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Full Length Research Paper

Influence of aseptic seedling explants on *in vitro* shoot multiplication of *Caralluma adscendens* var. *attenuata* Wight

V. Aruna[1], C. Kiranmai[2], S. Karuppusamy[2] and T. Pullaiah[2]*

[1]Department of Biotechnology, Chaitanya Bharathi Institute of Technology, Gandipet, Hyderabad-500075, Andhra Pradesh, India.
[2]Department of Botany and Biotechnology, Sri Krishnadevaraya University, Anantapur-515003, Andhra Pradesh, India.

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A protocol has been developed for *in vitro* shoot multiplication and plant establishment of *Caralluma adscendens* var. *attenuata*. Aseptic seedlings raised on Murashige and Skoog (MS) basal medium were used as a source of explants. Among different seedling explants cultured on different basal media, nodal explant on MS basal medium supplemented with BA 2 mg/L gave best shoot regeneration frequency. Nodal explants cultured on MS basal medium supplemented with BA 2 mg/L + 2iP 0.5 mg/L produced an average of 4.56 shoots. Microshoots were rooted on half strength MS basal medium with and without plant growth regulators. The best rooting occurred on semisolid half strength MS basal medium containing 0.6% agar and 0.1 mg/L NAA. The plantlets developed were hardened and established in natural soil with 73% survival rate.

Key words: Micropropagation, conservation, *in vitro* shoot multiplication, *Caralluma adscendens* var. *attenuata*.

INTRODUCTION

Caralluma adscendens var. *attenuata* is a succulent, medicinal plant belonging to the family Asclepiadaceae. It is a perennial herb, occurring wild in rocky and dry southern parts of India. Locally, it is known as 'Kundeti kommulu'. The juice of the plant along with black pepper is recommended in the treatment of migraine (Srinivasacharyulu, 1931). The herb was found to be a rich source of glycosides and flavonoids (Ramesh et al., 1999). Luteolin - 4'- O -neohesperidoside is more potent than ibuprofen and has significant anti-inflammatory action and antinociceptive activity (Ramesh et al., 1998). It is also reported to have significant antihyperglycemic activity (Venkatesh et al., 2003). The hypoglycemic effect of aqueous and alcohol extracts of whole plant was investigated by Jayakar et al. (2004). Natural populations of these plants are declining because of increase in demand in pharmaceutical market. Due to continuous harvesting of plant for its glycosides and as there are no organized agricultural cultivation or micropropagation methods of this plant, immediate measures are to be taken and a protocol was developed for micropropagation and conservation of wild species of *Caralluma*. So far there is only one report on micropropagation of *C. adscendens* var. *attenuata* from mature nodal explant (Aruna et al., 2009). The purpose of present study was to develop a rapid *in vitro* shoot multiplication protocol from aseptic seedling of this medicinal plant. Micropropagation studies on other species of *Caralluma* species include Sreelatha et al. (2009) on *Caralluma sarkariae,* Sreelatha and Pullaiah (2010) on *Caralluma stalagmifera* and Ugraiah et al. (2011) on *Caralluma bhupenderiana*.

*Corresponding author. E-mail: pullaiah.thammineni@gmail.com.

Abbreviations: IAA, Indole-3-acetic acid; **IBA,** indole-3-butyric acid; **Kn,** kinetin; **MS,** Murashige and Skoog (1962) medium; **NAA,** naphthalene acetic acid; **TDZ,** Thidiazuron; **2-iP,** 2-isopentyladenine; **2,4-D,** 2,4-Dichlorophenoxy acetic acid.

