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Micropropagation of an endangered medicinal plant *Ceropegia spiralis* L.

Sri Rama Murthy, K.*, Kondamudi, R. and Vijayalakshmi, V.

School of Conservation Biology and Plant Biotechnology, Department of Biotechnology, Montessori Mahila Kalasala, Vijayawada - 520 010, Andhra Pradesh, India.

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Micropropagation protocol was developed for *Ceropegia spiralis* L. a species threatened by over exploitation due to its medicinal importance and habitat destruction in Southern Peninsular India. Multiple shoots (14.37±0.12) induction was more successful using nodes as explants on MS medium supplemented with BAP 2.22 µM induced multiple shoots after fourth subculture. *In vitro* flowering was observed on 0.5 MS medium with 3% sucrose supplemented with IAA 11.54 µM. The thin cell layers were obtained from the nodes and internodes of the plant and were cultured on the medium supplemented with BAP 13.32µM + NAA 0.537µM induced 17.34±0.55 shoots showing extensive growth. Later on the organogenesis was also induced on the medium containing BAP 13.32µM + 2, 4-D 1.130 µM. *In vitro* tuber formation by culturing both individual and multiple shoots on MS medium with 3% sucrose supplemented with BAP and different auxins at different concentrations, individually and in combination. Shoots developed were rooted best on 0.5 MS with NAA 10.74 µM. Optimum shoot and root multiplication was obtained within 8 weeks. *In vitro* plantlets were successfully weaned and transferred to soil with 90 % survival rate. Successfully, acclimatized plants were reintroduced into nature for their recovery. The present paper describes a simple and efficient protocol for the rapid and large-scale *in vitro* propagation of *Ceropegia spiralis* through nodes. To the best of our acquaintance, this is the first report on the micropropagation of *Ceropegia spiralis* using this technique.

Key words: *Ceropegia spiralis* (L.), caulogenesis, thin cell layers, *In vitro* flowering, microtuber formation, *In vitro* rooting, acclimatization.

Introduction

The genus *Ceropegia* (Asclepiadaceae) was reported by 200 species distributed in the tropical and subtropical Asia, Africa, Australia and Malaysia and in the Canary and Pacific islands (Anonymous, 1992; Bruyns, 2003). In India 48 spp. were found, out of which 28 spp. are endemic to the peninsular

*Corresponding author: K. Sri Rama Murthy; e-mail: drksrmurthy@yahoo.com

region (Ansari, 1984; Ahmedulla and Nayar, 1986). *Ceropegia spiralis* L. is an annual herb grown wildly in south India and is in endangered category (Nayar and Sastry, 1987; Madhav Gadgil, 2004), popularly knew to villagers, herbalists, as “Nimmati gadda”. A slender erect or slightly twining herb up to 50 cm long, with depressed tubers. Leaves opposite, sessile, linear, entire, base and apex often curved and twisted at the tip, chartaceous. Flowers solitary or cymes, calyx lobes 5, linear, corolla lobes 5, greenish yellow with purple stripes below, obovate, beaks spirally contorted with long deciduous, purple hair, pollinia oblong. Fruits 2, slender, follicular mericarps. Flowering and fruiting are in between May to October months

Tuberous roots of many *Ceropegia* species are edible (Anonymous, 1992). The root tubers are the officinal parts known to contain an alkaloid called “Ceropegin” (Nadkarni, 1976) bitterness of the tubers was eliminated by boiling and then consumed (Mabberley, 1987). The *Ceropegia spiralis* root tubers known to contain starch, sugars, gum, albuminoids, fats, crude fiber and valuable constituents in many traditional Indian Ayurvedic drug preparations that are active against many diseases especially diarrhea, dysentery and the starchy tubers are useful as a nutritive tonic (Kirtikar and Basu, 1935; Reddy *et al.,* 2006; Chopra *et al.,* 1956). In this genus *C. bulbosa* and *C. candelabrum* also have medicinal properties (Jain and Defillips, 1991). Several reports were published on the *in vitro* studies of *Ceropegia* species i.e., *C. candelabrum* (Beena and Martin 2003; Beena *et al*., 2003), *C. bulbosa* var. *bulbosa* (Britto *et al.*, 2003), *C. bulbosa* (Goyal and Bhadauria, 2006), *C. jainii*, *C. bulbosa*, *C. bulbosa* var. *lushii* (Patil, 1998) and *C. sahyadrica* (Nikam and Savanth, 2007). However, to date, there are no reports on the micropropagation of *Ceropegia spiralis* through it is an important edible tuberous asclepiad in the Southern Peninsular India. The aim of the present investigation was to develop systems for *in vitro* propagation to conserve and domesticate the wild endemic taxa *C. spiralis*.

