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In vitro Callus Regeneration of *Caralluma bhupenderiana* Sarkaria - An Endangered and Endemic Medicinal Plant

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Abstract

An effective *in vitro* callus regeneration protocol was standardized for the endangered, endemic succulent plant *Caralluma bhupenderiana* Sarkaria. Explants from healthy stem segments were cultured on Murashige and Skoog (MS), (1962) medium, Gamborg's B_5 (B_5) and Woody Plant Medium (WPM) supplemented with 2,4-D 3mg/l for callus induction. Of all the media tested, MS medium with 2,4-D demonstrated the best callus response. Effect of different concentrations of auxins (Indole 3- Acetic Acid, Naphthalene Acetic Acid, Indole 3- Butyric Acid and Indole 3- Propionic Acid and 2,4-Dichlorophenoxy acetic acid) on callus induction was examined. However, 2,4-D (2mg/l) elicited the best callus induction. Plant regeneration was examined on MS medium fortified with 2,4-D (2mg/l) supplemented with various concentrations of cytokinins (Benzyl-6-adenopurine and Kinetin; 0.1 -0.4mg/l). Although significant shoot numbers were observed under the influence of 2mg/l 2,4-D and 2 mg/l BAP, the effect was profoundly increased upon second subculture in the same medium. The propagated shoots were kept for rooting on ½ strength MS medium supplemented with 0.1 mg/l NAA. Complete plants were developed and matured with a mean number of 12.06 ± 0.06 and root mean length of 3.00 ± 0.02. All the plantlets were acclimatized in the shade net house with a survival rate of 70%. The regenerated plants did not show any immediate notable phenotypic variation. The *in-vitro* protocol developed in the current study helps in the bulk production of *Caralluma bhupenderiana* and could be a good alternative non-conventional technique for conservation of rare and endemic species.

Key words: Callus induction, 2,4-Dichlorophenoxy acetic acid, *C. bhupenderiana*

Introduction

Biologically active compounds have gained great importance in the recent years due to their implications in promotion of good health and their role in prevention of various kinds of infections and diseases. Plants are vital sources of bioactive compounds. Due to the rapidly increasing demand for bioactive products, plants have become an easy target for isolation of such compounds (1). Hence plants are overexploited due to the ruthless collection of plants for their products and such dependency has resulted in depletion of many species. Further, overgrazing and industrialization also have added to the cause of quick eradication of plant species. In this process, majority of the traditional medicinal plants are under the extreme

limit of extermination. Hence, to overcome this problem, conservation of several valuable plant species is highly essential.

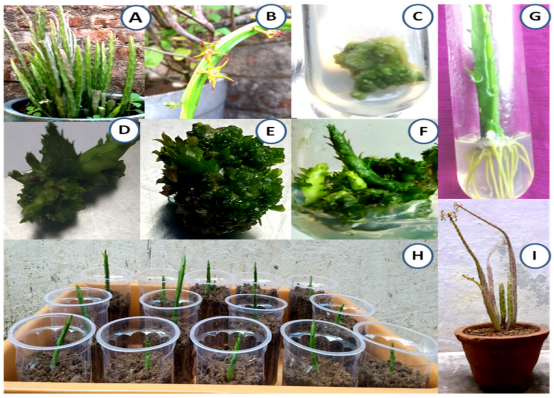
Members of Apocynaceae are known to be endowed with high amounts of bioactive compounds and are highly preferred by the pharmaceutical industry (2). *Caralluma bhupenderiana* Sarkaria is a member of the genus *Caralluma* and family Apocynaceae. *C. bhupenderiana* is an important medicinal plant that contains high number of bioactive compounds and is on the verge of its extinction. It is reported that about seven different varieties and 13 species of *Carallumas* are available in India (3). Majority of the species among these varieties found in India are endemic to southern parts of India and are enriched with high medicinal values (3).