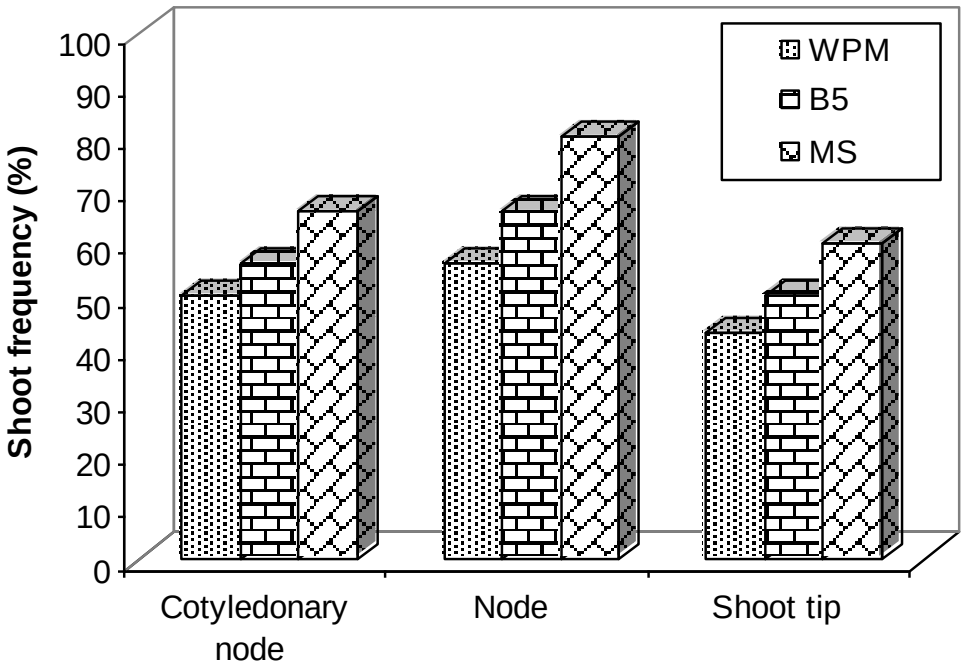

Figure 1. Effect of shoot regeneration frequency of different aseptic seedling explants of *Caralluma adscendens* var. *attenuata* on different types of media supplemented with BA 2 mg/L.

MATERIALS AND METHODS

Mature follicles of *C. adscendens* var. *attenuata* were collected near Sri Krishnadevaraya University campus, Anantapur, Andhra Pradesh, India. The seeds were treated with 1% Tween 20 (v/v) for 30 min with constant agitation, and seeds were washed thoroughly with distilled water about 4 to 5 times. Initially, seeds were dipped in ethanol for 60 s and then seeds were surface sterilized with 20% hydrogen peroxide (v/v) for 5 min. Immediately, surface sterilized seeds were washed about 4 to 5 times with sterile distilled water to remove all traces of hydrogen peroxide. The surface sterilized seeds were germinated on MS basal medium (Murashige and Skoog, 1962). Different seedling explants (shoot tip, node and cotyledonary node) were cultured on various media such as MS medium, B_5 medium (Gamborg et al., 1968) and WPM (Lloyd and McCown, 1980) fortified with BA 2 mg/L. MS medium supplemented with various concentrations of cytokinins (BA, Kn and 2iP) and its combinations were investigated to optimize hormonal requirement for shoot induction. *In vitro* microshoots of 4 to 5 cm length were cultured on auxin free full strength and half strength MS medium and also half strength MS medium with different concentrations of NAA. MS medium was fortified with 3 and 1% sucrose (w/v) for shoot and root induction, respectively. Media was solidified with 0.8% agar for shoot induction and 0.6% for rooting after adjusting the pH to 5.8. The medium was sterilized at 121°C under 15 lbs pressure for 15 min. The cultures were incubated at 25 ± 2°C with cool white fluorescent light of about 2000 lux with 16 h photoperiod. *In vitro* rooted plantlets cultured on hormonal media were washed with distilled water to remove adhering culture medium, and transferred to potted soil containing sterile vermiculite saturated with quarter strength MS medium. All plastic pots, lidded with plastic film, were incubated in a culture room at 27°C under a 16 h photoperiod provided with cool white fluorescent light. After 2 days, plastic films were gradually perforated to allow for acclimatization, then plastic film was fully removed and transferred to potted soil containing garden soil, peat mass and farmyard manure in 1:1:1 ratio. Acclimatized plants were kept under green house conditions for 2 weeks and then transferred to a field. All the experiments were repeated twice with 15 replicates each. The effect of different treatments was quantified and the data were analyzed using one way analysis of variance (ANOVA), and means were compared using the Tukey test at the 0.05% level of significance.

