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Original Research Article

Standardization and re-introduction of Critically Endangered *Ceropegia mahabalei* Hemadri and Ansari by *in vitro* propagation

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Abstract: A protocol is described for micropropagation of the tuberous, herbaceous, medicinally important and Critically Endangered *Ceropegia mahabalei* Hemadri and Ansari by *in vitro* culture of nodal segments. The effect of cytokinins (BAP, Kin and TDZ) and auxins (IBA, IAA and NAA) at various concentrations on *in vitro* regeneration were investigated. The highest number of nodes per explant (7.5±0.14) and maximum shoot length (8.02±0.12 cm) were found on full strength MS medium supplemented with BAP (8.88 µM). Whereas, IBA (9.84 µM) showed maximum root length (1.54±0.09 cm) and number of roots per shoot (5.8±0.37) with maximum shoot length (7.11±0.50cm). *In vitro* grown plantlets were acclimatized (100%) in potting material containing mixture of garden soil, coco peat and river sand (1:1:1/2) and showed normal development with flowering. The developed method can be employed for productive conservation of *C. mahabalei* through large scale *in vitro* propagation. These saplings were successfully reintroduced in their natural habitat with a survival rate of 42.67%.

Key words: *Ceropegia mahabalei*, Critically Endangered, Micropropagation, Reintroduction

Introduction

Ceropegia L., species are members of the family Asclepiadaceae (Apocynaceae). The genus *Ceropegia* L. is the largest genus of the tribe Ceropegiaeae with more than 200 species distributed in tropical and sub tropical regions of the old world, ranging from the Spanish Canary Islands in the west, through Central, Southern and Northern Africa, Madagascar, Arabia, India and South Asia to Northern Australia in the East (Good 1952; Bruyns 2003). In India, 55 species are present of which 38 species occur in Western Ghats (Yadav and Mayur 2008). The medicinal importance of the genus *Ceropegia* is due to the presence of important alkaloid, '*Ceropegin*' (Kirtikar and Basu 1935; Nadakarni 1976; Anonymous 1980; Jain and Defillips 1991). Most of the *Ceropegia* L. species are under threat due to unscrupulous collection or habitat degradation.

Ceropegia mahabalei Hemadri and Ansari is one of the endemic and Critically Endangered species of Western Ghats, India (Hemadri and Ansari 1971; Nayar and Sastry 1988; Singh *et al.,* 2001). It is locally known as "Gavati Kharpudi". The plant is herbaceous, erect (20-65 cm in height), flower is beautiful, attractive, single, pedicles and calyx hairy; corolla 5.5-10.0 cm long, base largely inflated, narrowed in neck, enlarging towards mouth, glabrous inside, lobes hairy within, connate at tips to form a beaked crown; corona biseriate, outer corona of five bidentate glabrous lobes, inner linear, erect. Fruit is pair of follicle, terete, glabrous, linear and tapering at the end. Flowering is observed in August-September and fruiting in September onwards (Almeida 2001; Singh *et al.,* 2001).

Tubers of this plant are rich in carbohydrates and are consumed by the local people (Jagtap and Singh 1999). They are also a good source of nutritive tonic (Kirtikar and Basu 1984). Percentage of successful pollination of this species is low due to endemic to Western Ghats, short life span and habitat degradation. Hence, survival rate and its natural population is depleting day by day. IUCN has also recommended large scale *in vitro* propagation of this plant species (Walter and Gillet 1998).

Attempts were made for the *ex situ* conservation of *C. mahabalei* (Deshmukh 2010). Micropropagation protocol of some *Ceropegia* species using different explants have been reported earlier (Muthukrishnan *et al.,* 2013). Tricotyledony was observed in *C. mahabalei* (Rajput *et al.,* 2012). However, micropropagation studies of *C. mahabalei*

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have not yet been reported. Considering this condition, present work was initiated to develop *in vitro* protocol for conservation of Critically Endangered *C. mahabalei*.

