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African Journal of Biotechnology Vol. 10(46), pp. 9328-9336, 22 August, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.2132
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

In vitro shoot multiplication and conservation of *Caralluma bhupenderiana* Sarkaria - an endangered medicinal plant from South India

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Accepted 30 March, 2011

An efficient protocol was described for the rapid *in vitro* multiplication of an endangered medicinal plant, *Caralluma bhupenderiana* Sarkaria, via enhanced axillary bud proliferation from nodal explants collected from young shoots of six-months-old plant. The physiological effects of growth regulators [6-Benzyladenine (BA), kinetin (Kn), 2-Isopentyl adenine (2iP), zeatin (Zn), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA)], different strengths of Murashige and Skoog (MS) medium and various pH levels on *in vitro* morphogenesis were investigated. The highest number (8.40 + 0.50) of shoots and the maximum average shoot length (3.2 ± 0.31 cm) were recorded on MS medium supplemented with BA (8.87 µM) at pH 5.8. Rooting was best achieved on half-strength MS medium augmented with NAA (2.69 µM). The plantlets regenerated *in vitro* with well-developed shoot and roots were successfully established in pots containing peat mass and garden manure in 1:1 ratio and grown in a greenhouse with 80% survival rate. The regenerated plants did not show any immediate detectable phenotypic variation.

Key words: Apocynaceae, asclepiadoideae, conservation, micro-propagation, endangered, *Caralluma bhupenderiana.*

INTRODUCTION

Caralluma R.Br. (*sensu lato.*) has been usually accepted to include about 120 taxa, with a wide African, Asian, South African and Southeast European distribution (Mabberley, 1993). It belongs to subtribe Stapeliinae (tribe Ceropegiae, sub family Asclepiadoideae and family Apocynaceae), which has its centre of origin in East Africa (Meve and Liede, 2004). A total of 13 species and seven varieties of *Caralluma* occur in India. Out of the 13 species of *Caralluma,* 11 species are solely endemic to South India (Jagtap and Singh, 1999). Many of them are rare and endangered specifically *Caralluma* *bhupenderiana, Caralluma sarkariae*, *Boucerosia truncato-coronata, Boucerosia procumbens* and *Boucerosia pauciflora* (Nayar, 1996). *Caralluma,* found in dry regions of the world, has paramount medicinal importance and has significant anti-inflammatory and antitumor activity (Deepak et al., 1997; Ramesh et al., 1999; Zakaria et al., 2001). The pregnane glycosides of *Caralluma* have been shown to possess antitumor and anti-cancer activities and in some studies *Caralluma* is reported to protect gastric mucosa and have antiulcer properties (Al-Harbi et al., 1994; Zakaria et al., 2002). The juicy stem of *C. tuberculata* is bitter tonic, febrifuge, stomachic and carminative useful in rheumatism and consumed as vegetable especially when cooked with minced meat (Shinwari et al., 2006). The plant had been utilized as a traditional anti-diabetic therapeutic agent equally well in both urban and rural population in Pakistan. It was observed that the administration of *C. sinaica* in different doses to healthy animals can cause

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Abbreviations: AA, Ascorbic acid; **BA,** 6-benzylominopurine; **IAA,** indole-3- acetic acid; **IBA,** indole-3-butyric acid; **Kn,** kinetin; **MS,** Murashige and Skoog (1962) medium; **NAA,** α-naphthalene acetic acid; **2-iP,** 2-isopentenyladenine.

significant decrease in glucose level (Habibuddin et al., 2008). In another report, it was observed that *C. fimbriata*, can be used in weight reduction (Lawrence and Choudhary, 2004). *Caralluma* species have been used for centuries in semi arid areas of Pakistan as emergency foods (Atal et al., 1980). *C. edulis* is known for its anti-diabetic properties (Wadood et al., 1989) and other *Caralluma* species for their antihyperglycemic activity (Venkatesh et al., 2003). The extracts of *C. attenuata* and *C. edulis* had hypoglycemic properties and provide synergistic effect in combination with the phlorizin extract which beneficially modify glucose transport, blood and urine glucose levels, blood insulin levels and helps in weight loss. Plants of *C. tuberculata* have been extensively used for the paralysis and joints pain and fever (Khan and Khatoon, 2008). The indiscriminate and destructive harvesting of many of these plants continues unabated despite increased governmental regulation, resulting in many species (especially those with slow growth) becoming endangered. *C. bhupenderiana* Sarkaria (subfamily: Asclepiadoideae, Family: Apocynaceae) is a succulent medicinal plant, depleted due to human activities of building constructions, road widening and completely eaten by sheep and goats in Vallanadu area in Palayamkotai, Thirunelveli dt, Tamil Nadu. In order to meet the increasing demand for *C. bhupenderiana* while conserving those in the wild, this study aimed at developing a simple, rapid and cost-effective protocol for its clonal propagation. To our knowledge, there is no detailed report on the micropropagation of *C. bhupenderiana.*