Almost all varieties of *Carallumas* are rich with bio active compounds like flavonoids (1), pregnane glycosides (4) that play a significant role in anti-cancer (5) and anti-inflammatory activity (6). Apart from these properties, majority of the *Carallumas* also used for prevention of diabetes, obesity, skin damage, skin infections, ulcers, antidote (7, 8). *Caralluma fimbriata* is also reported to prevent obesity and is used for weight reduction (9). Similarly, many of the species of this group are in high demand owing to their medicinal properties. Hence these plants are exploited and have been listed in the endangered list. *C. bhupenderiana* is another important medicinal plant that has not been much focused despite its high medicinal value and its endangered status. In order to protect these medicinally important plants from complete extinction, apart from natural conservation methods, alternate protocols for bulk production and multiplication of these plant species are essential. In vitro production and multiplication through tissue culture protocols have been developed for few of the *Caralluma* species. However, to our knowledge, no reports on micropropagation through callus cultures have been reported with regard to *C. bhupenderiana.* The objective of this study is to design an efficient protocol for the rapid and huge multiplication of *Caralluma bhupenderiana* Sarkaria through callus cultures.

Materials and Methods

C. bhupenderiana were collected from Vallanadu Hilly region, Tamilnadu (Fig. 3A) and were potted and maintained in a shade net house of Vikrama Simhapuri University, Kakutur. Healthy and matured stems having 10-15 internodes were collected from potted plants, washed under tap water for 5-10 min to remove dust and soil particles on the surface of stems. Stems were then washed with 1% (w/v) Tween 20 (Hi-media) for 5 min with constant rinsing and later washed with double distilled water for three times. Further treatments were carried inside the laminar air flow. Washed stems were treated with 70% alcohol for two minutes followed by three rinses with double distilled water to remove any traces of alcohol. The stems were then treated with 0.1 % (w/v) $HgCl_2$ for 5min, and washed with double distilled water for three times. The entire process is termed as surface sterilization. Surface sterilized stems were trimmed into equal pieces of 0.5-1cm in length.

Fig.3: Different stages of In vitro propagation of *C. bhupenderiana*



A. Natural Habitat; B. Inflorescence; C. Proliferation of the callus on MS + 2,4-D (2mg/l); D. Callus Induction on BAP (1mg/l) + 2,4-D (2mg/l); E. Regeneration from Shoot buds on 2,4-D (2mg/l) + BAP (2mg/l); F. Elongated

shoots; G. Rooting of developed shoots on ½ Strength MS + 0.1mg/L NAA; H. Acclimatized in vitro plant; I. Survived plant after potting.

Culture media, inoculation and culture conditions

Three basic culture media MS medium, B5 and WPM media were selected for the current study. The media were additionally supplemented with all the required macro and micro nutrients along with 3% sucrose. Agar (0.7% w/v) was used as a solidifying agent that helps in gelling. Plant growth regulators were also supplemented to this media. The pH of the medium was set to 5.7 ±1 using 0.1 NaOH and 0.1 HCl. To avoid any unwanted contamination, media was steam sterilized in an autoclave for 15 minutes at 121°C and 15 psi. Following this, the media was allowed to cool down and then stored in sterile room until further use. For the purpose of callus induction three different media namely MS (10), Gamborg's B_5 (B_5) and Woody Plant Medium (WPM) were selected and all the three media were fortified with 2,4-D at a concentration of 3mg/l. Depending on the shelf life and percentage of response basal medium was standardized for further callus induction. All the surface sterilized internodes were inoculated under sterile conditions in the Laminar Air Flow Chamber. There after maintained in the culture room at 24 ± 3° C, 16 hours light photo period under 2000 lux tube lights (Philips).

Callus induction and organogenesis

For the induction of callus healthy and surface sterilized internodes were selected and cultured on MS medium fortified with the different concentrations (0.1 to 4mg/l) of 2,4-D (2,4-Dichloro phenoxy acetic acid) IBA (Indole -3-Butyric Acid), IAA (Indole -3- Acetic Acid), IPA (Indole -3- Propionic Acid) and NAA (α-Naphthalene Acetic Acid) individually. For callus regeneration different combinations of auxins and cytokinins were examined. Each experiment was repeated thrice with 15 replicates in each set. Subcultures were done every 15 days. The induction of callus and regeneration of callus was observed regularly and recorded. While performing the sub-culturing of callus, the callus was taken out of the medium and weighed in sterile environment in order to estimate the percentage of callus produced.

