Original Article

High Frequency Axillary Bud Multiplication of *Caralluma stalagmifera* C.E.C. Fischer- A Medicinal Plant

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ABSTRACT

Objective: The objective of the present study was to develop a protocol for micropropagation of the medicinal plant *Caralluma stalagmifera* (Asclepiadaceae) from nodal explants.
Methods: Nodal explants of field grown plants were cultured on MS medium supplemented with BAP, 2-iP, GA_3 and auxins NAA, IAA and IBA. The regenerated shoots were rooted on half strength MS medium supplemented with IAA, IBA and NAA. They were acclimatised in plastic cups with polythene covers and then transferred to field.
Results and Conclusion: Axillary bud proliferation with a mean of 8.47 shoots/node was achieved when the explants were grown in an optimized Murashige and Skoog (MS) medium which were supplemented with the phytohormones BAP (2.0 mg/l) + 2-iP (2.0 mg/l) + NAA (0.5 mg/l). When the node segments grown *in vitro* were excised and cultured in a medium supplemented with the same concentration of the plant growth hormones that were used for the growth of the Axillary buds, more than 15 shoots developed in 30 days. Consecutive excision and culture of nodes grown *in vitro* greatly augmented the number of shoots which emerged as well as shoot elongation when the concentration of the growth hormones used in the MS medium was BAP (2.0 mg/l) + GA_3 (1.0 mg/l). Highest number of roots was observed when half strength MS medium enriched with NAA (0.5 mg/l) was used. Hardening process of the rooted plants was achieved in polycups having equal proportions of sterile peat mass, farmyard manure and soil, which resulted in a survival rate of 70%.

Keywords: Asclepiadaceae, Medicinal plant, C*aralluma stalagmifera*, Nodal explants, Shoot multiplication, Plant tissue culture, Micropropagation.

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INTRODUCTION

The dry and rocky regions of South India are home to *Caralluma stalagmifera* (Asclepiadaceae), a succulent and perennial herb having a sour taste. This plant as a whole has been used to extract various novel steroidal glycosides such as Indicosides I-II and Stalagmosides I-V along with the well-known steroidal glycosides such as Lasianthosides A-B and Carumbelloside III [1]. Butanol and aqueous extracts of *C. stalagmifera* have demonstrated anti-arthritic and anti-inflammatory activities in rats having kaolin induced arthritis and carrageenin induced paw oedema[2]. The genus *Caralluma* has certain plants which are prolific with esterified polyhydroxy pregnane glycosides having anti-tumour properties, while there are other plants in this genus which possess Cardenolides[3,4] and flavone glycosides[5,6]. According to the Indian Folkore reports and the practices of tribals and hunters, the extracts of *Caralluma* are known to stimulate weight loss by suppressing the appetite and this knowledge has been translated and marketed by the industry as GENASLIM, which is a famous pill for the obese people[7]. To meet the demands for medicines of herbal origin, there is a strong need to propagate elite plants such as *Caralluma*. Conventional means of cultivation of these plants provide very low yield due to lower rate of survival. Hence, adoption of alternate plant propagation methods such as *in vitro* propagation will ensure that the demands of the herbal medicinal industry are adequately met with. *In vitro* propagation assures the growth of true-to-type plants with limiting conditions of space and time. The present communication is the first report on *in vitro* propagation of *Caralluma stalagmifera* using nodes derived from mature plants.

MATERIALS AND METHODS

Collection of plant material and surface sterilisation

Caralluma stalagmifera was collected from Gooty hills, Anantapur distict, Andhra Pradesh, India and were potted in pots and maintained at Sri Krishnadevaraya University, Anantapur. The source of explants used for the study were young shoots that were growing actively. These explants were surface sterilized by first washing them under running tap water and then using 1% tween-20 (detergent) for 10 min. This was followed by a subsequent wash under running tap water. Then, the explants were transferred to the Laminar Air flow chamber for further steps of surface sterilization. The explants were washed with sterile double distilled water followed by a wash with 0.1% Mercuric chloride for 5 min and then subsequent wash steps with sterile double distilled water. Explants were then subjected to 70% ethanol treatment for 1 min. and 3-4 rinses in sterile double distilled water.

