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RESEARCH PAPER

Plant regeneration from cotyledon and leaf explants of *Ceropegia bulbosa* Roxb., an endangered medicinal plant

Balakrishnan Subbaiyan * & Vellaichamy Thangapandian

Balakrishnan Subbaiyan*
e-mail: bsubbaiyan@gmail.com
Vellaichamy Thangapandian
e-mail: drvthangapandian@gmail.com

PG and Research Department of Botany, Kongunadu Arts and Science College [Autonomous], Coimbatore, Tamil Nadu, India

* corresponding author

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ABSTRACT

An efficient protocol of callus induction, shoot multiplication and plant regeneration from cotyledon and leaf explants of *Ceropegia bulbosa* has been elaborated. The highest percentage (90.66) of callus induction was observed when cotyledonary explants cultured on MS medium having 0.5 mg/L 2,4-D in combination with 0.05 mg/L Kn followed by 86.25 % callus induction from leaf explant at 0.5 mg/l BAP in combination with 0.05 mg/l Kn. Shoot multiplication was observed, cotyledon derived callus cultured on 1.0 mg/L BAP in combination with 0.10 mg/L NAA could induce number of shoots (4.71) and their lengths (6.13 cm). Cotyledonary callus derived microshoots gave the highest rooting percentage (92.26 %) root numbers (13.14) and their lengths (8.87 cm) were observed on half-strength MS medium fortified with 0.4 mg/L IBA. *In vitro* derived plantlets were hardened in the greenhouse and successfully established into the soil.

Keywords: *Ceropegia*, cotyledon, 2, 4-D, plant regeneration, microshoots

РЕЗЮМЕ

Суббайан Б., Тангапандян В. Регенерация растений их семядолей и листьев *Ceropegia bulbosa* Roxb., редкого лекарственного растения. Разработан эффективный метод индукции каллюса, размножения побегов и регенерации растений из семядолей и эксплантатов листьев *Ceropegia bulbosa*. Наибольший процент (90,66) индукции каллюса наблюдался, когда семядольные эксплантаты культивировали на MS-среде, имеющей 0,5 мг/л 2,4-D, в комбинации с 0,05 мг/л Kn. 86,25 % индукции каллюса из эксплантата листьев отмечен при 0,5 мг/л BAP в сочетании с 0,05 мг/л Kn. Наблюдалось увеличение числа побегов: каллюс, полученный из семядолей, культивируемый на 1,0 мг/л BAP в сочетании с 0,10 мг/л NAA, мог индуцировать в среднем 4,71 побегов длиной 6,13 см. Побеги, полученные из каллюса семядоли на среде MS, обогащенной 0,4 мг/л IBA, дали наивысший процент корней (92,26 %) (общее число – 13,14) длиной в среднем 8,87 см. Полученные *in vitro* проростки прошли адаптацию в теплице и успешно внедрены в почву. *Переведено редакцией.*

Ключевые слова: *Ceropegia*, семядоля, 2, 4-D, регенерация растений, микропобеги

Ceropegia bulbosa Roxb. is an important medicinal plant that has tuber, which is traditionally used for urinary disorders and its decoction is also considered to be a digestive and healthy tonic (Swarnkar & Katewa 2008, Phulwaria et al. 2013). The plant is often harvested from the wild for its edible leaves and tubers, which are used locally as vegetable and food. It is growing in natural habitats with limited number of tubers that survive and being dormant in soil and produce the new plant in the next season. At the same time population of this species gets reduced due to continuous consumption of tubers by animals and drying or deterioration of tubers in drought and water logging conditions respectively. Continuous collection or over exploitation of *C. bulbosa* is must both for food and pharmaceuticals sources. It is under threat due to destructive collection of leaves and tubers or habitat degradation by grazing, scarcity of pollinators and poor seed setting quality, low viability and low germination capability (Goyal & Bhadauria 2006, Yadav & Kamble 2008). As these are the continuous problems in India, *C. bulbosa* classified under the RET category (BSI 2002, Goyal & Bhadauria 2006).

It is possible to conserve this species from drastic exploitation by applying effective propagation methods so as to remove from the RET list. Though *C. bulbosa* can be propagated through the seeds and tubers, it is not enough to establish its population in its own habitats as we expect. Early research reports proved that taxonomicaly close species *C. intermedia* (Karuppusamy et al. 2007), *C. odorata* (Srinivasarao et al. 2010) have poor seed germination capacity. Therefore, it is an urgent need for the establishment of an efficient *in vitro* regeneration method for their conservation. Plant tissue culture techniques are an alternate tool for the conservation of medicinal and RET listed plants (Thiyagarajan & Venkatachalam 2012).

Reinforcement of wild plant population using individuals raised *ex situ* is considered a wailed means of reducing the risk of threatened species (Bowes 1999). Many plant species respond well to *in vitro* conditions, including rich medium and also plant growth hormones (Cerabolini et al. 2004). Micropropagation of *C. juncea* (Krishnareddy et al. 2011) and *C. mahabalei* (Upadhye et al. 2014) were reported earlier.