Root initiation and multiplication:

Following the shoot regeneration, the next step is to initiate the rooting. In order to initiate the rooting, the *in vitro* regenerated shoots from the callus having 5-6 nodes were carefully separated and each individual shoot was transferred on to half strength MS medium supplemented with 0.1 to 0.5 mg/l concentrations of IAA, NAA and IBA. The tubes containing the transferred shoots were incubated for 4 weeks with 16 h photoperiod and the rate of rooting frequency was recorded.

Acclimatization and transplantation of the plantlets

Following the development of roots from the micro shoots under *in vitro* condition, they were picked out from the test tube carefully and the roots were washed under gentle flow of tap water for removing any remnants of the medium and agar deposits on the root surfaces. The regenerated shoots with healthy roots were planted in cups that were filled with sterilized coco peat, sand and soil at 1:1:1 ratio respectively. Water was supplied to plantlets every alternate day. To help in the process of acclimatization, the plantlets were covered with thick polythene bags to prevent loss of moisture through evaporation. Healthy plants after 30 days were transferred to soil pots to acclimatize and were maintained in the Shade net house provided with normal tap water every alternative day. Survival rate was recorded after 30 days.

Statistical analysis

Data are presented as mean ±SE. Data was analysed by one way ANOVA using Tukey's multiple comparisons test. Each experiment was repeated thrice with a minimum of 10 replicates in each set. $P < 0.05$ was considered to be statistically significant.

Mean values followed by identical superscripts were not statistically significant (P < 0.05).

Results and Discussion

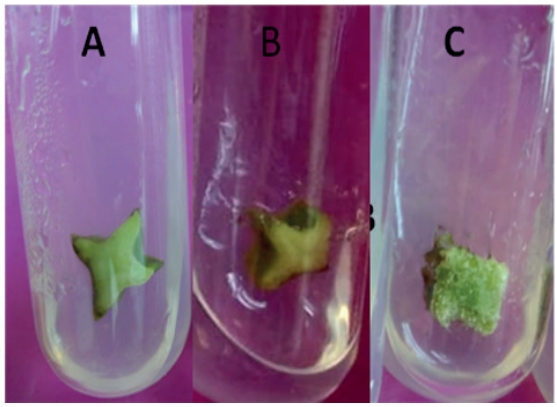
Selection of the basal medium for callus induction

The induction and regeneration of the callus is dependent upon the nutritional requirement of the cells. Hence, selection of basal medium is very important for carrying further experiments on micropropagation. To optimize the basal medium for induction of callus, stem segments of *C. bhupenderiana* were inoculated on B5, WPM and MS media, all fortified with 3mg/l 2,4-D. Among three supplemented media, MS medium fortified with 2,4-D produced better callus compared to B5 and WPM. The rate of callus induction and percentage of response was found to be significantly higher in MS medium fortified with 2,4-D (Fig.1) (Table.1). The stem segment turned into brown colour with slight morphological change in WPM medium (Fig.1B). A good response with inflated green and healthy callus was observed in the MS medium fortified with 2,4-D at a concentration of 3.0 mg/l (Fig.1C). MS medium is rich with essential salts as compared to B5 and WPM medium. This shows that proliferation of callus requires more salts. Hence MS Medium was selected for all further experiments like callus induction, organogenesis and rooting of *C. bhupenderiana*.

Table 1: Effect of different media on Callus induction in *C. bhupenderiana*

Medium	% of callus induced
B5 medium	53.33
WPM medium	33.33
MS medium	86.66

Fig.1: Effect of different types of medium on callus proliferation



A. B5 Medium; B. WPM Medium; C. MS medium

Consistent with our observations, MS medium was also found to be effective and favorable for callus induction in other plants such as *Asclepias syriaca* (11, 12), *Cryptolepis buchanani* (13, 14), *Calotropis gigantea* (15), *Gymnema sylvestre* (16), *Decalepis hamiltonii* (17), *Holostemma ada-kodien* (18), *Leptadenia reticulata* (19) and *Tylophora indica* (20).

