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Micropropagation of *Caralluma stalagmifera* var. *longipetala:* A rare succulent medicinal plant from Karnataka, India

Ramachandra Naik M.[1], Subbiah Karuppusamy[2]* and Y. L. Krishnamurthy[1]

[1]Department of P. G. Studies and Research in Applied Botany, Kuvempu University, Shankaraghatta, Karnataka 577451, India.
[2]Department of Botany, Centre for Botanical Research, The Madura College (Autonomous), Madurai – 625 011, Tamilnadu, India.

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An efficient *in vitro* protocol has been developed for the multiplication of shoots and conservation of a rare succulent medicinal plant *Caralluma stalagmifera* var. *longipetala* growing wildly in Karnataka State. Proliferation of multiple shoots was achieved on Murashige and Skoog's (MS) medium supplemented with various concentrations of 6-benzyladenine (BA), Kinetin (Kn), indole acetic acid (IAA), α- naphthalene acetic acid (NAA) and indole-3-butyric acid (IBA) alone or with various combinations from the nodal explants. The nodal explants cultured on medium containing BAP (2.0 mg/L) along with 0.5 mg/L Kn and 0.3 mg/L IAA produced the highest number of shoot sprouting (2.60±0.16) and maximum shoot length (3.96±0.20). The considerable frequency of callus induction and embryogenesis was noticed both in 1.0 mg/L NAA and 0.5-2 mg/L, 2, 4-D. The calli transferred to shoot induction medium containing the combination of hormones BAP (1.0 mg/L) plus IAA (0.2 mg/L) and NAA (0.1 mg/L) successfully regenerated *in vitro* shootlets. The *in vitro* rooting was achieved from both direct shoots regenerated from nodal explants and callus derived shootlets with NAA (0.2 mg/L). The *in vitro* rooted plantlets were successfully acclimatized (75%) in the greenhouse and gradually transferred to open field conditions.

Key word: Micro propagation, medicinal plant, *Caralluma stalagmifera* var. *longipetala.*

INTRODUCTION

Caralluma stalagmifera var. *longipetala* Karupp. & Pullaiah was originally described from Tamilnadu state (Karuppusamy and Pullaiah, 2007) and later it was recognized that its distribution was extended in Karnataka State of Southern India. The plant is a xerophytic succulent leafless medicinal herb belonging to Apocynaceae. The succulent stem of the plant is used to cure many ailments and have noted antiobesity properties (Karuppusamy et al., 2013). The herb is a rich source of flavonoidal glycosides and alkaloids (Kunert

*Corresponding author. E-mail: ksamytaxonomy@gmail.com.

et al., 2009) with significant anti-inflammatory, anti-oxidant and antimicrobial properties (Madhuri et al., 2011). It is also reported to have significant anti-arthritic activity in kaolin induced rats (Reddy et al., 1996). The chemical principles isolated from *C. stalagmifera* are steroidal glycosides, stalagmosides, carumbellosides and lasiathosides (Kunert et al., 2009).

Natural population of this plant species are declining day by day because of increase demand in the pharmaceutical market coupled with over-exploitation and habitat destruction. There are no formulated agronomic or cultivation techniques for these endemic succulent medicinal species until now. For the conservation of these important medicinal plants, several other wild *Caralluma* species have already been developed via *in vitro* multiplication protocol by various authors (Aruna et al., 2012; Ugraiah et al., 2011; Sreelatha et al., 2009). So far there is only one report available on the micropropagation of *C. stalagmifera* from *in vitro* grown seedling explants (Sreelatha and Pullaiah, 2010). Micropropagation of other related *Caralluma* species include on *Caralluma sarkariae* (Sreelatha et al., 2009), *Caralluma bhupenderiana* (Ugraiah et al., 2011) and *Caralluma adscendens* var. *attenuata* (Aruna et al., 2012). The purpose of the study was to develop a rapid *in vitro* shoot multiplication and callus regeneration protocol from plant materials collected from natural populations growing in wild.