As *Ceropegia bulbosa* is highly deserved for the modern drug development process and is permanently demanded as a stable food by many tribal's peoples, the status of the plant is continuously being either under a poorly available or non-available category. Consequently it is facing threats. Further, this condition develops pressure in commercial demand. Therefore, the steps taken to increase its population will ensure the availability of the greater biomass, which is more essential to meet the demand thereby it bestows the conservation of this species as well. Hence, the present investigation was taken as a challenge to have a mass propagation using the cotyledon and leaf explants excised from the seedlings emerged after seed treatments by breaking the seed dormancy for *in vitro* culture.

MATERIAL AND METHODS

Explant sterilization

Explants were excised from the healthy seedlings and these explants were transferred to 250 ml sterile conical flask. They were washed first under running tap water for 3–5 minutes and treated with 0.1 % (v/v) Tween-20 for 5 minutes followed by 0.5 % of Bavistin for 1 minute to remove the unwanted microorganisms adhered on them. Then they were repeatedly rinsed in distilled water. Further sterilization was done under aseptic environment in a laminar air-flow hood. Here the explants were surface sterilized with 20 % (v/v) ethanol for a minute and then by 0.1 % (v/v) $HgCl_2$ for 2–3 minutes. Finally, the explants were thoroughly washed (up to 6–8 times) with sterilized double distilled water. Thus pathogen free and healthy sterilized explants were inoculated for *in vitro* culture on the MS medium.

Culture media and conditions

MS basal medium containing different concentrations of plant growth regulators, auxins and cytokinins, were used either individually or in combination. After the media adjusted to optimum pH values range from 5.6 to 5.8 about 8 g/l agar was added. Finally MS medium, with necessary composition or in combination, was autoclaved at 121°C under 103 kPa for 30 minutes. This sterilized media was then transferred to an aseptic environmental condition provided at the working place of the laboratory. Cultures were incubated at 25±1°C with 16 hrs photoperiod under cool white fluorescent tubes (Philips L 58 W/640, 30 μmol m^{-2} S-1 PPF) and maintained between 75–80 % relative humidity.

Callogenesis (indirect organogenesis)

Aseptically excised cotyledon and leaf explants, from the seedlings emerged from the treated seeds, were cultured on MS basal medium supplemented with five different concentrations of (0.5, 1.0, 2.0, 3.0, 4.0 mg/l) 2,4 D, BAP, and TDZ alone or in combination with five different concentrations of Kn (0.05, 0.10, 0.20, 0.30 and 0.40 mg/l) for callus induction. Every 4th week the mature callus was subcultured on the same combination of fresh medium. At the end of the first 4th week culture, the efficiency of PGRs on callus induction was recorded by estimating the percentage of explants survival and days required for callus induction.

Effects of BAP, NAA and IBA on shoot regeneration

Mature callus emerged from the cotyledon and leaf explants were transferred into MS medium fortified with three different concentrations of (0.5, 1.0 and 2.0 mg/l) BAP and NAA alone or in combination of BAP or TDZ with three different concentrations of NAA (0.05, 0.10 and 0.20 mg/l) for evaluation of shoot regeneration. At the end of the 4th week, number of shoots generated by per explant and their lengths were calculated and recorded.

Effects of temperature and photoperiod on shoot multiplication

All the cultures were maintained at the culture room under 25±1°C temperature and 65–70 % relative humanity. The cultures were kept under white light at the intensity of 3000 lux provided from white fluorescence lambs (Philips, India) with 16 hrs photoperiod duration.

Root organogenesis

2 to 5 cm long *in vitro* regenerated shoots were cultured on half strength MS medium fortified individually with the similar concentrations (0.2, 0.4, 0.6, 0.8 and 1.0 mg/l) of IBA, IAA and NAA to check their root induction abilities. Percentage of root induction, number of roots regenerated per shoot explant and their lengths were recorded from the 4 weeks old culture.

Hardening for acclimatization

In vitro regenerated plantlets were individually potted and maintained at 90 % relative humidity in a mist chamber for three weeks. The acclimatized plantlets were subsequently transferred to individual pot that contained a mixture of same quantity of three different composition media, which were, Sand: Soil: Vermiculite (1:1:1, v/v), Forest soil: Soil: Vermiculite (1:1:1, v/v) and Garden soil: Soil: Vermiculite (1:1:1, v/v). All the pots were shifted to greenhouse and regularly watered. The survival percentage of the acclimatized plantlets was calculated and recorded from all the three treatments after two weeks of incubation period.

Statistical analysis

The cultures were periodically observed and morphological changes were recorded at regular intervals. Each experiment was repeated with 20 replicates. Callus induction, shoot and root regeneration and the number of shoots and roots emerged per explant and their lengths were recorded 4 weeks after the culture. Analysis of variance (ANOVA) was performed on all data to compare concentration and combination effects of the growth regulators. Means were segregated using Duncan's Multiple Range Test (DMRT).