Influence of auxins on callus induction

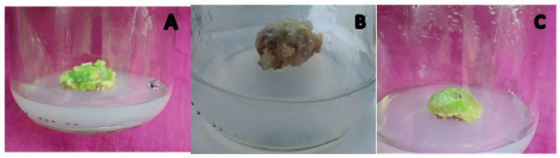
Stem segments of *C. bhupenderiana* were cultured on MS medium fortified with 2,4-D, IAA, IBA, NAA and IPA at various concentrations ranging from 0.1, 0.5, 1.0, 2 and 4mg/l under sterile conditions. Although MS medium fortified with all the auxins resulted in callus induction, the type and frequency of the callus varied. It was observed that the appearance and texture of the callus varied ranging from compact to friable (Fig. 2A & C). In some of the cases, embryogenic and browning of the callus was also observed (Fig. 2B). Healthy and green callus thus obtained under the influence of different concentrations of auxins were used for further subcultures. NAA at 1.0 mg/l resulted in small quantity of callus with compact texture. No callus was formed under the influence of lower concentrations of NAA. Under the influence of all concentrations of IPA, embryogenic callus was observed that eventually turned into brown colour after 2 weeks (Fig. 2B). Among the var-

ious auxins used in the current study, 2,4-D demonstrated highest response in terms of callus induction. 2,4-D at concentration of 0.1 mg/l to 2 mg/l, exhibited a dose dependent positive response resulting in green and healthy callus formation (Table. 2). The best response (86.6%) under 2,4-D was observed at a concentration of 2.0 mg/l, while such response was lowered at higher concentrations above 2mg/l (Table. 2). Our observations with respect to 2,4-D induced callus induction are similar to the findings of earlier reports that showed highest response in *Catharanthus roseus* (21), *Ceropegia candelabrum* (22).

Table.2: Effect of auxins on Callus induction from stem segments of *C. bhupenderiana*

Auxin	Concentration	% of callus induced
2,4-D	0.1	40
	0.5	46.6
	1.0	53.3
	2.0	86.6
	4.0	53.3
IAA	0.1	26.6
	0.5	33.3
	1.0	33.3
	2.0	26.6
	4.0	20
IBA	0.1	20
	0.5	26.6
	1.0	33.3
	2.0	40
	4.0	20
NAA	0.1	26.6
	0.5	26.6
	1.0	33.3
	2.0	20
	4.0	20
IPA	0.1	33.3
	0.5	33.3
	1.0	40
	2.0	33.3
	4.0	26.6

Fig.2: Types of calluses on different Auxins



A. Green friable nodular callus on 2 mg/l of 2,4-D;

B. Brown Embryogenic callus on IPA

C. Green compact callus on NAA

Organogenesis

Once the conditions were all standardized and set for maximal callus induction, the next step was to initiate morphological changes in the callus. The callus obtained from the ideal concentration of 2mg/l of 2,4-D was processed for organogenesis to obtain the regeneration of shoots. In order to test the organogenesis, two cytokinins namely Kinetin and BAP along with 2mg/l 2,4-D at different concentrations were examined. Among the two cytokinins tested, BAP showed significant response in shoot induction as compared to Kinetin. Kinetin at lower concentrations showed poor response while higher concentrations showed moderate response. Although, low concentrations of BAP showed reduced response, BAP at 2mg/l resulted in highest shoot numbers (19.9± 0.06) with a 100% response (Table.3). In consistence with our observations, other studies have shown that effective response in combination of auxins and cytokinins was obtained in plants such as *Oxystelma secamone* (23), *Aristolochia pyrotechnica* (24) and Leptadenia *pyrotechnica* (25).

