Research Article

Multiple shoot regeneration and alkaloid cerpegin accumulation in callus culture of *Ceropegia juncea* Roxb.

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ABSTRACT

This is the first report of *in vitro* propagation and alkaloid accumulation in callus cultures of *Ceropegia juncea* Roxb. a source of "Soma" drug in Ayurvedic medicine. Multiple shoots and callus induction was optimized by studying the influence of auxins [IAA (Indole-3-acetic acid), NAA (2-Naphthalene acetic acid) and 2,4-D (2,4-Dichlorophenoxyacetic acid.)] and cytokinins [BA (6-benzyladenine) and Kin (Kinetin)] alone and in combinations. The best response for multiple shoot induction was obtained in nodal explants on MS medium supplemented with 7.5 μM Kin (8.5 ± 3 shoots per explants). The shoots were rooted on half strength MS (Murashige and Skoog's) medium fortified with either IAA or NAA (0.5-2.0 μM). The plantlets were transferred directly to the field with 100 % success rate. Supplementation of MS medium with auxins and cytokinins enhanced the growth of callus but inhibited the shoot regeneration in nodal explants. Best callus induction and proliferation observed on MS + 1 μM 2,4-D+5 μM BA. However the maximum cerpegin content (470 μg/g dry weight) was recorded in dried callus derived on MS+10 μM IAA+5 μM BA. Quantitative TLC (Thin layer chromatography) studies of the callus revealed a phytochemical profile similar to that of naturally grown plants. The calli were maintained by subculturing at 4 weeks interval on fresh parent medium over a period of 34 months. The optimized *in vitro* propagation and callus culture protocol offers the possibilities of using organ/callus culture technique for vegetative propagation and production of cerpegin alkaloid. **[Physiol. Mol. Biol. Plants 2009; 15(1) : 71-77]** *E-mail : tdnikam@unipune.ernet.in*

Key words : *In vitro propagation, Pyridone alkaloid, Cerpegin, Callus, Ceropegia juncea*

INTRODUCTION

Ceropegia juncea Roxb. (Asclepiadaceae) is an important medicinal herb, which is used as a source of "Soma" a plant drug of the Ayurvedic medicine with a wide variety of uses (Asolkar *et al.,* 1992; Jagtap and Singh, 1999). The fleshy stem is used as a raw material for traditional and folk medicines for the treatments of stomach and gastric disorders (Jain, 1991). The alkaloid cerpegin was isolated and identified as pyridone type alkaloid, which are relatively rare in nature (Adibatti *et al*., 1991). The total alkaloidal fraction exhibited promising hepatoprotective, antipyretic, analgesic, local anesthetic, anti-ulcer, mast-cell stabilizing, tranquillsing and hypotensive activities and was devoid of side effects as noted out by the sub-acute toxicity studies (Adibatti *et al*., 1991). Although *C. juncea* is an important source of Ayurvedic drug, but due to the lack of proper cultivation practice, low number of seed formation, destruction of plant habitats and its removal is leading to a progressive devastation of the species.

Advanced biotechnological methods of culturing plant cells and tissues can be seen as an important tool for production of medicinally valuable metabolites and propagation and conservation of plant species, which could be used for sustainable production to suit commercial demand and thereby hopefully reducing the illegal trade of plants collected in nature (Thorpe, 2007). In the present study, we report a simple and reproducible method of micropropagation, induction of callus and its use for alkaloid production in *C. juncea* Roxb. facilitates the conservation of this important plant species and establishments of alkaloid cerpegin production from callus cultures.

MATERIALS AND METHODS

Materials

C. juncea was collected from a naturalized population in

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Satmala mountains (near Ellora caves), western Ghats, Maharashtra, India. These plants were grown in the botanical garden of the department of Botany, University of Pune and were used as a source of node and internode explants. The explants were washed thoroughly in sterile water; surface sterilized with 0.1 % (W/V) $HgCl_2$ solution for seven minutes, and rinsed four times with sterile distilled water.

Culture medium and culture condition

Sterilized node and internode explants (15 mm) were cultured on MS (Murashige and Skoog, 1962) medium supplemented with 0, 0.5, 1.0, 2.0, 5.0, 7.5, 10, 12.5, 18, 22 μM Cytokinins (BA, Kin) and auxins (IAA, NAA and 2,4-D), individually and in combinations. The pH of the media was adjusted to 5.8 before addition of agar (8 % w/v) The media were sterilized by autoclaving at 121 ^{0}C and 1.05 Kg cm^{-2} for fifteen minutes. The cultures were incubated in a growth room at 25 ± 2 ^{0}C and with 8 h light 16 h dark cycle and light intensity of 20-30 μmole $m^{-2}s^{-1}$ from cool light fluorescent tubes (Philips India Ltd., Mumbai, India).

