African Journal of Biotechnology Vol. 8 (17), pp. 4052-4057, 1 September 2009
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2009 Academic Journals

Full Length Research Paper

In vitro conservation of *Ceropegia intermedia* - an endemic plant of south India

Subbiah Karuppusamy*, Chadipiralla Kiranmai, Varimadugu Aruna and Thammineni Pullaiah

Department of Biotechnology and Botany, Sri Krishnadevaraya University, Anantapur – 515 003, Andhra Pradesh, India.

Accepted 18 January, 2007

The purpose of this study was developing *in vitro* techniques for conserving wild and endemic species of *Ceropegia intermedia* by axillary shoot multiplication. Murashige and Skoog (MS) medium with 6.66 µM N^6 benzyladenine (BA) was best for axillary bud proliferation inducing a mean of 5.51 shoots per node. Excision and culture of the nodal segments from the *in vitro* shoots on fresh medium with same concentration of BA facilitated development of more than 5 shoots per node. Subsequent subculture slightly enhanced up to third subcultures and decreased thereafter. Shoots developed were rooted best on half strength MS with 5.37 µM NAA. Plantlets established in pots exhibited 75% survival. Plantlets were successfully established in field and morphological characters were identical to mother plants.

Key words: *Ceropegia intermedia*, endemic species, axillary bud multiplication.

INTRODUCTION

Ceropegia (Asclepiadaceae) is a genus of climbers, herbs and rarely subshrubs distributed in tropical and subtropical Asia, Africa, Australia, Malaysia and in the Canary and Pacific Islands (Nayar, 1985). The tuberous roots of many *Ceropegia* species are edible (Mabberley, 1987) and many others are of medicinal value (Jain and Defilips, 1991). The root tubers contain starch, sugar, gum, albuminoids, fats and crude fiber and are valuable constituents in many traditional medi-cinal systems in India (Kirtikar and Basu, 1935). Active principle of tuberous roots contains an alkaloid cero-pegine which is active against diarrhoea and dysentery (Nadkarni, 1976).

Of the 44 species of *Ceropegia* found in India, 27 species are endemic to the Peninsular India (Ahmedullah and Nayar, 1986) which is distributed mainly in Western Ghats and most of them are enlisted under endangered category (Nayar and Sastry, 1983). *Ceropegia intermedia* are also endemic and endangered species of South India. Scanty population of this species is distributed in edges of moist deciduous forests in Tami-lnadu (Jagtap and Singh, 1999). As the species is a cross-pollinating one, the seed grown progenies of *C. intermedia* are not true-to-type. Low seed germination rate and habitat destruction threatens its population in natural habitat. Vegetative propagation by root tubers and stem cuttings is very arduous. Large scale propa-gation is a prerequisite for effective conservation of this endangered species (Arora and Bhojwani, 1989). Axillary bud multiplication is an effective alternative for clonal propagation. Until now, no *in vitro* studies have been reported on this endangered species. The present investigation reported the *in vitro* propagation of *C. intermedia* through axillary bud proliferation enhanced by benzyladenine.

*Corresponding author. E-mail: ksamylin@yahoo.co.in, Telephone: +91-08554-255561, Fax: +91-08554-2555244.

MATERIALS AND METHODS

Plant material for multiple shoot induction

Plants of *C. intermedia* were collected from Sirumalai hills of Eastern Ghats of Tamilnadu and grown in earthen pots under green house condition in Botanical Garden at Sri Krishnadevaraya University, Anantapur. The young shoots with six internodes were collected from the garden grown plants and washed with running tap water for 15 min. The nodes were cut (4 cm) separately and they were washed with Tween 20 (Merck, India) detergent solution (5% v/v) for 10 – 15 min. The surface sterilization of explants was followed by rinse with sterile distilled water 3 - 4 times to remove trace of detergent, rinsing in 80% ethanol for 30 s and finally treatment with mercuric chloride (0.1% w/v) ($HgCl_2$) for 5 min duration. To remove every trace of the sterilant, the shoot material was then washed with sterile distilled water at least 4 - 5 times. The shoot segments containing nodes (1 -1.5 cm) were prepared from the surface sterilized shoots and were used as explants. The whole process was carried out under the laminar air flow chamber.