Materials and methods

Plant materials

The wild *Ceropegia spiralis* (*Nimmati gadda*) was collected from Akashaganga of Tirumala hills, Eastern Ghats, India. The voucher photograph was deposited in the herbarium of Department of Biotechnology, Montessori Mahila Kalasala, Vijayawada, Andhra Pradesh, India. The nodes containing axillary buds were washed in the running tap water followed by a fungicide and bactericide each 0.3% for 10 min and with tween 20 (5% v/v for 4 min). Then with surface disinfectant $HgCl_2$ (0.1% w/v for 2 min) after repeated washes in double distilled water, the sterilized segments were then washed thoroughly with

sterilized distilled water, cut in to appropriate sizes, and cultured on nutrient medium. Before placing onto MS medium (1962) solidified with agar 0.9% (w/v) HiMedia Laboratories Pvt. Ltd. Mumbai and different growth regulators (BA, Kinetin, NAA, IAA, IBA) at different concentrations either alone or in combinations were added to the medium. In the present investigation all the media were autoclaved at 121˚C and 15 lbs pressure for 20 min after adjustment of the pH to 5.7±1 with 1N NaOH and 1N HCl.

All the cultures were maintained at 24±2˚C under 16hrs photoperiod with 3000 lux light intensity using fluorescent lights (Philips India Ltd.,) and 60-70% relative humidity within 250 ml Bottles and 25×150 mm culture tubes covered with the aluminum foil. When the hormones failed to induce a specific response (callus, adventitious shoots/roots) at the end of the first cycle, it was not considered as suitable combination. Twenty cultures were raised for each treatment and all experiments were repeated thrice.

Caullogenesis

The nodes of 0.5-1.0 cm containing axillary buds were inoculated on the MS medium (Murashige and Skoog, 1962) containing BAP and Kn alone at various concentrations for shoot proliferation, within 10 days rapid growth has been observed. Sub cultured after every 28-30 days, number of multiple shoots are in directly proportional up to five subcultures.

Organogenesis and thin cell layer cultures (TCLs)

Both the types of TCLs (lTCLs and tTCLs) were employed in the present investigation, the explants were excised from the nodal and internodal meristematic regions, 4 TCLs (2 from each side) were taken from either sides of the node. Only tTCLs response is considerable. The sections of the explant were placed on the MS medium supplemented BAP in combination with different auxins to induce the callus, organogenesis and somatic embryos.

In vitro flowering

Well-developed shoots were inoculated on the MS media supplemented with different compositions and combinations containing auxins alone for rooting and the plantlets were observed for flowering too. Hence, some of the plantlets were transferred to low light intensity as it favors the flowering. Here particularly, the temperature also plays a great role in the induction of the flowering as the chilling effect favors.

In vitro tuberization

In vitro tubers formation was done by culturing both individual and multiple shoots on MS medium with 3% sucrose supplemented with BAP and auxins at different concentrations, individually and in combination. The microtubers were observed after 15 days of incubation. Cultures treated with BAP 13.32 µM along with the combination of NAA 10.74 µM/ 2.68 µM to show the highest percentage of microtuber formation. The plants growing on the 0.5 MS medium containing NAA 10.74 µM + 3% sucrose were to exhibit aerial tuberization, whereas plants growing on 0.5 MS medium containing IBA 49.2 µM + 6% sucrose and 0.5 MS with IAA 11.54 µM were to produce tubberized roots.

In vitro rooting

Two to three centimeters of well-developed shoots were inoculated on the different media containing auxins alone. 0.5 MS medium with 3% sucrose supplemented with NAA 10.74 µM with 10 roots the Maximum root initiation (85%) was observed. The combinations of two different auxins (NAA+IBA data not presented) were also studied.

