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RESEARCH ARTICLE

In vitro callus induction and plant regeneration from internodal explants of *Caralluma stalagmifera* Fischer

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Abstract

An in vitro regeneration protocol through internodal callus culture has been standardized for the medicinal herb *Caralluma stalagmifera* Fischer. The explant induced callus on MS medium supplemented with various concentrations of growth regulators like 2,4-D, 2,4,5-T, 2,4,5-TP, Picloram, Dicamba, NAA, IAA, IBA, BAP, KN, 2-iP and Zeatin. The optimized callus induction occurred at the concentration of 2.0 mg/l 2,4-D. After initiation of callus, it was immediately transferred onto the regeneration medium for the plantlet regeneration. Shoots were regenerated from the surface of the callus on MS medium supplemented with BAP 2.0 mg/l + NAA 0.5 mg/l and regeneration of plantlets with 60% of frequency with 3.15 shoots/ explants were noted. The microshoots rooted well on ½ MS medium supplemented with NAA 0.5 mg/l. Regenerated shoots formed complete plantlets on medium containing 0.5 mg/l NAA and mature plants were established, acclimatized and thrived in green house conditions and 70% of the field capacity was observed. This protocol is suitable to produce number of plants from internodal explants of *Caralluma stalagmifera*.

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INTRODUCTION

Plants are a valuable source of a vast array of chemical compounds. Plants in addition to their aesthetic value constitute the major natural source of the food we eat, the air we breathe and the medicine to cure our many ills. Recently, the utilization of medicinal plants as a natural source of drugs is being increasingly encouraged (Di-Cosmo, 1990; Phillipson, 1990). Consequently, medicinal plants have been targeted for uncontrolled collection and destruction as a result of urbanization, overgrazing, pollution and expansion of cultivated areas (Namdeo 2007). Due to depletion of habitat and ruthless collection, medicinal plants are on the verge of extinction. Hence, the conservation of these valuable genotypes is imperative. Plant tissue culture technology holds great promise for micropropagation, conservation, and enhancement of the natural levels of valuable secondary plant products and to meet pharmaceutical demands and reduce the in situ harvesting of natural forest resources. For mass propagation of medicinal plant species in which conventional methods possess limitations, in vitro multiplication provides the way out.

Caralluma stalagmifera Fischer (Asclepiadaceae) is a succulent leafless herb growing in Gooty hills, Andhra Pradesh, Southern India. The stem of this herb is edible among the local people. The genus of *Caralluma* possesses of flavonoid glycosides (Meve and Heneidak, 2005) which are main phytochemical principle isolated from many *Caralluma* species in Southern India (Ramesh et al., 1999). *Caralluma* species are considered as a potential weight loss medicine apart from their antimicrobial, anti-nociceptive and anti-inflammatory activities (Zakaria et al., 2001). Due to its pharmaceutical demand, the species is threatened in its natural habitats.

Caralluma stalagmifera is endemic to south India. New steroidal glycosides, stalagmosides I – V (1-5) and indicosides I and II (7 and 8), together with the known compounds carumbelloside III, lasianthoside A, and

lasianthoside B, were isolated from whole plant of *Caralluma stalagmifera* (Olaf Kunert et al., 2006). The aqueous and butanol extracts of whole plant was tested on carrageenin induced rat paw- oedema and kaolin induced arthritis in rats. Both the extracts have shown significant anti-inflammatory and antiarthritic activities (Reddy et al., 1996).