RESULTS

Callus organogenesis

Application of different concentrations of plant growth regulators is essential to understand the optimum requirement for the callus induction in *C. bulbosa*. None of the callusing response was noted on the basal medium. BAP, 2,4-D, TDZ in combination with Kn were used in the medium to check their influence on callus induction ability of coty-

ledon and leaf explants of *C. bulbosa* two weeks after inoculation and these details are presented in Table 1. First callus induction was observed on 11 days after inoculation from the cotyledonary explants of *C. bulbosa* on the medium containing 0.5 mg/l BAP in combination with 0.05 mg/l Kn, followed by the same concentrations 2,4-D in combination with Kn, where the callus induction was noted 12 days after inoculation (Fig. 1).

The leaf explants required 14 to 17 days after culture to induce callus formation. 74.19 % callus formation was observed from cotyledon explants were cultured on MS medium fortified with 0.5 mg/l 2,4-D alone. The high percentage (90.66 %) of callus induction was observed, when cotyledonary explants were cultured on MS medium having 0.5 mg /l 2,4-D in combination with 0.05 mg/l Kn and followed by 86.25 % callus induction was observed from the same explants cultured on MS medium supplemented with 0.5 mg /L BAP in combination with 0.05 mg/l Kn. However, the leaf explants induced 76.23 % callus formation on MS medium fortified with 0.5 mg/l BAP in combination with 0.05 mg/l Kn and 69.22 % callus formation was observed on MS medium fortified with 0.5 mg /l 2,4-D in combination with 0.05 mg/l Kn. This investigation suggests that the 2,4-D, BAP in combination with Kn could exhibit the effective for callus formation from the cotyledonary explants of *C. bulbosa.*

Shoot regeneration

The matured callus was subcultured on the MS Medium contained different concentrations and combinations of

