

1. Varmah, J. C. and Bahadur, K. N., Country report and status of research of bamboos in India. *Indian For. Rec. (Bull.)*, 1980, **6**(1), 1–28.
2. Sur, K., Lahiri, A. K. and Basu, R. N., Hydration–dehydration treatment for improved seed storability of bamboo (*Dendrocalamus strictus* L.). *Indian For.*, 1988, **114**(9), 560–563.
3. Basavarajappa, B. S., Shetty, H. S. and Prakash, H. S., Membrane deterioration and other biochemical changes associated with accelerated ageing of maize seeds. *Seed Sci. Technol.*, 1991, **19**, 279–286.
4. Roberts, E. H., Predicting the storage life of seed. *Seed Sci. Technol.*, 1973, **1**, 499–514.
5. Ganguli, S. and Sen-Mandi, S., Effects of ageing on amylase activity and scutellar cell structure during imbibition in wheat seed. *Ann. Bot.*, 1993, **91**, 411–416.
6. Richa, Sharma, M. L. and Bala, N., Studies of endogenous levels of plant growth hormones in relation to seed viability in some bamboo seeds. *Indian J. Plant Physiol.*, 2006, **11**(4), 358–363.
7. Mahanna, H. T., Martin, G. C. and Nishijuna, C., Effect of temperature, chemical treatment and endogenous hormone content on peach seed germination and subsequent seedling growth. *Sci. Hortic.*, 1985, **27**, 63–73.
8. Choi, Y. H., Kobayashi, M. and Sakurai, A., Endogenous gibberellin A1 level and  $\alpha$ -amylase activity in germinating rice seeds. *J. Plant Growth Regul.*, 1996, **15**(3), 147–151.
9. Richa, Sharma, M. L. and Kaur, P., Effect of exogenous application of some plant growth regulators on enzyme activity with ageing of bamboo seeds. *J. Punjab Acad. Sci.*, 2000, **2**(1), 35–42.
10. Bernfeld, P.,  $\alpha$ - and  $\beta$ -amylase. *Methods Enzymol.*, 1955, **1**, 149–158.
11. Malik, C. P. and Singh, M. B., *Plant Enzymology and Histochemistry*, Kalyani Publishers, New Delhi, 1980.
12. Petruzzelli, L. and Taranto, G., Amylase activity and loss of viability in wheat. *Ann. Bot.*, 1990, **66**, 375–380.
13. Livesley, M. A. and Bray, C. M., The effects of ageing upon  $\alpha$ -amylase production and protein synthesis by wheat aleurone layers. *Ann. Bot.*, 1991, **68**, 69–73.
14. Mozer, T. J., Control of protein synthesis in barley aleurone layers by the plant hormones, gibberellic acid and abscisic acid. *Cell*, 1980, **20**, 479–485.
15. Das, G. and Sen-Mandi, S., Scutellar amylase activity in naturally aged and accelerated aged wheat seeds. *Ann. Bot.*, 1992, **69**, 495–501.
16. Richa and Sharma, M. L., Enhancing the germination of stored bamboo seeds using plant growth regulators. *Seed Sci. Technol.*, 1994, **22**(2), 313–317.
17. Puntarulo, S. and Boveris, A., Effect of natural and accelerated ageing on the hydroperoxide metabolism of soybean embryonic axes. *Plant Sci.*, 1990, **69**, 27–32.
18. Cakmak, I., Dragan, S. and Marschner, H., Activities of hydrogen peroxide-scavenging enzymes in germinating wheat seeds. *J. Exp. Bot.*, 1993, **44**, 127–133.
19. Gidrol, X., Lin, W. S., Degousee, N. and Kush, A., Accumulation of reactive oxygen species and oxidation of cytokinin in germinating soybean seeds. *Eur. J. Biochem.*, 1994, **224**, 21–28.
20. Basra, A. S., Singh, B. and Malik, C. P., Amelioration of the effect of ageing in onion seeds by osmotic priming and associated changes in oxidative metabolism. *Biol. Plant.*, 1994, **36**(3), 365–371.
21. Bewley, J. D., Seed germination and dormancy. *Plant Cell*, 1997, **9**, 1055–1066.
22. Sung, J. M. and Jeng, T. L., Lipid peroxidation and peroxide-scavenging enzymes associated with accelerated ageing of peanut seeds. *Physiol. Plant.*, 1994, **91**, 51–55.
23. Sung, J. M., Lipid peroxidation and peroxide-scavenging in soybean seeds during aging. *Physiol. Plant.*, 1996, **97**, 85–89.