RESULTS AND DISCUSSION

Explants remained green without response on MS basal medium without plant growth regulators. Aseptic seedling explants such as cotyledonary node, node and shoot tip explants were cultured *in vitro* on different media to regenerate shoots. Among three basal media, MS medium gave maximum response followed by B_5 and WPM with three aseptic seedling explants. Similarly, other members of Asclepiadaceae were also micro-propagated on MS medium (Komalavalli and Rao, 2000; Giridhar et al., 2005).

In Figure 1, positive morphogenetic response of three aseptic seedling explants resulted on three different media has been depicted. But maximum shoot regeneration frequency occurred in nodal explant than cotyledonary node and shoot tip. Nodal explant cultured on MS basal medium supplemented with BA 2 mg/L gave 80% shoot regeneration frequency after 30 days of culture. Similar response was also observed in *Gymnema sylvestre* (Komalavalli and Rao, 2000). All further experiments were conducted on MS medium with nodal explant.

The response of *in vitro* cultures to different concentrations of cytokinins alone was tested. MS

Table 1. Effect of different concentrations of cytokinins and its combinations on multiple shoot induction of *Caralluma adscendens* var. *attenuata* after 30 days of culture of MS medium with 3% sucrose.

Plant growth regulator (mg/l)			Shoot sprouting frequency (%)	Shoot number per explant Mean ± S.E	Shoot length (cm) Mean ± S.E
BA	Kn	2iP			
-	-	-	-	NR	NR
1.0	-	-	76	1.50 ± 0.15^{d}	1.39 ± 0.03^{fg}
2.0	-	-	83	3.83 ± 0.15^{b}	2.33 ± 0.04^{b}
3.0	-	-	70	2.03 ± 0.12^{cd}	2.99 ± 0.03^{a}
-	1.0	-	56	0.76 ± 0.11^{e}	0.99 ± 0.03^{h}
-	2.0	-	60	1.33 ± 0.13^{de}	1.38 ± 0.02^{fg}
-	3.0	-	66	1.83 ± 0.11^{cd}	1.82 ± 0.04^{de}
-	-	1.0	70	1.66 ± 0.12^{d}	1.53 ± 0.05^{ef}
-	-	2.0	80	2.13 ± 0.10^{cd}	2.39 ± 0.03^{b}
-	-	3.0	76	1.96 ± 0.10^{cd}	2.11 ± 0.04^{c}
2.0	0.1	-	63	1.43 ± 0.12^{d}	1.22 ± 0.05^{g}
2.0	0.5	-	66	1.76 ± 0.12^{d}	1.79 ± 0.04^{de}
2.0	1.0	-	73	2.13 ± 0.11^{cd}	2.01 ± 0.03^{c}
2.0	-	0.1	80	2.40 ± 0.14^{bc}	1.46 ± 0.03^{f}
2.0	-	0.5	90	5.16 ± 0.13^{a}	2.98 ± 0.03^{a}
2.0	-	1.0	73	2.00 ± 0.12^{cd}	1.68 ± 0.03^{e}

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

medium supplemented with BA 2 mg/L was more effective for shoot regeneration frequency and shoot number than other concentrations of BA, Kn, and 2iP on shoot production. Comparing the effect of cytokinins type (BA, Kn and 2iP) on shoot production, the best response was achieved on BA in Indian wild Strawberry (Indra and Upeandra, 2000). When there is increase or decrease in concentration of BA 2 mg/L, shoot number was decreased. But with increase in concentration of BA, there was increase in shoot length of *C. adscendens* var. *attenuata*. Among the three cytokinins tested, Kn showed least response (56%). 2iP at 1 mg/L resulted an average of 1.7 shoots, as the 2iP concentration was increased to 2 mg/L, shoot number increased to 2.2 shoots/explant. Further increase in concentration of 2iP resulted in decrease in shoot number and shoot length. As shown in Table 1, BA 2 mg/L produced an average of 3.83 shoots/ explant. Among three cytokinins, best shoot regeneration frequency with BA rather than KN and 2iP is in agreement with the findings that BA is needed for the *in vitro* propagation of latex producing plants (Sudha et al., 1998).

