Australian Journal of Crop Science AJCS
AJCS 6(1):41-45 (2012) ISSN:1835-2707

Research Note

In vitro regeneration of *Caralluma fimbriata* Wall. by organogenesis: a potent medicinal plant

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Abstract

In vitro regeneration of *Caralluma fimbriata*, an endemic Indian medicinal plant through organogenesis was achieved. The callus was initiated on the cut surface of inter nodal segments in the MS medium supplement with 2, 4 D and NAA (0.5and 1.0 mg/l). Then the callus was transferred into MS medium supplemented with BAP (1.0, 2.0 and 4.0 mg/l) along with auxins (2, 4 D and NAA (0.5 and 1.0 mg/l)). The green compact organogenic calli were observed on MS medium supplemented with 2, 4-D (0.5 mg/l) + (BAP 4.0 mg/l) produced 6.2 shoots/explant. The juvenile shoots were elongated in the MS medium supplemented with GA_3 (0.1mg/l) of an average shoot length of 6.55cm was observed. These *in vitro* raised shoots were rooted (6.0 roots/shoot) in the half strength MS medium supplemented with NAA (0.5mg/l). The rooted shoots were successfully transferred to a cup containing vermi-compost and garden soil (1:1) and acclimatized in plant growth chamber. The regenerated plantlets showed 82% of survival rate in the green house.

Keywords: Callus, *Caralluma fimbriata*, Regeneration, Plant Growth Regulators, Indirect Organogenesis.
Abbreviations: 2, 4-D_2, 4-dichlorophenoxyacetic acid; BAP_6-benzylaminopurine; GA_3_Gibberellic acid; IBA_Indole-3-Butyric acid; KIN_Kinetin; MS_Murashige and Skoog; NAA_1-Naphthaleneacetic acid

Introduction

The genus *Caralluma* (Asclipiadaceae) comprises about 200 genera and 2500 species. The member of this genus is a small erect fleshy plant which has four grooved round shaped stems devoid of leaves with small flowers of several dark colours. The species of *Caralluma* found in India are edible and forms a part of the traditional medicine system of the country (Al-Yaha et al., 2000). A total of 13 species and 7 varieties of *Caralluma* occur in India out of which 11 species are solely endemic to South India (Jagtap and Singh, 1999). *Caralluma* spp is commonly used in treatment of rheumatism, diabetes, leprosy, antipyretic and anthelmintic, tumor, fungal diseases, snake-scorpion bite, appetite and also antinociceptive activity (Ramesh et al., 1998; Badar et al., 2003; Venkatesh et al., 2003; Jayakar et al., 2004; Abdel-Sattar et al., 2007; Rebecca et al., 2007; Tatiya et al., 2010; Saivasanthi et al., 2011). *C. fimbriata* was reported as endemic and endangered medicinal plant of South India (Rajeswara et al., 2008), due to the large scale and indiscriminate collection which lead to the dwindling of this plant population. Hence *in vitro* culture methods are useful for conserving valuable, rare and endangered medicinal plants (Nalawade et al., 2003; Thomas and Shankar, 2009; Rahman et al., 2009; Thankappan and Patell, 2011). There was no report available to conserve this important endemic medicinal plant (*C. fimbriata)* till now. So the present study is undertaken to *in vitro* regenerate via callus mediated shoot organogenesis for its conservation.

Results and discussion

The internodal segments of *C.fimbriata* were collected from the actively growing part of lateral branching. The surface sterilized internodal segments (approximately 1.0-2.0 cm) were cultured on MS medium supplemented with different concentrations (0.5 and 1.0 mg/l) of NAA and 2, 4-D. On the whole, the explants type, its orientation in the culture medium and PGRs play a key role in regulating the differentiation process (Chawla, 2000). Selection of suitable explants at correct development stage plays a key role in the successful

Table 1. The effects of various concentrations of 2,4-D and NAA on callus induction in *C.fimbriata*

PGR (mg/l)		Percentage of regeneration (%)
2,4-D	NAA	
0.5	-	87.32±0.23[c]
1.0	-	98.21±0.32[a]
-	0.5	81.45±0.12[d]
-	1.0	92.23±0.25[b]

Values within each column were represented as means ± standard error. Means sharing the same letter are not significantly different (P = 0.05) using Duncan's multiple range test.