MATERIALS AND METHODS

Plant material and surface sterilization

Plants of *C. bhupenderiana* were collected from foot hills of Vallnadu, Thirunelveli dt, Tamil Nadu (Figure 1A and B) and grown in earthen pots in Botanical Garden at Sri Krishnadevaraya University, Anantapur. The shoot segments with six inter nodes were collected from the garden grown plants and washed with running tap water for 15 min. The nodes were cut (1 cm) separately and they were washed with Tween 20 (Merck, India) detergent solution (5% v/v) for 5 min. After thorough washing, the surface sterilization of explants was followed by rinse with sterile distilled water 3 to 4 times to remove trace of detergent, rinsing in 80% ethanol for 30 s and finally, treatment with mercuric chloride (0.1% w/v) ($HgCl_2$) for 3 min duration. To remove every trace of the sterilant, the shoot material was then washed with sterile distilled water at least 4 to 5 times.

Culture media and culture conditions

A culture medium containing MS (Murashige and Skoog, 1962) salts supplemented with macro-elements, micro-elements, 3% sucrose (Merck, India) and gelled with 0.8% (w/v) agar (Himedia, India). The pH of the medium was adjusted, to 5.8 by 1 N NaOH or 1 N HCl after adding the growth regulators. The media were steam sterilized in an autoclave under 15 psi and 121°C for 20 min. All of the cultures were incubated under 50 µMol M^{-2} S^{-1} light provided by cool white fluorescent lamp for a photoperiod of 16 h at 25 ± 2°C.

Shoot initiation and multiplication

For shoot induction, the nodal explants were cultured on MS medium supplemented with various plant growth regulators like 6-benzyladenine (BA), Kinetin (Kn), 2-Isopentenyl adenine (2iP) and Zeatin (Zn) at different concentrations (0.5, 1.0, 2.0, 3.0 and 5.0 µM) either individually or in combination with indole-3-acetic acid (IAA), indol-3-butyric acid (IBA) (or) α- naphthalene acetic acid (NAA) (0.1 and 0.5 µM). The effects of different strengths of MS medium (¼, ⅓, ½ and full strength), various pH levels (5.0, 5.4, 5.8 and 6.2) and different concentrations (50, 100, 200 and 500 mg/l) of ascorbic acid (AA) on morphogenesis were also assessed using optimal concentrations and combinations of BA (8.87 µM) and IAA (2.85 µM). All the cultures were subcultured on to the fresh medium after every four weeks. The frequency with which explants produced shoots, the number of shoots per explant and the shoot length were recorded after six weeks of culture.

Shoots with 5 cm in height were separated and individual shoots were transferred for rooting to half-strength MS medium containing different concentrations of NAA (α-naphthalene acetic acid), IAA (indole-3-acetic acid) and IBA (Indole-3-butyric acid). The cultures were incubated under 16 h photoperiod for 30 days until the micro shoots developed the roots. Then the rooting frequency was measured.

Acclimatization and transplantation of plantlets

The rooted plantlets were removed from the culture tubes and washed with tap water to remove traces of agar. Then, the plantlets were planted onto plastic cups containing a mixture of finely chopped peat mass and sterilized garden manure in 1:1 ratio. The plastic cups were covered with transparent polythene cover to maintain humidity until the development of new rudimentary leaves for 20 days. Then, the plastic cups were transferred to green house and polythene covers were removed. Quarter strength MS major salts solution poured with 7 days intervals up to 40 days of hardening and followed by pouring of tap water. Hardened plants were transferred to pots containing mixture of garden soil and forest humus (1:1 ratio). The pots were watered two days interval under green house condition. After 60 days, the frequency of survival was calculated.

Statistical analysis

Data were measured after 30 and 40 days for shoot multiplication and rooting, respectively. Mean values with the same superscript were not significantly different (p = 0.05%) according to Duncan's multiple range test (Duncan, 1955).