As the harvest of medicinal plants on a mass scale from their natural habitats for extraction of bioactive compounds for commercial use is leading to a depletion of plant resources, the conservation of these valuable genotypes is imperative. In recent years, there has been increased interest in in vitro culture techniques, which offer a viable tool for mass multiplication and germplasm conservation of rare, endangered, and threatened medicinal plants (Li et al., 2004 and Emma et al., 2005). Further, genetic improvement is another approach to augment the drug yielding capacity of plants (Tejavathi et al., 1999). A literature survey indicated that the in vitro protocol for internodal callus culture of this succulent herb was not yet standardized. In view of its medicinal importance and the lack of tissue culture reports, the present study reports the prime protocol for regeneration from internodal callus culture of *Caralluma stalagmifera.*

Materials and Methods

Plant material and explant source

Plants of *Caralluma stalagmifera* were collected from Gooty hills, Andhra Pradesh, India. These plants were collected along with roots, potted in pots and maintained at Botanical garden, Sri Krishnadevaraya University, Anantapur. Actively growing shoots (3-4 cm long) were used as an explant source for further work. The young shoots were thoroughly washed under running tap water for 5-10 min. to remove soil particles and then rinsed in a solution containing the surfactant, 1% Tween – 20 for 5 min. with constant agitation, then they were washed with sterilized double distilled water. Remaining steps of surface sterilization was carried out under aseptic conditions in laminar air flow chamber. Then the explants were immersed in 70% ethanol for 1 min. Later the explants were surface sterilized with 0.1% $HgCl_2$ solution for 5 min. and the explants were rinsed with several changes of sterilized double distilled water. Then the surface strerilized explants (internodes) were aseptically cut into appropriate sizes and blotted on a sterile filter paper disk and were carefully inoculated onto the culture media.

Culture media and culture conditions

The culture media consisted of MS salts (Murashige and Skoog, 1962) augmented with 3% (w/v) sucrose and gelled with 0.8% (w/v) agar (Hi-Media, India) and various auxins, 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4,5-Trichloro Phenoxy Acetic Acid (2,4,5-T), 2,4,5-Trichloro Phenoxy Picolinic Acid (2,4,5-TP), 4-Amino, 3,5,6-Trichloro Picolinic Acid (Picloram), 3,6-Dichloroanisic Acid (Dicamba), Naphthalene acetic acid (NAA), Indole-3-acetic acid (IAA), Indole-3-butyric acid (IBA) and cytokinins, 6-benzylaminopurine (BAP), Kinetin (KN), 6-(γ-γ-dimethylallylamino)-purine (2-iP) and 4-hydroxy-3-methyl-trans-2-butenylaminopurine (Zeatin) at different concentrations either alone or in combinations. All plant growth regulators were added to the medium before autoclaving. The p^H of the medium was adjusted to 5.8, followed by autoclaving at 121°C at 15psi (1.06 kg/cm^2) pressure for 15 min. The culture medium (15ml) was then dispensed into test tubes (25 X 150 mm) and 50 ml into culture bottles. The cultures were incubated under 16h photoperiod with cool white fluorescent tubes.

In vitro rooting and acclimatization

For in vitro rooting, individual microshoots 6-8 cm long were aseptically excised from the bottle and transferred to ½ strength MS medium supplemented with different concentrations of auxins i.e. NAA, IAA and IBA (0.1 mg/l to 3.0 mg/l) for root induction. The rooted plants were removed from culture tubes, washed with sterile double distilled water and transferred to plastic cups with sterile sand, soil and farmyard manure in 1:1:1 ratio. The potted plants were covered with perforated polythene covers. The plants were irrigated with ½ strength MS salts without sucrose for 10 days and kept in culture room. After 15 days the polythene covers were removed and well developed plants were first transferred into green house conditions and after 30 days the plants were shifted to field conditions and 70% of the survival rate was recorded.

Statistical analysis

A minimum of 15 culture tubes were raised for each combination and all experiments performed three times. Analysis of variance (ANOVA) and mean separations were carried out using Tukey's test at 0.05% level of significance.