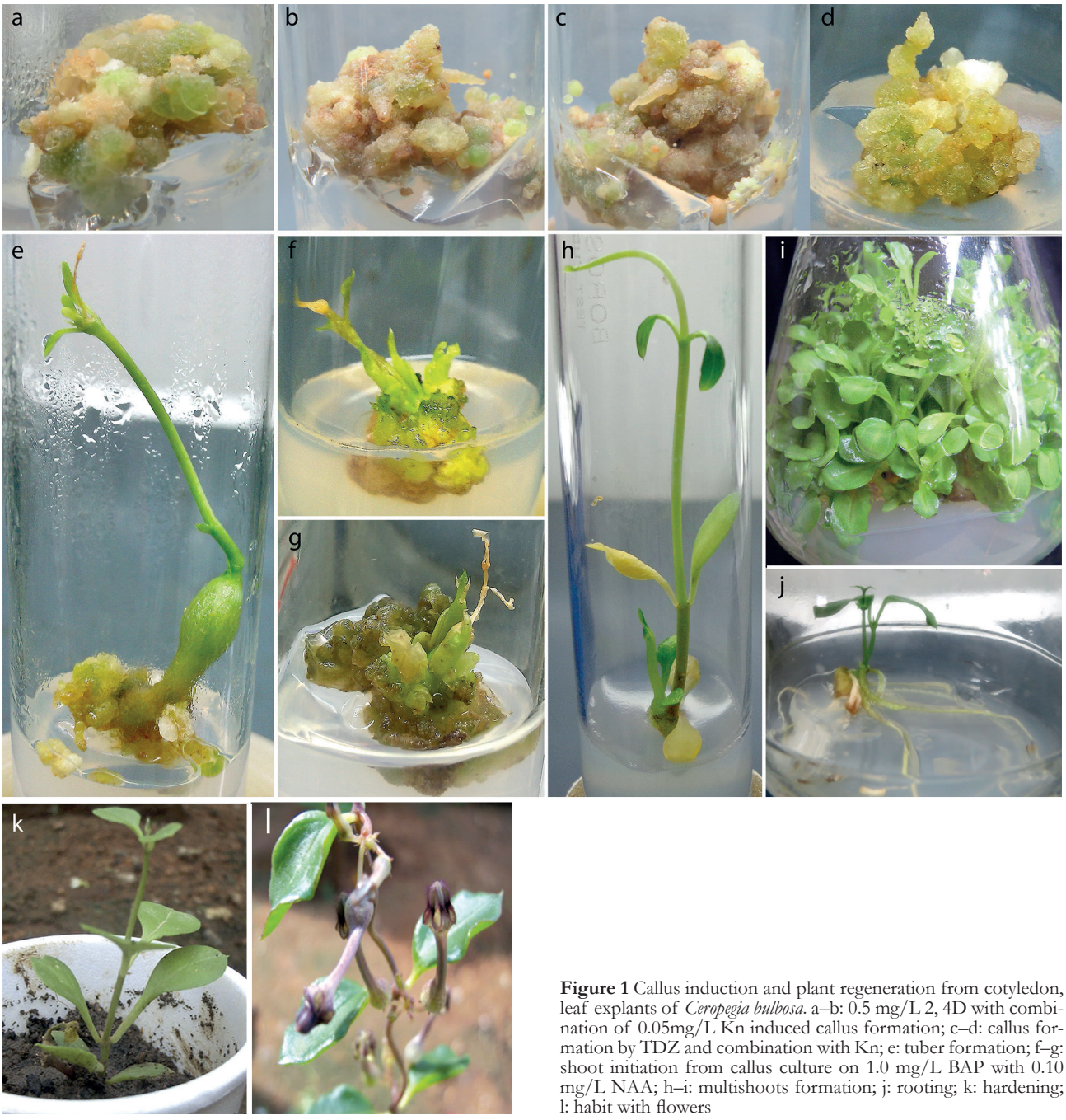

Figure 1 Callus induction and plant regeneration from cotyledon, leaf explants of *Ceropegia bulbosa*. a–b: 0.5 mg/L 2, 4D with combination of 0.05mg/L Kn induced callus formation; c–d: callus formation by TDZ and combination with Kn; e: tuber formation; f–g: shoot initiation from callus culture on 1.0 mg/L BAP with 0.10 mg/L NAA; h–i: multishoots formation; j: rooting; k: hardening; l: habit with flowers

Table 1. Callus induction efficiency of cotyledon, leaf segments excised from the seedlings emerged from the treated seeds on MS medium supplemented with different concentrations of BAP, 2,4-D and TDZ in combination with Kn. 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

S. No.	Plant growth regulators (mg/L)				Days required for callus induction		Percentage of callus induction	
	BAP	2,4-D	TDZ	Kn	Cotyledon	Leaf	Cotyledon	Leaf
1	0.5	0.0	0.0	0.0	12	13	73.44±0.62^{ef}	68.40±0.64^{c}
2	1.0	0.0	0.0	0.0	13	15	71.07±1.0^{g}	63.96±0.81^{f}
3	2.0	0.0	0.0	0.0	24	23	66.20±0.87^{h}	65.29±0.64^{e}
4	0.0	0.5	0.0	0.0	11	14	74.19±0.89^{e}	71.15±0.81^{b}
5	0.0	1.0	0.0	0.0	16	17	64.34±1.12^{j}	67.26±0.56^{d}
6	0.0	2.0	0.0	0.0	19	22	63.34±1.40^{k}	57.16±1.10^{hi}
7	0.0	0.0	0.5	0.0	12	15	64.44±0.48^{i}	64.98±0.57^{ef}
8	0.0	0.0	1.0	0.0	17	18	62.54±0.76^{lm}	57.86±0.96^{hi}
9	0.0	0.0	2.0	0.0	19	23	60.30±0.62^{m}	60.33±0.83^{g}
10	0.5	0.0	0.0	0.05	11	15	86.25±0.36^{b}	76.23±0.87^{a}
11	1.0	0.0	0.0	0.10	13	17	77.57±0.84^{d}	61.54±0.97^{fg}
12	2.0	0.0	0.0	0.20	15	19	72.20±1.27^{fg}	56.24±0.97^{ij}
13	3.0	0.0	0.0	0.30	19	26	66.96±1.21^{h}	57.18±0.72^{i}
14	4.0	0.0	0.0	0.40	23	27	62.18±0.62^{lm}	46.74±0.84^{m}
15	0.0	0.5	0.0	0.05	13	15	90.66±0.04^{a}	69.22±0.59^{bc}
16	0.0	1.0	0.0	0.10	15	18	80.47±0.55^{c}	56.42±1.08^{ij}
17	0.0	2.0	0.0	0.20	17	21	74.29±0.86^{e}	59.23±0.87^{g}
18	0.0	3.0	0.0	0.30	22	26	62.18±0.71^{lm}	55.49±0.26^{j}
19	0.0	4.0	0.0	0.40	26	29	56.99±0.93^{n}	51.25±0.87^{k}
20	0.0	0.0	0.5	0.05	14	16	76.91±0.22^{de}	62.21±0.96^{f}
21	0.0	0.0	1.0	0.10	16	19	72.18±0.62^{fg}	57.08±0.21^{gh}
22	0.0	0.0	2.0	0.20	18	21	65.40 ± 0.59^{hi}	62.93±1.00^{f}
23	0.0	0.0	3.0	0.30	23	26	63.81±0.42^{k}	52.54±1.09^{k}
24	0.0	0.0	4.0	0.40	27	29	56.29±1.46^{n}	48.58±0.91^{l}

Table 2. Shoot induction efficiency of cotyledon and leaf derived callus of *C. bulbosa* seedlings emerged from the treated seeds on MS medium containing different concentrations and combinations of auxins and cytokinins. 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