Culture media

For culture media, MS medium (Murashige and Skoog, 1962) was used for shoot bud proliferation from nodal explants of *C. intermedia*. Axillary bud induction and multiplication of shoots were examined using MS medium variously supplemented with BA, KN, TDZ and Zeatin alone. For rooting, half strength MS medium supplemented with various concentrations of auxins IAA, IBA, and NAA were examined.

Culture conditions

MS medium was fortified with 20 g/l sucrose (Qualigens, India) and gelled with 0.8% (w/v) agar (Sd-fine chemicals, India), and the pH was adjusted to 5.8 after adding the growth regulators. The media were steam sterilized in an autoclave under 15 psi and 121° C under a 16 h photoperiod supplied by cool white fluorescent tubes. At least fifteen cultures were raised for each treatment and all the experiments were performed three times.

Effect of cytokinins on shoot proliferation

MS basal media containing different concentrations of cytokinins (BA, KN, TDZ and zeatin) were investigated for their effects on shoot multiplication from nodal explants. Subculture was carried out at 4 weeks intervals, and shoot induction frequency was measured after 4 weeks. Repeated subculture was carried out on MS medium supplemented with BA 6.66 µM for shoot bud proliferation.

Shoots with 5 cm in height were separated and individual shoots transferred to rooting medium half strength MS medium containing different concentrations of IAA (indole-3-acetic acid), IBA (indole-3-butyric acid) and NAA (naphthalene acetic acid). The cultures were incubated under 16 h photoperiod for 30 days until the microshoots developed the roots. Then the rooting frequency was measured.

Acclimatization and transplantation of plantlets

The rooted plantlets were removed from the culture tubes and washed with tap water to remove trace of agar. Then the plantlets were planted onto plastic cups containing a mixture of finely chopped peatmass and sterilized garden manure in 1:1 ratio. The plastic cups were covered with transparent polythene cover to maintain humidity until the development of new leaves for 10 days. Then the plastic cups were transferred to green house and polythene covers were removed. Quarter strength MS major salts solution poured with 5 days intervals up to 40 days of hardening and followed by pouring of tap water. Hardened plants were transferred to pots containing mixture of garden soil and forest humus (1:1 ratio). The pots were watered two days interval under green house condition. After 60 days the frequency of survival were calculated.

Statistical analysis

Data were measured after 30 and 40 days for shoot multiplication and rooting respectively. Mean values with the same superscript were not significantly different (p = 0.05%) according to Duncan's Multiple range test (Duncan, 1955).

RESULTS AND DISCUSSION

Effect of cytokinins on shoot multiplication

The percentage of initial explants responding to culture varied according to the type and concentration of cytokinins (Table 1). All concentrations of tested cytokinins facilitated axillary bud induction with various frequencies of response. Axillary nodes remained green and fresh but failed to sprout on MS media without cytokinins. BA was the most efficient cytokinin for the axillary bud initiation and subsequent proliferation. BA alone at 6.66 µM induced a mean of 5.57 shoots per explant with 100 percent response (Table 1; Figures 1A and B). As BA concentration increased, shoot numbers decreased signi-