Media and Culture conditions

Stem segments, 1.0 cm long, containing the axillary buds were aseptically cut from the surface sterilized shoot pieces and inoculated onto the sterilized nutrient medium, one explant/tube. The damaged ends were removed. The basal medium (MS) used was that Murashige and Skoog[8] supplemented with 3% (w/v) sucrose and 0.8% agar. Depending on the experiment the basal medium was further supplemented with cytikinins (BAP and 2-iP), Gibberellic Acids (GA_3) and auxins (NAA, IAA and IBA) alone or as a combination mentioned in Tables 1-2. Bacteriological agar was added to the medium after it's pH was adjusted to 5.8. The gelled medium was dispensed into 150 mm × 25 mm rimless

culture tubes (20ml medium/ tube) and autoclaved at 1.06 kg/cm-2 pressure and 121 ^{o}C for 15 min. Each treatment consisted of 15 culture replicates and each experiment was performed in triplicates. The temperature of the culture room was maintained at 25^{o}C with cool fluorescent white light (50µ mol m^{-2} S^{-1}) for a photoperiod of 16 hours.

Shoot multiplication and rooting

Regenerated shoots having more than two nodes were excised from the primary cultures and subcultured on medium supplemented with BAP 2.0 mg/l + 2-ip 2.0 mg/l + NAA 0.5 mg/l. Sub culture was repeated every 30 days for shoot production. Cultures were grown in 25 mm × 150 mm culture tubes or in 300ml culture bottles. To induce rooting, full and half strength MS medium supplemented with various concentration of growth hormones such as IAA, IBA and NAA (0.1 mg/l to 3.0 mg/l) was used in which excised regenerated shoots were inoculated. For induction of rooting regenerated shoots were excised and cultured on full strength and half strength MS medium with various concentrations of NAA, IAA and IBA (0.1 mg/l to 3.0 mg/l).

Acclimatization of plantlets

The rooted plantlets were transplanted in plastic pots having sand, peat mass and farmyard manure in the ratio of 1:1:1 after thoroughly washing them. These plantlets after transplanting were subjected to irrigation with ½ strength MS basal liquid medium devoid of sucrose, after which they were covered with polythene covers.

After 10 days the polythene covers removed and well developed plants were transformed to green house conditions and then they were transferred outdoors after acclimatization for a period of two weeks.

Statistical analysis

Analysis of variance (ANOVA) was used to analyze the data statistically and the means obtained were compared using the Tukeys test at a probability level of significance of 0.005%.

RESULTS AND DISCUSSION

Explants cultured on basal medium did not show any growth and eventually necrosed after 15 days as in *Gymnema elegans*[9], *Tylophora indica*[10] and *Cynanchum callialatum*[11]. Externally applied hormones for bud sprouting was necessary. MS medium containing BAP (2.0 mg/l) was more effective than 2-iP (2.0 mg/l) for inducing proliferation of axillary buds. In contrast 2-iP was more effective than BAP to induce shoot formation *in Decalepis hamiltonii*[12]. The shoots obtained were longer in MS medium which was supplemented with 2-iP (2.0 mg/l) when compared to supplementation with the growth hormone BAP (Table - 1). 2-iP was combined with BAP at different concentrations, maximum response with mean number of 2.88 shoots /explant and shoot length of 2.80 cm was observed when the combination of growth hormones used was BAP 2.0 mg/l + 2-ip 2.0 mg/l (Table 1).

Different combination of cytokinins in the culture media can increase the number of shoots formed as against using a single cytokinin (Prasad *et al.*[13]) To understand the effect of different growth hormones on the regeneration of shoots from nodal explants, various experiments were performed. Assuming that combined effect of cytokinins, auxins and GA_3 could improve further multiplication rate of shoots, a combined effect of different concentrations and combinations were studied (Table 2).

A maximum of 1.54 shoots / explant with 4.10 cm of maximum shoot length was observed when nodal explants of *Caralluma stalagmifera* cultured on medium

supplemented with BAP 2.0 mg/l+ GA_3 1.0 mg/l (Table 2) (Fig 1A). This is the maximum length of shoots observed out of all the concentrations of GA_3 with BAP 2.0 mg/l (Table 2).