S. No.	Plant growth regulators (mg/L)			Cotyledon derived callus			Leaf derived callus		
	BA	NAA	IBA	% of culture response	No. of shoots/ callus	Average shoot length (cm)	% of culture response	No. of shoots/ callus	Average shoot length (cm)
1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2	0.5	0.0	0.0	64.41±1.31^{h}	3.11±0.04^{ij}	4.25±0.54^{e}	63.36±1.13^{i}	2.51±0.50^{g}	3.15±0.13^{i}
3	1.0	0.0	0.0	68.06±0.24^{f}	3.29±0.08^{ij}	5.04±0.18^{cd}	65.02±1.0^{fg}	2.59±0.30^{g}	4.70±0.52^{e}
4	2.0	0.0	0.0	65.89±0.82^{g}	2.91±0.09^{k}	3.96±0.06^{f}	60.47±1.07^{jk}	2.91±0.09^{f}	4.02±0.21^{ef}
5	0.0	0.5	0.0	58.0±0.07^{l}	3.06±0.05^{ij}	5.56±0.45^{bc}	61.88±1.14^{j}	3.01±0.23^{de}	3.50±0.50^{gh}
6	0.0	1.0	0.0	63.51±0.54^{hi}	3.85±0.26^{ef}	5.25±0.54^{bc}	65.66±0.73^{fg}	3.16±0.27^{d}	3.98±0.12^{g}
7	0.0	2.0	0.0	61.59±0.56^{k}	3.54±0.46^{gh}	3.66±0.76^{g}	60.29±0.63^{k}	2.98±0.10^{e}	4.0±0.12^{ef}
8	0.5	0.05	0.0	75.58±0.54^{c}	4.67±0.18^{b}	5.81±0.49^{ab}	70.67±0.58^{cd}	4.26±0.69^{ab}	5.14±0.07^{b}
9	1.0	0.10	0.0	90.91±0.72^{a}	4.71±0.26^{a}	6.13±0.10^{a}	82.44±0.76^{a}	4.37±0.30^{a}	5.78±0.30^{a}
10	2.0	0.20	0.0	71.89±0.82^{d}	4.36±0.45^{cd}	5.41±0.44^{bc}	58.54±0.46^{l}	3.81±0.24^{c}	5.07±0.49^{bc}
11	0.0	0.5	0.05	72.7±0.67^{d}	3.80±0.17^{fg}	5.0±0.11^{cd}	64.55±0.78^{hi}	4.18±0.63^{b}	4.84±0.18^{d}
12	0.0	1.0	0.10	76.57±0.01^{c}	4.03±0.17^{def}	5.32±0.23^{bc}	72.30±0.81^{b}	4.15±0.11^{bc}	5.05±0.37^{bc}
13	0.0	2.0	0.20	62.73±0.67^{j}	3.95±0.37^{cd}	5.08±0.20^{e}	66.59±0.56e^{f}	3.30±0.27^{cd}	4.22±0.23^{e}
14	0.5	0.0	0.05	71.56±0.49^{de}	4.18±0.31^{cde}	5.14±0.07^{de}	68.23±1.00^{d}	2.91±0.07^{f}	3.76±0.50^{h}
15	1.0	0.0	0.10	81.56±0.50^{b}	4.32±0.46 ^{b}	5.17±0.09^{de}	71.24±1.09^{c}	3.18±0.64^{cd}	3.81±0.46^{h}
16	2.0	0.0	0.20	70.59±0.44^{de}	4.26±0.18 ^{cde}	4.59±0.26^{e}	67.89±0.39^{de}	3.06±0.58^{de}	3.44±0.59^{hi}

BAP and NAA with IBA to test the shoot proliferation in *C. bulbosa*. MS medium having 1.0 mg/l BAP alone induced 68.06 % shoot initiation, 3.29 shoots per explant and 5.04 cm shoot length from the cotyledon derived callus. The highest percentage (90.91 %) of shoot induction was observed on MS medium fortified with 1.0 mg/l BAP in combination with 0.10 mg/l NAA followed by 81.56 % shooting at 1.0 mg/l NAA in combination with 0.10 mg/l IBA from the cotyledon derived callus. The maximum number of adventitious shoots (4.71) and shoot length (6.13 cm) was recorded from the cotyledon derived callus, when they were cultured on MS medium supplemented with 1.0 mg/l BAP in combination with 0.10 mg/l NAA and the same concentration also produced 4.37 number of shoots and 5.78 cm shoot length from the leaf derived callus (Table 2).

Rooting

Half strength MS medium fortified with different concentration of IBA, IAA and NAA were used for root induction. Percentage of root induction, number of roots produced per *in vitro* shoot and root lengths were recorded 4 weeks after culture. Cotyledon derived *in vitro* shoots could able to produce higher percentage (92.26 %) of root regeneration on the half-strength MS medium fortified with 0.4 mg/l IBA. And moreover this concentration could able to produce maximum number roots (13.14) and root length

Table 3. Effect of different concentration of auxins on root regeneration abilities of the 45 days old cotyledon and leaf callus emerged shoot of *C. bulbosa*. 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