Callus induction, maintenance and plant regeneration

The greenish friable calli from the nodal and internodal explants were maintained by repeated subculturing after every four weeks. Subculturing was done with 300 ± 10 mg of fresh calli samples on agar-solidified extensive callus-inducing medium (Table 2). For regeneration studies, calli were cultured on solidified MS medium fortified with various levels of BA/Kin (5.0-12.5 μM) alone and in combination with auxins IAA/NAA (5.0-12.5 μM).

Growth measurements

Pre-weighed fresh calli were dried separately at 45 ^{0}C in hot air oven until constant dry weight was obtained. Results were expressed as dry weight (g) per culture. Cerpegin was extracted from the calli grown on various media.

Rooting of shoots and transfer of plants to the field conditions

For root induction, individual shoots 3-5 cm in length and having 2-3 nodes were separated and transferred directly to soil; to MS medium devoid of growth regulators and fortified with different concentrations (0.5-2.0 μM) of IAA and NAA individually. The *in vitro* rooted shoots were washed with slow running tap water to remove agar and transferred to pots containing garden soil.

Extraction of alkaloid cerpegin and chromatographic separation

Cerpegin was extracted from powdered callus samples for 24 h in 90 % ethanol (v/v) by using a rotary shaker (Adibatti *et al*., 1991) The filtrate was condensed *in vacuo* to a syrupy mass and mixed with 10 ml 1 % sulphuric acid. The acidic extract was cooled and mixed with 10 ml chloroform in a separating funnel. The acidic phase was separated out and made basic (pH 10) by adding 4-6 ml ammonia solution. The basic aqueous phase was again extracted with chloroform. The chloroform layer was separated and evaporated to dryness. The dried sample was dissolved in 3 ml chloroform and the total aliquot was applied on a TLC plate (0.2-0.3 mm thick with silica gel 'G', E-Merk). The plates were developed in a solvent mixture of chloroform:methanol:20 % aqueous ammonia (30:60:2). The separated alkaloid spot was recognized by application of Dragendroff's reagent at the edge of the TLC plate. The silica particles with alkaloids were scrapped from the TLC plates, dissolved in chloroform, and separated by centrifugation. The cerpegin fraction was evaporated to dryness and the weight of cerpegin was taken and expressed as μg/g of dry weight. The reference alkaloid fraction was obtained from the naturally grown plant material by following a similar procedure.

Statistical analysis

All the experiments were arranged in completely random design (CDR) and conducted at least thrice. Data from each of the experiments were subjected to analysis of varience (ANOVA). Means differing significantly were compared using Duncans (1955) Multiple Range Test (DMRT) at 5 % probability level.

RESULTS AND DISCUSSION

Callus cultures

Effect of various auxins (IAA, NAA and 2,4-D) and cytokinins (BA, Kin) at different concentrations were studied by adding them individually as well as in combinations (Table 1, 2). The response varied with the type of explants, growth regulators and their concentrations (Table 1). Additions of 1.0-7.5 μM IAA and 1.0-5.0 μM NAA induced formation of direct roots, mainly from the cut portions of the explants. Inclusion of higher levels of IAA (10.0-12.5 μM) or NAA (7.5-12.5 μM) elicited formation of calli from the surface of the

Table 1. Effect of growth regulators on *in vitro* shoot induction in *C. juncea* after 4 weeks of culture.

Growth regulators (μM)					Nodal explants which form shoot %	Number of shoots/explant Mean±SE	
BAP	Kin	IAA	NAA	2,4-D			
0.5					80.4	1.0±0.0[a]	
1.0					100	1.1±0.3[ab]	
2.0					100	1.6±0.3[ab]	
5.0					100	2.4±0.3[bc]	
7.5					100	5.6±0.3[e]	
10.0					81.4	3.4±0.9[c]	★
12.5					66.6	2.8±1.1[bc]	★
	0.5				84.7	1.1±0.3[a]	
	1.0				91.3	2.0±0.4[ab]	
	2.0				100	4.1±0.4[c]	
	5.0				100	6.9±0.5[d]	
	7.5				100	8.5±0.3[e]	
	10.0				100	3.9±0.8[bc]	★
	12.5				62.3	2.5±0.7[ab]	★
	7.5	0.5			98.6	5.0±0.8[c]	
	7.5	1			95.6	4.1±0.9[bc]	
	7.5	2			64.7	2.0±1.5a	
	7.5		0.5		96.4	4.5±1.04[b]	★★
	7.5		1		65.6	4.4±0.70[b]	★★
	7.5		2		-	-	★★
	7.5			0.5	94.7	1.4±1.3[bc]	
	7.5			1	-	-	★★
	7.5			2	-	-	★★