**Values presented represent Mean ± SE. Mean*

Table-3: Effect of 2,4-D (2mg/l) and with cytokinins on organogenesis of *C. bhupenderiana*

Plant Growth regulators used for morphogenesis			Organogenesis from callus	
	BAP	KN	% of response	No. of Shoots/ explant (Mean ± SE)
2,4-D 2mg/l	0.1	--	60	3.11±0.11^{e}
	0.5	--	80	5.16 ± 0.11^{c}
	1.0	--	93.3	7.14 ± 0.09 ^{b}
	2.0	--	100	19.9 ± 0.06^{a}
	4.0	--	73.3	3.81 ± 0.12^{d}
	--	0.1	NR	NR
	--	0.5	53.3	1.125 ± 0.13^{f}
	--	1.0	53.3	1.25± 0.16^{f}
	--	2.0	60	1.11 ± 0.11^{f}
	--	4.0	60	1.22 ± 0.14^{f}

values followed by different alphabets are statistically significant (P<0.05) and those with same alphabet are not significant.

In-vitro rooting and acclimatization

Healthy in vitro shoots measuring 5 to 6 cm that were regenerated from the callus were selected and inoculated individually into the $^{1}/_{2}$ strength MS medium supplemented with 0.1, 0.2, 0.3 mg/l concentrations of different auxins IAA, IBA and NAA. The inoculated tubes were incubated in culture room and maintained under controlled environment with 16 h of photoperiod and were regularly observed for any root sprouting. After three weeks of incubation, it was observed that root formation occurred under the influence of all three auxins. However, NAA at a concentration of 0.1mg/l induced the highest number of roots with 100% response. Such response was also associated with maximal root length (Table. 4). Further, it was observed that auxins at higher concentrations (above 0.3 mg/l) resulted in callus formation. Our results are in line with the earlier findings in few of the Asclepiadaceae members like *Decalepis hamiltoni* (26), *Decalepis arayalpathra* (27), *Caralluma adscendens* (28), *Ceropegia intermedia* (29), *Caralluma tuberculata* (30), *Caralluma stalagmifera* (31).

Table 4: Rooting response of regenerated shoots of *C. bhupenderiana* in half strength MS medium supplemented with different concentrations of auxins

Concentration of Auxins (mg/l)			% Response	No. of Roots/Shoot Mean ± SE	Length of roots (cm) Mean ± SE
IAA (mg/l)	IBA (mg/l)	NAA (mg/l)			
0.1	--	--	86.6	4.23 ±0.12^{c}	1.83 ± 0.03^{d}
0.2	--	--	86.6	2.69± 0.17^{e}	1.66 ± 0.06^{d}
0.3	--	--	73.3	1.18 ± 0.11^{g}	1.71± 0.03^{d}
--	0.1	--	80	3.08 ± 0.08^{e}	1.62 ± 0.08^{d}
--	0.2	--	86.6	1.56 ± 0.05^{f}	1.86 ± 0.02^{c}
--	0.3	--	73.3	1.09 ±0.09^{g}	1.75 ±0.02^{d}
--	--	0.1	100	12.06 ± 0.06^{a}	3.00 ± 0.02^{a}
--	--	0.2	93.3	7.42 ±0.24^{b}	2.05 ±0.03^{b}
--	--	0.3	80	3.91 ± 0.08^{d}	1.33 ± 0.07^{e}

**Values presented represent Mean ± SE. Mean values followed by different alphabets are statistically significant (P<0.05) and those with same alphabet are not significant.*

After three weeks healthy shoots along with developed roots were removed carefully from the test tube and washed gently under slow flow tap water to remove any remnants of the media around the roots. These were planted in plastic cups filled with coco peat, sand and soil at 1:1:1 ratio respectively (Fig 3H). The planted cups were covered with thick polythene bags to prevent the loss of moisture through evaporation. In order to provide nutrients, the plants were sprinkled with half strength MS medium weekly once. After 30 days healthy plants were transferred to pots filled with manure and black soil and were maintained in the shade net house and watered every alternative day. The acclimatized *C. bhupenderiana* plants successfully survived with a survival rate of 70%.

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Conflict of Interest

Authors do not have any conflict of interest.

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