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Research Article

# ***In vitro* conservation protocol of *Ceropegia bulbosa*: An important medicinal and threatened plant species of Western Rajasthan**

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Abstract

*In vitro* regeneration protocol has been standardized for highly medicinal and threatened succulent *Ceropegia bulbosa* Roxb. The paper focuses on morphogenic response of nodal explant when cultured on MS media. Murashige and Skoog medium supplemented with 6-benzyladenine (BA) ( $2.0 \text{ mg l}^{-1}$ ) was found optimum for axillary shoot bud induction with 83.4 % response. Further shoots were multiplied through repetitive (3-4 times) transfer of the original explant and by subculture of the *in vitro* generated shoots. Maximum number of shoots  $5.7 \pm 0.78$  with shoot length of  $3.6 \pm 0.82$  cm was achieved on MS medium augmented with combination of  $0.25 \text{ mg l}^{-1}$  BA +  $0.25 \text{ mg l}^{-1}$  KN +  $0.1 \text{ mg l}^{-1}$  IAA and additives ( $50.0 \text{ mg l}^{-1}$  ascorbic acid,  $25 \text{ mg l}^{-1}$  each of citric acid, arginine and adenine sulphate). For *ex vitro* rooting, pulse treatment of IBA  $250 \text{ mg l}^{-1}$  for 3 min was found optimum. The rooted shoots were successfully hardened in the green house condition (RH 75-80% at  $26-28^\circ\text{C}$ ) and about 80 % shoots were transferred to the garden.

Keywords

*Ceropegia bulbosa*; khedula; herbal medicine; *ex vitro* root

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## Introduction

*Ceropegia bulbosa* (Roxb.) commonly known as khedula, belongs to family Apocynaceae (previously known as Asclepiadaceae), and is a medicinally important plant of Thar Desert of Rajasthan. The root tubers are edible and contain cerpegin alkaloid (Mabberley, 1978). The plant tubers known for several medicinal properties and used for treatment of diarrhoea and dysentery. Tribal women used the tubers to promote fertility and vitality. *C. bulbosa* is measured as a good source of compounds in curing disease of kidney particularly in urinary bladder stone disease (Swarnkar and

Katewa, 2008). Khan and Pradhan (2012) reported the antiurolithic activity of *Ceropegia*.

Due to increased anthropozoogenic activity and livestock populations the status of wild plant predominantly those used in medicines have been affected seriously (Singh *et al.*, 2009). Because of the pharmaceutical importance of alkaloid cerpegin and its uses as anti-ulcer, hepatoprotective, analgesic, antipyretic, hypotensive, tranquillising and mast-cell stabilizing activity (Adibatti *et al.*, 1991), *C. bulbosa* is overexploited and this plant is now considered as a threatened plant (Dhir and Shekhawat, 2014, Walter and Gillet, 1998).

Conventionally *C. bulbosa* is propagated mainly by seeds, however, germination and viability of seeds is very poor (Goyal and Bhadauria, 2006), so it is not a competent method for the conservation of this important medicinal plant. Furthermore, the conventional methods of propagation are not able to complete the demand of pharmaceutical companies (Shekhawat *et al.*, 2002, 2009). Consequently, there is an urgent need to establish an efficient *in vitro* regeneration method for the conservation of *C. bulbosa*. Plant tissue culture is a helpful method for the propagation and conservation of endangered as well as medicinally important plants (Mathur and Shekhawat, 2013, Singh *et al.*, 2012; Thiyagarajan and Venkatachalam, 2012, Jana and Shekhawat, 2010 a,b). Some attempts have been reported on the *in vitro* propagation of *C. bulbosa* (Dhir and Shekhawat, 2014; Phulwaria *et al.*, 2013). Micropropagation techniques offer the possibility of producing fair amount of identical and superior quality plants in a short stretch of time and limited space for obtaining a biomass for restoration of the species, moreover, *in vitro* technology is also used for production of biologically active compounds (Dhir *et al.*, 2014; Wang *et al.*, 2013; Dhir and Shekhawat, 2013, 2012; Jana and Shekhawat, 2012).

