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# *In vitro* Shoot Multiplication of Medicinally Important *Caralluma stalagmifera* Fischer

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**Abstract:** ***An efficient shoot multiplication protocol was developed for Caralluma stalagmifera Fischer a medicinal plant belonging to the family Asclepiadaceae. Shootlets were regenerated from nodal explants of Caralluma stalagmifera through axillary shoot proliferation. The induction of multiple shoots from nodal segments was the highest in MS medium supplemented with BAP 2.0 mg/l + NAA 0.5 mg/l. For rooting different concentrations of NAA, IBA and IAA were used and highest rooting percentage (73%) was recorded on ½ strength medium with 0.5 mg/l NAA. The rooted plantlets were hardened and successfully established in soil.***

**Keywords:** *Caralluma stalgmifera, in vitro* regeneration.

**Abbreviations:** BAP: 6-Benzyl aminopurine; KN: 6-Furfuryl aminopurine; IAA: Indole-3-acetic acid; IBA: Indole-3-butyric acid; NAA: Naphthalene Acetic acid.

## 1. Introduction

*Caralluma stalagmifera* Fischer belongs to family Asclepiadaceae. There are about 10 variable species of *Caralluma* that occur in India mainly adapted to dry habitats (Rathore *et al.*, 2008). It is an erect succulent herb. The young shoots of this plant are edible and cooked as vegetable, also used in pickles and preserved. *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).

Plants belonging to this genus are rich in esterified polyhydroxy pregnane glycosides, flavone glycosides. Indian folklore records its use as a potent appetite suppressant and weight loss promoter. Some *Caralluma* species are used in the treatment of obesity. The extract of *Caralluma* sp. in the form of capsules has been released under trade name GENASLIM for body weight control (Lawrence and Choudary, 2004).

Medicinal plants are of great concern to the researchers in the field of biotechnology not only for rapid propagation but also for production of valuable secondary metabolites. Axillary bud multiplication is the simplest and most reliable way to produce clonal plants of elite species as exemplified in many medicinal plants. Seed derived progenies are not true to type due to cross – pollination. Propagation through seeds is not reliable due to low span of viability. Tissue culture studies on *Caralluma edulis* (Kaur et al., 1992; Rathode et al., 2008). *Caralluma adsendens* var. *attenuata*, *Caralluma adscendens* var. *fimbriata*, *Caralluma adscendens* var. *adscendens* (Aruna *et al.*, 2009) have been reported. This paper describes an efficient and rapid propagation method of *Caralluma stalagmifera* using nodal explants.

## 2. Materials and Methods

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 with 5-6 nodes were used as explant source for multiplication. The young shoots were washed under running tap water to remove soil particles for 5-10 minutes followed by detergent (1% Tween - 20) for 5 min. 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 1min. 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 explants were dissected, damaged ends were remove and blotted on a sterile filter paper disk and inoculated on medium for shoot induction.

The basic nutrient medium used for the present study MS (Murashige and Skoog, 1962) with 3% sucrose. The medium was solidified with 0.8% agar after adjusting $p^H$ to 5.8 and poured approximately 15 ml per tube (150 X 25 mm Borosil, India). The tubes were sealed with aluminum foil and sterilized at 121ºC and 1.06 $kg.cm^{-2}$ pressure for 15 min. The cultures were incubated at 25 ± 2ºC with irradiance of 50µ mol $m^{-2}$ $S^{-1}$ for 16h photoperiod.

Then the MS medium fortified various concentrations of growth regulators (BAP, KN, NAA, IAA and IBA) at different concentrations either alone or in combinations. The *in vitro* rooting was carried out on ½ strength MS medium supplemented with various concentrations of auxins i.e., NAA, IAA and IBA.

*In vitro* raised shoots after rooting were transformed to small pots containing sterile sand, soil and farmyard manure in

1:1:1 ratio and subsequently to the field. The experiments were setup in a complex randomized design. All the experiments were repeated thrice with 15 replicates per treatments. Data was statistically analysed by analysis of variance (ANOVA) and means were compared by Tukey's Test at 0.05% level of significance.