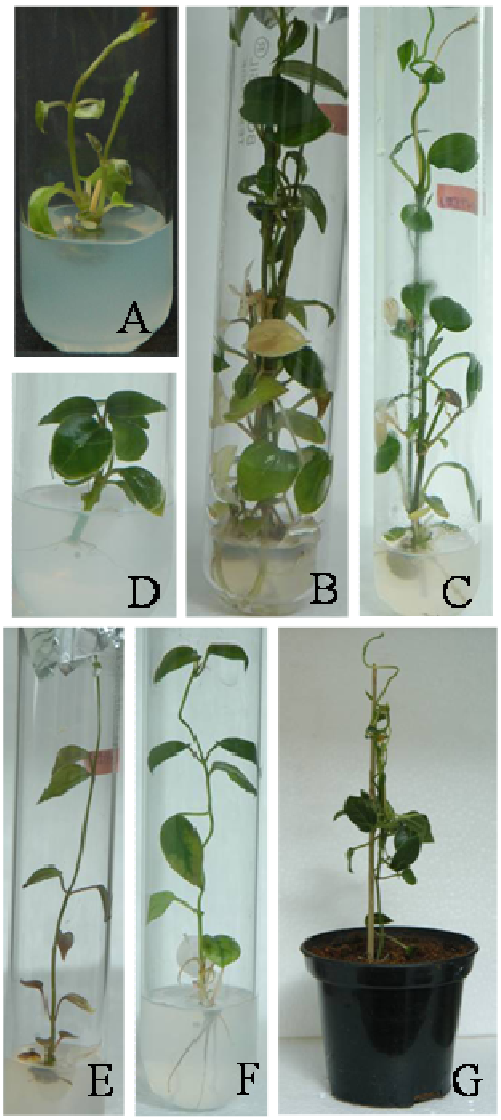

Figure 1. Micropropagation of *Ceropegia intermedia* through axillary bud multiplication. **A.** Axillary bud initiation on MS + BA 6.66 µM after 15 days culture. **B.** Axillary bud multiplication on MS + BA 6.66 µM after 30 days culture. **C.** Axillary bud multiplication on MS + KN 6.07 µM after 30 days culture. **D.** Axillary bud multiplication on MS + Zeatin 2.28 µM after 30 days culture. **E.** Axillary bud growth on MS + TDZ 2.27 µM after 30 days culture. **F.** *In vitro* rooting on ½ MS + NAA 5.37 µM after 40 days culture. **G.** Normal growth of micropropagated plant after one month of growth in green house condition.

Table 1. Axillary bud multiplication of *Ceropegia intermedia* on MS medium supplemented with different cytokinins after 30 days culture.

Cytokinins (µM)				Response (%)	No. of shoots/explant (mean ± SE)
BA	KN	TDZ	Zeatin		
Cytokinins free MS				No response	No response
4.44	--	--	--	80	2.13 ± 0.12^{c}
6.66	--	--	--	100	5.51 ± 0.18^{a}
8.87	--	--	--	100	3.31 ± 0.14^{b}
13.3	--	--	--	100	1.57 ± 0.09^{d}
22.2	--	--	--	90	1.46 ± 0.09^{d}
--	4.65	--	--	70	1.53 ± 0.09^{d}
--	6.07	--	--	80	2.66 ± 0.12^{c}
--	9.29	--	--	100	2.22 ± 0.14^{c}
--	23.2	--	--	90	1.37 ± 0.07^{d}
--	--	2.27	--	90	1.53 ± 0.10^{d}
--	--	4.54	--	100	1.66 ± 0.08^{d}
--	--	9.08	--	90	1.97 ± 0.07^{d}
--	--	22.7	--	80	1.88 ± 0.49^{d}
--	--	--	2.28	80	2.13 ± 0.10^{c}
--	--	--	9.12	90	1.71 ± 0.09^{d}
--		--	9.12	90	2.13 ± 0.08^{c}
--		--	22.8	90	1.33 ± 0.07^{d}

Data represent the mean of 15 replicates.
Means followed by different letters are significantly different at 5% level.

ficantly. Acceptable results (80%) are also obtained with KN 6.97 µM (2.66 shoots), and also gave shoots with longer internodes (Figure 1C). Zeatin induced a mean of 2.13 shoots at 2.28 µM, but all other concentrations induced a single stunted slightly vitrified shoot (Figure 1D). All concentrations of TDZ containing cultures induced a mean of single vitrified shoots invariably (Figure 1E). The shoot multiplication rate was highest in BA 6.66 µM when compared to all other tested cytokinins. Therefore, BA 6.66 µM was excluded from the subsequent subculture, multiplication and transplantation experiments.

In vitro propagation of plants belonging to Asclepiadaceae has also been shown to have optimum overall growth in MS medium containing BA (Komalavalli and Rao, 2000). Thus the degree of shoot multiplication varied considerably with the BA concentration. In the present study, 6.66 µM BA supplemented MS medium promoted maximum shoot multiplication frequency, whereas KN did not improve the shoot numbers significantly. Similar response was also observed in the shoot multiplication of *Ceropegia jainii* and *Ceropegia bulbosa* (Patil, 1998), *Ceropegia candelabrum* (Beena et al, 2003), and *Decalepis arayalpathra* (Gangaprasad et al., 2005). Superiority of BA for shoot multiplication in Asclepiadaceae has been reported in many other studies (Sudha et al., 1996; Raghu Ramulu et al., 2002). However, the poor performance of KN in our study is contradictory to the report on *Hemidesmus indicus*, another member of Asclepiadaceae (Patnaik and Debata, 1996).