Results and Discussion

For the induction of callus and regeneration of shoots, internodal explants of *Caralluma stalagmifera* were inoculated on MS medium supplemented with different concentrations of auxins and cytokinins like 2,4-D, 2,4,5-T, 2,4,5-TP, Picloram, Dicamba, NAA, IAA, IBA and cytokinins, BAP, KN, 2-iP and Zeatin either individually or in

combinations. Callus was produced from internodal explants in all hormonal treatments. But, morphology and growth of the callus varied with different level of auxin concentrations. The highest frequency (75%) of green compact callus was observed on MS medium containing 2,4-D 2.0 mg/l (Fig 1A). When the concentration was increased to 3.0 mg/l to 5.0 mg/l, callus changed to brown profuse, creamish friable callus and gradual decreases in the percentage of callus formation. The internodal segments produced green callus in medium containing 2,4,5-T 1.0 mg/l with 70% of response. But further increase in concentration there was decrease in the percentage of response and also change in nature of callus was observed. MS medium fortified with 2,4,5-TP at 1.0 mg/l showed maximum response (60%) with green friable callus.

Among different concentrations of Picloram tested, maximum response was observed at 2.0 mg/l and callus morphology was green friable callus. However, at lower concentrations (0.1 mg/l to 0.5 mg/l) produced pale green scanty callus and at 1.0 mg/l produced pale green embryogenic callus. But further increased in more than 2.0 mg/l there was decrease in the response and also change in nature of callus was observed. Internodal segments cultured on MS medium fortified with different concentrations of Dicamba produced maximum frequency of response (55%) at 2.0 mg/l with green embryogenic callus. Among the different concentrations of NAA, maximum response was observed at NAA 2.0 mg/l with 50% of response with green compact callus with roots. At lower and higher concentrations callus productions was reduced. MS medium supplemented with IAA, at lower concentrations 0.1 mg/l to 0.5 mg/l explants did not respond and increased concentrations of IAA 1.0 mg/l produced green compact callus with roots with 50% of response. Only two concentrations of IBA produced callus with 30% and 40% response. In remaining concentrations there was no response and explants remained green after 50 days of culture.

In our present study out of various auxins used for callus induction 2,4-D 2.0 mg/l resulted maximum response and NAA, IAA and IBA gave least response compared to other auxins. The effectiveness of 2,4-D in inducing callus was reported by Sharma and Chandel (1992), Sharma et al. (1995), Taha et al. (2008), Patil (1998) and Beena and Martin (2003).

Synergistic effect of 2,4-D (2mg/l) in combination with cyotokinins on callus induction

Based on the percentage of response and the rate of callus induction 2,4-D 2.0 mg/l was found to be optimum. Above and below this concentration the rate of callus induction or percentage of cultures responding decreased. Hence further experiments were carried out with 2.0 mg/l 2,4-D. Internodal explants were cultured on MS basal medium supplemented with 2,4-D 2.0 mg/l in combination with BAP (0.5mg/l, 1.0mg/l and 2.0 mg/l), KN (0.5 mg/l, 1.0 mg/l and 2.0 mg/l), 2-iP (0.5 mg/l, 1.0 mg/l and 2.0 mg/l) and Zeatin (0.5 mg/l, 1.0 mg/l and 2.0 mg/l) for callus induction.

The combination of BAP 0.5 mg/l and 2,4-D 2.0 mg/l elicited the highest response of callus formation with internodal segments (80%) (Fig 1B) and the combination of 2,4-D 2.0 mg/l + BAP 1.0 mg/l elicited the second highest response of callus formation with internodal segments (75%) of *Caralluma stalagmifera*. Internodal segments cultured on 2,4-D 2.0 mg/l + KN 0.5 mg/l produced green compact callus. Light green friable callus resulted on 2,4-D 2.0 mg/l + KN 1.0 mg/l with 65% response. Among various combination of 2,4-D and 2-iP, 2,4-D 2.0 mg/l + 2iP 0.5 mg/l produced light green friable callus. Increase in concentration of 2,4-D 2.0 mg/l + 2-iP 1.0 mg/l and 2,4-D 2.0 mg/l + 2-iP 2.0 mg/l produced green compact and green profuse callus with maximum response of 60% and 70% respectively. Internodal segments cultured on 2,4-D 2.0 mg/l + Zeatin 0.5 mg/l produced green profuse callus with 60% response. Where as 2,4-D 2.0 mg/l + Zeatin 1.0 mg/l produced green friable callus with maximum response (65%). A combination of 2,4-D 2.0 mg/l + BAP 2.0 mg/l + 2-iP 2.0 mg/l produced green compact callus and when 2-iP is replaced by Zeatin 0.5 mg/l in the above combination the same internodal segments produced green friable callus.