MATERIALS AND METHODS

Plant material, surface sterilization and inoculation

Succulent plants of *C. stalagmifera var. longipetala* were collected from Muddapura of Chitradurga District in Karnataka and plants were maintained in pots containing mother soil under polyhouse condition in the Kuvempu University campus, Shivamogga (Figure 1A and B). The tender shoot segments with six to eight inter nodes were collected from the potted plants and washed with running tap water for 15 min to remove the soil particles and other dust particles. The internodes were cut into small pieces and rinsed with 1% (v/v) Tween 20 (Merck, Bangalore, India) for 5 min. They were further rinsed in distilled water three times and taken into the laminar air flow chamber where they were rinsed with sterile double distilled water. The explants were immersed in 30% ethanol for 3 min and again washed with sterilized double distilled water. It was followed by Mercuric chloride ($HgCl_2$ 0.1% (v/v)) treatment for 2 min. After sterilization, the explants were thoroughly rinsed with several changes of sterile double distilled water. The explants were trimmed into pieces of about (0.6 mm to 10 mm) and then inoculated into culture media. Murashige and Skoog's media (HiMedia, Mumbai, India) media was fortified with 3% (w/v) sucrose and 0.8% of agar for solidification. The pH of media was adjusted to 5.8 prior to the addition of 0.8% agar and autoclaved.

Shoot initiation and multiplication

For the induction of shoots, nodal explants were cultured on MS medium amended with various plant growth regulators like 6-benzyladenine (BA), Kinetin (Kn), and α- naphthalene acetic acid (NAA) (HiMedia, Mumbai, India) at different concentrations (0.5, 1.0, 2.0, 3.0 and 5.0 mg/L). Cultures were subcultured on to the fresh medium with every 30 days period of intervals. The *in vitro* response was measured in the frequency of shoot multiplication, the number of shoots per explants and the shoot lengths at the end of six week old cultures.

Callus induction and multiplication

Internodal segments of *C. stalagmifera* var. *longipetala* were cultured on MS medium fortified with auxins like 2, 4-dichlorophenoxyacetic acid (2, 4 D) and α- naphthalene acetic acid (NAA) in different concentrations (0.5 to 2.0 mg/L) alone. Regenerative and embryogenic calli were transferred to fresh MS medium supplemented with different concentration of BAP (0.5 to 2.5 mg/L), IAA (0.2 mg/L) and NAA (0.1 and 0.3 mg/L) alone or in combinations for the regeneration of shoots.

Root initiation and multiplication

The regenerated *in vitro* shoots (4 to 5 cm height) were separated and callus induced shoots with 2 to 3 cm height were isolated and transferred for root induction on to half-strength MS medium containing different concentrations of NAA, IAA and IBA (Indole-3-butyric acid). The cultures were maintained under 16 h photoperiod for one month until the micro shoots initiated the roots. *In vitro* rooting response were measured with number of roots and mean length of roots (Ugraiah et al., 2011).

Acclimatization and transplantation of plantlets

In vitro rooted plantlets were removed from culture tubes with at least two roots of 2 to 4 cm length. They were washed carefully with tap water to remove traces of agar and then transferred to the pots containing different potting mixtures namely: cocopeat (HiMedia, Mumbai, India), cocopeat + sand + soil (1:2:1) and cocopeat + sand (1:1). The planted pots were covered with transparent polythene to maintain humidity until the development of new rudimentary leaves and sprouting new roots (Aruna et al., 2012). After a month they were removed and the plants were maintained in the lab temperature conditions for 15 more days. After two months of hardening, the plants were transferred to new pots containing humus soil, kept in polyhouse for one month. During the first 15 days of acclimatization neither watering nor any fertilizers was provided to plants. The hardened plants were planted on nursery bed with frequent watering in natural condition.

Statistical analysis

The experiments were randomized and repeated three times. Each treatment consisted of 15 replicates. Data were statistically analyzed by analysis of variance (ANOVA) and mean readings were compared by Tukey's test at 0.05% probability level.