Acclimatization

Micro shoots with well-developed root system were transferred directly to small pots containing sterile vermiculite and coco peat in (1:1) ratio rejuvenated growth within 20 days. Survival rate of the plantlets and plantlets established in the field were checked and compared to mother plants. Starting from a single plant, 650 rooted shoots obtained within four months.

Experimental design and data statistical analysis

All experiments were carried out in a randomized design, twenty replicates were raised for each treatment and experiments were repeated thrice. Visual observation were made every day with 5 and 10x magnified lenses. The growth of the plant was recorded every day. The data were analyzed statistically using one way analysis of variance (ANOVA), and the data means ± SD of at least three different experiments were represented and compared using Tukey-Kramer multiple comparisons test with the level of significant $P = 0.05$.

Results and discussion

Caullogenesis

The morphogenetic responses of nodal explants to various cytokinins (BAP, Kinetin) were evaluated (Table 1). Nodal explants cultured on growth regulator free MS medium showed no sign of bud break even after two weeks. Addition of a cytokinin was essential to induce bud break and multiple shoot formation from the explant. In the present study, the presence of an axillary bud was essential for the development and induction of multiple shoots in *C. spiralis*. Of the two cytokinins tested, BAP was more effective than Kn.

The nodal segments responded by an initial enlargement of the dormant axillary buds followed by bud break within a week, and multiple shoot induction and proliferation (Fig. 1A) with in 4 weeks of culture on BAP containing media. The new shoots developed adjacent to the axillary shoots. The frequency of axillary shoot proliferation and the number of shoots per explant increased with increasing concentration of BAP up to some extent, (Table 1). BAP 2.22 µM showed the highest shoot regeneration ability and number of regenerated shoots were (14.37±0.12). Thus, BAP when added singly in the medium was the most effective plant growth regulator. Indicating, the cytokinin specificity of nodal explants of *C. spiralis* for multiple shoot formation has been reported earlier for several *Ceropegia* species. These results are in consonant with the multiple shoot induction in *C.jainii and C.bulbosa* (Patil, 1998), *C. bulbosa* var. *bulbosa* (Britto *et al*., 2003; Goyal and Bhadauria, 2006), *C. sahyadrica* (Nikam and Savanth, 2007). In contrast to the synergistic effect of BAP in combination with an auxin has been reported in *C. candelabrum* (Beena *et al.,* 2003) and other Asclepiad *Holostemma annulare* (Sudha *et al.,* 1998), *Hemidesmus indica* (Sreekumar *et al.,* 2000), *Holostemma ada-kodien* (Martin, 2002). The endogenous levels of growth regulators in the members of Asclepiadaceae might be responsible for the observation of variation in the response and growth regulator requirement for *in vitro* shoot regeneration.

Thin cell layer culture and Organogenesis

The tTCLs are capable to produce more number of shoots. However, there is no report on plant regeneration system using TCL culture in Asclepiadaceae. This is the first report from the *C. spiralis*. In the current investigation, the shoot regeneration was noticed using tTCLs isolated from the node explants. The combination of BAP 13.32 µM+ NAA 0.537 µM was quite suitable for adventitious shoot (17.34 ± 0.55) development from callus (Table 2 and Fig.

1B). The tTCL explants from *in vitro* grown plants swelled after 4-5 days of culture due to small amount of light green callus proliferation on the sub epidermal area. Shoots regeneration occurred from tTCL explants that appeared green and formed a peripheral crown of buds which elongated rapidly within a week. By this technique, we have produced more than 20 plants from a single node. In the present study, tTCLs were quite active in callogenesis. This callus was organogenic and embryogenic capable to produce bipolar structures called somatic embryos, which in turn gave rise to emblings (Fig. 1F). 2,4-D at (0.45, 1.13, 2.26, 4.52 µM) in combination with BAP produced extensive callus. Similar results were also reported in *Lilium longifolium* (Nhut *et al.,* 2001), Tobacco spp. (Cremer-Molenaar *et al.,* 1994), *Chrysanthemum* (Teixeira and Fukai, 2003) *Heliconia psittacorum* (Goh *et al.,* 1995), *Pelargonium* (Gill *et al*., 1992).