RESULTS AND DISCUSSION

The morphogenetic responses of nodal segment explants to various cytokinins (BA, Kn, 2iP and Zn) are summarized in Table 1. Placing explants in a medium without growth regulators (control) induced 1 to 2 shoots. However, the multiplication rate and shoot number were higher in cultures supplemented with plant growth regulators. The percentage of response varied with the type of growth regulator used and its concentration. All

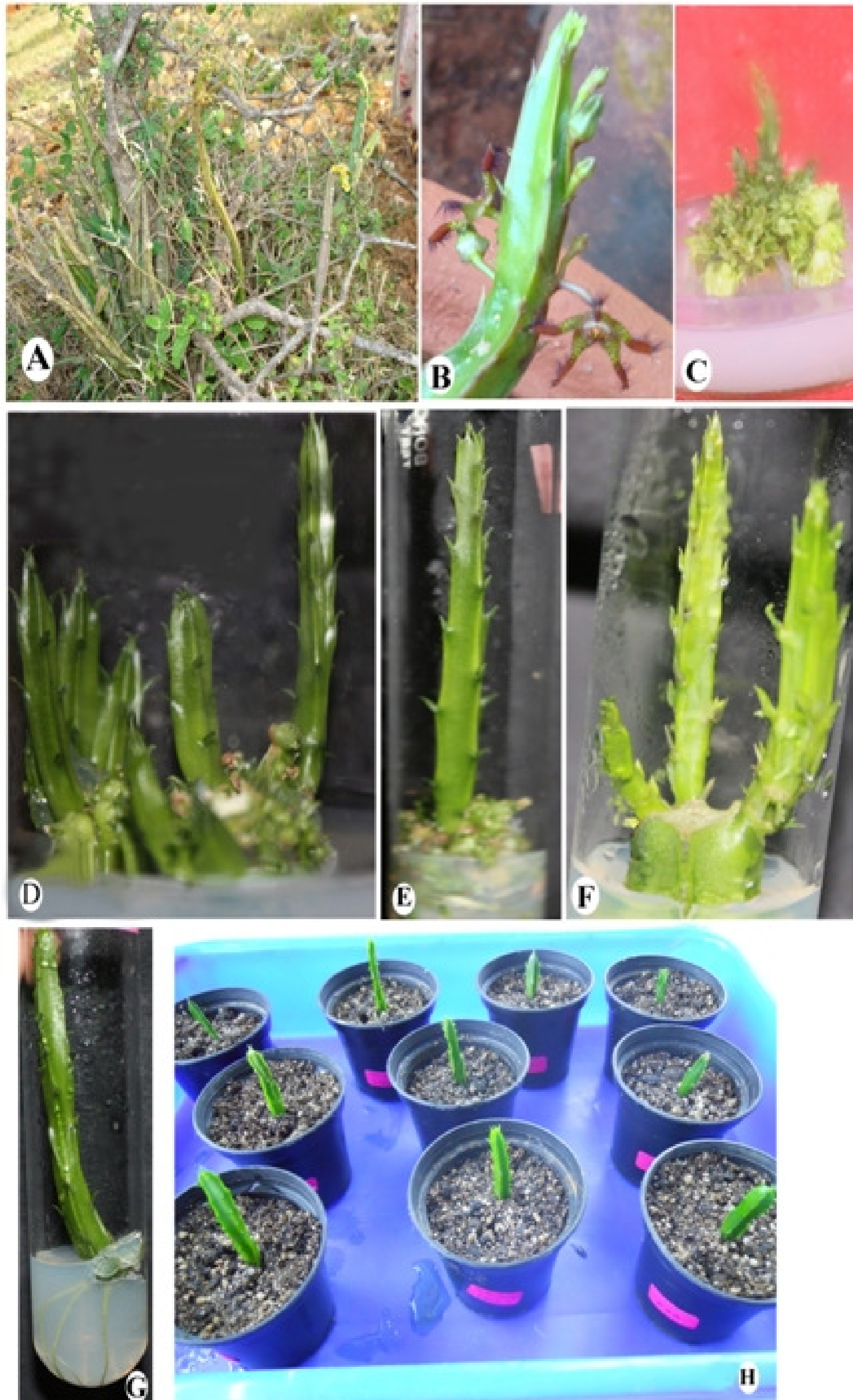

Figure 1. *In vitro* propagation of *C. bhupenderiana*. (A) Natural habit; (B) inflorescence of *C. bhupenderiana*; (C) swelling of the dormant axillary bud; (D) multiple shoot induction from medium containing 8.87 µM BA; (E) multiple shoot induction from medium containing 4.65 µM Kn; (F) multiple shoot production on MS medium supplemented with 8.87 µM BA and 2.69 µM NAA; (G) rooted shoot just before transfer to soil. The shoots were extracted from half-strength MS medium supplemented with 8.87 µM + 2.85 µM IAA + 100 mg/l AA; (H) acclimatized plants growing in plastic cups 45 days after transfer to soil.