Values are means ± standard errors of three independent experiments consisting of a total of 21 explants.
Means within a column followed by the same letters are not significantly different at 5 % level by Duncan's multiple range test.
★Callus mediated shoot formation, ★★Extensive callus formation.

explants, which was accomplished by the formation of numerous root hairs. Incorporation of 2,4-D (1.0-5.0 μM) resulted only in callus formation, which was extensive with 2.0 μM 2,4-D. Nodal explants regenerated shoots on the medium with 0.5-1.0 μM BA/Kin (described in later section). Higher levels of BA/Kin (12.0, 18.0 μM) were also effective for induction of calli in both the explants, but at very high levels (22.0 μM), features of hyperhydricity were observed.

Out of 175 combinations of auxins and cytokinins, extensive callus proliferation was noticed on eleven combinations (Table 2). Significant callus production from both the explants was obtained on MS containing 1.0 μM 2,4-D together with 5.0 μM BA or MS containing 1.0 μM 2,4-D in conjunction with 5.0 μM Kin. A considerable callus growth was obtained with other combinations of growth regulators in the range of 5 to 12.5 μM (Table 2, fig. b). Callus was nodular and greenish

Table 2. Effect of growth regulators on growth and cerpegin production in the callus of *C. juncea* after 5 weeks of culture.

Growth regulator (μM)					Growth of callus culture (Dry weight) (g)	Cerpegin in callus (μg/g D.W.)
BA	Kin	IAA	NAA	2,4-D		
12.5					0.32±0.01[b]	220±0.6[c]
	12.5				0.30±0.01[a]	212±1.0[b]
		10.0			0.37±0.01[d]	280±0.6[e]
			10.0		0.34±0.01[e]	275±0.6[d]
				2.0	0.39±0.01[e]	170±1.0[e]
5.0		10.0			0.52±0.02[e]	470±1.0[k]
	5.0	10.0			0.52±0.01[e]	461±1.0[j]
2.0			7.5		0.50±0.02[d]	460±1.0[j]
	2.0		7.5		0.50±0.02[d]	450±0.6[h]
5.0				1.0	0.55±0.02[f]	380±0.5[g]
	5.0			1.0	0.54±0.02[f]	370±0.6[f]

Values are mean±standard error of three independent experiments consisting of a total of 33-callus cultures. Mean values followed by the same letters were not significantly different at the 5% level by Duncan's multiple range test.

in colour except on 2,4-D-containing medium, where the callus was white. Previous studies show that in *C. jainii* and *C. bulbosa* (Patil, 1998); the highest callus induction frequency from nodal segment occurred on MS medium supplemented with 9.05 μM 2,4-D. Similarly in *Ceropegia candelabrum,* optimum callus induction was noticed from leaf and internode explants grown on MS supplemented with 4.52 μM 2,4-D (Beena *et al.*, 2003). A ratio of auxins and cytokinins greater than one in the nutrient medium was effective for the induction of callus in most of the members of Asclepiadaceae viz. *Calatropis gigantea* (Roy *et al*., 2000), *Hemidesmus indicus* (Sarasan *et al*., 1994), *Tylophora indica* (Bapat and Rao, 1984; Benzamin *et al*., 1979), *Leptadenea reticulate* (Hariharan *et al.*, 2000) and *Ceropegia candelabrum* (Beena *et al*., 2003), and *Ceropegia bulbosa* (Patil, 1998). The results of the present investigation suggest that for callus induction and proliferation in *Ceropegia juncea,* the desired ratio of auxins (NAA and IAA) and cytokinin (BA and Kin) was > 1, while for 2,4-D, BA and Kin it was <1. In contrast Manjula *et al*., (2000) reported that in *Tylophora indica* cytokinin alone was sufficient for induction and subsequent growth of callus and the addition of auxin along with cytokinin inhibited the growth of callus.

