetic acid.*

Introduction

The genus, *Ceropegia* under the family Apocynaceae, represented by approximately 200 species, which are usually tuberiferous erect herb and climbers which are distributed in tropical and subtropical Asia, Africa, Australia and Pacific Islands [1-2]. Presently the genus is represented by 50 species of which about 38 species were recorded in Western Ghats. Out of these 38 species of *Ceropegia* in Western Ghats about 15 are narrow endemic and all of them are highly threatened [3-4], *Karthikeyan et al.* [5] reported about 56 species, of which 2 subspecies and 3 varieties in India.

The *Ceropegia juncea Roxb* is an important medicinal, which is used as a source of "Soma", a plant drug contained ayurvedic medicine with a wide variety of uses [6]. The alkaloid ceropegin was isolated and identified as pyridone type alkaloid, which is relatively rare in nature [7]. The decoction of the whole plant is used for the treatment of liver disorders, ulcerative condition and fever. The plant paste is topically used as an anaesthetic agent [8-9]. Plant tissue culture technique would minimize the damage to remnant populations of RET medicinal plants. There are some reports on *in vitro* propagation studies of *Ceropegia* species using different explants [10-13].

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The high quality production of callus formation based upon the different explants like cotyledon, leaf, nodal, internode etc., and this process done with suitable plant growth regulators for regenerate the subsequent plantlets. Hence, the seed germination of this plant very poor but in pharmaceutical way, the plant has more medicinal uses so the plant will conserve methods that are *in vitro* propagation. The main objective of the present study mentioned the successful protocol for callus induction, shoot regeneration and complete plantlet development in *C. juncea* using nodal explants

Materials and Methods

Nodal explants were transferred into 250mL sterile conical flask and thoroughly washed under running tap water for 15-20min to remove the soil particles. Then explants treated with 0.1% (v/v) *Tween-20* for 5 minutes and followed by treated with 0.5% of *Bavistin* for the removal of unwanted microorganisms and rinsing 1-2 minutes distilled water. Further, the explants were transferred into aseptic environment in a laminar air-flow chamber. Next step is the explants surface was sterilized with help of 20% (v/v) ethanol (1 minute] and then followed by 0.01% (v/v) of $HgCl_2$ (2-3 minutes). Finally, the explants were washed with sterile double, distilled water (6-8 times). Pure and healthy sterilized explants were inoculated into MS medium.

Culture media and conditions

MS basal medium containing different concentration of plant growth regulators like auxins and cytokinins either individually or combined with 3% sucrose. After the media adjusted pH value 5.6 to 5.8 and 8g/L agar was added. Finally, MS medium was autoclaved at 121°C and 103kPa for 20 minutes. After that media sterilized then transferred into aseptic environment lab condition. Cultures were incubated at 22±1°C with 16/8h photoperiod by cool white fluorescent tubes (Philips L. 58 W/640, 30µmol·m^{-2}·s^{-1} PPF) and 75-80% relative humidity.

Callogenesis (Indirect Organogenesis)

Aseptically excised nodal explants were cultured on MS basal medium supplemented with various concentrations of alone 2,4-D, TDZ (0.5, 1.0 and 2.0 mg/L) or in combined with five different concentrations of BAP and KN (0.05, 0.10, 0.20, 0.30 and 0.40mg/L) for callus formation. Every four week's the mature callus was subcultured on the same combination of fresh medium. During subculture each callus was cut into the size 2–4mm. First four week's of culture, the efficiency of PGRs on callus induction was estimated by recording the percentage of explants survival and days required for callus induction.

$$\text{Frequency of callus} = \frac{\text{Number of explants responded}}{\text{Total number of explants inoculated}} \times 100 \qquad (1)$$

Plant regeneration from callus cultures

Mature callus were transferred into MS medium, fortified with BAP and NAA (0.5, 1.0 and 2.0mg/L) alone or in combination of IBA (0.05, 0.10 and 0.20mg/L) for evaluation of shoot regeneration. After four week's, number of shoots produced per cultured explant and length of shoots were recorded.

Rooting

The length of regenerated shoots 2 to 5cm were cultured on half strength MS medium fortified with various concentration of IBA, IAA and NAA (0.5, 1.0, 1.5, 2.0 and 2.5mg/L) for induction rooting purpose. Percentage of root induction, number of roots and root lengths were recorded after four week's culture.