Table 1. Effect of various concentrations of BA, Kn, 2iP and Zeation on shoot formation in mature nodal explant of *C. bhupenderiana* cultured on MS medium with 3% sucrose.

Plant growth regulator (µM)	Shoot sprouting frequency (%)	Mean shoot number per explant ±SE	Mean length of shoots (cm) ± SE
BA			
2.22	40	1.03 ± 0.09 [d]	2.00 ± 0.04 [c]
4.44	66	3.90 ± 0.21 [bc]	2.86 ± 0.49 [c]
8.87	80	8.40 ± 0.50 [a]	5.60 ± 0.14 [a]
13.31	73	2.40 ± 0.16 [b]	1.93 ± 0.04 [d]
22.19	46	1.40 ± 0.16 [bd]	2.36 ± 0.20 [b]
Kn			
2.32	-	NR	NR
4.65	40	1.50 ± 0.00 [b]	2.06 ± 0.41 [b]
9.29	60	2.50 ± 0.16 [a]	1.63 ± 0.28 [a]
13.94	53	2.60 ± 0.40 [a]	2.16 ± 0.33 [c]
23.2	26	1.46 ± 0.24 [b]	2.00 ± 0.37 [cd]
2iP			
2.46	48	1.46 ± 0.12 [cd]	1.83 ± 0.23 [ab]
4.92	57	1.86 ± 0.12 [a]	1.93 ± 0.12 [ab]
9.84	75	1.66 ± 0.17 [b]	1.46 ± 0.18 [d]
14.76	64	1.80 ± 0.08 [a]	1.60 ± 0.08 [abc]
24.61	50	1.46 ± 0.28 [c]	2.13 ± 0.17 [a]
ZN			
2.28	42	NR	NR
4.56	53	1.10 ± 0.08 [de]	2.13 ± 0.36 [b]
9.12	68	1.96 ± 0.30 [a]	1.93 ± 0.24 [bc]
13.68	57	1.50 ± 0.08 [b]	1.86 ± 0.28 [bc]
22.81	48	1.13 ± 0.24 [bc]	2.73 ± 0.04 [a]

Means + SE, n =45. Means followed by the same letter are not significantly different by the DMR test at 0.05% probability level; NR, No response.

concentrations of BA (2.22, 4.44, 8.87, 13.37 and 22.19 µM), Kn (2.32, 4.65, 9.29. 13.94 and 23.2 µM), 2iP (2.46, 4.92, 9.84, 14.76 and 24.61 µM) and Zn (2.28, 4.56, 9.12, 13.68 and 22.81) alone facilitated shoot bud differentiation. Swelling of the dormant axillary bud took place within ten days and then, differentiation into multiple shoots occurred after four weeks (Figure 1C). Among the various cytokinins tested, BA was found to be more efficient than others with respect to initiation and subsequent proliferation of shoots (Table 1). Of the various levels of BA tested, 8.87 µM proved to be most effective, as in this medium an average of 8.40 ± 0.50 shoots were developed per explant in 80% of cultures (Figure 1D). Upon lowering the concentration of each cytokinin, a reduction in the number of shoots per culture was recorded. Similarly, at a higher concentration (22.19 µM) the number as well as the percent response was drastically reduced (Table 1). Acceptable results (75%) were also obtained with Kn 9.84 µM (2.6 shoots) and also gave shoots with longer internodes (Figure 1E). 2iP induced a mean of 2.5 shoots at 9.29 µM but all other concentrations induced a single stunted slightly vitrified shoot. All concentrations of Zeatin containing cultures induced a mean of single vitrified shoot invariably (Table 1). A callus occasionally formed at the base of the explant retarding axillary bud formation and the subsequent growth of shoots. Therefore, precautions were taken to remove such callus growth while sub culturing.

Nodal segments containing axillary buds have quiescent or active meristems depending upon the physiological stage of the plant. These buds have the potential to develop into complete plantlets. The conventional method used for the vegetative propagation of stem cuttings relies on the axillary bud taking over the function of the main shoot in the absence of a terminal bud. In nature, these buds remain dormant for a specific period depending on the growth pattern of the plant.