RESULTS AND DISCUSSION

Effect of cytokinin on shoot regeneration

The success of micropropagation depends on the selection of suitable explants, media composition, types of growth regulators, their concentrations and combinations

Figure 1. *In vitro* propagation of *C. stalagmifera* var. *longipetala*. **A)** Inflorescence of *C. stalagmifera* var. *longipetala*. **B)** Shoot induction from MS medium containing BA 2.0 mg/L. **C)** Rooting of regenerated shoots containing 0.2 mg/L NAA. **D)** Rootlet plant. **E)** Callus induction from medium containing 1.5 mg/l 2,4-D. **F)** Callus to shoot induction. **G)** Multiple shoots Containing BA 0.5 mg/L Kn+ 0.3 mg/l IAA. **H)** Shoot and callus induction from medium containing 0.5 Kn and NAA 0.5 mg/L. **I)** Coco peat planted pots. **J)** Polythin covered pots. **K)** Plants growing in forest soil under playhouse.

with culture conditions. The effect of cytokinins and its concentration on bud breaking from nodal explants cultured on MS basal medium is given in Table 1. The MS basal medium fortified with BAP 2.0 mg/L was found to have the best shoot sprouting, number and length of shoots without basal callus formation from nodal explants. The shoot buds sprouted on Kn containing medium showed only limited growth even if they were maintained for longer period of subculture. MS medium is the most efficient medium for shoot proliferation of *Caralluma* spp. such as *Caralluma adscendens*, *Caralluma bhubenderiana* (Ugraiah et al., 2011) and *Caralluma lasiantha* (Aruna et al., 2012). Nodal explants selected for shoot proliferation gave positive

Table 1. Effect of various concentrations of BAP and Kn on shoot formation in mature nodal explants of *C. stalagmifera* var. *longipetala* cultured on MS medium with sucrose (3%)

Hormone	Concentration (mg/L)	Shoot sprouting frequency (%)	Mean shoot number per explants ± SE	Mean length of shoots (cm) ± SE
BAP	0.5	32.5	1.35±0.09[b]	1.68±0.15[bc]
	1.0	40	1.40±0.11[b]	2.25±0.15[b]
	1.5	57.5	1.75±0.12[b]	2.56±0.18[b]
	2.0	80	2.25±0.17[a]	3.11±0.20[a]
	2.5	65	2.05±0.15[a]	2.77±0.22[b]
Kn	1.0	12	1.20±0.09[c]	1.42±0.10[c]
	1.5	32.5	1.45±0.11[b]	2.02±0.11[b]
	2.0	40.5	1.40±0.12[b]	2.50±0.12[b]
	2.5	55	1.80±0.13[b]	2.70±0.23[b]
	3.0	47.5	1.70±0.12[b]	2.62±0.15[b]

Means ± SE, n = 30. Means followed by the same letter in a column are not significantly different by the Tukey's test at 0.05% probability level.

morphogenic response with 80% of bud break and emergence of bud within a week on MS medium supplemented with BAP 2.0 mg/L and with 3% of sucrose; the highest number of shoots (2.25 shoots/explants) with mean length (3.11 cm), whereas from the Kn containing medium nodal explants obtained 1.80 shoots/explants with mean length of 2.7 cm in Kn 3.0 mg/L fortified cultures. There are several examples in literature showing the nodal explants are most effective in succulent Asclepiads (Sreelatha et al., 2009). The response of nodal explants treatment results are presented in Table 1. Out of these treatments, MS medium fortified with BAP 2.0 mg/L had a better shoot sprouting frequency (80). High concentration of BAP above 3.0 mg/L resulted in reduced shoot sprouting and frequency of response (Personal communication). Kn containing cultures produced less than 2 shoots/explants (Table 1).

Effect of cytokinins with auxin on shoot multiplication

C. stalagmifera var. *longipetala* nodal explants were cultured on MS medium supplemented with various concentrations of cytokinins (BAP 1.5 to 2 mg/L, Kn 0.2 to 1.5 mg/L) and auxins (NAA 0.2 to 1 mg/L, IAA 0.3 to 0.6 mg/L) (Table 2). The effect of BAP on multiple shoots proliferation has been demonstrated in Asclepiads (Aruna et al., 2009, 2012). Combination of 1.5 mg/L BAP + 0.6 mg/L Kn produced highest number of shoot sprouting frequency of about 76%, the mean shoot/explants was 2.05±0.17 and mean shoot length was 3.07±0.17 cm. Similarly the combination of BAP 1.5 mg/L +NAA 0.3 mg/L produced sprouting frequency of about 53.3% (Table 2). Mean shoot number per explants was about 1.40±0.11 and the mean shoot length is 2.12±0.14 cm. Basal callus formed in all the shoots were healthy (Figure 1B).