Table 1. Influence of Cytokinins on the shoot regeneration in *Ceropegia spiralis.*

PGRs	PGR in µM	Shoot no Mean ± SD	Shoot length Mean ± SD
BAP	0.0444	5.50±0.12^{c}	4.87±0.82$^{c\,d}$
	0.222	3.58±0.08^{d}	8.54±0.17$^{b\,c}$
	0.444	6.35±0.08^{c}	13.49±0.22^{a}
	0.888	5.23±0.12^{c}	6.57±0.09^{c}
	1.332	5.21±0.07^{c}	7.50±0.14^{c}
	1.776	5.84±0.10^{c}	7.23±0.15^{c}
	2.22	14.37±0.12^{a}	6.56±0.16^{c}
	2.664	10.76±0.11^{b}	8.42±0.10$^{b\,c}$
	3.108	9.64±0.08^{b}	9.00±0.24^{b}
	3.552	8.79±0.12$^{b\,c}$	10.30±0.34^{b}
Kn	0.0456	3.49±0.09^{d}	8.52±0.18$^{b\,c}$
	0.228	2.34±0.11^{d}	5.75±0.25^{c}
	0.456	2.67±0.12^{d}	5.49±0.28^{c}
	0.912	5.11±0.12^{c}	7.48±0.21^{c}
	1.368	4.10±0.11^{c}	7.04±0.26^{c}
	1.824	4.15±0.09$^{c\,d}$	6.63±0.23^{c}
	2.28	4.54±0.08$^{c\,d}$	5.57±0.19^{c}
	2.736	4.59±0.14$^{c\,d}$	5.71±0.22^{c}
	3.192	4.44±0.08$^{c\,d}$	6.05±0.21^{c}
	3.648	5.50±0.12^{c}	6.44±0.15^{c}

Data indicate mean ± standard deviation following by the same letter was not significantly different by the Tukey-Kramer multiple comparisons test at 0.05% probability. Twenty replicated were used per treatment experiments were repeated thrice.

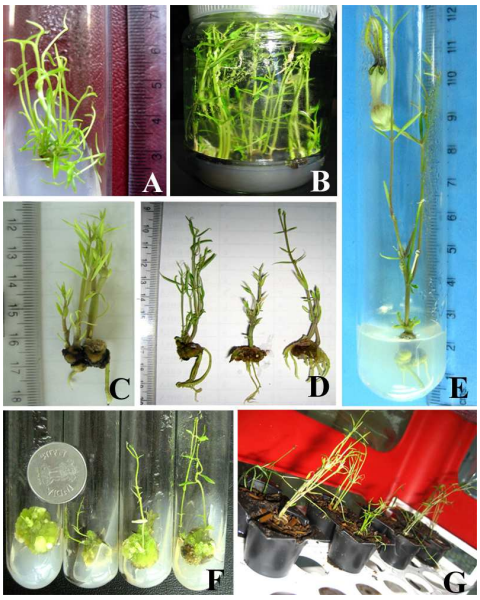

Fig.1. Micropropagation of *Ceropegia spiralis* A: Multiple shoot initiation from nodal explant cultured on MS medium fortified with BAP 2.22 µM. B: Plantlets raised from TCLs on MS medium containing BAP 13.32 µM+NAA 1.11 µM. C: *In vitro* tuberization of *C.spiralis* on MS salts + 3% sucrose + BAP 13.32 µM + NAA 2.68µM D*: In vitro* raised shoots showing roots on 0.5 MS medium supplemented with NAA 10.74 µM. E: Flowering *in vitro* on the 0.5 MS salts + 3% sucrose + IAA 11.54 µM F: Different stages of shoot buds regeneration from callus, derived from the *in vitro* grown nodes cultured on MS + BAP 13.32 µM + 2,4-D 1.13 µM G: Acclimatized *in vitro* grown plantlets of *Ceropegia spiralis*.

Table 2. Effect of BAP and Auxins on shoot regeneration from *Ceropegia spiralis* thin cell layers.