Materials and Methods

Plant material collection and surface sterilization

Wild plant of *C. mahabalei* collected from Ralegan, Sub division Junnar, District Pune, Maharashtra, India (Fig.1A) was maintained as mother plant in the botanical garden of Agharkar Research Institute (ARI), Pune. Actively growing shoots of mature plant of *C. mahabalei* were used to initiate the aseptic culture. Standard surface sterilization procedure was adopted for aseptic inoculation. The shoot segments were defoliated and thoroughly washed under running tap water for 15 min to remove superficial contamination, soil and other cell debris. Explants were washed with detergent Tween 20 (5% v/v) for 10 min. Followed by treatment of fungicide Bavistin (1% w/v) for two min. Thereafter, explants were disinfected with alcohol (70%v/v) for 1 min followed by treatment of mercuric chloride (0.1% w/v) for 1 min under aseptic conditions. After each treatment explants were rinsed with sterile distilled water for three to five times. The sterile explant with single node was inoculated on enrichment medium.

Media and Incubation Conditions

For all set of experiments full strength Murashige and Skoog (MS) basal medium (Murashige and Skoog 1962) containing salt, macro and micro nutrients supplemented with sucrose (3% w/v) as carbon source was used. Gelling agent used was agar (0.8% w/v). The pH adjusted for medium was 5.8 (± 0.1), with the help of 1N NaOH or 0.1N HCl before autoclaving and sterilized at 121°C and 15 lbs pressure for 15 min. Every chemical used for experiments were of analytical grade (SD Fine Chemicals and Sigma, India). All cultures were incubated at 25 ± 2°C with 16 hrs photoperiod provided by cool white fluorescent tubes 45µMol m^{-2} s^{-1} (Philips, India). After every 40 days, all cultures were transferred on fresh medium.

Shoot Induction and Multiplication

For establishment of culture, surface sterilized, defoliated explants with single node were aseptically cultured on full strength MS basal medium supplemented with different concentrations of various cytokinins like 6-benzylaminopurine (BAP; 2.21-17.75µM), 6-furfurylaminopurine/Kinetin (Kin; 2.32-18.57µM) and thidiazuron (TDZ; 2.27-18.16µM). MS medium without any growth hormone was considered as control. After every ten days interval, observations were recorded for number of shoots per explants and average shoot length.

Root Induction and in vitro Flowering

Well established and healthy shoots with two to four nodes were cultured on full strength MS medium supplemented with various root inducing growth hormones. Effectiveness of indole-3-butyric acid (IBA; 2.46-19.68µM), α-naphthalene acetic acid (NAA; 0.54-5.37µM) and indole-3-acetic acid (IAA; 2.85-22.80µM) were checked for root induction and data of 40 days were recorded for percentage of root induction, average number of roots, average root length and average shoot length after interval of every ten days.

Effect of high concentration of sucrose on *in vitro* flowering is also observed. For this purpose, full strength MS medium was supplemented with different concentrations of NAA (0.54-5.37µM) and IBA (2.46-19.68µM) in combination of 5% and 6% sucrose.

Data Analysis

Morphological changes in culture were recorded at regular period. All experiments were repeated thrice with ten replicates per treatment. The experiments were conducted in a completely randomized design. Data were subjected to one way Analysis Of Variance (ANOVA) and comparison of means was made with the Dunnett multiple comparison test using Graph Pad Prism 6 software. Data were presented in the form of mean ±SE.

Plant acclimatization

Hardening is the most crucial step for successful survival of sapling; while transferring from *in vitro* to *ex vitro* conditions. Plantlets with well developed shoots and sturdy roots of *C. mahabalei* were removed from culture medium and gently washed by water to remove culture medium and treated with Bavistin (1% w/v) for 5 min to avoid fungal infection. Different permutation and combinations were done in sterile potting material to obtain maximum survival rate of hardened plantlets. Treated plants were then planted in thermocol cups

containing mixture of sterile garden soil, coco peat and river sand (1:1:1/2). The potted plants were kept in the polyhouse under controlled conditions (humidity 80-90% and temperature 25-30°C) for 40 days. And observations were recorded at the interval of ten days. To make plants photosynthetically active and ease acclimatization in field, they were kept under direct sunlight for 10-15 days. 500 saplings were hardened in polyhouse. In the monsoon season of year 2013 with the help of Forest department, Maharashtra; 450 saplings were reintroduced in their natural habitat in Junnar sub-division, Pune District, Maharashtra, India. Remaining 50 saplings were maintained as control in ARI, Pune.