S. No.	Plant growth regulators			% of rooting		No. of roots/shoot		Root length (cm)	
	IBA	IAA	NAA	Cotyledon	Leaf	Cotyledon	Leaf	Cotyledon	Leaf
1	0.2	0.0	0.0	83.48±0.57^{c}	86.59±0.62^{c}	12.36±0.31^{b}	12.02±0.68^{ab}	8.37±0.74^{ab}	6.06±0.80^{b}
2	0.4	0.0	0.0	92.26±0.68^{a}	89.12±0.83^{a}	13.14±0.52^{a}	12.42±0.44^{a}	8.87±0.28^{a}	7.47±0.56^{a}
3	0.6	0.0	0.0	75.44±1.20^{f}	76.03±0.21^{d}	11.22±1.07^{bc}	10.45±0.54^{c}	7.59±0.61^{c}	5.87±1.02^{bc}
4	0.8	0.0	0.0	64.07±0.89^{g}	72.01±1.18^{e}	10.39±0.42^{cd}	9.01±0.57^{e}	6.65±0.67^{d}	5.03±0.30^{c}
5	1.0	0.0	0.0	61.03±1.00^{ij}	61.71±0.48^{hi}	9.93±1.06^{def}	8.74±0.54^{ef}	6.14±0.35^{de}	4.80±0.60^{cd}
6	0.0	0.2	0.0	80.10±1.07^{d}	72.69±0.74^{e}	11.15±0.78^{bc}	9.43±0.40^{d}	6.91±0.67^{cd}	5.51±0.29^{bc}
7	0.0	0.4	0.0	90.02 ±0.68^{b}	87.24±0.67^{b}	12.82±0.52^{b}	11.79±0.70^{b}	8.32±0.28^{b}	6.05±0.07^{b}
8	0.0	0.6	0.0	72.25±0.61^{ef}	69.78±1.36^{f}	10.96±0.94^{c}	7.76±0.61^{gh}	6.0±0.12^{e}	5.01±0.13^{c}
9	0.0	0.8	0.0	62.24±0.68^{hi}	65.27±1.28^{g}	9.23±0.67^{ef}	8.00±0.12^{f}	5.26±0.35^{f}	4.21±1.29^{de}
10	0.0	1.0	0.0	57.24±0.76^{k}	62.22±1.05^{h}	9.42±0.17^{ef}	6.96±0.06^{i}	5.42±0.51^{ef}	4.05±0.47^{e}
11	0.0	0.0	0.2	60.35±1.02^{j}	59.31±0.63^{i}	9.00±0.88^{f}	7.04±0.03^{h}	5.03±0.49^{g}	4.29±0.28^{de}
12	0.0	0.0	0.4	63.74±0.97^{gh}	61.15±0.70^{hi}	9.58±0.44^{def}	8.26±0.24^{ef}	6.37±0.54^{de}	4.49±0.83^{d}
13	0.0	0.0	0.6	77.24±0.70^{e}	76.77±1.35^{d}	10.65±0.63^{cd}	9.32±0.50^{d}	6.87±0.28^{cd}	5.02±0.35^{c}
14	0.0	0.0	0.8	55.18±1.28^{k}	62.32±0.94^{h}	8.75±0.77^{fg}	7.25±0.79^{h}	5.11±0.95^{g}	3.95±0.89^{f}
15	0.0	0.0	1.0	52.25±1.41^{l}	57.95±1.21^{i}	7.98±0.97^{g}	6.21±0.62^{i}	4.96±0.35^{h}	3.66±0.35^{g}

(8.87 cm) followed by 90.02 % rooting and 12.82 roots and 8.32 cm long root was observed on the medium containing 0.4 mg/l IAA from the same explant of *C. bulbosa*. The moderate rooting percentage (89.12 %), average number of roots (12.42) and root length (7.47 cm) were noted from the leaf callus derived shoots on 0.4 mg/l IBA containing medium (Table 3). Hardening experiments is one of the important establishment processes of the *in vitro* raised plantlets of *C. bulbosa* using different hardening media composition to determine their survivability rate. The highest survivability percentage (93.3 %) was shown by the cotyledon derived callus plantlets, when they were growing on the hardening media composed of Forest soil: Soil: Vermiculite in 1:1:1 ratio followed by 83.3% survivability was observed from leaf derived callus plantlets on same hardening media.

DISCUSSION

Observations of the present study indicated that the suitable combinations of auxin and cytokinins were important for caullogenesis from the cotyledon and leaf explants of *C. bulbosa*. Cotyledon explants of *C. bulbosa* showed higher percentage of callus induction and multiple shoot formation on the MS medium containing auxin and cytokinin. In the present study, it was observed that the MS medium fortified with 0.5 mg/l 2,4-D in combination with 0.05 mg/l Kn was superior in organogenic potential compared to other growth regulator combinations in the culture of *C. bulbosa* (Table 1). A number available research reports pointed out that combination of auxins and cytokinins could bring the capability of *in vitro* propagation in tissue culture. The induction of caulogenesis in cultures requires an adjustment in the levels of auxins and cytokinins based on the type of explants. The 2,4-D is the main synthetic auxin used to induce the callogenesis. Because, one of its main characteristics is the capacity to efficiently stimulate the cell division in plant cell culture (Gaspar et al. 1996, Feher et al. 2003).