However, using tissue culture, the rate of shoot

Table 2. Effect of different combination of BA, NAA, IAA and IBA on shoot regeneration of mature nodal explant of *C. bhupenderiana*.

Plant growth regulator				Shoot sprouting frequency (%)	Mean shoot number per explant ±SE	Mean length of shoot (cm) ±SE	Basal callusing
BA	NAA	IAA	IBA				
8.87	0.54	-	-	75	3.40 ± 0.08 [b]	4.2 ± 0.15 [a]	-
8.87	2.69	-	-	90	4.63 ± 0.24 [a]	2.00 ± 0.08 [bc]	+
8.87	-	0.57	-	75	2.66 ± 0.20[c]	2.03 ± 0.20 [c]	+
8.87	-	2.85	-	80	4.0 ± 0.30 [a]	3.03 ± 0.16 [b]	-
8.87	-	-	0.49	55	1.56 ± 0.17 [e]	1.76 ± 0.12 [d]	-
8.87	-	-	2.46	70	2.00 ± 0.28 [cd]	1.70 ± 0.29 [de]	-

Means ± SE, n=45. Means followed by the same letter are not significantly different by the DMRT at 0.05% probability level; (-) sign indicates no callusing; (+) signs represent the intensity of callusing.

multiplication can be greatly enhanced by performing axillary bud culture in a nutrient medium containing suitable cytokinin or cytokinin and auxin combinations. Due to continuous availability of cytokinin, shoots formed by the bud already present in the explant (nodal segment) develop into axillary buds, which then grow directly into shoots. Multiple shoot formation following the *in vitro* culture of nodal segments has proved to be an effective method of mass multiplication. The effect of BA on multiple shoot bud differentiation has been demonstrated in a number of cases using a variety of explants (Karuppusamy and Pullaiah, 2007; Thomas and Shankar, 2009; Tavares et al., 2010). In the present case, BA also proved to be more effective than other cytokinins. Among the different levels of BA tested, 8.87 µM produced the maximum number of shoots from nodal explants. Kn, 2iP and Zn were less effective than BA. Similar response was also observed in the shoot multiplication of *C. edulis* (Rathore et al., 2008), three varieties of *Caralluma adscendens* (Aruna et al., 2009), *Ceropegia jainii*, *Ceropegia bulbosa* (Patil, 1998), *Ceropegia candelabrum* (Beena et al., 2003) and *Decalepis arayalpathra* (Gangaprasad et al., 2005). However, the poor performance of Kn in this study is contradictory to the report on *Hemidesmus indicus*, another member of Asclepiadoideae (Patnaik and Dabata, 1996).

Effect of auxin and cytokinin

The efficiency of the optimal concentration of BA with various auxins (NAA, IAA and IBA) was also evaluated for multiple shoot induction (Table 2). BA with NAA was found to be the most effective combination for shoot regeneration and multiplication. Nodal explants cultured on MS medium supplemented with BA 8.87 µM and NAA 2.69 µM exhibited 90% shoot regeneration (Figure 1F). Upon increasing the concentration of NAA up to 10.74 µM, a gradual decrease in regeneration frequency and the number of shoots per explant was recorded. The elevated concentration of NAA (10.74 µM) resulted in little callusing at the cut end thus, reducing the percent shoot regeneration and the number of shoots per explant. Among the various combinations of BA and IAA used, the highest shoot regeneration frequency (80%) and number of shoots per explant (4.0 ± 0.30) along with the maximum shoot length (3.0 ± 0.16 cm) were recorded on MS medium supplemented with BA 8.87 µM + IAA 2.85 µM after six weeks of inoculation (Table 2). Among the BA and IBA combinations, the maximum frequency (70%) of shoot bud formation and the greatest number of shoots (2.0 ± 0.28) per explant were obtained on MS medium containing BA 8.87 µM with IBA 4.9 µM.

A low concentration of auxin along with a high concentration of cytokinin was most promising for the induction and multiplication of shoots in *C. bhupenderiana* and MS medium supplemented with BA 8.87 µM in combination with NAA 2.69 µM proved most effective for direct shoot regeneration. The synergistic effect of BA in combination with an auxin has been demonstrated in many medicinal plants from the Asclepiadoideae such as *Gymnema sylvestre* (Reddy et al., 1998), *Holostemma annulare* (Sudha et al., 1998), *Hemidesmus indicus* (Sreekumar et al., 2000), *Holostemma ada-kodien* (Martin, 2002), *Leptadenia reticulata* (Arya et al., 2003), *C. candelabrum* (Beena et al., 2003), *Tylophora indica* (Thomas and Philip, 2005; Faisal et al., 2007), *Huernia hystrix* (Amoo et al., 2009) and *Sarcostemma brevistigma* (Thomas and Shankar, 2009). In accordance with these reports, the present study also exemplifies the positive modification of shoot induction efficacy obtained by employing a low concentration of auxin in combination with a cytokinin.