Cerpegin (1,1-dimethylfuro[3,4-C]pyridine-3,4(1H,5H)-dione), the alkaloid present in root tubers of *C. bulbosa* (Nadkarni, 1976), was identified as pyridine, which is a rare alkaloid found in nature (Adibatti *et al.*, 1991). Cerpegin is recognized to have analgesic properties (Sukumar *et al.*, 1996). The aqueous extract of edible *C. bulbosa* contains polyphenols, potassium, steroids, and sugars. Plant contain greater amount of antioxidants which belong to phenolic compounds (flavonoids and phenolic acids), nitrogen-bearing compounds (amines, cyanogenic glycosides, alkaloids and non-protein amino acids), ascorbic acid and carotenoids (Dhir and shekhawat, 2014).

The aim of the present research was to develop a micropropagation protocol for *Ceropegia bulbosa* using nodal explant.

## Material and methods

### Plant Material and Disinfection

Plants of *Ceropegia bulbosa* were procured from Jaisalmer and Barmer district of Rajasthan (India) during rainy season and maintained in the green house of the plant biotechnology unit, Botany Department, Jai Narain Vyas University, Jodhpur. The fresh shoot sprouts were used as explant. The shoots were cut into 4-5 cm long segments (each with 2-3 nodes) and were prior treated with 0.1% (w/v) Bavistin for 9-10 min, followed by 6-7 times washing with sterile water and subsequently surface sterilized with 0.1% (w/v) HgCl<sub>2</sub> for 1-2 min. Shoot segments were washed with at least five to six changes of

autoclaved distilled water under aseptic environment in laminar air flow bench so that no traces of sterilant leftover.

### Basal Medium and culture conditions

MS medium supplemented with 0.8% w/v agar and 3% w/v sucrose was used as a basal medium. Prior to autoclaving the medium, pH was adjusted at 5.8. Test tubes with 15 ml and conical flasks with 50 ml of medium were capped using non absorbent cotton plugs. The medium was autoclaved at 121°C and 1.06 kg cm<sup>-2</sup> for 15 min. All cultures were incubated at 26±2°C, 16 h d<sup>-1</sup> photoperiod at irradiance of 40-50 μ mol m<sup>-2</sup> s<sup>-1</sup> PFD and 60-70% RH.

### Shoot induction

Nodal shoot segments of *C. bulbosa* were inoculated singly on MS medium (Murashige and Skoog, 1962) containing 0.8% agar, 3% sucrose and different concentrations of BA (6-benzyl aminopurine) and KN (Kinetin) (0.5-2.0 mg l<sup>-1</sup>). Medium was also enriched with additives namely ascorbic acid (50 mg l<sup>-1</sup>) and 25 mg l<sup>-1</sup> each of citric acid, adenine sulphate and l-arginine.

### Shoot multiplication

The cultures were multiplied by two different approaches, (1) the mother explants (after removing the newly formed microshoots) were transferred to fresh nutrient medium containing 1.0 mg l<sup>-1</sup> BA and 0.1 mg l<sup>-1</sup> IAA for three passages and (2) subculturing of shoots differentiated *in vitro* during repeated transfer. The culture medium used for multiplication was augmented with 0.25-1.0 mg l<sup>-1</sup> of BA, 0.25 mg l<sup>-1</sup> of KN and 0.1 mg l<sup>-1</sup> of IAA. Data of shoot number and shoot length as well as frequency of explants producing shoot were recorded after every 4 weeks of culture.

### Ex vitro rooting of in vitro raised shoots

*In vitro* regenerated shoots were rooted by *ex vitro* method. The newly generated shoots were first taken out from culture vessel and separated, washed gently with sterile water to remove adhered agar and their bases were treated with different concentrations of IBA, NAA and NOA (100-500 mg l<sup>-1</sup>) for different time intervals. Shoots pulse treated with root inducing auxin were inoculated in polycarbonate capped bottles containing sterile soilrite (supplied by Kel Perlite, Bangalore, India) and is a mixture of Irish peat moss with horticulture grade perlite and exfoliated vermiculite) moistened with small amount of one-fourth strength of MS salts solution for *ex vitro* root induction. The bottles were kept in the green house for rooting near pad section (RH 75-80% at 26-28°C) in green house.