Culture medium enriched with BAP 2.0 mg/l + 2-iP 2.0 mg/l + NAA 0.5 mg/l demonstrated the best result with 88 % of response after 20 days of culture. On this combination explants produced 8.47 shoots/ explant with an average shoot length of 2.50cm (Fig 1B). Decreased or increased concentration of NAA reduced the shoot number as well as length (Table 2). Where as combination with IAA produced 6.22 shoots/explant and 2.30cm of shoot length at 1.0 mg/l with 73 % of response (Table 2). Use of IBA in place of IAA was tested in *Caralluma stalagmifera*. BAP 2.0 mg/l + 2-iP 2.0 mg/l +IBA 0.5 mg/l produced maximum number of 6.10 shoots/explant with 1.55 cm of shoot length (Table 2). Addition of GA_3 to cytokinins and auxins combinations reduced shoot number in nodal explants of *Caralluma stalagmifera* (Table 2).

In our present study combination with cytokinins (BAP and 2-iP), NAA continued to give better results than IAA and IBA (Table 2). *Holostemma annulare*[14] showed similar results. Medicinal plants belonging to Asclepiadaceae such as *Gymnema elegans*[9] *Holostemma ada kodien*[15] and *Hemidesmus indicus*[16] have been used in experiments to understand the synergistic effect of cytokinins in combination with auxins. Tideman and Hawkar[17] have reported that during the propagation of latex producing plants, the presence of NAA resulted in suppression of shoot formation and increase in callus formation. In our present study best results were obtained from two cytokinins (BAP + 2-iP) and auxins (NAA, IAA and IBA) combinations. Malathy and Pai[18] used a combination of two cytokinins and auxins in MS medium for enhanced axillary bud proliferation.

Shoot formed in the medium containing BAP 2.0 mg/l + 2-iP 2.0 mg/l + IAA IBA in combination with different concentrations of GA_3 were used as explants to subculture them onto the medium enriched with BAP 2.0 mg/l + 2-iP 2.0 mg/l + NAA 1.0 mg/l. This resulted in significant multiplication of shoots though the concentration of plant growth hormones did not have any impact on the length of the shoots (Table 2). When these shoots which developed were subcultured in culture medium having GA_3 and BAP in the concentration of 1 mg/l and 2 mg/l respectively, there was optimal shoot elongation with 40 days (Fig 1C). Enhanced shoot multiplication in subsequent culture is in accordance with published literature on Asclepiadaceae medicinal plants like *Hemidesmus indicus*[16], *Holostemma ada-kodien*[15], *Asclepias erosa*[19] and *Ceropegia candelabrum*[20]. Patnaik and Debata[21] have described that repeated subculture of *Hemidesmus indicus* did not result in proliferation of shoots.

In order to enhance shoot multiplication different auxins were combined with the optimized cytokinin concentration. Experiments involving *Hemidesmus indicus*[21] and *Asclepias erosa*[19] have expounded the fact that auxins at low concentration could modify positively the shoot induction response when combined with cytokinins. The addition of GA_3 to culture media resulted in a response which was very much comparable to that as seen with auxins, thereby implying that GA_3 can be a replacement for auxins for shoot induction[22]. Hence, shoot propagation would be ideally accomplished with an optimized ratio of gibberelins and cytokinins. But in the present study the combination of GA_3 and cytokinin was generally less satisfactory for the induction of shoots than that of

auxins + cytokinins. However, GA_3 was preventive for shoot regeneration in sugar beet floral axillary buds[23].

Subculture of the shoot explants on to fresh medium was performed once in 4 weeks to provide an uninterrupted supply of shoots with no decrease in their morphogenetic potential for a longer period.