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## A protocol for multiplication and restoration of *Ceropegia fantastica* Sedgw.: a critically endangered plant species

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*Ceropegia fantastica* Sedgw. (Asclepidaceae) is a critically endangered, endemic species in Western Ghats of India. The fruit and seed setting are very low and *in vitro* propagation is the only viable alternative for its sapling raising and restoration of this plant's population in the wild natural environment through reintroduction. Attempts have been made here for regeneration of this species through *in vitro* technique using nodal segments as explants and up to 13 multiple shoots were initiated on Murashige and Skoog's (MS) basal medium supplemented with 6-benzyl aminopurine ( $1.5 \text{ mg l}^{-1}$ ). Shoots were multiplied by routine periodic subcultures. The shoots of 3–4 cm length were isolated and rooted on MS basal medium (without  $\text{CaCl}_2$ ) containing indole-3-butyric acid ( $1 \text{ mg l}^{-1}$ ). The rooted plantlets were hardened and successfully established in pots. More than 250 hardened plantlets in two successive years were transferred to their natural habitats of Western Ghats.

**Keywords:** *Ceropegia fantastica*, nodal explants, restoration, Western Ghats.

THE genus *Ceropegia* includes more than 200 species distributed in the Old World ranging from South-East Asia, India, Madagascar, Tropical Arabia, Canary Islands, Africa except Mediterranean region, New Guinea and Northern Australia. In India, about 50 species are present and most of them are endemic to Western Ghats<sup>1,2</sup>, which is one of the centres of diversity of *Ceropegia*<sup>3,4</sup>. The tubers of many *Ceropegia* species contain starch, sugar, gum, albuminoids, fats and crude fibre which are useful as a nutritive tonic<sup>5</sup>. The tuberous roots of many species of *Ceropegia* are edible and are eaten by local inhabitants and animals<sup>6</sup>, causing threat to their survival in the nature. The bitter principle of the root is due to the presence of an alkaloid called Ceropegine<sup>7</sup>. Additionally, propagation either by seed or by vegetative techniques is rather difficult and cumbersome (unpublished work). Habitat modification is one of the major causes for reduction of natural population of this species. Because of these constraints, their distribution is strictly confined mainly to the highly protected areas. The majority of endemic species are grown in limited areas and some of them are

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known only from their specific localities and several are on the verge of extinction<sup>8</sup>. *Ceropegia fantastica* is a twining herb with tuberous roots and is endemic to Goa, Karnataka and Maharashtra region of Western Ghats of India and grows on a lateritic soil in open semi-evergreen forests and among shrub vegetation<sup>2</sup>. Rapid *in vitro* multiplication using nodal buds is a potent technique for mass multiplication which is known to be efficient for conservation of threatened plant species<sup>9</sup>. Several constraints such as scarcity of pollinators, poor seed set and low seed viability may be responsible for its rarity. *In vitro* propagation is the solitary appropriate alternative for its sapling raising and re-establishment of population in wild through new plantations. The present study describes *in vitro* propagation of *C. fantastica* through axillary bud culture as an alternative method to achieve a higher rate of shoots multiplication and regeneration of plantlets and successful transplantation of tissue culture plants (Figure 1). It is the first step towards the conservation and recovery of this valuable plant species.

The tubers of *C. fantastica* were collected from the localities of Netravali (South Goa) and Gavase (Kolhapur district, Maharashtra) in Western Ghats and maintained in pots in the polyhouse of Botanical Garden of Shivaji University, Kolhapur. The nodal segments from *in vitro* grown seedlings and shoots sprouted from tubers were used as explants for the culture establishment. The explants were washed with running tap water for 30 min followed by a wash with a liquid soap (Labolene) (5% v/v) for 10 min. The explants were further repeatedly washed in double distilled water followed by surface sterilization with mercuric chloride (0.1% w/v) for 7 min and rinsing 4–5 times in sterile distilled water to remove the traces of mercuric chloride. The seeds were germinated on MS basal medium and on ½ strength Murashige and Skoog (MS) medium (Table 1) and nodal segments from the aseptic seedlings were used to establish the cultures. The nodal segments of about 1 cm length containing an axillary bud were excised and cultured on the nutrient medium.

MS medium<sup>10</sup> with major and minor salts, vitamins, FeEDTA, inositol (100 mg l<sup>-1</sup>), 30 g l<sup>-1</sup> sucrose and 2 g l<sup>-1</sup> Gelrite (Gellan Gum, Sigma) was used as the basal medium. The pH of the medium was adjusted to 5.8 before autoclaving. The media were steam sterilized in an autoclave under 15 psi and 121°C for 20 min. All cultures were maintained at 25 ± 2°C with 16 h light/8 h dark photoperiods (light intensity of 25 µmol m<sup>-2</sup> s<sup>-1</sup>, Philips TL 34).