Results and Discussion

Shoot Induction and Multiplication

The morphogenetic responses of nodal explants to various cytokinins (BAP, Kin and TDZ) were evaluated (Table 1). Shoot initiation and multiplication rate was variable with type and concentration of cytokinins. Among the three cytokinins, BAP was found to be most effective followed by Kin and TDZ. Similar results for shoot induction and proliferation have been obtained in other threatened and medicinal plants (Patil 1998; Beena *et al.,* 2003; Karuppusamy *et al.,* 2009; Chavan *et al.,* 2011). Maximum response was obtained on full strength MS basal medium supplemented with BAP (8.88µM) giving maximum number of shoots per explant (7.56±0.14) and highest shoot length per explant (8.02±0.12cm) (Fig. 1B, C). Full strength MS medium supplemented with Kin (11.61µM) showed significant shoot response with an average number of shoots per node (4.0±0.21) and average shoot length per explant (4.82±0.26 cm). But obtained shoots were thin and delicate. Though TDZ did not enhance shoot induction significantly, it was able to induce average shoots per node (2.8±0.29) and shoot length per explant (3.48±0.28cm) at 2.27µM concentration. Effect of TDZ leads to irregular and stunted shoot growth. Similar response was also observed in some other species of *Ceropegia* (Beena *et al.,* 2003; Karuppusamy *et al.,* 2009; Chavan *et al.,* 2011). Nodal explants cultured on growth regulator free medium failed to induce shoot. Therefore, BAP (8.88µM) was used for subsequent sub cultures and multiplication experiments. Up to sixth subculture, multiplication rate was 8 shoots per explant. After that multiplication rate declined.

Table 1: Effect of different cytokinins on shoot induction of *Ceropegia mahabalei* Hemadri and Ansari

GROWTH HARMONES	CONCENTRATION (µM)	AVG SHOOT LENGTH OF EXPLANT ± SE (cm)	AVG NO OF NODES / EXPLANT ± SE
Growth Hormone Free		-	-
BAP	02.21	4.55±0.25**	3.36±0.22**
	04.44	5.25±0.23**	4.16±0.22**
	06.66	6.00±0.20**	5.16±0.17**
	08.88	8.02±0.12**	7.56±0.14**
	11.10	5.57±0.16**	5.01±0.18**
	13.31	4.68±0.15**	4.03±0.16**
	15.53	4.38±0.24**	3.56±0.24**
	17.75	4.18±0.25**	3.36±0.23**
KINIETIN	02.32	2.34±0.34**	1.70±0.33*
	04.64	3.45±0.29**	2.40±0.22**
	06.96	3.78±0.33**	2.80±0.29**
	09.28	4.17±0.36**	3.20±0.29**
	11.61	4.82±0.26**	4.00±0.21**
	13.93	3.95±0.41**	3.20±0.24**
	16.25	3.20±0.40**	2.40±0.33**
	18.57	2.80±0.53*	1.90±0.37*
TDZ	02.27	3.48±0.28**	2.80±0.29**
	04.54	2.98±0.19**	2.30±0.16**
	06.81	2.81±0.11**	2.30±0.15**
	09.08	2.60±0.21**	1.80±0.20**
	11.35	2.19±0.20**	1.40±0.16**
	13.62	1.40±0.26*	0.80±0.13*
	15.89	1.20±0.29	0.70±0.15*
	18.16	1.00±0.29	0.60±0.16

Values represents mean± SE from 10 replicates per treatment and all the experiments were thrice. The values are significantly different $^{**}P \leq 0.01$ and $^{*}P \leq 0.05$ level when compared by Dunnett multiple comparison test.