The combination of BAP (2.0 mg/L) + Kn (0.5 mg/L) + IAA (0.3 mg/L) yielded maximum shoot regeneration frequency of 90%. Number of shoots per explant was 2.60±0.16 and 3.96±0.20 cm. The mean shoot length followed by other combination of hormones such as BAP (2.0 mg/L) + Kn (1.0 Mg/L) + IAA (0.3 mg/L) showed regeneration frequency of about 86% and the number of shoots per explants was 2.10±0.18 and 3.32±0.17. The mean shoot length, of other combination of cytokinins BAP with auxins showed that the medium resulted in moderate callus formation (Table 2). The combination of BAP (2 mg/L) + NAA (0.5 mg/L) showed lower frequency of shoot sprouts (53.3%) with basal callus (Figure 1G).

Callus induction and somatic embryogenesis

The intermodal explants cultured on various concentration of 2,4-D (0.5, 1.0, 1.5, 2.0) and NAA (0.5, 1.0, 1.5, 2.0) become swollen and generally dedifferentiated and developed friable callus after two weeks of culture. Among the different auxins tested 2,4-D at 1.5 mg/L favored the best response of callus production (90%) (Table 3). NAA supplemented cultures showed good callus production but there was no further shoot regeneration or somatic embryogenesis when other auxins in different combinations were added (Table 3). The calli subcultured onto media containing a combination of 2,4-D (1.5 mg/L) with BAP (1.0 mg/L), IAA (0.2 mg/L) and NAA (0.1 mg/L) produced maximum number of globular embryos on the surface within four weeks (Figure 1E). In *Asclepias,* studies demonstrated the need of 2, 4-D (2 mg/L) and BA (0.1 mg/L) for callus induction (Vyapari et al., 1993). Other Asclepiad members like *Tylophora indica* and *Hemidesmus indicus* produced

Table 2. Effect of different combination of BAP, Kn, NAA, and IAA on shoot regeneration of mature nodal explant of *C. stalagmifera* var. *longipetala*

BAP	Kn	NAA	IAA	Shoot sprouting frequency (%)	Mean shoot number per explants ± SE	Mean length of shoots (cm) ± SE	Formation Basal Callus (CP)
1.5	0.2			66.6	1.75±0.13^{b}	2.80±0.15^{b}	-
1.5	0.4			73.3	2.15±0.13^{a}	2.98±0.20^{b}	-
1.5	0.6			76	2.05±0.17^{a}	3.07±0.17^{a}	-
1.5	0.8			70	1.95±0.15^{bc}	2.82±0.15^{b}	-
1.5		0.3		53.3	1.40±0.11^{c}	2.12±0.14^{b}	+
1.5		0.5		50	1.55±0.11^{c}	2.34±0.13^{b}	+
1.5		1.0		43.3	1.40±0.11^{c}	1.94±0.09^{bc}	++
	0.5	0.5		40	1.45±0.11^{c}	1.39±0.10^{c}	++
	0.3	0.5		46.6	1.50±0.11^{c}	2.52±0.13^{b}	++
	0.5	1.0		36.6	1.35±0.13	1.40±0.10^{c}	++
2.0	1.0			83.3	2.05±0.17^{a}	3.22±0.24^{a}	-
2.0	1.5			73.3	2.05±0.16^{a}	3.11±0.09^{a}	-
2.0		0.2		70	1.85±0.11^{b}	2.84±0.13^{b}	-
2.0		0.4		66.6	1.80±0.12^{b}	2.72±0.19^{b}	+
2.0		0.5		53.3	1.50±0.10^{c}	2.45±0.17^{b}	++
2.0	0.5	0.5		70	1.85±0.13^{b}	3.01±0.11^{a}	+
2.0	1.0	0.5		66.6	1.60±0.10	2.88±0.14^{b}	+
2.0	0.5		0.3	90	2.60±0.16^{a}	3.96±0.20^{a}	-
2.0	1.0		0.3	86	2.10±0.18^{a}	3.32±0.17^{a}	-
2.0	0.5		0.6	70	1.85±0.12^{b}	2.87±0.17^{b}	-

Means ± SE, n = 30. Means followed by the same letter in a column are not significantly different by the Tukey's test at 0.05% probability level; NR – no response.