MS medium supplemented with various cytokinins and auxins such as 6-benzyl aminopurine (BAP), 1-naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA), kinetin (KN) and thiodiazuron (TDZ) (Table 2) were studied for their effects on shoot multiplication. MS medium without growth hormones served as the control. Subcultures were carried out at four weeks intervals. The

efficiency of each medium variant was recorded after four weeks in terms of explants response and the number of shoots per explant. *In vitro* raised shoots with 3–5 cm length were separated and individual shoots were transferred for rooting on MS medium containing various auxins such as IBA, IAA and NAA (Table 3) and the number of roots per explant was recorded after four weeks. MS medium without growth hormones served as the control.

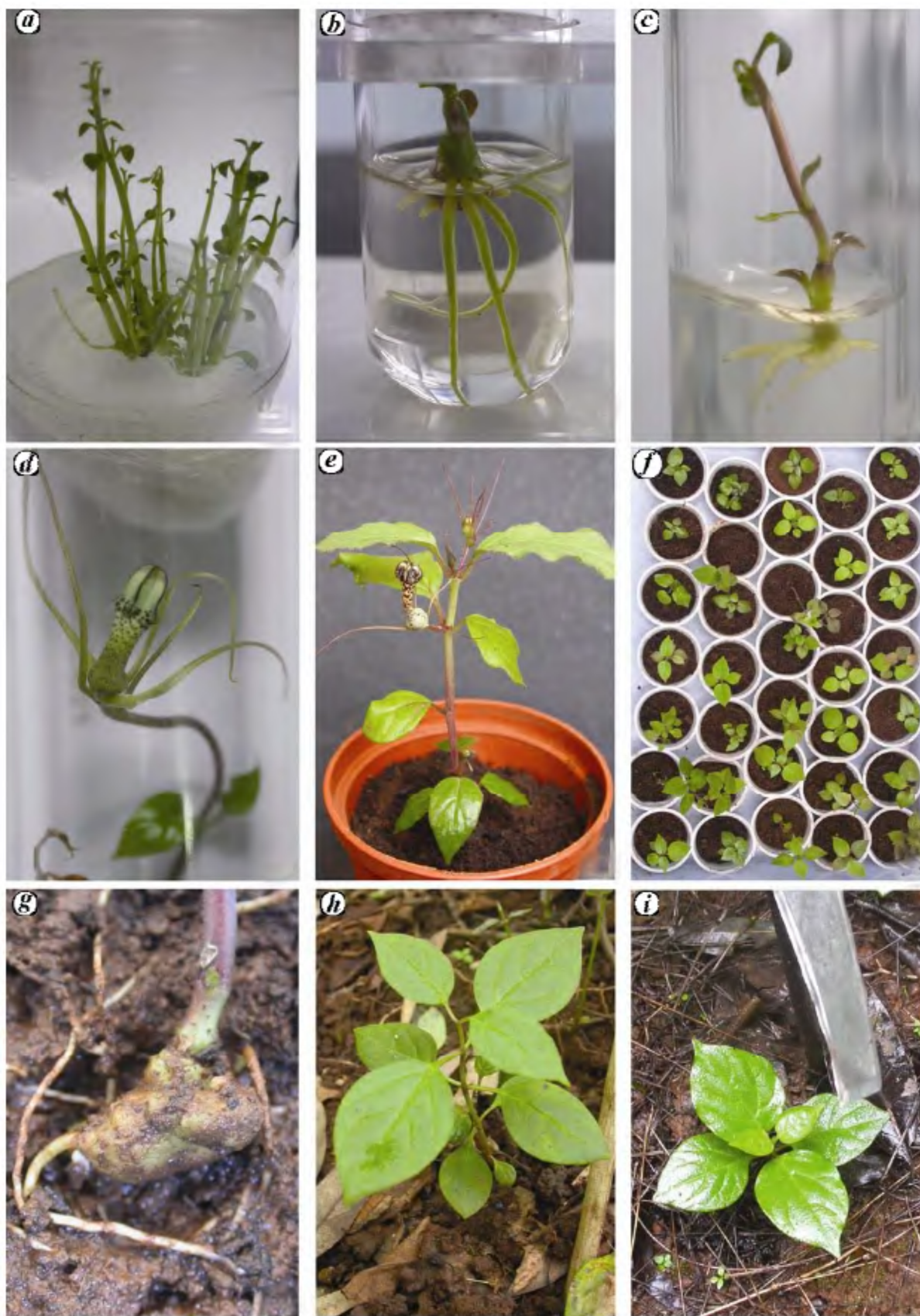
The effect of different treatments was quantified and the data were analysed by Dunnett multiple comparisons test by using one way ANOVA. All experiments were repeated at least thrice with 10 replicates. The results were recorded after four weeks of culture.