To increase shoot number and shoot quality, nodal explants were cultured on combination of BA 2 mg/Lwith different concentration of 2iP and KN. With the addition of two cytokinins, there is increase in shoot number. But combination of BA and KN produced less shoot number compared to BA 2 mg/L, whereas combination of BA 2 mg/L and 2iP 0.5 mg/L resulted in maximum number of 5.16 shoots/explant with average shoot length of 2.98 cm.

After four weeks of incubation shoots were transferred to fresh medium. During subculture, basal axillary buds of the developed axillary buds also underwent initiation. There are several reports in which enhanced shoot multiplication occurred during subculture in Asclepiads like *G. sylvestre* (Komalavalli and Rao, 2000), *Ceropegia candelabrum* (Beena et al., 2003), *C. adscendens* var. *adscendens*, *C. adscendens* var. *attenuata* and *C. adscendens* var. *fimbriata* (Aruna et al., 2009) (Figure 2).

Shoots with 4 to 5 cm length were excised and rooted on full strength and half strength MS basal medium without hormones. After 15 days of incubation, rooting was observed on half strength MS basal medium. In order to increase root number and reduce the time for rooting, different concentrations of NAA was added to half strength MS basal medium. Maximum number of rooting resulted on NAA 1.0 mg/L with basal callusing as shown in Table 2. However, survival of plantlets required strong, healthy, long roots, which were obtained from half strength MS basal medium supplemented with NAA 0.1 mg/L in *Caralluma*. The reason for the reduced survival in higher concentrations of NAA treatments may be due to poor vascular connection of the root with the stem because on the intervention of callus. The positive response of rooting in the present study is similar to observations of other member of Asclepiadaceae *Decalepis arayalpathra* (Sudha et al., 2005). Similar

Table 2. Effect of full, half strength MS and different concentration of NAA on *in vitro* rooting of *Caralluma adscendens* var. *attenuata* after 30 days.

Treatment	Rooting frequency (%)	Number of roots per shoot Mean ± S.E	Root length (cm) Mean ± S.E
Full strength MS medium	66	4.67 ± 0.23^{d}	3.58 ± 0.03^{b}
Half strength MS medium	80	5.73 ± 0.24^{d}	4.31 ± 0.04^{a}
Half strength MS +NAA 0.1 mg/L	90	9.36 ± 0.24^{c}	4.19 ± 0.05^{a}
Half strength MS + NAA 0.5 mg/L	76	13.20 ± 0.32^{b}	2.11 ± 0.03^{c}
Half strength MS + NAA 1.0 mg/L	66	15.10 ± 0.37^{a}	1.37 ± 0.03^{d}
Half strength MS + NAA 2.0 mg/L	-	CP	CP