## 3. Results and Discussion

Culture medium devoid of growth regulators (control) failed to stimulate bud break in explants even when the cultures were maintained beyond the normal one month period. The explants in the control remained fresh and green for about two weeks, but thereafter started to dry up.

MS medium supplemented with different concentrations of BAP/ KN individually or in combinations and also BAP in combination with auxins NAA/IAA/IBA resulted initiation of axillary buds (Table 1). Nodal explants were inoculated on MS medium fortified with different concentrations of BAP (0.1 mg/l to 8.0 mg/l) and KN (0.1 mg/l to 8.0 mg/l) alone and BAP (2.0 mg/l) with NAA, IAA and IBA (0.1 mg/l to 2.0 mg/l).

Out of these treatments, medium fortified with BAP 2.0 mg/l alone had better shoot sprouting frequency of 80 % with 2.54 shoots/ explant and attained a length of 1.80 cm without basal callus (Fig 1A). Increased concentrations of BAP resulted in reduced number of shoots (Table 1).

Of the different concentrations of KN used KN 2.0 mg/l induced 2.07 shoots with 1.26 cm of length. Number of shoots on medium supplemented with KN was lesser as compared to BAP. BAP was most favorable cytokinin for initiation and multiplication of axillary buds. The superior activity of BAP compared to KN is reported in many plants i.e., *Ceropegia bulbosa* (John Britto et al., 2003; Patil, 1998), *Ceropegia sahyadrica* (Nikam and Savant, 2007), *Ceropegia jainii* (Patil, 1998), *Gymnema sylvestre* (Komalavalli and Rao, 2000), *Holostemma annulare* (Sudha et al., 2000).

Addition of different levels of KN to medium with the optimal level of BAP (2.0 mg/l) did not enhance the number of shoots (Table 1). In order to enhance shoot multiplication, different auxins were combined with the optimized cytokinin concentration. Nodal segments of *Caralluma stalagmifera* cultuted on various concentrations of BAP + NAA, Shoot number increased on the media containing BAP 2.0 mg/l + NAA 0.5 mg/l and a maximum number of 6.72 shoots/explant (Fig. 1B ) (Table 2) with 4.16 cm of length.

In a number of cases cytokinin alone is enough for optimal shoot multiplication (Garland and Stoltz, 1981). However axillary shoot proliferation in some species may be promoted by the presence of an auxin and cytokinin. Assuming that combined effect of auxin and cytokinin could improve further multiplication rate of shoots, different concentrations and combinations were studied.

Out of various treatments of BAP + IAA, BAP 2.0 m/l + IAA 1.0 mg/l produced 5.13 shoots/ explants with 3.02 cm of shoot length. Among various treatments of BAP and IBA 2.81 shoots/explant achieved from the medium supplemented with BAP 2.0 mg/l + IBA 0.5 mg/l with an average shoot length 3.50cm (Table 2).

So, in our present investigation among different combinations of BAP and auxins tested for shoot regeneration NAA gave maximum number of shoots when compared to other auxins and the order of response is NAA> IAA> IBA. Shoot number and sprouting frequency with cytokinin and auxin treatment was much satisfactory when compared to cytokinin alone and combinations.

A synergistic effect of BAP in combination with an auxin has been demonstrated in many medicinal plants of Asclepiadaceae viz. *Caralluma edulis* (Rathore et al., 2008), *Ceropegia bulbosa* var. *bulbosa* (John Britto et al., 2003), *Ceropegia candelabrum* (Beena et al., 2003), *Hemidesmus indicus* (Sree Kumar et al., 2000) and *Cryptolepis buchanani* (Prasad et al., 2004). In consanance with their observations, low concentrations of an auxins in combination with a cytokinin positively modifies the shoot induction frequency. However, inhibitory effect by addition of auxin to cytokinin has been reported in *Ceropegia sahyadrica* (Nikam and Savant, 2007).