Plant acclimatization

The well-developed plantlets were carefully removed from the culture media and washed with distilled water for the removal of traces from the plantlets. They have used different hardening media substrates for hardening purposes. The pots contained autoclaved soil, sand and vermiculate they are taken alone or combined. These pots were kept in mist chamber and humidity level of chamber 85–90%. After day, the MS medium sprayed to plantlets, other coming alternate day. After 14 days the water will supply to every pots. End of this process well develop plantlets transferred in green house condition.

Statistical analysis

The cultures were observed periodically and morphological changes were recorded at regular intervals. Each experiment was repeated with 20 replicates. First four culture callus induction percentage was recorded. In shoot and rooting experiments, percentage, numbers and length were recorded after four week's. Analysis of variance (ANOVA) was performed on all data to compare concentration effect of growth regulators and influence of days. Means were separated using Duncan's Multiple Range Test (DMRT).

Results and Discussions

Callus induction

Nodal explants of *C. juncea* was inoculated on MS medium with various levels of 2,4-D and TDZ alone or in combination with BAP and KN needs to induce the callus. After 14 days the callus growth started. From the study 67.80 % of callus formation was observed on MS medium fortified with 0.5mg/L 2,4-D alone but any different morphogenic characters did not occur. Green colored callus with nodular structure formed on the surface of callus because of activation of the 2,4-D combinations with BAP or Kn. In MS basal medium contained 0.5mg/L 2,4-D with combination of 0.05mg/L BAP can be observed the high percentage (73.53%) of callus induction (Table 1). *Nikam* and *Savant* [14] and *Phulwaria et al.* [11] who are reported that the optimum percentage of callusing was observed from explants cultured on MS medium fortified with 2,4-D and combination of BAP as in case of *Ceropegia juncea* and *Ceropegia bulbosa* respectively.

On the other hand the same concentration of plant growth regulators could induce the callus in huge size and the green color blotches are scattered on the surface of the callus in 4^{th} week. The callus formation started on the basal cut of the nodal explants. The occurrence of green spots on surface of callus which are considered as meristematic centers it indicates capacity of the callus to produce the adventitious shoots [15-16]. Recently, different concentration of auxins and combination with cytokinins could induce green nodules on the surface of callus in many plant species [17-19]. From the result the high concentration of 2,4-D and TDZ (3.0mg/L and 4.0mg/L) combination with BA and Kn (0.30mg/L and 0.40mg/L) the callus grows slowly but other steps of subculture the growth was stunned. Then the callus color changed whitish green into brown in color for the reason of callus cells were died because of the used high concentration of plant growth regulators. The effective role of combined treatments of BAP with 2,4-D on callus formation of Asclepediaceae members are also reported in earlier, *Ceropegia candelabrum* [20], *Tylophora indica* [21] and *Caralluma lasiantha* [22].

Shoot proliferation and Multiplication

Among the BAP and NAA alone or combination with IBA tested for shoot proliferation and multiplication of *C. juncea*. From the results of the present study showed that the shoots regenerated from the organogenic callus. MS medium supplemented with 1.0mg/L BA alone to induce the shoot initiation percentage (65.66%), shoot number (3.48) and shoot length (5.04cm) but not sufficient numbers shoots produced. On the other hand MS medium fortified with 1.0mg/L of BAP and addition of 0.10mg/L NAA could produce the high percentage of 82.44% shoot initiation, numbers of shoots (5.37) and shoots length (6.13cm) and followed 1.0mg/L

NAA and combination with 0.10mg/L IBA induced 72.30% of shoot initiation, numbers of shoots (4.15) and shoots length (5.32cm), (Table 2 and Fig. 1g).

Table 1. Effect of different concentrations and combination of 2, 4-D, TDZ, BAP and KN in MS medium on callus induction from nodal segments of treated seedlings of *Ceropegia juncea*