Table 3. Effect of different combination of 2.4.D, BAP, NAA, and IAA on callus induction of mature thin explant of *C. stalagmifera* var. *longipetala*

Growth regulator (mg/L)		Callus frequency (%)	Basal callusing	Callus nature	Embryos treatment with BAP+IAA+NAA (mg/l) (subculture)	Number of shoot sprouting (%)
2.4.D	0.5	75	++	Crystal white	0.5.0+0.2+0.1	2.53±0.17
	1.0	85	+++	Greenish white	1.0+0.2+0.1	3.47±0.24
	1.5	90	+++	Greenish white	1.5+0.2+0.1	2.67±0.22
	2.0	80	++	white	2.0+0.2+0.1	CP
NAA	0.5	60	+	white	1.0+0.2+0.3	CP
	1.0	70	++	Crystal white	1.5+0.2+0.3	CP
	1.5	55	+	Crystal white	2.0+0.2+0.3	CP
	2.0	35	+	Cream white	2.5+0.2+0.3	CP

CP-Callus production.

callus on 2, 4-D and BAP (Thomas and Philip, 2005; Sarasan et al., 1994). The somatic embryos were induced within two weeks of subculture with media containing NAA and 2, 4-D at different concentrations (Stephan and Jayabalan, 2001; Inamdar et al., 1990).

Rooting of *in vitro* regenerated shoots

In *C. stalagmifera* var. *longipetala*, half strength MS medium supplemented with auxins such as IAA, IBA and NAA at different concentrations showed varied effect on rooting (Table 4). The experiment was aimed at the induction of rooting in basal portion of *C. stalagmifera*var. *longipetala* microshoots; NAA was found more effective than the IAA and IBA hormones. The NAA showed positive response of rooting in the present study, which is similar to the observations on other Asclepiads such as *Decalepis arayalpathra* (Sudha et al., 2005) and *Euphorbia tirucalli*

Table 4. Rooting response of *in vitro* regenerated shoots of *C. stalagmifera*var. *longipetala*in half strength MS media containing NAA, IAA and IBA in various concentrations with sucrose (1%) after one month.

NAA	IAA	IBA	Percentage of root response	Mean number of roots per shoot ± SE	Mean length of roots (cm) ± SE	Degree callusing (CP)
0.1			80	3.75±0.35^{c}	4.17±0.29^{b}	
0.2			90	6.10±0.56^{a}	5.90±0.47^{a}	
0.3			85	4.80±0.50^{ab}	4.40±0.40^{b}	
0.4			50	2.60±0.36^{d}	2.82±0.24^{d}	++
0.5			CP	-	-	+++
	0.2		75	3.80±0.25^{c}	3.82±0.38^{cd}	
	0.3		80	4.05±0.34^{ab}	3.25±0.33^{d}	
	0.4		60	2.80±0.19^{d}	2.50±0.22^{d}	
	0.5		40	2.10±0.22^{d}	1.28±0.13^{d}	+
	1.0		CP	-	-	+++
		0.2	65	3.05±0.33^{cd}	2.48±0.19^{d}	
		0.4	55	2.75±0.37^{d}	1.75±0.13^{d}	
		0.6	35	2.10±0.18^{d}	0.99±0.10^{e}	+
		0.8	CP	-	-	++
		1.0	CP	-	-	+++

(Uchida et al., 2004). The concentrations of 0.2 mg/L NAA induced on an average 6.10±0.56 root/shoot respectively and 5.90±0.47 root lengths (Table 4, Figure 1D). Other concentrations like NAA at 0.1 0.3 and 0.4, mg/L gradually decreased root numbers and root length and also NAA 0.5 mg/L induced the basal callus.

0.3 mg/L of IAA induced 4.05±0.34 root / shoot (80%) and 3.25± 0.33 root lengths followed by other growth hormones; IAA (0.2, 0.4, and 0.5 mg/L). The concentration of 0.2 mg/L IBA induced 3.05±0.33 root / shoot (65%) and 2.48±0.19 root lengths respectively (Table 4). Effective response of IBA in rooting has been reported for medicinal plants like *Ceropegia bulbosa* (Britto et al., 2003,) *Ceropegia candelabrum* (Beena et al., 2003) and *Decalepis hamiltonii* (Giridhar et al., 2005). The plantlets resumed good growth after four weeks of transplantation and acclimatization processes, out of 80% of plants transferred, 75% survived after three weeks of transfer under the green house condition.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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