Excision and culture of nodal segments from *in vitro* derived shoots on MS medium supplemented with same concentrations of BAP (2.0 mg/l) + NAA (0.5 mg/l) developed more than 12 shoots with 25 days. Subsequent culture increased the rate of shoot multiplication on the same medium (Fig 1C). Subculture was done every third week. Increased rate of shoot multiplication in successive cultures has also been reported in medicinal plants like *Caralluma edulis* (Rathore et al., 2008), *Hemidesmus indicus* (Sree Kumar et al., 2000), *Ceropegia candelabrum* (Beena et al., 2003) and *Gymnema sylvestre* (Komalavalli and Rao 2000). However, Patnaik and Debata (1996) in *Hemidesmus indicus* have observed reduction in number of shoots during repeated subcultures.

Microshoots were transferred to full or half strength MS medium for root induction. Half strength MS medium was superior to full strength MS medium for root induction. *In vitro* rooting was successfully achieved from micro shoots cultured on half strength MS medium with various concentrations of auxins like NAA, IAA and IBA (0.1 mg/l to 3.0 mg/l) as shown in Table 3. Reduction of MS salt solution to ½ strength enhances root formation in shoot lets. The favorable effects of low concentration of macro and micro nutrients on rooting is probably due to the decreased requirement of nitrogen for rhizogenesis. ½ strength MS medium fortified with auxins at lower concentrations facilitated better rooting (Table 3). Among different auxins NAA was superior to IBA and IAA. ½ strength MS medium fortified with NAA ( 0.5 mg/l) was best for *in vitro* rooting and developed a mean number of 8.42 roots/shoots (Table 3) with maximum root length (Fig 1D). Efficiency of NAA at lower concentrations in *in vitro* rooting has been reported in medicinal plants like *Caralluma adsendens* var. *attenuata*, *Caralluma adscendens* var. *fimbriata*, *Caralluma adscendens* var. *adscendens* (Aruna et al., 2009), *Cryptolepis buchanani* (Prasad et al., 2004), *Decalepis*

*arayalpathra* (Sudha et al., 2005) and *Leptadenia reticulata* (Farzin et al., 2007).

Microshoots with a proper root system were ready for field transfer. Microshoots were transferred to potting mixture (soil, sand and farmyard manure in 1:1:1 ratio) filled in plastic cups (Fig 1E) and then field conditions through step wise hardening process. Initially the plantlets were covered with polythene bags to maintain high humidity and irrigated every two days once with ½ strength MS salts with free of sucrose. They were kept in a separate room under observations with ambient temperature and normal day light photoperiod. The bags were removed periodically for gradual hardening. After two weeks they were taken out side the room and kept in a shady place under normal temperature and light. After eight weeks when the plantlets had achieved height, they were transferred in the soil and watered with tap water. The rooted plants were successfully established in soil with 70% survival rate.

In conclusion, the outlined procedure offers a potential system for conservation, improvement and propagation of *Caralluma stalagmifera*. MS medium containing 2.0 mg/l BAP + 0.5 mg/l NAA is the best for shoot proliferation. The use of axillary nodes for micropropagation is beneficial than other explant types. ½ strength MS basal medium supplemented with 0.5 mg/l NAA is the best for *in vitro* rooting.