Effect of ascorbic acid

In another experiment, nodal explants were cultured on growth regulators BA (8.87 µM) + IAA (2.85 µM) containing medium supplemented with various concentration of ascorbic acid (50 to 500 mg/l) (Table 3). Results obtained after six weeks of incubation revealed that 100 mg/l ascorbic acid in the presence of BA 8.87 µM and

Table 3. Effect of ascorbic acid on shoot regeneration from nodal segments of *C. bhupenderiana* in MS medium after six weeks of culture.

Treatment	Regeneration (%)	Mean number of shoot/explant	Mean shoot length (cm)
BA (8.87 µM) + IAA (2.85 µM) + AA (50 mg/l)	90	6.5 ± 0.1[b]	4.8 ± 0.20[a]
BA (8.87 µM) + IAA (2.85 µM) + AA (100 mg/l)	93	8.6 ± 0.71[a]	5.2 ± 0.31[a]
BA (8.87 µM) + IAA (2.85 µM) + AA (200 mg/l)	80	4.5 ± 0.31[c]	4.0 ± 0.21[b]
BA (8.87 µM) + IAA (2.85 µM) + AA (500 mg/l)	63	3.4 ± 0.29[c]	3.5 ± 0.15[b]

Data represent mean ± SE. Means followed by the same letter within columns are not significantly different (P = 0.05) using DMRT.

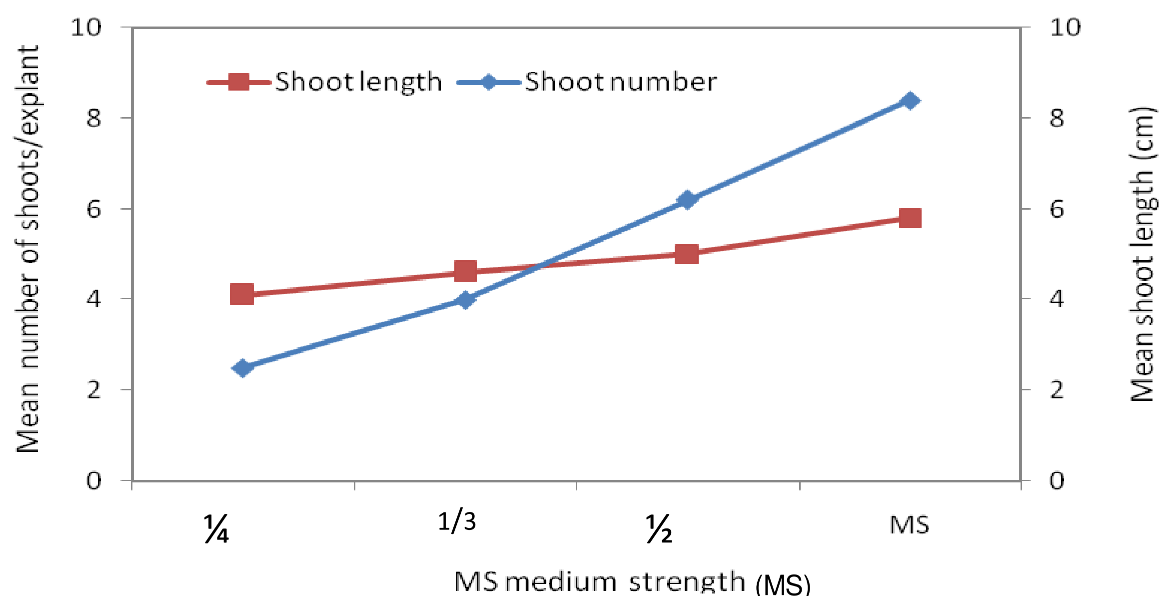

Figure 2. Effect of medium strength on shoot regeneration from nodal explants of *C. bhupenderiana* supplemented with BA (8.87 µM) + IAA (2.85 µM) + AA (100 mg/l). Data represent the mean ± SE.