Growth of callus (in terms of dry weight) derived from both node and internode explants remained more or less constant after every subculture on a fresh medium, for a period of more than 34 months. Similar results have been reported in other members of Asclepiadaceae, viz. callus cultures of *Tylophora indica* (Bapat and Rao,1984); *Hemidesmus indicus* (Sarasan *et al*., 1994) and *Calatropis gigantea* (Roy *et al*., 2000); *Tylophora indica* (Benzamin *et al*., 1979) and *Asclepias syrica* (Wilson and Mahlberg, 1977) were maintained on subculture for 5,3,5 and 12 months respectively. Thus, in the members of Asclepiadaceae, including *Ceropegia juncea* Roxb., calli can be maintained for prolonged periods.

Shoot regeneration

Nodal explants were cultured on growth regulator free and 0.5 μM BA or Kin or NAA or IAA fortified MS medium, where in 84 % of cases, direct formation of a single healthy shoot was observed after 18 days of culture initiation. Increase in concentration of BA or Kin (5.0-7.5 μM) increase the percent response and number of shoots per explant. However, 7.5 μM Kin was superior than BA, as the average number of shoots per explant (8.5±0.3) (Fig. 1a) was more than the mean number of shoots obtained with MS containing 7.5 μM BA (5.6±0.3) (Table 1). However, supplementation of BA in MS medium has been effective in the induction of multiple shoots from nodal explants of *Ceropegia candelabrum* (Beena *et al*., 2003), *C. jainni*, *C. bulbosa* (Patil, 1998), *C. sahyadrica* (Nikam and Savant, 2007).

Higher concentration of BA or Kin (18.0 and 22.0 μM) caused callusing on the entire surface of the explant in the first two weeks of culture. In the third weeks, 1-2 callus-interspersed shoot buds developed, which were weak and slow growing. The callus was friable and greenish in colour. Evidence of hyperhydricity was observed on the shoots produced in the presence of more than 18 μM Kin or BA.

Experiments were done to compare shoot regeneration from internode explants on the similar media. There were minor differences in callusing response but no shoot regeneration was observed. Lack of shoot regeneration potential was also reported in root internode and leaf

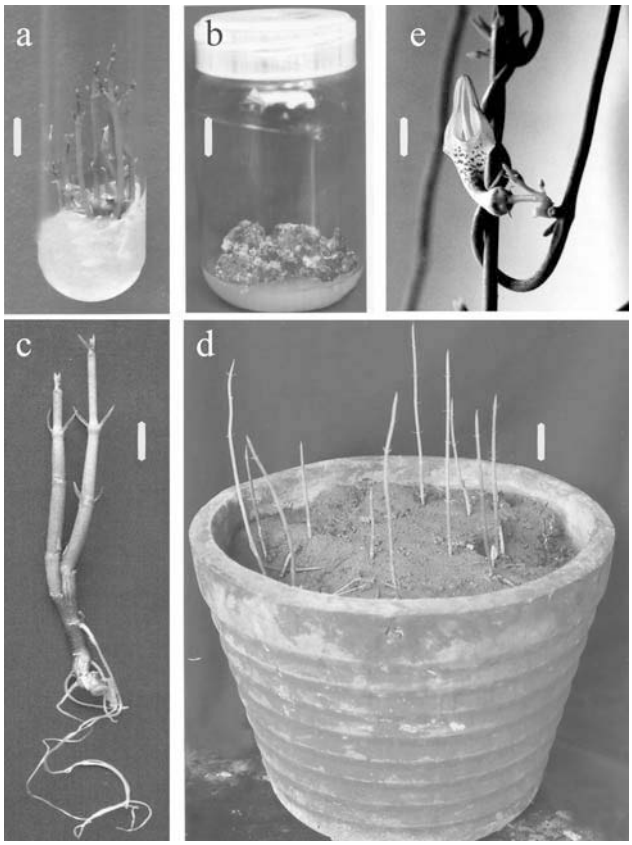

Fig. 1. *In vitro* multiplication and callus culture in *Ceropegia juncea*. **a.** Shoot proliferation in nodal explant on MS + 7.5 µM Kin. (bar = 10mm). **b.** Callus culture on MS + 10.0 µM IAA + 5.0 µM BA (bar = 10mm). **c.** Rooted shoot on MS + 2.0 IAA µM (bar = 20mm). **d.** Plant in soil under natural conditions (bar = 20mm). **e.** Flowering branch after 8 months growth (bar = 10mm).

explants of *C. candelbrum* (Beena *et al*., 2003), *C. sahyadrica* (Nikam and Savant, 2007), *Hemidesmus indicus* (Sarsan *et al*., 1994) *Tylophora indica* (Majunla *et al*., 2000), *Gymnema sylvestre* (Komallavalli and Rao, 2000), when cultured on MS medium fortified with BA, Kin, NAA and 2,4-D alone and in combination.