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

| *Plant growth regulators (mg/l)* | *Shoot sprouting frequency (%)* | *Shoot No. per explant Mean ± SE* | *Shoot length (cm) per explant Mean ± SE* |
|---|---|---|---|
| **BAP** | | | |
| 0.1 | 60 | $1.06 \pm 0.02^{f}$ | $1.09 \pm 0.02^{de}$ |
| 1.0 | 75 | $1.55 \pm 0.02^{c}$ | $1.16 \pm 0.02^{d}$ |
| 2.0 | 80 | $2.54 \pm 0.04^{a}$ | $1.80 \pm 0.02^{d}$ |
| 3.0 | 55 | $1.76 \pm 0.03^{bc}$ | $1.44 \pm 0.03^{c}$ |
| 5.0 | 50 | $1.50 \pm 0.02^{cd}$ | $1.24 \pm 0.02^{cd}$ |
| 8.0 | 40 | $1.32 \pm 0.03^{d}$ | $1.07 \pm 0.02^{de}$ |
| **KN** | | | |
| 0.1 | 30 | $1.12 \pm 0.02^{e}$ | $1.04 \pm 0.01^{de}$ |
| 1.0 | 45 | $1.27 \pm 0.03^{de}$ | $1.12 \pm 0.01^{d}$ |
| 2.0 | 65 | $2.07 \pm 0.02^{ab}$ | $1.26 \pm 0.03^{cd}$ |
| 3.0 | 55 | $1.36 \pm 0.02^{d}$ | $1.18 \pm 0.02^{d}$ |
| 5.0 | 50 | $1.10 \pm 0.02^{ef}$ | $1.06 \pm 0.02^{de}$ |
| 8.0 | 0 | NR | NR |
| **BAP + KN** | | | |
| 2.0 + 0.1 | 50 | 1.25 ± 0.01 | 1.29 ± 0.01 |
| 2.0 + 0.5 | 65 | 1.33 ± 0.02 | 1.38 ± 0.02 |
| 2.0 + 1.0 | 70 | 1.80 ± 0.01 | 1.86 ± 0.01 |
| 2.0 + 2.0 | 80 | 2.40 ± 0.02 | 2.00 ± 0.01 |

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 BAP 2.0 mg/l in combination with different concentrations of auxins on shoot proliferation from mature explants of *Caralluma stalagmifera*

| *Growth regulators* | | | | Shoot sprouting Frequency (%) | Number of shoots/explant Mean ±S.E. | Shoot length Mean± S.E. |
|---|---|---|---|---|---|---|
| *BAP* | *NAA* | *IAA* | *IBA* | | | |
| 2.0 | 0.1 | - | - | 70 | 3.12 ± 0.02 | 2.06 ± 0.02 |
| 2.0 | 0.5 | - | - | 85 | 6.72 ± 0.01 | 4.16 ± 0.04 |
| 2.0 | 1.0 | - | - | 65 | 2.81 ± 0.01 | 1.71 ± 0.03 |
| 2.0 | 2.0 | - | - | 52 | 1.08 ± 0.01 | 1.34 ± 0.04 |
| 2.0 | - | 0.1 | - | 60 | 1.81 ± 0.01 | 1.37± 0.04 |
| 2.0 | - | 0.5 | - | 65 | 2.22 ± 0.03 | 1.88 ± 0.03 |
| 2.0 | - | 1.0 | - | 75 | 5.13 ± 0.02 | 3.02 ± 0.03 |
| 2.0 | - | 2.0 | - | 55 | 1.24 ± 0.02 | 1.22 ± 0.02 |
| 2.0 | - | - | 0.1 | 65 | 1.78 ± 0.01 | 2.38 ± 0.02 |
| 2.0 | - | - | 0.5 | 78 | 2.81 ± 0.01 | 3.50 ± 0.02 |
| 2.0 | - | - | 1.0 | 60 | 1.56 ± 0.02 | 1.84 ± 0.01 |
| 2.0 | - | - | 2.0 | 55 | 1. 43 ± 0.02 | 1.41 ± 0.03 |

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 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 (A - E) *In vitro* shoot multiplication of medicinally important *Caralluma stalagmifera* Fischer.

A. Axillary bud initiation on MS+ BAP 2.0 mg/l

**B.** Multiple shoots formed on MS+ BAP 2.0 mg/l + NAA 1.0 mg/l.

**C.** Enhanced number of shoots during 1st sub culture on medium as above.

**D.** Rooting of *in vitro* regenerated shoots on ½ strength MS medium with NAA 0.5 mg/l.

**E.** Plantlets in small cups (After 20 days).

**Figure 1**