Da Silva et al. (2003) noted that 83.2 % and 86.4 % callus formation from the cotyledon and hypocotyl explants of *Glycine wightii*, when they were cultured on MS medium fortified with 1.0 mg/l 2,4-D in combination with 0.10 mg/l Kn. It was reported that leaf explants of *Catharanthus roseus* cultured on MS medium having 4.06 µM 2,4-D in combination with 2.3 µM Kn could produce good friable, high biomass callus (Garg 2010). The present study is in harmony with the works of Da Silva et al. (2003) and Garg (2010), who used same combination of the 2 plant growth regulators (2,4-D + Kn). 2,4-D in combination with Kn could induced callus formation in many plant species; *Tylophora indica* (Sadguna et al., 2013) and *Rauvolfia serpentina* (Gupta et al., 2014).

The highest percentage (82%) of callus induction was observed, when cotyledon explants of *Decalepis hamiltonii* were cultured on MS medium supplemented with 0.5 mg/l BAP in combination with 0.05 mg/l Kn (Samydurai et al. 2016). However, the moderate percentage (86.25 %) of callus induction in the present study was observed using cotyledon explants cultured on MS medium supplemented with 0.5 mg/l BAP in combination with 0.05 mg/l Kn. The results observed in this study are in agreement with the report of Samydurai et al. (2016).

The lower concentration of 2,4-D, BAP and TDZ (0.5 and 1.0 mg/l) could induce huge size callus. It was found that there was some green color blotches scattered on the surface of the 4 weeks old callus. The occurrence of green spots on surface of the callus is considered as meristematic centers that indicate the capacity of the callus to produce the adventitious shoots in subsequent culture (Nabors et al. 1982, Ishii 1982). Recently, some workers noticed the appearance of green nodules on the surface of the callus of many plant species, when they were getting callus using different concentration of auxins in combination with cytokinins (Chakradhar & Pullaiah 2014, Dhir & Shekhawat 2014). The callus property of the present study is in agreement with the callus nature work of Chakradhar & Pullaiah (2014), Dhir & Shekhawat (2014).

In the present study a considerable amount of callus formation was observed from the low concentrations of plant growth hormones containing MS medium, when the cotyledon and leaf explants were cultured. Among the three explants used, higher percentage of callus induction was observed from cotyledon explant. In addition, the color of the callus emerged from the all the three explants on high

concentrations of 2,4-D, BAP and TDZ (2.0 and 3.0 mg/l) containing MS medium turned from green colour to brown colour after 3 weeks of culture. Shoot developments were not possible from these kinds of callus. Similar types of callus were found during *in vitro* propagation of *Sarcostemma acidum* (Rathore & Shekawat 2013).

Compact green calli were transferred to MS medium augmented with varied levels of plant growth regulators to study their effect on shoot regeneration. Fully green coloured callus was not able to regenerate the shoots. Some of them remained whitish green colour patches over the surface and hard in texture only had the capacity of shoot regeneration. The data presented in table 1 indicated that percentage of plant regeneration varied with different hormonal concentrations. Maximum number of shoots and shoot length was recorded from the cotyledon derived callus on MS medium fortified with 1.0 mg/l BAP in combination with 0.1 mg/l NAA.

Muthukrishnan et al. (2015) reported the highest percentage of shoot induction from the internode explant derived callus of *Ceropegia thwaitesii*, when they were cultured on MS medium having 1.0 mg/l BAP in combination with 0.10 mg/l NAA. This combination could induce the maximum number of shoots (19.43) and shoot length (2.99 cm). Epicotyl explant derived callus of *C. bulbosa* was cultured on MS medium supplemented with 1.0 mg/l BAP in combination with 0.10 mg/l NAA, which could produce 25.5 shoots and 4.46 cm shoot length (Phulwaria et al. 2013). The result of the shoot induction study is in agreement with the results of Muthukrishnan et al. (2015) and Phulwaria et al. (2013), who used very similar concentration and combination of (1.0 mg/l) BAP and (0.10 mg/l) NAA.

Singh et al. (2011) achieved shoot proliferation (6.8±1.0) from the hypocotyl derived callus of *Catharanthus roseus* that were cultured on MS medium supplemented with 1.5 mg/l BAP in combination with 1.0 mg/l NAA. Shoots were regenerated from the internodal derived callus of *Caralluma stalagmifera*, when they were cultured on MS medium supplemented with 2.0 mg/l BAP in combination with 0.5 mg/l NAA. This concentration and combination could induce 60 % of shoot regeneration and produce 3.15 shoots per explant (Sreelatha et al. 2015). In the present study the highest percentage (90.91 %) of shoot initiation, number of adventitious shoots (4.71) and shoot length (6.13 cm) were observed, when the cotyledon derived callus was cultured on MS medium acuminated with 1.0 mg/l BAP in combination with 0.10 mg/l NAA. The result of our study is in agreement with in the works of Singh et al. (2011) and Sreelatha et al. (2015), who used BAP in combination with NAA resulted in synergistically increase of shootings *in vitro* propagation. The inclusion of BAP in combination with NAA in the culture medium has been reported to be necessary for inducing shoot multiplication in some Asclepideaceae members such as *Ceropegia bulbosa* var. *bulbosa* (Patil 1998), *C. intermedia* (Karuppusamy et al. 2009) and *Wattakaka volubilis* (Vinothkumar et al. 2015).