S.no	Plant growth regulators				Days required for callus formation inoculation	Percentage of callus induction
	2,4-D	TDZ	BAP	KN	Node	
1	0.5	0.0	0.0	0.0	14	67.80±0.64^{c}
2	1.0	0.0	0.0	0.0	15	63.86±0.81^{e}
3	0.0	0.5	0.0	0.0	16	64.49±0.64^{f}
4	0.0	1.0	0.0	0.0	17	56.66±1.10^{hi}
5	0.5	0.0	0.05	0.0	15	73.53±0.87^{a}
6	1.0	0.0	0.10	0.0	17	65.56±0.56^{d}
7	2.0	0.0	0.20	0.0	19	63.48±0.57^{e}
8	3.0	0.0	0.30	0.0	26	56.76±0.96^{hi}
9	4.0	0.0	0.40	0.0	27	59.13±0.83^{g}
10	0.0	0.5	0.05	0.0	16	68.52±0.59^{bc}
11	0.0	1.0	0.10	0.0	19	60.54±0.97^{f}
12	0.0	2.0	0.20	0.0	21	55.54±0.97^{ij}
13	0.0	3.0	0.30	0.0	26	56.28±0.72^{i}
14	0.0	4.0	0.40	0.0	29	45.84±0.84^{m}
15	0.5	0.0	0.0	0.05	15	69.25±0.81^{b}
16	1.0	0.0	0.0	0.10	18	55.62±1.08^{ij}
17	2.0	0.0	0.0	0.20	21	58.53±0.87^{g}
18	3.0	0.0	0.0	0.30	26	54.79±0.26^{j}
19	4.0	0.0	0.0	0.40	29	50.75±0.87^{k}
20	0.0	0.5	0.0	0.05	18	60.81±0.96^{f}
21	0.0	1.0	0.0	0.10	20	58.08±0.21^{gh}
22	0.0	2.0	0.0	0.20	22	61.53±1.00^{f}
23	0.0	3.0	0.0	0.30	26	49.18±0.91^{k}
24	0.0	4.0	0.0	0.40	30	51.24±1.09^{k}

Each value represents the mean ± SD, n = 20. Mean followed by the same letters in each column are not significantly different at $P < 0.05$ according to Duncan's multiple range test.

Table 2. Effect of different concentration and combinations of auxins and cytokinins in MS medium on shoot initiation of *C. juncea* from nodal derived callus

S.	Plant growth regulators				Node callus	
no	BAP	NAA	IBA	% of shooting	No. of shoots	Shoot length
1	0.5	0.0	0.0	63.36±1.13^{i}	2.51±0.50	4.25±0.54^{de}
2	1.0	0.0	0.0	65.66±0.73^{fg}	3.48±0.64^{cde}	5.04±0.18^{cd}
3	2.0	0.0	0.0	60.47±1.07^{jk}	3.06±0.58^{de}	3.96±0.06^{d}
4	0.0	0.5	0.0	61.88±1.14^{j}	3.81±0.23^{bcd}	5.56±0.45^{bc}
5	0.0	1.0	0.0	64.55±0.78^{hi}	3.26±0.27^{def}	5.25±0.54^{bc}
6	0.0	2.0	0.0	60.29±0.63^{k}	2.98±0.10^{de}	3.66±0.76^{e}
7	0.5	0.05	0.0	70.67±0.58^{e}	4.26±0.69^{b}	5.81±0.49^{abc}
8	1.0	0.10	0.0	82.44±0.76^{a}	5.37±0.30^{a}	6.13±0.10^{a}
9	2.0	0.20	0.0	58.54±0.46^{l}	3.81±0.24^{bcd}	5.41±0.44^{bc}
10	0.0	0.5	0.05	71.02±1.0^{c}	4.18±0.63^{bc}	5.0±0.11^{cd}
11	0.0	1.0	0.10	72.30±0.81^{b}	4.15±0.11^{bc}	5.32±0.23^{bc}
12	0.0	2.0	0.20	66.59±0.56e^{f}	3.30±0.27^{de}	5.08±0.20^{cd}
13	0.5	0.0	0.05	68.23±1.00^{d}	2.91±0.07^{f}	3.76±0.50^{d}
14	1.0	0.0	0.10	71.24±1.09^{c}	2.59±0.30^{f}	3.44±0.59^{e}
15	2.0	0.0	0.20	67.89±0.39^{de}	2.91±0.09^{ef}	3.81±0.46^{d}

Each value represents the mean ± SD, n = 20. Mean followed by the same letters in each column are not significantly different at $P < 0.05$ according to Duncan's multiple range test.