Root Induction

Root induction response was compared on full strength MS medium supplemented with various concentrations of auxins (NAA, IBA and IAA) (Table 2). MS medium without any auxin was treated as control which did not show any root development. Root initiation was observed from twelfth day of incubation. Out of three auxins, IBA was found to be the most effective giving 100 % response. Similar results for root induction were obtained in other species of *Ceropegia* (Chandore *et al*., 2010; Muthukrishnan *et al.,* 2012). The highest number of roots per shoot (5.8±0.37) and maximum root length per shoot (1.54±0.09 cm) with average shoot length of 7.11 cm was obtained on full strength MS medium enriched with 9.84µM

IBA (Fig.1D). NAA also showed significant response on 4.3µM concentration giving highest number of roots per shoot (3.13±0.24) and maximum root length per shoot (1.01±0.03 cm) with average shoot length of 5.54cm. But the roots observed were very thick and brittle. In case of full strength MS medium supplemented with IAA, roots developed were very thin, small and delicate. As a result, very less number of roots per shoot (2.5±0.23) and root length per shoot (0.9±0.05cm) were obtained. Hence, MS medium fortified with IBA (9.84µM) was used for root development of *C. mahabalei* plantlets.

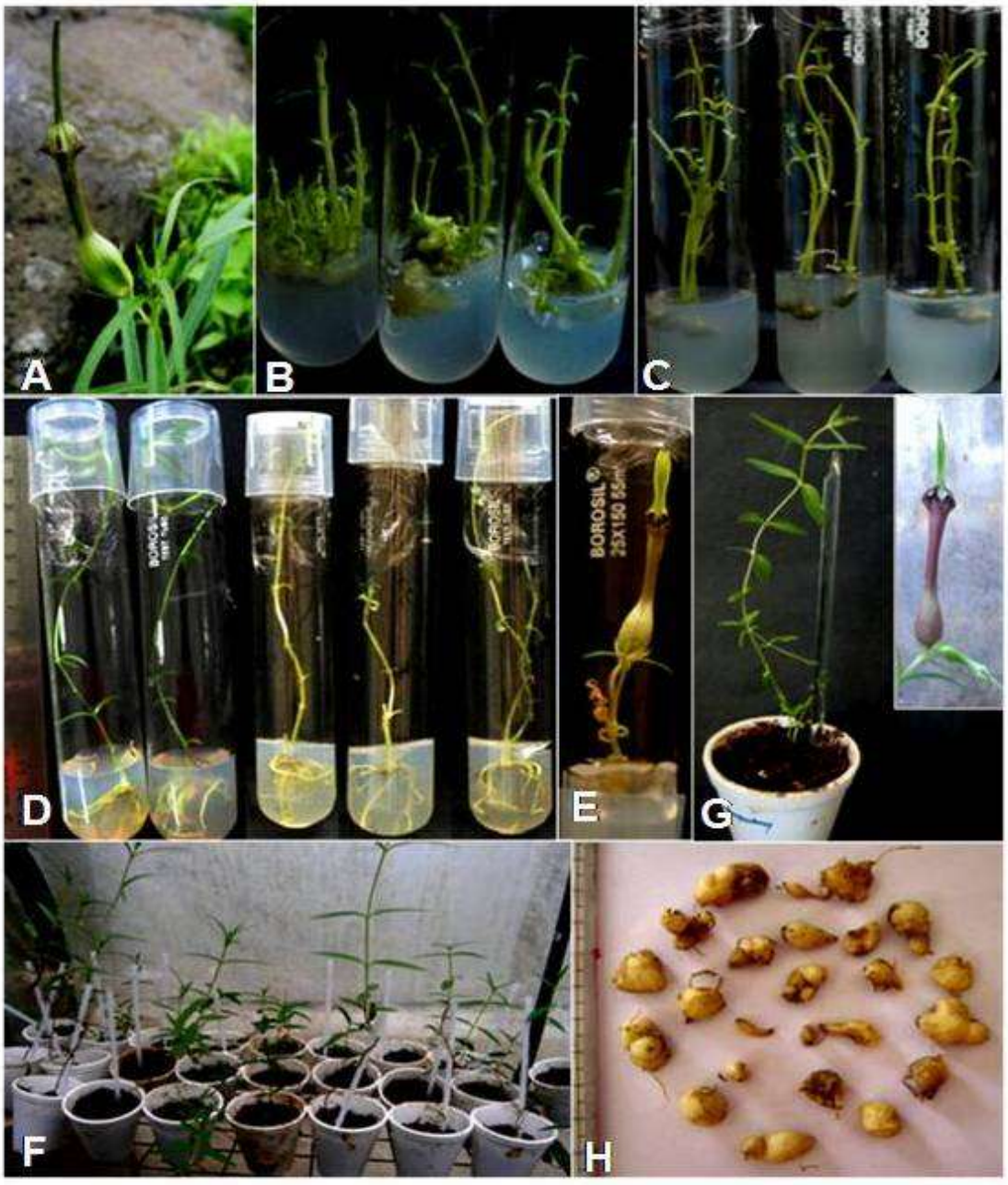

Figure 1: Micropropagation of *Ceropegia mahabalei* Hemadri and Ansari (A) *Ceropegia mahabalei* in natural habitat, (B-C) Initiation and multiplication of shoot (MS medium + 8.88µM BAP), (D) *In vitro* root development (MS medium + 9.84µM IBA), (E) *In vitro* flowering (MS medium + 3.22µM NAA with 6% sucrose), (F) Hardened plants in polyhouse with flowering and (G) Tubers harvested after hardening.