So, in our present investigation among these combinations 2,4-D (2.0 mg/l) +BAP (0.5 mg/l) gave maximum response when compared to other combinations. Datta et al. (2002) reported that the highest percentage of callus was induced by the combination of 2,4-D and BAP in *Beta palona*. There are reports where optimum callus development was seen on 2,4-D with various cytokinins in *Hemidesmus indicus* (Sarasan et al., 1994) and *Tylophora indica* (Faisal et al., 2005). Vyapari et al. (1993) demonstrated the need of 2,4-D 2.0 mg/l and BAP 0.1 mg/l for callus induction in *Asclepias tuberosa*.

Influence of plant growth regulators on organogenesis

Callus was obtained from mature internodal explants after an incubation period of 20-25 days, further this callus was sub cultured for shoot regeneration onto MS medium supplemented with various concentrations of cytokinins and auxins like BAP, 2-iP, KN, Zeatin and NAA either singly or in combinations. Among the various concentrations of BAP used BAP 2.0 mg/l produced 1.52 shoots / explant with 70% of response. Out of four concentrations of 2-iP used shoot bud regeneration (50%) was observed on 2-iP 2.0mg/l. KN 0.5mg/l and Zeatin 0.1mg/l showed 1.13 shoots / explant and 1.10 shoots / explant with 50% and 55% of response (Table 1).

Among different combinations used for plant regeneration BAP 2.0 mg/l + NAA 0.5mg/l produced 3.15 shoots/explant with maximum regeneration capacity (60%) in 20 days of culture (Fig 1C) (Table 1). When these shoots were subcultured on the same media there was increase in the shoot number.

Without supply of exogenous plant growth regulators regeneration of shoots was not observed. In vitro organogenesis depends on the application of exogenous phytohormones. The requisite concentration of each type of regulant differs greatly according to the kind of plant being cultured and cultural conditions. In the present study 2.0 mg/l BAP alone proved to be best for shoot development from callus, whereas combinations of BAP 2.0 mg/l + NAA 0.5mg/l found to be effective in *Caralluma stalagmifera* (present study).

The efficiency of BAP in inducing adventitious shoots over KN was proved in *Hemidesmus indicus* (Sreekumar et al., 2000), *Ceropegia jainii* (Patil, 1998), *Ceropegia bulbosa* (John Britto et al., 2003) and *Ceropegia sahyadrica* (Nikam and Savant, 2007).

Auxins and cytokinins are able to bring shoot or root formation from callus but the effective concentrations of these regulators may vary. The combination of auxin and cytokinin was found to be effective for shoot bud formation from callus in *Hemidesmus indicus* (Sharma and Yelne, 1995), *Holostemma ada–koiden* (Martin, 2002), *Tylophora indica* (Faisal et al., 2005), *Ceropegia bulbosa* (John Britto et al., 2003) and *C. candelabrum* (Beena and Martin, 2003).

In vitro rooting and Acclimatization

The regenerated shoots derived from callus were transferred to various rooting media and 15 days after inoculation root formation was induced from basal cut portion of shoot. The shoots were transferred to hormone free MS and ½ MS media which did not produce any roots. Reduction of MS salt solution to ½ strength enhanced root formation in plantlets supplemented with auxins. Of the three auxins tested (NAA, IAA and IBA) NAA 0.5 mg/l was found to be effective for root induction. The maximum frequency of (75%) of root formation (8.42 roots/shoot) was achieved in the same media composition (Table 2) (Fig 1D). A similar mode of response was observed in the other members of asclepiads like *Decalepis arayalpathra* (Sudha et al., 2005), *Ceropegia candelabrum* (Beena and Martin, 2003) and *Caralluma adsendens* var. *attenuata*, *Caralluma adscendens* var. *fimbriata*, *Caralluma adscendens* var. *adscendens* (Aruna et al., 2009).