BAP µM/l	Auxins in µM/l				No of shoots Mean ± SD	Length of shoots Mean ± SD	Basal Callusing
	2,4-D	IAA	IBA	NAA			
13.32	0.452				6.79± 1.52^{c}	4.50±0.18 ^{d}	++++
13.32	1.130				11.35±2.56 ^{b}	4.25±0.36 ^{d}	++++
13.32	2.26				6.42± 0.57 ^{c}	1.10±0.33 ^{d}	++++
13.32	4.52				3.58±0.23 ^{d}	1.28±0.33 ^{d}	++++
13.32		0.577			5.17±0.32 ^{d}	11.90±0.84 ^{b}	+++
13.32		1.44			3.59±0.35 ^{d}	8.20±0.24 ^{c}	+++
13.32		2.88			5.89±0.35 $^{c d}$	5.05±0.14 ^{c}	++
13.32		5.77			7.01±0.53 ^{c}	8.56±0.19 ^{b}	+++
13.32			0.49		2.04±0.3 ^{d}	0.59±0.18 ^{d}	+
13.32			1.23		5.56±0.58 ^{c}	5.43±0.52 ^{c}	+++
13.32			2.46		6.05±0.57 ^{c}	15.09±0.66 ^{a}	++
13.32			4.92		10.50±0.62 ^{b}	8.29±0.12 ^{bc}	+
13.32				0.537	17.34±0.55 ^{a}	10.32±0.25 ^{b}	++++
13.32				1.342	6.00±0.48 ^{c}	9.87±0.50 ^{b}	+++
13.32				2.685	7.34±0.36 ^{c}	6.53±0.14 ^{c}	+++
13.32				5.37	5.44±0.42 ^{d}	7.04±0.33 ^{c}	++++

Data indicate mean ± standard deviation following by the same letter was not significantly different by the Tukey-Kramer multiple comparisons test at 0.05 % probability. Twenty replicated were used per treatment experiments were repeated thrice.

In vitro flowering

The tissue culture of *C. spiralis* was not only favored rapid multiplication but was also characterized by seasonal *in vitro* flowering that synchrony with the plants growing in the wild. *In vitro* flowering was observed on ½MS medium with 3% sucrose supplemented with IAA 11.54 µM. Flowers were formed within a period of 35 days when the explants were transferred to the rooting medium and observed 33% rooting with a mean of 6.06±0.05 (Table 3 and Fig. 1E). The induced flower buds attained full bloom only when the cultures were transferred to low light intensity regime 2000 lux within a period of 4 days. It was observed that under 16/8 h light / dark cycle buds failed to bloom and died off. Therefore, day length plays a crucial role in flower induction both *in vivo* and *in vitro* possibly due to altered photosynthetic turnover on flowering (Pierike, 1967). *In vitro* flowering in *Ceropegia* spp. were also reported in C. *bulbosa* var. *bulbosa* (Britto *et al*., 2003) and *C. jainii* (Patil., 1998) and other species *Lycopersicon esculentum* (Sheeja and Mandal, 2003), *Spathoglottis plicata* (Murthy *et al.,* 2006). Thus, the present results are comparable to those of the early study even though all the flowers were observed on the rooting media, only some combinations were favourable for the enlargement of the flowers. This investigation, MS medium of full and quarter strength supplemented with IBA 49.2 µM with 6% sucrose was not at all suitable for the flowering. The half strength medium with IBA and Kn combinations failed in the induction of flowering and rooting.

Table 3. Effect of different auxins on *in vitro* flowering in *Ceropegia spiralis.*

Conc. of the PGR in µM/l				Media	Sucrose	Flowers	Response
IBA	IAA	NAA	Kn	strength	%	Mean ± SD	%
49.2				0.50	6%	16.19±0.49 [a]	50 [c]
4.92				0.50	3%	2.21±0.34 [d]	33 [c]
9.84				0.50	3%	5.18 ±0.27 [c]	50 [c]
49.2				0.50	3%	3.10±0.19 [d]	33 [c]
	5.77			0.50	3%	1.14±0.17 [d]	16 [d]
	11.54			0.50	3%	2.11±0.16 [d]	33 [c]
	46.16			0.50	3%	1.16±0.23 [d]	16 [d]
		5.37		0.50	3%	1.07±0.19 [d]	16 [d]
		10.74		0.50	3%	10.14±0.37 [b]	84 [a]
		53.7		0.50	3%	2.04±0.36 [d]	33 [c]
4.92		26.85		0.50	6%	1.16±0.44 [d]	16 [d]
9.84		21.48		0.50	6%	1.13 ±0.26 [d]	16 [d]
14.76		16.11		0.50	6%	3.06±0.18 [d]	33 [c]
19.68		10.74		0.50	6%	4.98±0.18 [c]	50 [c]
24.6		5.37		0.50	6%	3.00±0.10 [d]	33 [c]