The efficient root induction of regenerated shoots determines the success of *in vitro* cultures. Of the three auxins tested, IBA was found to be superior to IAA and NAA. Vinothkumar & Senthilkumar (2015) reported that elongated *in vitro* shoots of *Wattakaka volubilis* produced well developed long roots, when they were cultured on half strength MS basal medium supplemented with 0.6 mg/l IBA and 3 % (w/v) sucrose.

When *in vitro* shoots of *C. candelabrum* were cultured on half strength MS medium having 0.49 μM IBA produced 100 % rooting with 6.9 roots/explant (Beena et al. 2003). The highest percentage of rooting (96±1.9 %) was observed, when microshoots of *C. panchganiensis* were cultured on half strength MS medium supplemented with 7.36 μM IBA, where 9.3 roots/microshoots and 3.6 cm root length Chavan et al. (2013). When *in vitro* derived shoots of *C. attenuata* were cultured on half strength MS medium having 2.46 μM IBA, they could induce the highest percentage (100) of root induction and number of (9.8) roots (Chavan et al. 2011). The results of the present study are in agreement with the works of Chavan et al. (2013) and Chavan et al. (2011).

When half strength MS medium was enriched with 0.6 mg/l IAA, it could produce desirable percentage of rooting and number of roots as it was noted in the present investigation. Nikam et al. (2007) reported the highest percentage of rooting, number of roots and root length in *C. hirsuta*, when the nodal explants derived *in vitro* shoots were cultured on the half strength MS medium fortified with 2.0 μM IAA. When the microshoots of *Caralluma tuberculata* were cultured on half strength MS medium supplemented with 5.07 μM IAA, they were produced 3.0±0.15 roots/microshoot (Rehman et al. 2014).

Present investigation revealed that higher concentrations of auxins induced the callus formation at the base of the nodal segments explants during root organogensis. Sahoo et al. (1997) and Saha et al. (2010) stated that root development was getting delayed on higher IBA, NAA and IAA concentration containing medium. They further said that at the same time the callus formation took place at the base of the shoots of some other plant species (Sahoo et al. 1997, Saha et al. 2010). Similarly, Nikam et al. (2008) reported that the higher concentrations of IBA, NAA and IAA resulted in swelling and callus formation at the base of the shoots of *C. odorata* and *C. maccannii*.

Chavan et al. (2011) stated that MS medium was supplemented with increasing concentration of IAA could produce the callus formation in *C. attenuata* during *in vitro* root formation. Baksha et al. (2007) explained that the increasing concentrations of (1.0 mg/l) NAA and IBA could decrease root induction percentage and number of roots per explant, however, it could increase callus formation too. Similarly, the higher concentrations of IBA, IAA and NAA could induce the callus formation rather than rooting. Thus, the present study is in agreement with the works of Baksha et al. (2007), Nikam et al. (2008) and Chavan et al. (2011).

It has been identified that IBA found to have root induction potential compared to the other auxins. However, 0.6 mg/l IBA had a cumulative action, which played a great role in root induction and elongation in *C. bulbosa*. Though, the explants were cultured on all the three auxins (IBA, IAA and NAA), NAA and IAA exhibited poor response in root

induction in *C. bulbosa*. In contrast, some researchers said that NAA and IAA had root organogenesis ability. It has been reported that *in vitro* derived shoots of *Brachystelma glabrum* cultured on half strength MS medium having 0.5 mg/l NAA showed the highest rooting percentage (80 %), root numbers (5.3) and root (6.4 cm) length (Lakshmi et al. 2017). The best rooting response was obtained from the *in vitro* shoots of *Caralluma bhupenderiana* in a medium containing 2.69 μM NAA (Ugraiah et al. 2011). Nikam et al. (2008) stated that MS medium was supplemented with 2.0 μM IAA showed the highest rooting percentage (74.6 %) and root numbers (2.8). However, they could able to get significant root induction when NAA was combined with IBA. But, in this present investigation, the best root induction was obtained on MS medium supplemented with 0.4 mg/l IBA in *C. bulbosa*.

Best rooting was also observed in many Asclepideaceae members such as *Gymnema sylvestre* (Komalavalli & Rao 2000), *Ceropegia candelabrum* (Beena et al. 2003), *C. thwaitesii* (Muthukrishnan et al. 2012), *C. bulbosa* and *C. juncea* (Subbaiyan et al. 2015). MS medium fortified either with individual different concentrations of auxins or in combination with cytokinins could induce callus, somatic embryogenesis, proliferations of shoot and root, tuber formations and enhance the secondary metabolites productions as reported by many researchers (Ghanti et al. 2004, Desai et al. 2014).

As *in vitro* callogenesis and shoot multiplication process were achieved using cotyledonary explants of *C. bulbosa*, this kind of *in vitro* propagation should have been useful for the conservation as well as mass propagation of *C. bulbosa*. It can also be possible to enhance the secondary metabolite, cerpegin using cell suspension culture. However, an effort will be taken to improve mass propagation on low cost to reinstate this plant into its wild habitats.

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