Table2: Effect of different auxins on root induction of *Ceropegia mahabalei* Hemadri and Ansari

GROWTH HARMONES (µM)			AVG SHOOT LENGTH/ EXPLANT (cm)	AVG ROOT LENGTH/ EX PLANT (cm)	AVG NO OF ROOTS/ EXPLANT	% RESPONSE
NAA	IBA	IAA				
Growth Hormone Free			-	-	-	-
00.53			2.49±0.24**	0.40±0.04**	1.46±0.19**	80
01.07			3.15±0.32**	0.42±0.05**	1.53±0.19**	76.67
01.61			3.46±0.22**	0.44±0.05**	1.73±0.19**	80
02.14			3.68±0.24**	0.47±0.04**	2.26±0.24**	90
02.68			4.01±0.32**	0.51±0.04**	2.40±0.26**	86.67
03.22			4.43±0.28**	0.60±0.04**	2.73±0.26**	93.33
03.75			4.54±0.18**	0.70±0.05**	2.86±0.20**	96.67
04.29			5.54±0.23**	1.01±0.03**	3.13±0.24**	100
04.83			4.23±0.17**	0.80±0.04**	2.53±0.19**	96.67
05.37			3.39±0.27**	0.60±0.04**	2.33±0.21**	93.33
	02.46		3.45±0.21**	0.40±0.05**	2.50±0.27**	93.33
	04.92		4.01±0.22**	0.53±0.06**	3.56±0.40**	93.33
	07.38		5.94±0.50**	0.89±0.13**	4.90±0.46**	93.33
	09.84		7.11±0.50**	07.2±0.09**	5.80±0.37**	100
	12.30		6.69±0.43**	1.15±0.11**	4.50±0.42**	100
	14.76		5.58±0.39**	0.91±0.10**	3.53±0.21**	100
	17.22		5.17±0.25**	0.85±0.09**	2.30±0.29**	100
	19.68		4.31±0.31**	0.51±0.05**	2.10±0.21**	93.33
		02.85	4.05±0.17**	0.52±0.04**	2.06±0.20**	100
		05.70	5.05±0.23**	0.90±0.05**	2.50±0.23**	100
		08.55	4.50±0.19**	0.74±0.03**	2.06±0.17**	100
		11.40	4.05±0.17**	0.57±0.03**	1.80±0.18**	96.67
		14.25	3.64±0.15**	0.43±0.04**	1.63±0.18**	86.67
		17.10	3.48±0.14**	0.35±0.04**	1.46±0.15**	90
		19.95	3.00±0.13**	0.28±0.03**	1.23±0.13**	86.67
		22.80	2.76±0.11**	0.18±0.02**	1.00±0.11**	80

Values represents mean± SE from 10 replicates per treatment and all the experiments were thrice. The values are significantly different $^{**}P \leq 0.01$ and $^{*}P \leq 0.05$ level when compared by Dunnett multiple comparison test.