**Table 1.** Effect of different plant growth regulators and their concentrations on bud breaking from nodal shoot segment of *C. bulbosa*

| Conc. of BA (mg <sup>l</sup> <sup>-1</sup> ) | Conc. of KN (mg <sup>l</sup> <sup>-1</sup> ) | Response (%) | Shoot number Mean ± SD | Shoot length (cm) Mean ± SD |
|--|--|--------------|------------------------|-----------------------------|
| Control                                      | -  | -            | -                      | -                           |
| 0.5  | -  | 39.6         | 1.1±0.22               | 0.72±0.17                   |
| 1.0  | -  | 46.2         | 1.2±0.26               | 1.5±0.32                    |
| 1.5  | -  | 55.8         | 1.6±0.29               | 2.1±0.59                    |
| 2.0  | -  | 83.4         | 2.4±0.55               | 2.6±0.73                    |
|  | 0.5  | 00.0         | 0.0±0.0                | 0.0±0.0                     |
|  | 1.0  | 42.5         | 1.0±0.22               | 1.5±0.26                    |
|  | 1.5  | 51.4         | 1.7±0.29               | 1.8±0.37                    |
|  | 2.0  | 54.9         | 2.1±0.42               | 1.6±0.30                    |



**Figure 1.** Shoot bud induction, multiple shoot production and rooting protocols for *Ceropogia bulbosa*. (A): *C. bulbosa* grown in green house condition, (B): Bud breaking from nodal segments on MS medium containing BAP (2.0 mg<sup>l</sup><sup>-1</sup>) after 2 weeks, (C-E): Multiple shoot induction on MS medium containing BAP (0.25 mg<sup>l</sup><sup>-1</sup>) + kinetin (0.25 mg<sup>l</sup><sup>-1</sup>) + IAA (0.1 mg<sup>l</sup><sup>-1</sup>), (F): *Ex vitro* rooted shoots pre-treated with IBA (100 mg<sup>l</sup><sup>-1</sup>) for 3-4 min., (G): Successfully hardened plants of *C. bulbosa*.

### Acclimatization of regenerated plantlets

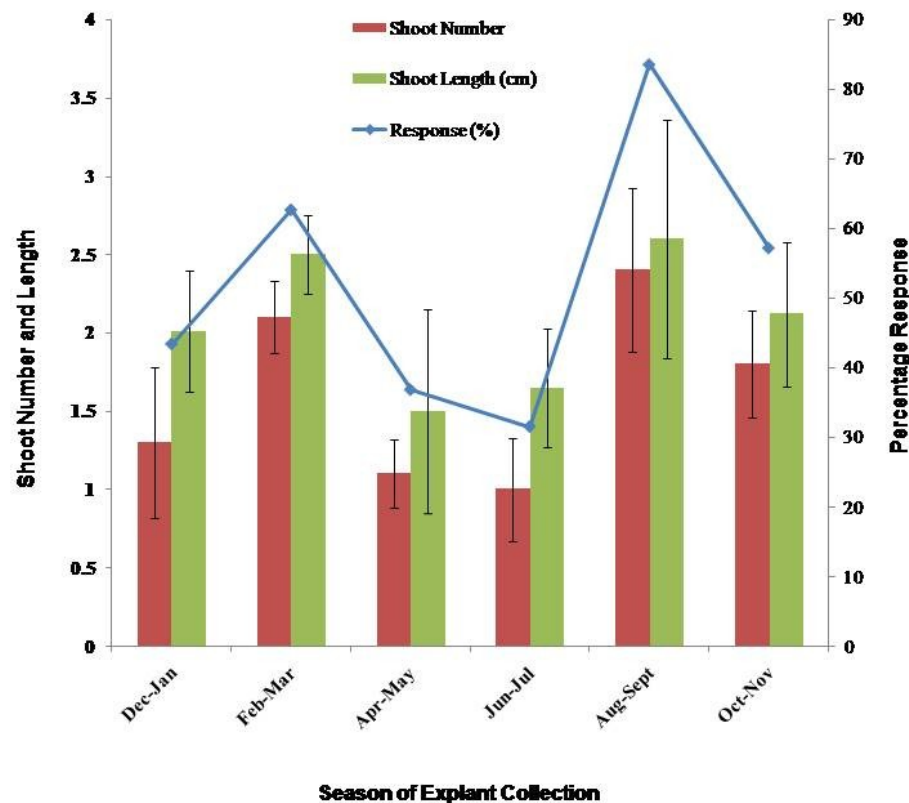
The rooted plantlets were hardened in the green house by regular loosening the caps of bottle containing plantlets after 8-10 days of root induction. After that caps were removed completely, allowing the plants to face the green house conditions for 20-25 days. The bottles were subsequently transferred from pad section of the greenhouse having high relative humidity (75-80%) and low temperature (26-28°C) towards the fan section where relative humidity was