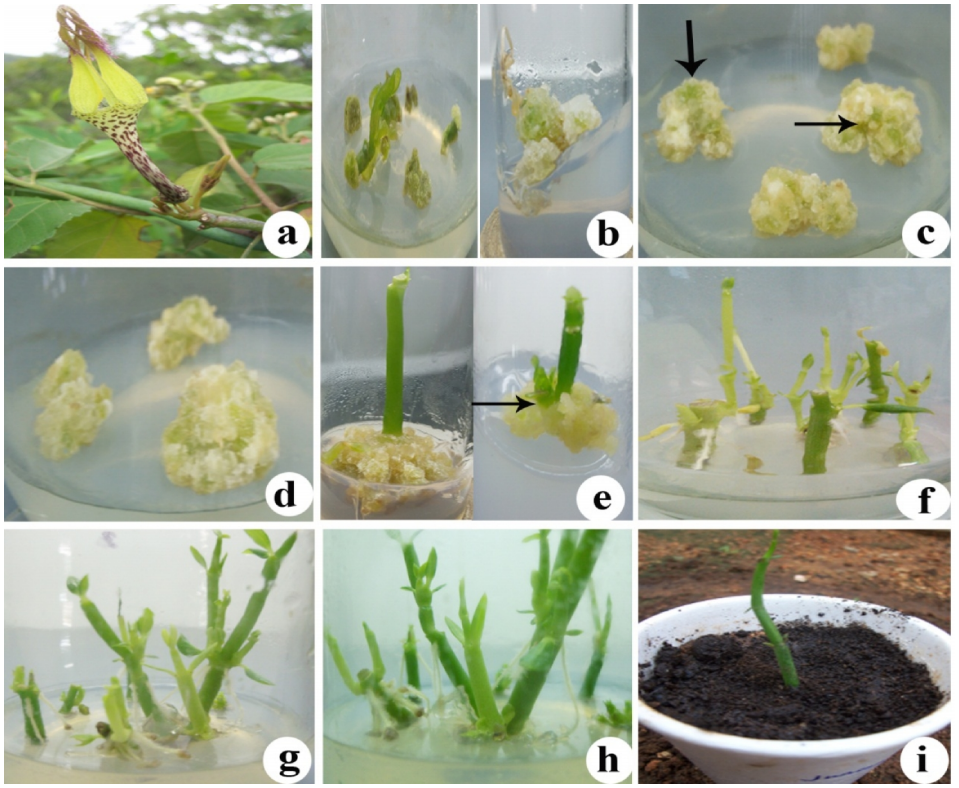

Fig. 1. Callus induction and plant regeneration from nodal explants of *Ceropegia juncea*:
a. Flower of *C. juncea*, b. Callus initiation from nodal explant,
c and d. green callus at 0.5mg/L 2,4-D and combination with 0.05mg/L BAP, e. shoot initiation from callus;
f and g. Multishoots formation at 1.0mg/L BAP and combination with 0.10mg/L NAA;
h. Rooting at 0.5mg/L IBA; i. Hardening

The ability of shoot multiplication was observed, when explants were cultured on combined treatments of various concentrations of BAP and NAA. *Phulwaria et al*. [11] noticed the maximum numbers of shoot was achieved when proliferated callus was cultured on MS medium supplemented with BAP either alone or in combination with IAA or NAA. Maximum numbers of shoots were regenerated on MS medium containing 1.0mg/L BAP and 0.1mg/L NAA. From the study the result is agreement with the report of *Phulwaria et al*. [11]. *Dhir* and *Shekhawat* [18] mentioned that the combination of 8.88µM BAP with 0.54µM NAA and 8.88µM BAP with 0.27µM NAA produced highest number of shoots in *C. bulbosa* var. *bulbosa* and *C. bulbosa,* var. *lushii* respectively. The best performance of shoot regeneration and multiplication was noticed by using of tTCLs isolated from the node callus explants of *Ceropegia spiralis* cultured on MS medium fortified with BAP 13.32µM and combination of NAA 0.537µM [23]. The effect of BAP and NAA on shoot regeneration and multiplication has also been demonstrated in a number of cases [24-26].