Table 3: Plantation Location and survival rate of reintroduced *Ceropegia mahabalei* Hemadri and Ansari saplings

S.No	LOCATION	ELEVATION (meter)	NO OF SAPLINGS PLANTED IN 2013	NO OF SAPLINGS PLANTED IN 2014	TOTAL SURVIVAL
1	Shindewadi	913.79	120	54	
2	Ralegan	915.31	80	37	
3	Tambe	865.63	100	44	42.67%
4	Tambe	874.47	50	15	
5	Utshir	995.47	100	42	

Effect of sucrose concentration of in vitro flowering

Initiation of *in vitro* flower buds was observed on high concentration (5% and 6%) of sucrose in two weeks. First completely developed flower was recorded in third week of inoculation, on full strength MS medium supplemented with NAA (3.22µM) in combination with 6 % sucrose (Fig.1E). The length of flower measured 8 cm long. Similar results were recorded for other species of *Ceropegia* using various concentrations of sucrose, BAP and spermine (Patil 1998; Nair *et al.,* 2007; Chavan *et al.,* 2011). The study reveals that increased concentration of carbohydrates in growth medium induces flowering.

Plant acclimatization

Plantlets with well-developed shoots and roots were transfered to small thermocol cups containing different types of sterile potting material (garden soil, coco peat and river sand) and their survival percentage was calculated after 40 days (Fig.2). The maximum plants (100%) were acclimatized in potting material containing mixture of garden soil, coco peat and river sand (1:1:1/2). Plantlets grown only on coco peat also showed 90% survival rate up to 40 days but died due to absence of nutrients.

The saplings reintroduced in their natural habitat showed 42.67% survival rate (Table 3). Whereas, saplings which were kept in polyhouse as control, grew vigorously with

100% survival rate (Fig.1F). These saplings exhibited true characters similar to the field grown plants and showed flowering too (Fig. 1G). Tubers were harvested after completion of plant life cycle and maintained in ash for next growing season (Fig. 1H).

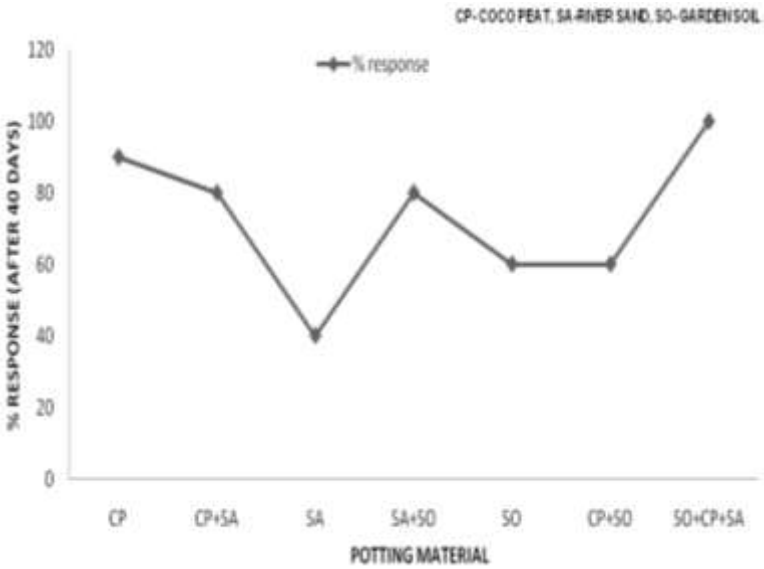

Figure 2: Effect of different potting material during acclimatization of *Ceropegia mahabalei* Hemadri and Ansari

Low survival rate of reintroduced sapling in nature as compared to control is due to abiotic factors like irregular rainfall, temperature variation and biotic factors like animal grazing, tuber consumption as food and medicinal use by local people. Another reason for demolishing of species is lack of pollination due to habitat destruction (Deshmukh 2010).

Conclusion

The successful micropropagation protocol developed here provides an effective means for the rapid propagation and conservation of an endemic, Critically Endangered, medicinally important, edible and potential ornamental plant, *C. mahabalei.* The amplified multiplication and cost effectiveness makes this protocol truly beneficial. Furthermore, this developed protocol for mass multiplication can be applied for conservation of other Critically Endangered *Ceropegia* species.

Ex situ conservation of *C. mahabalei* is also important to increase its natural population. To overcome the problem of changing environmental conditions, biotic and abiotic factors plants should be conserved in protected areas. Also efforts should be made toward promoting awareness among local communities.

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