In general, the effect of BA was slightly more pronounced than the effect of KN both in terms of axillary bud breaking and shoot multiplication per explant. The dependence of cytokinins and shoot multiplication frequency has already been established in *H. indicus* (Patnaik and Debata, 1996), *Holostemma annulare* (Sudha et al., 1996), *Holostemma ada-kodien* (Martin, 2002), and *C. bulbosa* (John Britto et al., 2003).

The main objective of this study was to establish a rapid micropropagation system for *C. intermedia* through axillary shoot multiplication. As the plant is a seasonally dormant twining climber, the first step was to select the best cytokinin for shoot bud induction. Nodal explants were used in this experiment because they provide identical clones with desired traits, which is important for conservation of rare and endangered taxa (Gangaprasad et al., 2005).

Shoot growth and quality on MS media supplemented with various concentration of different cytokinins was considerably varied in each cytokinin type. The shoots had only 1 - 3 internodes on average with thick leaves and more or less vitrified appearance in zeatin containing medium (Figure 1D). Growth of the regenerated shoots was optimal at a concentration of 4.44 – 13.3 µM BA (8.5 cm average), and the appearance of shoots was also normal. Growth response of shoots regenerated on KN containing

Table 2. Effect of subculture of *Ceropegia intermedia* microshoots on MS medium supplemented with BA 6.66 µM after 30 days interval.

Subculture	Shoots/explant (mean ± SE)	Length (cm) of shoots (mean ± SE)
1st	5.9 ± 0.19[c]	4.3 ± 0.08[b]
2nd	6.3 ± 0.18[b]	5.1 ± 0.09[a]
3rd	7.0 ± 0.19[a]	4.8 ± 0.05[a]
4th	4.5 ± 0.12[d]	4.1 ± 0.03[b]
5th	4.1 ± 0.12[d]	4.0 ± 0.03[b]

Data represent the mean of 15 replicates.
Means followed by different letters are significantly different at 5% level.

media was more or less similar to BA supplemented media. However, shoots regenerated on TDZ containing media developed slightly vitrified shoots and long internodes with slight pigmentation (Figure 1E).

In the presence study, the micropropagated shoots exhibited leaf and shoot bud abscission. Similar phenolmenon has also been encountered during *in vitro* shoot multiplication of other Asclepiadaceae members viz. *H. indicus* (Patnaik and Debata, 1996), *Gymnema sylvestre* (Komalavalli and Rao, 2000), and *C. candelabrum* (Beena et al., 2003), where addition of aminoacids, calcium and iron was essential to overcome the problem. In the present study, however, such supplements were not necessary because shoots showing leaf drop did not die. These shoots rooted normally and bore fresh leaves when transferred to pot after rooting.

In the present study, there is no need for separate shoot elongation medium as medium contains BA 6.66 µM itself shoots were elongated in normal growth. But some other Asclepiadaceae members, shoot elongation took place with medium containing BA and NAA combination in *G. sylvestre* (Komalavalli and Rao, 1997; Reddy et al., 1998), *Tylophora indica* (Sharma and Chandel, 1992), and *Decalepis hamiltonii* (Bais et al., 2000).

Subculture and shoot multiplication

Following the above step, shoot multiplication was continued by realizing successive subcultures of axillary buds on fresh medium of identical composition in order to determine the maximum number of times this could be done to obtain a large number of shoots without affecting their appearance and quality. For subculture, new shoots excised from *in vitro* developed microshoots cut into segments contained single node and cultured on MS medium supplemented with 6.66 µM BA. The sprouting rate of axillary buds remained stable (around 90%) during the four subcultures made on media containing 6.66 µM BA, with a slight tendency to increase as the number of transfers grew (Table 2). In subsequent subcultures, the number of shoots increased for the next 2 - 3 subcultures and reduced thereafter (Table 2). On the same media, the fifth subcultures affected 35% of explants by vitrification. The best multiplication rates were obtained during the third subculture in the media containing 6.66 µM BA. Hence from a single nodal explant it was possible to obtain a significant number of new shoots within a period of 3 months through limited subcultures (Table 2). The shoot multiplication at an enhanced pace by subse-quent subcultures observed in this study is in agreement with report on other Asclepiadaceae members, such as *G. sylvestre* (Komalavalli and Rao, 2000), *H. indicus* (Sreekumar et al., 2000), *H. ada-kodien* (Martin, 2002), and *D. arayalpathra* (Gang-aprasad et al., 2005). However, Patnaik and Debata (1996) reported a reduction in shoot numbers on *H. indicus*.