Rooting

The raised roots from microshoots to enhance the plantlets, different concentrations of auxins (IBA, IAA and NAA) were tested (Table 3). In *in vitro* shoots produce healthy roots from half-strength MS medium fortified with 0.5mg/L IBA. Half-strength MS medium fortified with IBA (0.5mg/L) was found to initiation of healthy roots from *in vitro* shoots. And moreover this concentration maximum number roots (9.57) and length of roots (5.32) were achieved in MS medium and followed 0.5mg/L IAA induced number roots (8.87) and length of roots (4.38

cm) were recorded (Table 3, Fig. 1h). From the study it reveals root induction of C. *juncea* is poor, when in vitro shoots culture on different concentration of NAA and IAA. NAA and IAA were poor for induction of roots in *C. juncea*. However, they showed significant root induction when combined with IBA. IBA is the most effective for root induction PGR's to the comparison of NAA and IAA. The formation in vitro roots from microshoots on this medium were found to be thicker which has an added advantage during hardening purpose. In microshoots shoots were produced the new and thick roots, they were used to during hardening purpose. Generally, IBA is more active PGR's for root induction in such plants; they are *Ceropegia noorjahaniae* [12], *Ceropegia bulbos*a; *Ceropegia juncea* [13], *Wattakaka volubilis* [27] and *Ceropegia thwaitesii* [28].The healthy *in vitro* rooted plantlets were taken into plastic cups for hardening. Hardening media having sterilized sand:soil:vermiculite in the ratio of 1:1:1 (Fig. 1i). After the pots will transferred into greenhouse condition and regularly watered for acclimatization, transferred plantlets were well established in a soil. Finally, the successful survival rate 85% was recorded.

Table. 3. Effect of different concentration of auxins on root regeneration abilities of the 45 days old Node callus emerged shoots of *C. juncea*

S.no	Plant growth regulators			Node		
	IBA	IAA	NAA	% of rooting	No of root	Length of Root
1	0.5	0.0	0.0	77.9 ± 1.21^{a}	9.57 ± 0.31^{a}	5.32 ± 0.50^{a}
2	1.0	0.0	0.0	70.29 ± 1.45^{c}	8.52 ± 0.49^{abc}	4.83 ± 0.25^{ab}
3	1.5	0.0	0.0	66.61 ± 0.86^{d}	6.25 ± 0.54^{ef}	4.21 ± 0.87^{bc}
4	2.0	0.0	0.0	61.92 ± 0.86^{f}	7.74 ± 0.31^{a}	3.18 ± 0.41^{def}
5	2.5	0.0	0.0	59.75 ± 1.43^{g}	6.18 ± 0.95^{ef}	3.83 ± 0.51^{cde}
6	0.0	0.5	0.0	73.24 ± 0.91^{b}	8.87 ± 0.79^{ab}	4.38 ± 0.65^{bc}
7	0.0	1.0	0.0	64.44 ± 1.15^{e}	7.44 ± 0.69^{cd}	4.04 ± 0.72^{bcd}
8	0.0	1.5	0.0	55.37 ± 0.54^{h}	6.44 ± 0.56^{de}	3.36 ± 0.11^{def}
9	0.0	2.0	0.0	55.05 ± 1.00^{h}	5.83 ± 0.50^{efg}	2.90 ± 0.31^{fg}
10	0.0	2.5	0.0	50.98 ± 1.01^{i}	5.44 ± 0.58^{efg}	2.88 ± 0.33^{fg}
11	0.0	0.0	0.5	51.81 ± 1.07^{l}	3.66 ± 0.38^{h}	2.88 ± 0.33^{fg}
12	0.0	0.0	1.0	66.24 ± 1.09^{d}	5.15 ± 0.05^{fg}	2.92 ± 0.06^{fg}
13	0.0	0.0	1.5	54.22 ± 0.87^{h}	4.93 ± 0.34^{g}	$2.99\pm0.19e^{fg}$
14	0.0	0.0	2.0	49.15 ± 0.87^{j}	3.69 ± 0.62^{h}	2.44 ± 0.52^{fg}
15	0.0	0.0	2.5	46.27 ± 1.05^{k}	2.85 ± 0.28^{h}	2.30 ± 0.50^{g}

Each value represents the mean ± SD, n = 20. Mean followed by the same letters in each column are not significantly different at $P < 0.05$ according to Duncan's multiple range test a,b,c,d,e,f,g, h and i – indexed from figure 1a-i

Conclusion

This study describes an *in vitro* callus formation and shoot multiplication was achieved from nodal explants of *C. juncea*, a rare medicinal plant. It is concluded that the best callus growth obtained by inclusion of 0.5mg/L 2,4D with 0.05mg/L BAP. Further, the result showing high percentage of shoot multiplication was observed in a 1.0mg/L BA with 0.10mg/L NAA. This protocol can be definitely useful for bulk production of this rare medicinal species that can be reintroduced into natural habitat. Hence, it is sure that this protocol can serve the purpose of the needy people and make this species available in its own habitats.

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