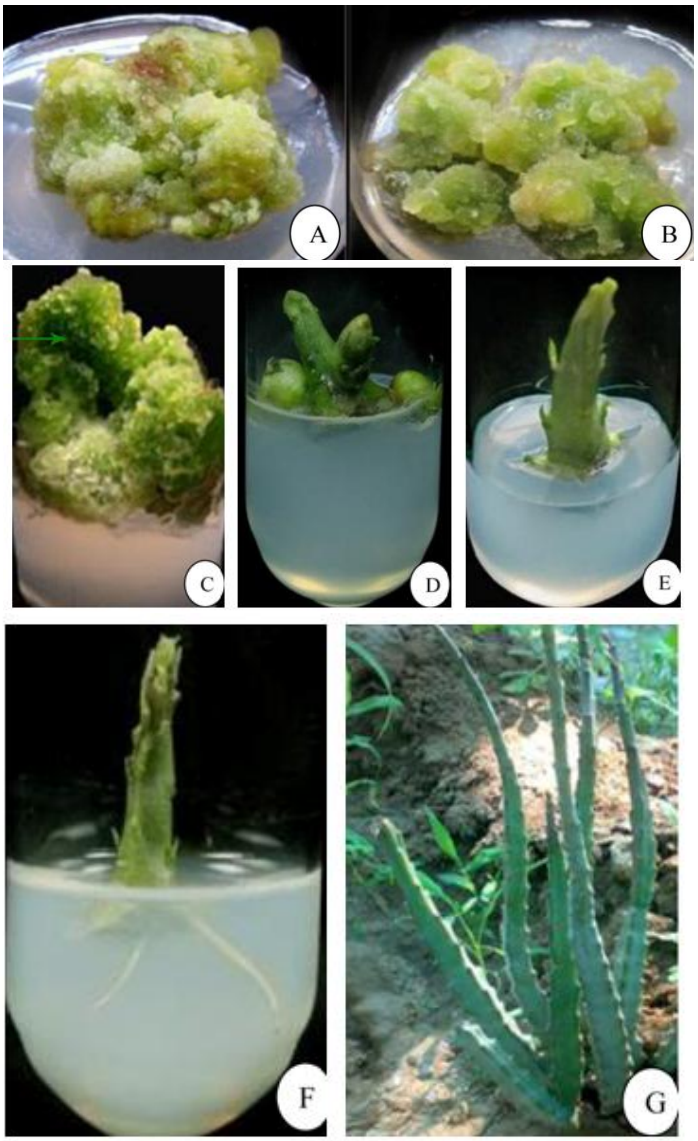

Fig 1. Induction of callus, shoot regeneration and complete plant from internodal segments of *C. fimbriata* (A) and (B): Pale green callus formation in the NAA and 2,4-D; (C) Organogenic callus formation along with small shoot nodules (Arrow indicates shoot primodia) in MS+ 2, 4-D 0.5 mg/l + BAP 4.0 mg/l; (D) Multiple shoots formation from organogenic callus; (E) Shoot elongation by GA_3 (0.1mg/l); (F) Root induction by NAA (0.5mg/l); (G) Acclimization in garden.

establishment of culture under *in vitro* conditions. Morphological integrity of explants along with the proper choice of plant growth regulators strongly influence induction of optimal callus and shoot regeneration (Khawar et al., 2005). The internodal segments bulged three to four times after the inoculation in 5-7 days. The pale green calli were obtained from the cut surface of the intermodal segments in both NAA and 2, 4-D concentrations (0.5 and 1.0 mg/l) at the 20^{th} day of inoculation (Table 1 and Fig. 1A, 1B) and later transferred into the medium containing BAP (1.0, 2.0 and 4.0 mg/l) in combination with different concentrations (0.5 and 1.0mg/l) of NAA and 2, 4-D for organogenic callus. Similarly there were many reports available to substantiate the auxin-cytokinin combination for the induction of organogenic callus in various systems like *Astragalus adsurgens* (Luo et al., 1999), *Lavandula* (Dronne et al., 1999), Eucalyptus (Bandyopadhyay et al., 1999), *Valeriana edulis* (Castillo et al., 2000), *Lathyrus sativus* (Zambre et al., 2002), Diffenbachia (Shen et al., 2007). The occurrence of green organogenic callus is considered as a prerequisite for organogenic regeneration (Ezura et al., 2000; Zambre et al., 2002). In the present study the organogenic calli (green compact nodular) were obtained significantly, after the second and third subculture (15 days duration) in the medium containing MS+ 2, 4-D 0.5 mg/l + BAP 4.0 mg/l (Fig.1C). The subsequent subculture induced a maximum of 6.2 shoots/explants at the same medium within seven weeks (Fig. 1D and Table 2).