Rooting

Excised axillary node derived *in vitro* shoots (4 - 6 cm long) were rooted only upon transfer to half strength MS medium containing auxins, whereas no rooting was noted in hormone free ½ MS medium. Half strength MS medium supplemented with different auxins at different concentrations showed varied effect of rooting (Table 3). Of the three auxins tested, NAA 5.37 µM was most effective for root induction with minimum basal callus formation (Figure 1F). Rooting was observed from the cut ends of the microshoots within 15 days. The effectiveness of NAA in rooting has been reported for a few Asclepiadaceae species *D. hamiltonii* (Reddy et al., 2001; Anitha and Pullaiah, 2002). However, a single root emerged after 20 days in the presence of IBA that continued its linear growth without branches. Extensive callusing at the base without root formation and thin, delicate roots with intervention of callus were noticed when the medium was supplemented with different concentration of IAA. The shoots were cultured on ½ MS fortified with 5.37 µM NAA to improve the overall growth of roots and to reduce the basal callusing and time duration of root induction. In contrast, a drastic inhibitory effect of root induction was observed in ½ MS containing 9.80 µM IBA. But in a few Asclepiadaceae taxa reported the best rooting response in IBA containing medium (Martin, 2002; Beena et al., 2003; Gangaprasad et al., 2005).

Table 3. Effect of different auxins on rooting response of *Ceropegia intermedia* microshoots cultured on ½ MS medium after 40 days.

Auxin	concentration (µM)	No. of roots/shoot (mean ± SE)	Length (cm) of roots (mean ± SE)	Basal callusing
Auxin	Free medium	No rooting		
IAA	0.57	$1.4 \pm 0.08^{b*}$	2.1 ± 0.04^{c}	+
	2.85	2.5 ± 0.08^{a}	3.1 ± 0.11^{b}	+
	5.71	2.8 ± 0.22^{a}	1.3 ± 0.06^{c}	+
	11.4	$1.6 \pm 0.07^{b*}$	1.9 ± 0.07^{cd}	+
IBA	0.49	$1.0 \pm 0.04^{c*}$	1.1 ± 0.05^{c}	
	2.46	$1.3 \pm 0.07^{bc*}$	1.7 ± 0.07^{c}	
	4.90	$1.1 \pm 0.05^{c*}$	1.8 ± 0.06^{c}	+
	9.80	$1.0 \pm 0.04^{c*}$	1.4 ± 0.07^{c}	+
NAA	0.54	$1.3 \pm 0.07^{bc*}$	1.4 ± 0.08^{c}	
	2.69	2.2 ± 0.10^{ab}	2.1 ± 0.12^{c}	
	5.37	$2.4 \pm 0.67^{a*}$	4.3 ± 0.10^{a}	
	10.7	$1.0 \pm 0.03^{c*}$	2.4 ± 0.08^{c}	+

Data represent the mean of 15 replicates.
Means followed by different letters are significantly different at 5% level (* $p < 0.001$).
+ indicate basal callusing.

Acclimatization

During hardening, the gradual exposure of plants to conditions out side the polythene covering helped to conserve and develop a proper balance of relative humidity and thereby increased the rate of survival. About 74 well rooted plants (6 - 10 cm height) with 5 - 6 nodes and 10 expanded leaves were successfully transferred to soil. The regenerated plants did not show any detectable variation in morphological or growth characteristics when compared with the donor plants (Figure 1G).

Conclusion

In conclusion, the outline of protocol offers a potential system for improvement, conservation and micro-propagation of *C. intermedia* from nodal explants. MS medium containing 6.66 µM BA is the best for shoot proliferation. The use of axillary nodes for micro-propagation is beneficial than other explant types. Half strength MS basal medium supplemented with 5.37 µM NAA is the best for root induction.

REFERENCES

Anitha S, Pullaiah T (2002). *In vitro* propagation of *Decalepis hamiltonii*. J. Trop. Med. Plants. 1: 227-232.

Arora R, Bhojwani SS (1989). *In vitro* propagation and low temperature storage of *Saussurea lappa* C B Clarke, an endangered medicinal plant. Plant Cell Rep. 8: 44-47.