Data indicate mean ± standard deviation following by the same letter was not Significantly different by the Tukey-Kramer multiple comparisons test at 0.05 % probability. Twenty replicated were used per treatment experiments were repeated thrice.

In vitro tuberization

Cultures treated with BAP 13.32 µM along with the combination of NAA 2.682 µM showed the 84 percentage of microtuber formation (Table 4 and Fig. 1C). The similar effects were observed in, *Dioscorea japonica* (Islam *et al.*, 2008) performed better at low concentration of NAA along with a higher concentration of sucrose and the presence of BAP. On ½ MS medium with 3% sucrose supplemented with IBA at 4.92, 9.84, 49.2 µM and IAA at 5.77, 11.54 µM showed no response with respect to tuberization. In another case, on full strength MS medium with 3% sucrose containing BAP at 13.32 µM with the IBA concentrations of 0.49, 1.23, 2.46, 4.92 µM also showed no response. However, the 3 % sucrose has a remarkable role in the tuberization, it was found to be optimum for microtuber development in *C. spiralis*. Whereas an increase or decrease in sucrose concentration resulted in inhibition in microtuber formation. Whereas the plants growing in the medium containing IBA and Kn were able to produce the tubers with low intensity. In *Dioscorea bulbifera* (Mantell and Hugo, 1989), *D. cayenensis* and *D. rotundata* (Ovono *et al*., 2007), *Ceropegia* spp., (Patil, 1998) increased levels of sucrose (8%) has negative effect in the formation of the micro tubers independently of the presence of Kn. But dissimilar effects has been reported in case of *C. jainii*, *C. bulbosa* var. *bulbosa*, var. *lushii* (Britto *et al.*, 2003; Patil, 1998) and *D. alata* and *D.bulbifera* (Mantell and Hugo, 1989). The internodal regions of the plant were able to form aerial tubers on 0.5 MS supplemented with IBA 49.2 µM with 6% sucrose. Single root tuberization also observed that some of the roots were getting tubberized after rooting. 0.5 MS + IAA 11.54 µM with 3% sucrose.

In vitro rooting

Various combinations of media were used for *in vitro* rooting and the results were presented in Table 5 and Fig.1D. Shoots were developed on 0.5 MS supplemented with NAA 10.74 µM. IBA concentration increased, the formation of root initials also increased up to 49.2 µM. When compared to other auxins NAA also showed the highest rooting efficiency. IBA induced more number of root initials, whereas the roots induced in the NAA were long but less in number. However, the combinations of two different auxins IBA+NAA had the cumulative action played a great role in the induction and elongation of roots. As the strength of the medium decreased, the plant was able to absorb sufficient amount of the nutrients by bringing physiological changes in the form of roots. The rooting medium containing IBA 14.76 µM along with NAA 16.11 µM was capable of producing starchy tubers in *Ceropegia spiralis*.

Table 4. Influence of medium strength, sucrose concentration and plant growth regulators on *in vitro* tuberization in *Ceropegia spiralis*.