In vitro regenerated plantlets with well developed shoots and roots were washed with water and were transferred into plastic pots containing soil, sand and farmyard manure in 1:1:1 ratio. The potted plants were covered with polythene cover to ensure high humidity and irrigated every two days with ½ strength MS salts solution free of sucrose for 10-15 days. Then they were transferred into earthen pots for 10 days and then transferred to normal soil and water with tap water. The rooted plants were successfully established in soil with 70% survival rate in *Caralluma stalagmifera* (Fig 1E).

Tissue culture technology offers an alternative method for the conservation of germplasm as well as micropropagation of medicinally important plant resources. Presently there is great demand for the use of plant based medicaments in place of synthetic drugs. As a result of non-scientific exploitation, most of the medicinal plant resources are being threatened and are on the verge of extinction. Therefore, application of this technology provides the raw materials required for the isolation of drugs by the pharmaceutical industries without depleting natural plant resources.

Table 1

Effect of various plant growth regulators on morphogenic response of callus induced from internodal segments of mature explants of *Caralluma stalagmifera* cultured on MS medium

Plant growth regulators used for morphogenesis					**Type of explant (Internodal segments)**	
BAP	**2-iP**	**KN**	**Zeatin**	**NAA**	**% of responses**	**No. of shoots/ explant Mean ± SE**
0.1	-	-	-	-	0	NR

0.5	-	-	-	-	40	1.06 ± 0.02 ^{d}
1.0	-	-	-	-	50	1.15 ± 0.06 ^{e}
2.0	-	-	-	-	70	1.52 ± 0.09 ^{b}
-	0.1	-	-	-	0	NR
-	0.5	-	-	-	40	1.04 ± 0.02 ^{de}
-	1.0	-	-	-	45	1.05 ± 0.02 ^{d}
-	2.0	-	-	-	50	1.22 ± 0.04 ^{bc}
-	-	0.1	-	-	35	1.00 ± 0.00 ^{e}
-	-	0.5	-	-	50	1.13 ± 0.02 ^{c}
-	-	-	0.1	-	55	1.10 ± 0.02 ^{d}
-	-	-	0.5	-	40	1.04 ± 0.01 ^{d}
2.0	2.0	-	-	0.5	55	2.19 ± 0.05 ^{b}
2.0	-	-	0.1	0.5	50	1.22 ± 0.03 ^{bc}
2.0	-	-	-	0.5	60	3.15 ± 0.03 ^{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. NR - No response.