The individual use of cytokinins was not found to give optimum results in most of the systems; the best result was obtained when KN or BAP was employed in combination with an auxin. Multiple shoot induction using an auxin-cytokinin combination is a common protocol, as it has been reported for several organogenesis systems (Nikam and Savant, 2000; Sudha et al., 2000; Martin, 2002; Beena, 2003; Park et al., 2011). The synergistic effects of BAP and NAA were reported in *Morus indica*, where a combination of these plant growth regulators induced axillary bud sprouting at a higher frequency than when BAP alone was used (Chitra and Padmaja, 1999). In the present study, higher concentration of cytokinin (BAP) and low concentration of auxin (2, 4-D) induced organogenic calli from the explants (inter nodal segments). Similar results were observed in Pelargonium (Saxena et al., 2000) and *Sarcostemma brevistigma* (Thomas and Shankar, 2009). In case of shoots which inoculated on the MS medium supplemented with 0.1 to 1.0 mg/l GA_3, the maximum shoot elongation (97.6 %) was obtained at the concentration 0.1mg/l GA_3 (6.55 cm) (Fig.1E and Table 3). Moreover, in the rooting medium which was MS supplemented with different concentrations (0.5 and 5.0 mg/l) of auxins (NAA and IBA). The maximum response (72.3 %) and root length (3.0 cm) of the elongated shoots were achieved in MS+ 0.5 mg/l NAA (Table 4). In this medium, the roots were shorter, thicker and brown coloured (Fig. 1F). Acclimatized plantlets were healthy and well developed when transferred to soil. The plants were grown as high as 60 cm with 82% survival rate and no morphological difference from the parent plants were observed after eight months (Fig.1G).

Materials and methods

Plant material

The plants were collected from Narthamalai hills, Pudukkottai Dt, Tamil Nadu, India and authenticated by Botanical Survey of India, Coimbatore(Specimens No. BSI/ SRC/ 5/ 23/ 09-10/ Tech-1569). The plants were maintained under natural day light and watered at the green house of medicinal garden at Anna University of Technology, Tiruchirappalli, Tamil Nadu. After the 4^{th} week young shoots were observed in the potted plants. The actively growing internodal parts were used as the explants sources for organogenesis.

Table 2. The effects of various concentrations of 2, 4-D, NAA and BAP on shooting percentage, mean number and length of shoots per internodal explant of *C.fimbriata* after 14 weeks of culture

PGR (mg/l)			Regeneration (%)	No.of Shoots/explants	Mean length of shoots/explants (cm)
BAP	2,4-D	NAA			
1	-	-	21.30±0.50^{ih}	1.40±0.30^{fe}	2.40±0.12^{e}
2	-	-	32.66±0.81^{f}	1.90±0.05^{d}	3.40±0.23^{c}
4	-	-	11.33±0.52^{l}	1.33±0.20^{gf}	1.50±0.21^{h}
1	0.5	-	44.66±0.75^{d}	2.50±0.50^{c}	3.23±0.15^{d}
2	0.5	-	54.66±0.52^{b}	3.50±0.50^{b}	4.20±0.12^{b}
4	0.5	-	76.33±0.81^{a}	6.20±0.10^{a}	6.50±0.10^{a}
1	1	-	23.03±0.56^{h}	1.30±0.40^{gf}	1.80±0.23^{fg}
2	1	-	32.33±0.24^{fe}	1.60±0.30^{fe}	1.60±0.34^{g}
4	1	-	28.33±0.43^{g}	1.57±0.20^{fe}	0.80±0.35^{jk}
1	-	0.5	37.66±0.51^{e}	1.24±0.31^{gf}	1.81±0.10^{f}
2	-	0.5	47.03±0.20^{c}	1.87±0.41^{e}	1.50±0.16^{h}
4	-	0.5	21.30±0.10^{ih}	1.63±0.32^{f}	0.81±0.32^{j}
1	-	1	17.33±0.52^{k}	0.81±0.19^{g}	0.50±0.14^{l}
2	-	1	21.33±0.17^{i}	1.39±0.28^{fg}	0.89±0.20^{i}
4	-	1	200.4±0.23^{j}	1.21±0.34^{gf}	0.56±0.31^{k}

Values within each column were represented as means ± standard error. Means sharing the same letter are not significantly different (P = 0.05) using Duncan's multiple range test.