comparatively low (50-60%) and temperature was high (32±2°C) for further acclimatization. After 4-5 weeks, *ex vitro* rooted plantlets were transferred to polybags filled with sand, garden soil and organic manure (1:1:1). After 7-8 weeks under the greenhouse conditions, plantlets were shifted to the earthen pot containing field soil. For acclimatization experiments, 20 replicates were taken for each treatment and experiments were performed three times. These pots were transferred and maintained in nursery.



**Table 2.** Effect of different plant growth regulators and their concentrations on shoot multiplication of *C. bulbosa*

| Conc. of BA (mg l <sup>-1</sup> ) | Conc. of KN (mg l <sup>-1</sup> ) | Conc. of IAA (mg l <sup>-1</sup> ) | Shoot number Mean ± SD | Shoot length (cm) Mean ± SD |
|-----------------------------------|-----------------------------------|------------------------------------|------------------------|-----------------------------|
| Control                           |                                   |                                    | 0.0±0.0                | 0.0±0.0                     |
| 0.25                              | -                                 | 0.1                                | 2.7±0.32               | 0.95±0.29                   |
| 0.5                               | -                                 | 0.1                                | 2.9±0.56               | 1.6±0.39                    |
| 1.0                               | -                                 | 0.1                                | 3.2±0.62               | 2.0±0.45                    |
| 0.25                              | 0.25                              | 0.1                                | 5.7±0.78               | 3.6±0.82                    |
| 0.5                               | 0.25                              | 0.1                                | 4.3±0.68               | 2.1±0.46                    |
| 1.0                               | 0.25                              | 0.1                                | 3.4±0.66               | 1.2±0.33                    |

**Figure 2.** Effect of different collection seasons on percent response, number and length of shoots of *Ceropogia bulbosa*

### Experimental design, data collection and statistical analysis

All the experiments were performed according to completely randomised block design (RBD) for single factor experiments (Compton and Mize, 1999), with a minimum of 20 replicates per treatment and were repeated thrice. The results are expressed as mean ± SD of three experiments. Observations were noted after 4 weeks of interval.

### Results and discussion

#### Surface sterilization and culture establishment

The nodal shoot segments collected during rainy season (August-September) was found to be the best for initiation of culture (Fig. 2). Bud breaking was observed after 10-12 days of explant

inoculation on MS medium augmented with different concentrations of cytokinins.

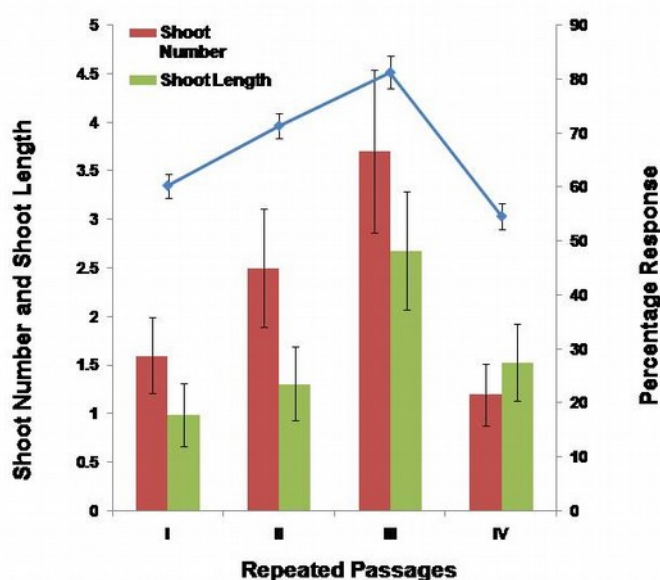
MS medium augmented with 2.0 mg l<sup>-1</sup> BA was found optimum for bud breaking and from each node, 2-3 shoots were regenerated on this medium (Fig. 1B). The frequency of the shoot regeneration was comparatively low on MS medium supplemented with KN (Table 1).