IAA 2.85 µM gave the maximum response with regards to overall shoot growth in terms of number of shoots and shoot length (Figure 1G). Increasing the concentration of ascorbic acid to 500 mg/l suppressed the regeneration ability and the shoots formed were vitrified. Similarly, a stimulative effect of ascorbic acid was also reported (Sharma and Chandel, 1992; Neelam and Chandel, 1992; Faisal et al., 2007 and Lakshmi et al., 2010).

Effect of medium strength and pH

The effect of the strength of the MS medium on shoot proliferation was also examined with ¼ MS, ⅓ MS, ½ MS and full-strength MS (Figure 2). The shoot proliferation was found to be highest for full-strength MS medium and poorest on ½ MS. The effect of different medium pH levels (5.0, 5.4, 5.8 and 6.2) was also tested for MS medium supplemented with BA (8.87 µM) + IAA (2.85 µM) + AA (100 mg/l) (Figure 3). The optimum pH for shoot proliferation and elongation was found to be 5.8 and multiplication was inhibited in more acidic media.

The shoots produced in this way were either subcultured for further multiplication or transferred onto a rooting medium.

Rooting of regenerated shoots

The shoots regenerated *in vitro* were transferred to half-strength MS medium. Root formation from the basal cut portion of the shoots was observed one week after transfer to the rooting medium. The presence of an auxin (NAA, IAA and IBA) at a low concentration in half-

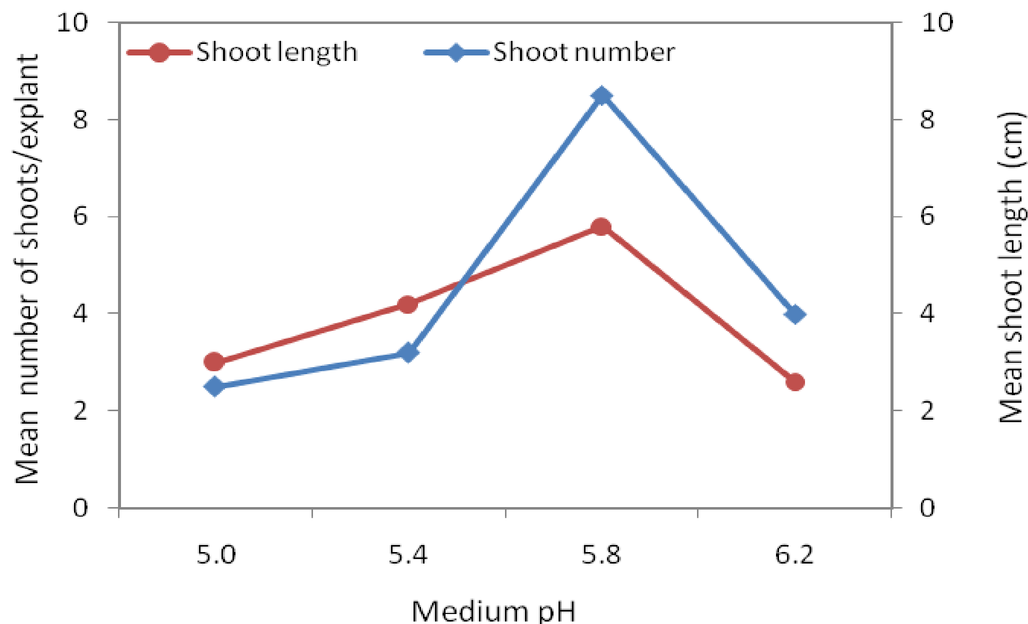

Figure 3. Effect of medium pH on shoot regeneration from nodal explants of *C. bhupenderiana* supplemented with BA (8.87 µM) + IAA (2.85 µM) + AA (100 mg/l). Data represent the mean ± SE.

strength MS medium was found to be more effective for rooting (Figure 1G) and the best rooting was achieved in this medium fortified with 0.5 µM NAA; fairly good shoot numbers (4.86 ± 0.26) and root lengths per shoot (4.00 ± 0.21) were obtained (Table 4). No rooting was observed from the base of any microcutting prior to the first week of culture. Rooting frequency increased gradually over time and reached a maximum after six weeks of culture.

To optimize the rooting response of plantlets raised *in vitro,* different auxins (NAA, IAA and IBA) were tested at various concentrations. In general, NAA was observed to induce a strong rooting response; this has been used to promote rooting in a wide range of plant species and is readily available around the world. The rooting of this plant was significantly affected by the concentration of NAA. The best rooting response was obtained in a medium containing NAA 2.69 µM. The success of NAA in promoting efficient root induction has been reported for a few Asclepiadoideae species *Decalepis hamiltonii* (Reddy et al., 2001; Anitha and Pullaiah, 2002), three varieties of *C. adscendens* (Aruna et al., 2009) and *Ceropegia intermedia* (Karuppusamy et al., 2009).