TABLE - 2

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

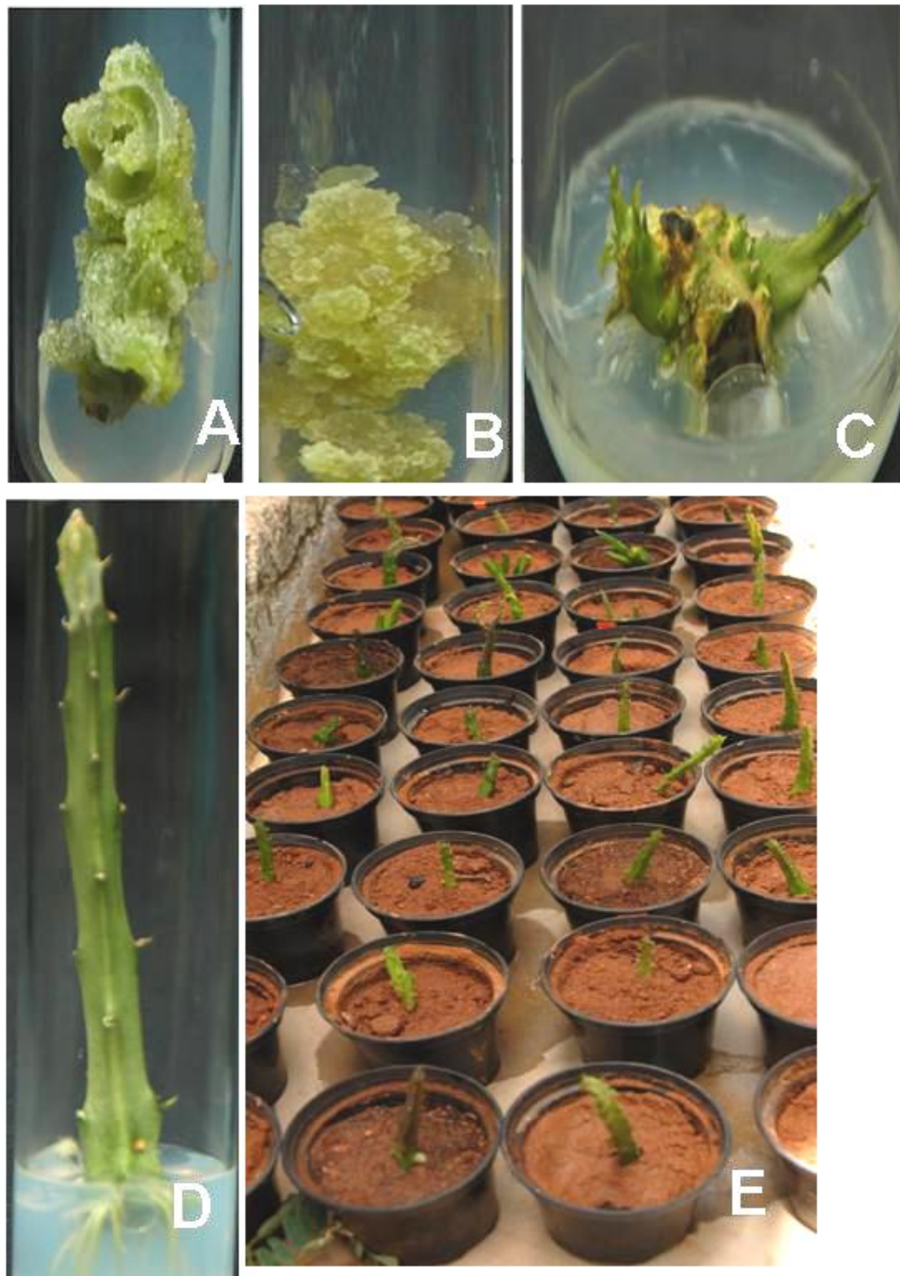
Concentration of Auxin mg/l			% of response	Number of Roots/shoot Mean ± SE	Length of roots (cm) Mean ± SE
NAA	IAA	IBA			
0.10	-	-	65	4.47 ± 0.05 ^{b}	2.13 ± 0.02 ^{b}
0.50	-	-	73	8.42 ± 0.04 ^{a}	3.59 ± 0.02 ^{a}
1.00	-	-	60	2.51 ± 0.01 ^{c}	1.83 ± 0.01 ^{bc}
2.00	-	-	-	CP	CP
3.00	-	-	-	CP	CP
-	0.10	-	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

Legend of Figures

Figure 1A – E. In vitro callus induction and plant regeneration from internodal explants of *Caralluma stalagmifera* Fischer


A
B
C
D
E

A. Callus proliferation from internodal explants on MS + 2,4-D 2.0 mg/l.
B. Callus induction from internodal segments cultured on MS + BAP 0.5 mg/l + 2,4-D 2.0 mg/l.
C. Shoot buds regeneration from organogenic callus on MS + BAP 2.0 mg/l + NAA 0.5 mg/l.
D. Rooting of in vitro regenerated shoots on ½ strength MS medium + NAA 0.5 mg/l.
E. Acclimatized in vitro grown plants.

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