Addition of IAA (0.5-5.0 µM) and NAA (0.5-2.0 µM) to the medium containing 7.5 µM Kin and BA did not improve the multiple shoot formation (Table 1). On the contrary, superiority of BA or Kin and NAA or IBA in combination has been reported for micropropagation from nodal explant in *Gymnena sylvestre* (Komalavalli and Rao, 2000), *Tylophora indica* (Sreekumar *et al*., 2000) *Holostema annulalarae* (Sudha *et al*., 1998). Cumulative work of many years in plant tissue culture on members of Asclepiadaceae suggested that among various types of explants (root, hypocotyls, cotyledon, leaf, node, internode etc) mostly nodal explants responded well for caulogenesis on synthetic media supplemented with cytokinins.

Shoot differentiation in internode-derived fresh and maintained calli on MS fotified with different auxins and cytokinins, combinations and concentrations, as described in material and methods, was unsuccessful. However, two to three weeks after inoculation, 100 % of friable, white-grayish callus culture became greenish and had many distinct dark green portions on the surface. Occasional differentiation of shoot bud in nodal calli occurred on MS+7.5 µM BA+50 mg/l casein hydrolysate, but shoot differentiation was inconsistent and occurred only in about 18 % of the callus cultures (1-3 shoots per culture). In *Calatropis gigantia* (Roy *et al*., 2000) and *Hemidesmus indicus* (Sarasan *et al*., 1994), green nodular callus developed from the stem and leaf explants showed shoot bud regeneration upon subculturing on MS with Kin (1.5-2.0mg/l) and CM (10%). In *Typophora indica* (Benjamine *et al*., 1979), callus induced from root and stem explants developed shoot buds, but leaf derived callus was unable to respond for shoot regeneration. This might be due to the lack of inducing factor in the calli or some inherent inhibitory factor present in the calli.

Cerpegin in callus

The alkaloid content curve had a pattern similar to the growth curve for the callus (Fig. 2) on MS medium containing 10 µM IAA in conjunction with 5.0 µM BA. The growth curve of callus had a normal pattern with initial lag phase of two weeks, followed by three weeks exponential phase and at the end, the sixth and seventh week represented the senescent phase, (Fig. 2). The alkaloid content was slightly higher in the first week

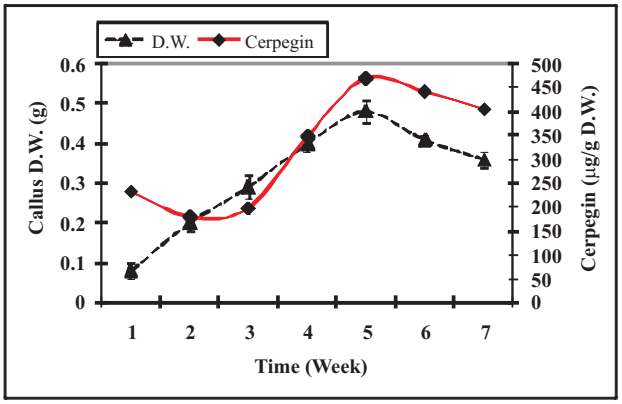

Fig 2. Growth curve of stem callus culture and ceropegin content curve of the callus of *Ceropegia juncea.*

than second week of culture. This might be due to the initial alkaloid content of the inoculum of fourth week of the culture used for the experiment. From the end of the third week, the cerpegin content, which increased progressively upto the end of the fifth week and later the content decreased in the sixth and seventh week (Fig. 2). The decline in the growth of callus and alkaloid content might be due to depletion of nutrients and/or other limiting factors. The highest total alkaloid content (470±1.0 µg/g dry weight) in the callus was obtained after 5 weeks of culture. The alkaloid content in the stem of naturally grown plants was about 0.5 mg/gm dry weight.