Acclimatization

Plantlets with 4 to 5 rudimentary leaves and well developed roots were successfully hardened inside the growth room in a selected planting substrate (garden soil, soil rite or vermiculite) for four weeks and eventually established in natural soil (Figure 1H). Of the three different types of planting substrates examined, the percentage survival of the plantlets was highest (80%) in vermiculite (Table 5). About 80% of the regenerated plants survived following transfer from vermiculite to natural soil and no detectable variation with respect to morphology or growth characteristics was observed.

The period of transition during the process of hardening after transfer from the *in vitro* to the *ex vitro* environment is considered to be the most important step in tissue culture. Due to the heterotrophic mode of nutrition, a lack of adaptation or exposure to the outside environment, during laboratory to land transfer micropropagated plants are first placed in the hardening chamber. In general, during the period of hardening, care was taken over the physical (temperature, light intensity, relative humidity, air current, atmospheric CO_2) and other factors (mineral nutrition, pH and texture of soil) employed. One important factor during acclimatization is the type of potting material used. Of the three different types of planting substrates used (garden soil, soil rite and vermiculite), the highest survival rates for the micropropagated plants were achieved in vermiculite.

Conclusions

In conclusion, we have established a direct *in vitro* culture system for an important medicinal plant *C. bhupenderiana*, which should enable the large-scale nursery production of this valuable medicinal plant for the landscape. The outline of protocol offers a potential system for improvement, conservation and micropropagation of *C. bhupenderiana* from nodal explant. MS

Table 4. Rooting response of *in vitro* regenerated shoots of *C. bhupenderiana* in half strength MS containing NAA, IAA and IBA in various concentrations with 1% sucrose after 25 days.

Plant growth regulator (mg/l)			Percentage of response	Mean number of roots per shoot ± SE	Mean length of root (cm) ± SE	Degree of callusing
NAA	IAA	IBA				
0.54	-	-	62	2.90 ± 0.21 c	3.13 ± 0.12 b	-
1.07	-	-	75	2.26 ± 0.04 cd	1.43 ± 0.04 e	-
1.61	-	-	60	2.70 ± 0.29 c	2.50 ± 0.21 c	-
2.15	-	-	66	3.70 ± 0.08 b	2.20 ± 0.21cd	+
2.69	-	-	88	4.86 ± 0.26 a	4.00 ± 0.21 a	-
5.37	-	-	-	CP	CP	+ +
-	0.57	-	45	1.83 ± 0.18 dc	2.36 ± 0.33bc	-
-	1.14	-	68	3.20 ± 0.24 b	2.40 ± 0.32 b	-
-	1.71	-	71	3.10 ± 0.21 bc	2.50 ± 0.21 b	-
-	2.28	-	48	2.27 ± 0.14 c	0.95 ± 0.03 d	-
-	2.85	-	80	4.23 ± 0.33 a	3.40 ± 0.16 a	-
-	5.71	-	-	CP	CP	+ +
-		0.49	40	1.93 ± 0.04 b	2.00 ± 0.00 b	-
-		0.98	46	2.50 ± 0.08 a	2.56 ± 0.04 a	-
-		1.48	-	CP	CP	+
-		1.97	-	CP	CP	+ +
-		2.46	-	CP	CP	+ +
-		4.90	-	CP	CP	+ + +

Means ± SE, n=45. Means followed by the same letter are not significantly different by the DMR test at 0.05% probability level; (-) sign indicates no callusing, number of (+) signs represents the intensity of callusing; CP , callus production.

Table 5. Evaluation of different planting substrates for hardening of plantlets of *C. bhupenderiana* raised *in vitro.*

Planting substrate	Number of plant transferred	Number of plant survived	Survival (%)
Garden soil	100	53	53
Soil rite	100	70	70
Vermiculite	100	80	80

medium containing BA (8.87 µM) is the best for shoot proliferation. The use of axillary nodes for micro-propagation is beneficial than other explant types. Half strength MS basal medium supplemented with NAA (2.69 µM) is the best for root induction.

ACKNOWLEDGEMENTS

Authors are thankful to the Department of Science and Technology (DST) Government of India, New Delhi for its financial assistance.

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