When shoot explants of 5.0 cm length were subcultured onto the rooting medium which contained half strength MS medium enriched with different concentrations of the 3 auxins: IBA, NAA and IAA, only NAA proved to show significant root induction potential compared to the other auxins (Table 3). Extensive callusing at the base with out root formation was noticed respectively when high concentration of NAA, IAA and IBA supplemented to the media in *Caralluma stalagmifera*. Root induction was possible with all auxins. However, strong root systems were obtained on NAA supplemented medium, with 0.5 mg/l concentrations exhibiting the best response (Table 3) (Fig 1D). At this concentration 73 % shoots developed roots with an average 8.42 roots/shoot. Though auxins are known to be the ideal plant hormones responsible for root induction, the extent of rooting induced considerably varies from one plant to the other[24]. The positive response of rooting in the present study is similar to observation of other members of Asclepiads *Decalepis arayalpathera*[25] and *Ceropegia candelabrum*[20].

Hardening process of the plantlets that were regenerated *in vitro* was initiated by first washing the plantlets with water a couple of times to clear all culture medium that was attached to the plantlets, followed by which the plantlets were potted in 10 cm pots having equal quantities of peat mass, sand and farmyard manure. These plantlets were incubated in the culture room for sometime. The potted plants were covered with polythene cover to ensure high humidity and irrigated every two days with ½ strength MS macro salts free of sucrose for 2-3 weeks. The plantlets were exposed to outside conditions gradually from the polythene covering, thus maintaining an optimal balance of relative humidity and thereby enhancing their rate of survival. After 15 days the hardened plantlets were transferred to the green house, where 75% of the plantlets survived (Fig 1E). There were absolutely no aberrations in the phenotype of the plants in the green house that were regenerated *in vitro*.

CONCLUSION

Thus, we can infer that the protocol which we have described in this research work can be used to micropropogate *Caralluma stalagmifera* successfully using the techniques of axillary bud multiplication that ensures the aggrandization of the endemic medicinal plant.

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Conflict of Interest

We declare no conflict of interest.

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Table 1: Effect of various concentrations of BAP and 2-iP on multiple shoot induction from mature nodal explants of *Caralluma stalagmifera* cultured on MS medium.

Plant growth regulators (mg/l)	Shoot sprouting frequency (%)	Shoot No. per explant Mean ± SE	Shoot length (cm) Mean ± SE
BAP			
0.1	60	$1.06 \pm 0.02a^{i}$	1.09 ± 0.02^{h}
1.0	75	1.55 ± 0.02^{e}	1.16 ± 0.02^{g}
2.0	80	2.54 ± 0.04^{ab}	1.80 ± 0.02^{b}
3.0	55	1.76 ± 0.03^{d}	1.44 ± 0.03^{e}
5.0	50	1.50 ± 0.02^{e}	1.24 ± 0.02^{f}
8.0	40	1.32 ± 0.03^{f}	1.07 ± 0.02
2-iP			
0.1	30	1.16 ± 0.04^{h}	1.08 ± 0.02
1.0	60	1.33 ± 0.04^{f}	1.47 ± 0.04^{e}
2.0	70	1.92 ± 0.02^{c}	2.21 ± 0.04^{ab}
3.0	50	1.58 ± 0.04^{e}	1.22 ± 0.03^{f}
5.0	45	1.31 ± 0.03^{f}	1.21 ± 0.03^{f}
8.0	40	1.18 ± 0.04^{d}	1.10 ± 0.02^{h}
BAP + 2-iP			
2.0 + 0.1	60	1.28 ± 0.02^{g}	1.40 ± 0.02^{e}
2.0 + 0.5	65	1.35 ± 0.01^{f}	1.51 ± 0.01^{d}
2.0 + 1.0	75	2.18 ± 0.01^{b}	1.68 ± 0.01^{c}
2.0 + 2.0	88	2.88 ± 0.01^{a}	2.80 ± 0.01^{a}

Values represent mean ± standard error of 15 replicates per treatment in three repeated experiments. Means followed by the same letter not significantly different by the Tukey test at 0.05% probability level.