Ahmedullah M, Nayar MP (1986). Endemic Plants of Indian region Vol1. Botanical Survey of India, Calcutta, India. p.119.

Bais HP, George J, Ravishankar GA (2000). *In vitro* propagation of *Decalepis hamiltonii* Wight & Arn., an endangered shrub, through axillary bud cultures. Curr. Sci. 79: 408-410.

Beena MR, Martin KP, Kirti PB (2003). Hariharan, M.; Rapid *in vitro* propagation of medicinally important *Ceropegia candelabrum*. Plant Tissue Organ Cult. 72: 285-289.

Duncan DB (1955). Multiple range and multiple *F*-test. Biometrics 11: 1-42.

Gangaprasad A, Decruse SW, Seeni S, Nair GM (2005). Micropropagation of ecorestoration of *Decalepis arayalpathra* (Joseph &Chandra) Venter–An endemic and endangered ethnomedicinal plant of Western Ghats. Ind. J. Biotechnol. 4: 265-270.

Jagtap AP, Singh NP (1999). Fascicles of Flora of India, Fascicle 24.) Botanical Survey of India, Calcutta, India. pp. 211-241.

Jain SK, Defillips RA (1991). Asclepidaceae. In: Algonae MI (ed.). Medicinal plants of India Vol. 1. Reference Publication Inc., Michigan, USA. pp. 144-152.

John Britto S, Natrajan E, Arockiasamy DI (2003). *In vitro* flowering and shoot multiplication from nodal explants of *Ceropegia bulbosa* Roxb. var. *bulbosa*. Taiwania 48: 106-111.

Kirtikar KR, Basu BD (1935). Indian medicinal plants Vol. 3. M/s Bishen Sing Mahendrapal Singh, New Delhi, India. pp. 1638.

Komalavalli N, Rao MV (1997). *In vitro* propagation of *Gymnema sylvestre* W & A - a rare medicinal plant. Ind. J. Exp. Biol. 35: 1088-1092.

Komalavalli N, Rao MV (2000). *In vitro* Propagation of *Gymnema sylvestre* - A multipurpose medicinal plant. Plant Cell Tissue Organ Cult. 61: 97-105.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassay for tobacco tissue cultures. Physiol. Plant. 15: 473-497.

Nadkarni KM (1976). Indian Materia Medica Vol.1. Popular Prakashan, Bombay, India. pp. 303-304.

Nayar MP (1985). Meaning of Indian flowering plant names. Bishen singh Mahendrapal singh, Dehra Dun, India. p.78.

Nayar MP, Sastry ARK (1983) Red Data Book of Indian Plants. Botanical Survey of India. Calcutta. p. 58.

Mabberley DJ (1987). The Plant book. Cambridge University Press, Cambridge. p. 114.

Raghu Ramulu D, Murthy KSR, Pullaiah T (2002). *In vitro* propagation of *Cynanchum callialatum*. J. Trop.Med. Plants 3: 233-238.

Reddy PS, Gopal GR, Sita GL (1998). *In vitro* multiplication of *Gymnema sylvestre* R.Br. – A important medicinal plant. Curr. Sci. 75: 843-845.

Reddy BO, Gridhar P, Ravishankar GA (2001). *In vitro* rooting of *Decalepis hamiltonii* Wight & Arn., an endangered shrub by auxins and root-promoting agents. Curr. Sci. 81: 1479-1481.

Patnaik J, Debata BK (1996). Micropropagation of *Hemidesmus indicus* (L.) R.Br. through axillary bud culture. Plant Cell Rep. 15: 427-430.

Sharma N, Chandel KPS (1992). Effect of ascorbic acid on axillary shoot induction in *Tylophora indica* (Burm.f.) Merrill. Plant Cell Tissue Organ Cult. 29: 109-113.

Sreekumar S, Seeni S, Pushpangadan P (2000). Micropropagation of *Hemidesmus indicus* for cultivation and production of 2-hydroxy 4-methoxy benzaldehyde. Plant Cell Tissue Organ Cult. 62: 211-218.

Sudha CC, Krishnan PN, Pushpangadan P (1996). *In vitro* propagation of *Holostemma annulare* (Roxb.) Schum K, A rare medicinal plant. In Vitro Cell Dev. Biol. Plant 33: 57-63.