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

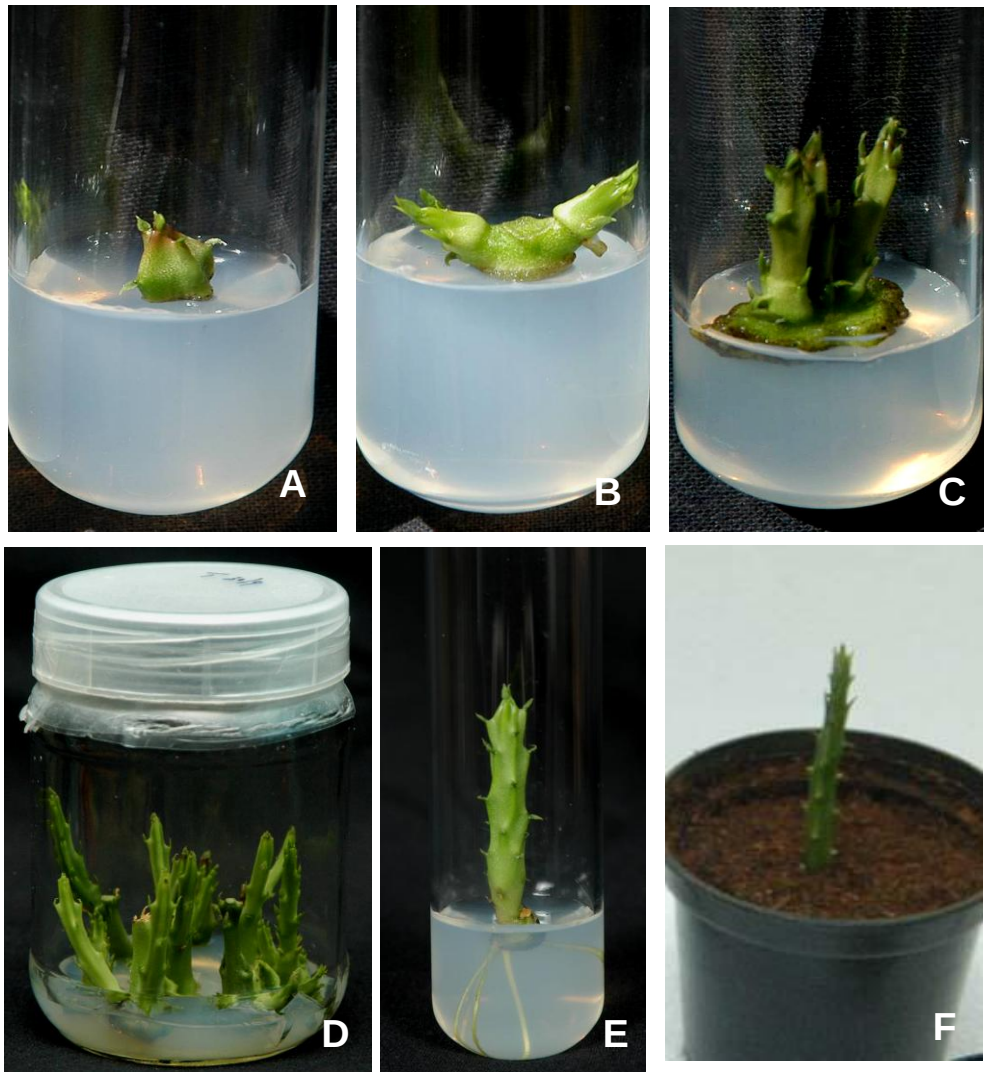

Figure 2. Micropropagation of *Caralluma adscendens* var. attenuata. (A) Effect of BA 2 mg/L on shoot regeneration from shoot tip explants cultured on MS medium after 20 days. (B) Effect of BA 2 mg/L on shoot regeneration from cotyledonary nodal explant cultured on MS medium after 20 days. (C) Effect of BA 2 mg/L on shoot regeneration from nodal explants cultured on MS medium after 20 days. (D) Effect of BA 2 mg/L + 2iP 0.5 mg/L on shoot regeneration from nodal explants cultured on MS medium. (E) Effect of NAA 0.1 mg/L on *in vitro* rooting after 25 days. (F) Acclimatized *in vitro* grown plant of *Caralluma adscendens* var. *attenuata.*

observations were also reported in *Euphorbia tirucalli* (Uchida et al., 2004). *In vitro* regenerated plantlets with well developed shoots and roots were transferred to pots containing peat moss, farmyard manure and garden soil in 1:1:1 ratio. The plantlets were successfully established in soil with 73% survival rate in *C. adscendens* var. *attenuata.* Plantlets did not show any morphological abnormalities during an observation period of 3 months in field conditions.

The present study revealed that the *in vitro* plantlet production was achieved on MS medium with BA 2 mg/L + 2iP 0.5 mg/L and 3% sucrose. Roots induced on NAA 0.1 mg/L showed better field survival. The protocol developed here could be used for shoot multiplication and conservation of this medicinal plant.

Conclusion

In conclusion, we have established a direct *in vitro* culture system for an important medicinal plant *C. adscendens* var. *attenuata*, which should enable the large-scale nursery production of this valuable medicinal plant.

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