Table 2: Effect of different combinations of BAP, 2-iP, NAA, IAA, IBA and GA_3 on shoot regeneration of mature nodal explants *Caralluma stalagmifera*

Plant growth regulators (mg/l)						Shoot sprouting frequency (%)	No. of shoots/ explant Mean ± SE				Shoot length (cm) Mean ± SE			
BAP	2-iP	NAA	IAA	IBA	GA_3									
2.0	-	-	-	-	0.5	60	1.26	±	0.02	e	2.64	±	0.03	b
2.0	-	-	-	-	1.0	75	1.54	±	0.02	d	4.10	±	0.02	a
2.0	-	-	-	-	2.0	58	1.30	±	0.03	de	2.27	±	0.03	b
2.0	2.0	0.1	-	-	-	65	5.18	±	0.02	b	1.78	±	0.02	bc
2.0	2.0	0.5	-	-	-	88	8.47	±	0.04	a	2.50	±	0.01	b
2.0	2.0	1.0	-	-	-	75	4.26	±	0.04	bc	1.68	±	0.02	c
2.0	2.0	0.5	-	-	1.0	70	2.34	±	0.05	c	3.07	±	0.02	ab
2.0	2.0	-	0.5	-	-	68	2.84	±	0.03	c	1.60	±	0.04	c
2.0	2.0	-	1.0	-	-	73	6.22	±	0.02	ab	2.30	±	0.03	b
2.0	2.0	-	-	0.1	-	65	2.62	±	0.04	cd	1.50	±	0.02	c
2.0	2.0	-	-	0.5	-	70	6.10	±	0.02	ab	1.55	±	0.02	c
2.0	2.0	-	1.0	-	1.0	71	1.79	±	0.03	d	2.60	±	0.05	b
2.0	2.0	-	-	0.5	1.0	70	1.60	±	0.03	d	2.46	±	0.05	b

Values represent Mean ± SE, of 15 replicates per treatment in three repeated experiments. Mean followed by the same letter are not significantly different by the Tukey test at 0.05% probability level.

Table 3: Effect of various auxins on rooting response from *in vitro* regenerated shoots of *Caralluma stalagmifera* cultured on MS half strength after 30 days

Concentration of Auxin mg/l			% of response	Number of Roots/shoot Mean ± SE				Length of roots (cm) Mean ± SE			
NAA	IAA	IBA									
0.10	-	-	65	4.47	±	0.05	b	2.13	±	0.02	b
0.50	-	-	73	8.42	±	0.04	a	3.59	±	0.02	a
1.00	-	-	60	2.51	±	0.01	c	1.83	±	0.01	bc
2.00	-	-	-	CP				CP			
3.00	-	-	-	CP				CP			
-	0.10	-	58	1.30	±	0.02	de	1.80	±	0.02	bc
-	0.50	-	60	2.41	±	0.02	c	1.70	±	0.02	c
-	1.00	-	68	3.36	±	0.03	bc	3.12	±	0.02	a
-	2.00	-	55	1.35	±	0.02	de	1.50	±	0.02	d
-	3.00	-	-	CP				CP			
-	-	0.10	45	1.28	±	0.03	e	1.45	±	0.02	d
-	-	5.00	60	1.39	±	0.02	de	1.70	±	0.02	c
-	-	1.00	65	3.21	±	0.02	bc	2.58	±	0.02	ab
-	-	2.00	50	1.45	±	0.02	d	1.76	±	0.02	c
-	-	3.00	-	CP				CP			

Values represent mean ± standard error of 15 replicates per treatment in three repeated experiments. Means followed by the same letter not significantly different by the Tukey test at 0.05% probability level. CP – Callus Production

Figure 1: A – E. High Frequency axillary bud multiplication of *Caralluma stalagmifera*

a. Maximum shoots elongation observed on MS + BAP 2.0 mg/l + GA_3 1.0 mg/l.
b. *In vitro* regeneration of nodal explants with multiple shoot development on MS medium supplemented with BAP 2.0 mg/l + 2-ip 2.0 mg/l + NAA 0.5 mg/l.
c. Enhanced shoot length observed after sub culture on the MS medium containing BAP 2.0 mg/l + GA_3 1.0 mg/l.
d. *In vitro* rooting if *in vitro* raised shoots on ½ strength MS medium fortified with NAA 0.5 mg/l after 30 days.
e. Acclimatization of *in vitro* grown plants of *Caralluma stalagmifera*.