Conc. of the PGR in µM/l						Medium strength	Sucrose %	Diameter of the tuber Mean± SD
Kn	IBA	IAA	NAA	2-4,D	BAP			
	49.2					0.50	6%	1.54±1.50 [b]
		46.16				0.50	3%	0.50±0.06 [d]
			5.37			0.50	3%	1.47±0.22 [cb]
			10.74			0.50	3%	2.10±0.28 [a]
			53.7			0.50	3%	0.00±0.00 [d]
4.56	4.92					0.50	3%	0.89±0.17 [c]
9.12	9.84					0.50	3%	1.44±0.21 [b]
		0.577			13.32	Full	3%	0.54±0.08 [d]
		1.44			13.32	Full	3%	0.63±0.10 [d]
		2.88			13.32	Full	3%	0.74±0.07 [cd]
		5.77			13.32	Full	3%	0.55±0.07 [d]
			0.537		13.32	Full	3%	0.00±0.00 [d]
			1.342		13.32	Full	3%	2.00±0.48 [a]
			2.685		13.32	Full	3%	2.00±0.48 [a]
			5.37		13.32	Full	3%	0.00±0.00 [d]
				0.452	13.32	Full	3%	0.71±0.14 [cd]
				1.13	13.32	Full	3%	0.74±0.06 [c]
				2.26	13.32	Full	3%	0.80±0.23 [c]
				4.52	13.32	Full	3%	0.53±0.15 [d]

Data indicate mean ± standard deviation following by the same letter was not significantly different by the Tukey-Kramer multiple comparisons test at 0.05 % probability. Twenty replicated were used per treatment experiments were repeated thrice.

Table 5. Influence of different combinations of Auxins on Rooting in *Ceropegia spiralis*.

Conc. of the PGR in µM/l				Media strength	Sucrose %	Total no of roots Mean ± SD	Response %
IBA	IAA	NAA	Kn				
49.2				0.25	6%	6.56±0.09 [b]	85 [a]
49.2				0.50	6%	7.31±0.08 [b]	50 [c]
4.92				0.50	3%	2.33±0.10 [c]	56 [c]
9.84				0.50	3%	2.50±0.07 [c]	33 [c]
49.2				0.50	3%	6.50±0.06 [b]	33 [c]
	11.54			0.50	3%	6.06±0.05 [b]	33 [c]
	46.16			0.50	3%	3.04±0.07 [c]	33 [c]
		5.37		0.50	3%	8.70±0.06 [a]	66 [bc]
		10.74		0.50	3%	8.44±0.07 [a]	85 [a]
		53.7		0.50	3%	7.01±0.08 [b]	85 [a]
4.92		26.85		0.50	6%	4.58±0.08 [b]	85 [a]
9.84		21.48		0.50	6%	4.52±0.08 [b]	85 [a]
14.76		16.11		0.50	6%	7.34±0.10 [b]	90 [a]
19.68		10.74		0.50	6%	4.48±0.06 [b]	88 [a]
24.6		5.37		0.50	6%	4.76±0.08 [b]	80 [a]

Data indicate mean ± standard deviation following by the same letter was not significantly different by the Tukey-Kramer multiple comparisons test at 0.05 % probability. Twenty replicated were used per treatment experiments were repeated thrice.

Acclimatization

The well developed plantlets were transferred to the cups containing autoclaved vermiculite and coco peat in 1:1 ratio. The plantlets were acclimatized in a mist chamber. The humidity 95 % was maintained in the initial days. Later on, the percent of humidity was decreased by pricking the mist chamber cover with the needle. On 30th day, the plants were transferred to the pots. The survival rate of plantlets was 85% (Fig. 1G). The plantlets were successfully adapted to the natural environment and exhibited its similarity with that of mother plants.

The present study reports successful micropropagation protocol that can be employed in the propagation of endemic taxa *Ceropegia spiralis* and helps in conservation and domestication.

Abnormalities noticed

In our investigation, the *in vitro* raised shoots exhibited leaf and shoot tip abscission. Similar effect was noticed during the *in vitro* multiplication of other Asclepiadaceae members like *Ceropegia candelabrum* (Beena *et al*., 2003); *Hemidesmus indica* (Patnaik and Debata, 1996); *Gymnema sylvestre* (Komalavalli and Rao, 2000). Shoot tip abscission occurred at the node region below the shoot tip. Nevertheless, the shoots resumed growth by the initiation of axillary bud of the node at which the shoot tips abscised as reported in *Ceropegia candelabrum* (Beena *et al*., 2003); *Holostemma adakodien* (Martin, 2003). Leaf and shoot tip abscission effected the multiplication of shoots. Accumulation of ethylene in the culture tubes could be caused necrosis and abscission of leaves and shoot tips.

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