Table 3. The effects of various concentrations of GA_3 on regeneration percentage and length of shoot per explant of *C.fimbriata* after 3 weeks of culture

GA_3 (mg/l)	Percentage of Regeneration	length of shoot (cm)
0.1	97.6±0.50^{a}	6.55±0.62^{a}
0.5	81.3±0.57^{b}	5.03±0.15^{b}
1.0	78.3±0.56^{c}	4.43±0.12^{c}

Values within each column were represented as means ± standard error. Means sharing the same letter are not significantly different (P = 0.05) using Duncan's multiple range test.

Table 4. The effects of various concentrations of auxins on rooting percentage, mean number and length of roots per shoot explant of *C.fimbriata* after 3 weeks of culture

PGR	(mg/l)	Rooting (%)	No. of roots/Shoot	Mean length of roots/shoot (cm)
NAA	IBA			
0.5	-	72.32±0.23^{a}	6.0±0.41^{a}	3.0±0.27^{a}
5.0	-	35.23±0.32^{c}	2.0±0.32^{c}	2.3±0.11^{b}
-	0.5	50.45±0.12^{b}	2.4±0.45^{b}	1.3±0.36^{d}
-	5.0	33.23±0.25^{d}	2.3±0.21^{bc}	1.5±0.12^{c}

Values within each column were represented as means ± standard error. Means sharing the same letter are not significantly different (P = 0.05) using Duncan's multiple range test.

Culture conditions

MS salts, vitamins (Murashige and Skoog, 1962), sucrose 3% (g/l) and agar 0.8% (g/l) were used in the experiments. The pH of the medium (MS salts, vitamins, sucrose and hormones) were adjusted to 5.7±0.1 before the addition of agar and autoclaved at 121°C for 15 min. All the culture was incubated at 24±1°C under 16/8 h photoperiod with an irradiance of 75 µmol/ m^{-2} s^{-1} by cool fluorescent lights.

Callus induction

The young explants were washed with running tap water for 5-10 min to remove soil particulars and other extraneous fine particles and were then washed thoroughly with Tween 20 in running tap water for 15 min. Followed by five rinses with distilled water. The explants were treated with 0.1% (w/v) Bavistin antifungal agent for 10 min, followed by five rinses of distilled water and then treated with 0.5 % (v/v) sodium hypochloride for 15 min, followed by five rinses with sterile distilled water and then the explants were dipped in 0.1 % $HgCl_2$ (w/v) for 5 min. After surface sterilization, the explants were thoroughly rinsed with sterile distilled water. Furthermore, the explants were trimmed into pieces (approximately 1-2 cm) and then inoculated into MS supplemented with various concentrations (0.5 and 1.0 mg/l) of 2, 4-D and NAA.

Regeneration

The calli obtained from the concentrations (0.5 and 1.0 mg/l) of 2, 4-D and NAA were transferred into the respective concentrations combined with BAP (1.0, 2.0 and 4.0 mg/l). In another set of an experiment, the internodal segments were inoculated in the MS+BAP (1.0, 2.0 and 4.0 mg/l). The percentage of shoot formation and the mean number of shoots

per explants were recorded in both BAP and its combination with auxins after 14 weeks. The clump of shoots obtained from the organogenic calli were separated and divided into single shoots and then inoculated in MS medium supplemented with GA_3 (0.1, 0.5 and 1.0 mg/l). The elongated shoots were transferred into the MS medium supplemented with various concentrations (0.5 and 5.0 mg/l) of NAA and IBA. The rooting percentage, the mean number and length of roots were recorded after 3 weeks. Rooted plantlets were carefully removed from the culture tubes, washed with water and transferred to cup filled with soil (vermi-compost and garden soil (1:1 by volume)) and hardened in a plant growth chamber (Narang Scientific Pvt. Ltd., New Delhi, India). The plants were finally transferred into greenhouse and observed its morphological characteristics for 8 months.

Statistical analysis

Each treatment had three replicates containing 50 explants and all the experiments were repeated thrice. The data on various parameters were subjected to one-way Analysis of Variance (ANOVA) using SPSS version 16.0 (SPSS Inc., Chicago, USA). The significance of differences among means was carried out using Duncan's multiple range test at P = 0.05 (Gomez and Gomez, 1976).

Conclusion

The present study demonstrates an organogenic propagation route via adventitious shoot development in *C.fimbriata*, a potent endemic medicinal plant. This technique will be useful in the conservation of the potent endemic medicinal plant.

Acknowledgements

The authors thank the DST SERC, New Delhi, India for providing financial assistants.

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