Similar pattern of growth curve and total alkaloid content was recorded for the callus grown on other combinations of growth regulators (Table 2). At every subculture, callus growth and alkaloid production in the callus maintained on eleven different media (Table 2) was compared with alkaloid content in the stem biomass of plants grown in the garden. It demonstrated that the presence of auxins and cytokinins together in the nutrient medium had synergetic effect on the growth of callus and alkaloid production. The callus growth and alkaloid content was more or less doubled in the callus grown on media containing both auxin and cytokinin together. Nevertheless, the type of auxin and cytokinin and their concentration affected the growth and content of alkaloid (Table 2).

The callus obtained on MS supplemented with 5.0 µM BA and 1.0 µM 2,4-D together showed highest growth rate but it was less efficient in alkaloid production (Table 2). On the other hand, the growth rate of callus on MS containing 10.0 µM IAA in conjunction with 5.0 µM BA was comparatively slightly lower, but the callus was more potent in alkaloid production (Table 2, Fig. 1b). The pyridone type of alkaloid cerpegin content in *Ceropegia juncea* and earlier reports on nicotine content in callus and cell culture of *Nicotiana* species (Ramawat and Merillon, 1999) and for other type of secondary metabolite production in the species of *Ephedra* (Ephidrin), *Lithospermum* (Shikonin), *Datura, Hyoscymus*, *Atropa* (tropane alkaloid) and *Papavar* (morphin), suggest that the IAA or NAA were more effective than 2,4-D, BA and Kin. (Ramawat and Merillon, 1999).

Rooting of regenerated shoots

Shoots longer than 3 cm when placed on rooting media (Table 3) showed 100 % rooting within two-three weeks of subculture (1-3 roots per shoots). Rooting occurred from the basal end of the shoots cultured on medium without growth regulators. However, better results were obtained after the addition of auxins (IAA,NAA and IBA) at 1.0-5.0 µM; where rooting was initiated within first weeks of culture. IAA was the most effective auxin (2.0 µM) in terms of percent shoots forming roots (100%) and root length (12-14 cm fig.1c). IBA favored rooting in other asclepiad medicinal plants such as *Ceropegia candelabrum* (Beena *et al*., 2003), *Hemidesmus indicus* (Sreekumar *et al*., 2000), *Holostema ada-hodein* (Martin, 2002), *Holostema annulalarae* (Sudha *et al*., 1998), and *Gymnena sylvestre* (Komalavalli and Rao, 2000).

Hardening

The rooted plantlets established well upon transfer

Table 3. Effect of different auxins on rooting of *in vitro* developed shoots in *C. juncea* after 4 week of culture

Growth regulators (µM)	Frequency of Shoots forming Roots %	Number of roots/shoot Mean ± SE
IAA		
0.0	100	1.5±0.5[a]
0.5	100	2.1±0.5[b]
1.0	100	2.9±0.4[c]
2.0	100	3.5±0.3[b]
5.0	100★	2.1±0.3[b]
7.5	100★	8.2±0.7[ab]
NAA		
0.5	100	2.4±0.2[c]
1.0	100	2.7±0.4[c]
2.0	100	3.4±0.1[a]
5.0	100★	3.0±0.2[b]
7.5	100★	9.3±0.4[d]
IBA		
0.5	100	2.0±0.3[d]
1.0	100	2.8±0.4[d]
2.0	100	2.9±0.3[d]
5.0	100★	5.7±0.2[d]
7.5	100★	9.4±0.7[d]

Values are means ± standard errors of three independent experiments consisting of a total 21 shoots.
★Callus mediated root formation
Means within column followed by same letters are not significantly different at the 5% level Duncan's multiple range test.

directly to soil in rainy and winter season. However, in summer season, hardening for one week in shade was necessary for 100 % survival in the field conditions. (fig.1d).The plants flowered normally and were morphologically indistinguishable from the donor plant. (fig.1e) The earlier reported rate of survival of *in vitro* raised plantlets of members of Asclepiadaceae upon transfer to field conditions was mostly in the range of 90-100 % (Sreekumar *et al*., 2000; Komalavalli and Rao, 2000; Beena *et al*., 2003). We speculate that the higher survival rate of plantlets in this study and also from earlier reports is due to the in-built capacity of Asclepiadaceae members to grow even under extreme drought conditions.

In conclusion, the *in vitro* micropropagation approach could support domestication and commercial cultivation of *Ceropegia juncea* Roxb. The cells/tissues/ organs or plantlets could be explored for alkaloid cerpegin and to improve its method of production and utilization.

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