#### Multiplication of shoots

After the *in vitro* raised shoots were excised from the mother explants and cultured on the fresh medium, the shoot number increased considerably up to the third repeated transfers which reduced thereafter (Fig. 3). The maximum response was observed during the third subculture. Repeated transfer of the mother explants help in increasing the shoot number may be because of suppression

**Table 3.** Effect of different concentrations of root inducing auxins on *ex vitro* rooting of shoots of *C. bulbosa*

| Concentration of IBA (mg <sup>l</sup> <sup>-1</sup> ) | Concentration of NAA (mg <sup>l</sup> <sup>-1</sup> ) | Concentration of NOA (mg <sup>l</sup> <sup>-1</sup> ) | Response (%) | Root number Mean ± SD | Root length (cm) Mean ± SD |
|---|---|---|--------------|-----------------------|----------------------------|
| 100   |   |   | 52.4         | 2.0±0.28              | 0.87±0.13                  |
| 250   |   |   | 73.5         | 3.1±0.56              | 2.3±0.44                   |
| 500   |   |   | 41.7         | 2.2±0.45              | 2.0±0.34                   |
|   | 100   |   | 54.5         | 1.4±0.32              | 1.4±0.20                   |
|   | 250   |   | 52.8         | 2.0±0.41              | 1.7±0.25                   |
|   | 500   |   | 26.4         | 1.8±0.37              | 1.3±0.19                   |
|   |   | 100   | 46.8         | 1.3±0.17              | 0.9±0.14                   |
|   |   | 250   | 32.6         | 1.7±0.38              | 1.1±0.16                   |
|   |   | 500   | 23.1         | 1.9±0.42              | 1.3±0.20                   |

**Figure 3.** Effect of repeated subculture of mother explant on percent response, number and length of shoots of *Ceropogia bulbosa*

of apical dominance during subculture that induced basal meristematic cells to form new shoots (Shekhawat and Shekhawat, 2011).

In another approach of shoot multiplication, amplified shoots were achieved by subculturing of *in vitro* produced shoots (separated from mother explant) on fresh culture medium. The maximum number ( $5.7 \pm 0.78$ ) of shoots was produced on MS medium containing a combination of BA ( $0.25 \text{ mg l}^{-1}$ ), KN ( $0.25 \text{ mg l}^{-1}$ ) and IAA ( $0.1 \text{ mg l}^{-1}$ ) with average shoot length of  $3.6 \pm 0.82 \text{ cm}$ , within 25-30 days (Fig. 1C-E) (Table 2).

### **Ex vitro rooting and hardening of in vitro cloned shoots**

In order to minimize the cost and time, experiments were designed to achieve *ex vitro* rooting. *In vitro* produced shoots were harvested and washed with water to remove adhered agar to avoid bacterial and fungal contamination. The basal part of these shoots was treated with different concentrations of IBA, NAA and NOA for 3-4 min and subsequently

transfers to bottles containing soilrite and placed near pad section of the green house. The maximum frequency (73.5 %) of root induction with  $3.1 \pm 0.56$  roots with root length of  $2.3 \pm 0.44 \text{ cm}$ , was recorded when the shoots were treated with  $250 \text{ mg l}^{-1}$  of IBA for 3 min (Fig. 1F; Table 3). It is well established for various plant species that IBA is more efficient for inducing roots under *ex vitro* conditions (Patel *et al.*, 2014; Phulwaria *et al.*, 2013).

### **Acclimatization and pot transfer**

The shoots regenerated under *in vitro* conditions could be successfully rooted *ex vitro* and hardened in the green house. 80% plantlets were hardened after 30-40 days of rooting. *Ex vitro* rooting help the *in vitro* raised plant in acclimatization because they have lateral root system and high root number/length, which increase percentage transplant survival rate (Yan *et al.*, 2010). There are many reports on *ex vitro* rooting suggesting that, *ex vitro* rooted plants showed better survival in natural environmental conditions (Shekhawat *et al.*, 2012;

Phulwaria *et al.*, 2013b). The acclimatized plantlets were transferred to earthen pots containing sand, garden soil, and organic manure and after a month plants were transferred to nursery and exposed to natural conditions (Fig. 1G).

## Conclusion

*In vitro* propagation protocol of *Ceropegia bulbosa* has been successfully standardized and a combination of BA with KN and IAA was found optimum for multiple shoot induction. The process described in the study could be employed for large scale multiplication, propagation and conservation of germplasm of this important medicinal and threatened plant species.

## Competing Interest

The authors declare that they have no competing interests.

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