The rooted plantlets were first hardened in the culture room in pots with mixture of soil and coco-pit (1 : 1) supplemented with ½ strength MS basal medium without sucrose, agar and growth regulators for two weeks. Then they were transferred in the polyhouse and irrigated with water regularly for four weeks and successfully established in pots with 65% success rate. To test the survival of the hardened plants in their natural habitat, 50 and 200 plants were planted in the first and second year respectively. The monitoring of the plant growth and development confirmed their successful restoration, paving a way for a large scale transplantation of tissue culture raised plants.

To obtain uniform and aseptic explants, *in vitro* seed germination was carried out. Seed germination showed improved results, i.e. 90% on 1/2 strength MS basal medium which was better than MS basal, i.e. 75%. After eight weeks, *in vitro* raised seedlings of 2–3 cm length were used as explants for multiplication.

MS medium supplemented with 0.5–3.0 mg l<sup>-1</sup> BAP was used for shooting. Among the tested formulations, MS medium with 1.5 mg l<sup>-1</sup> BAP was found best suited for the axillary bud proliferation and up to 13 shoots were observed after 4 weeks of culture (Figure 1a). In *Ceropegia jainii* and *Ceropegia bulbosa*, BAP alone was reported as a most effective cytokinin<sup>11</sup>. The number of shoots significantly reduced with further increase in the concentration, i.e. 7.4, 3.2, 2.1 on MS basal medium supplemented with BAP 2, 2.5 and 3 mg l<sup>-1</sup> respectively. Combinations of other growth regulators such as NAA, IBA, KN and TDZ were also effective in axillary bud proliferation, however, the efficiency of BAP was found to be superior in the present study. In addition, occasional *in vitro* flowering was noticed on MS medium provided with TDZ 0.5 mg l<sup>-1</sup> (Figure 1d), as has been reported earlier in *C. jainii* on 1/2 strength MS medium with 0.5 mg l<sup>-1</sup> of BAP and 50 µg l<sup>-1</sup> of spermine<sup>11</sup>.

The *in vitro* regenerated shoots of 3–5 cm length were excised and were planted for developing plantlets by means of *in vitro* rooting. For this, the MS medium supplemented with IBA, IAA and NAA was used. Root primordia emerged from the shoot base within 15–20 days after transfer and subsequently developed into roots



**Figure 1.** *In vitro* propagation of *Ceropegia fantastica*. **a**, Axillary bud multiplication on MS + 1.5 mg/l BAP. **b**, **c**, Rooting on MS (without  $\text{CaCl}_2$ ) + 1 mg/l IBA. **d**, *In vitro* flowering on MS + 0.5 mg/l TDZ. **e**, Hardened plant with flowering, **f**, Hardened plants before plantation stage. **g**, Tuber formation in field. **h**, Plants in natural habitat (first year). **i**, Plants in natural habitat after first showering (second year).

## RESEARCH COMMUNICATIONS

**Table 1.** Performance of seed germination of *Ceropegia fantastica* Sedgw. on MS basal medium

Medium	Germination (%)
1/2 Strength MS basal	90
MS basal	75

**Table 2.** Effect of different plant growth regulators on shoot induction and multiplication from nodal explants of *Ceropegia fantastica* Sedgw.

Growth regulators (mg l <sup>-1</sup> )					Shoots/explant (mean ± SE)
BA	NAA	IBA	KN	TDZ	
Growth regulator free (MS)					00
0.5					2.7 ± 0.30**
1.0					4.5 ± 0.30**
1.5					10.2 ± 0.48**
2.0					7.4 ± 0.52**
2.5					3.2 ± 0.71**
3.0					2.1 ± 0.60**
1.0	0.5				3.3 ± 0.30**
1.0	1.0				2.9 ± 0.37**
1.0	1.0	1.0			1.3 ± 0.15*
			0.5		0.7 ± 0.15*
			1.0		1.1 ± 0.17*
			2.0		1.7 ± 0.21**
			3.0		1.3 ± 0.15*
				0.1	0.6 ± 0.21*
				0.5	1.5 ± 0.22**
				0.5	1.1 ± 0.17*
2.0		0.1			5.1 ± 0.43**
2.0		0.5			4.1 ± 0.37**

Values represent mean ± SE of 10 replicates per treatment and all the experiments were repeated thrice. Values are significantly different \**P* < 0.05 and \*\**P* < 0.01 level when compared by Dunnett multiple comparisons test using one way ANOVA.

**Table 3.** Effect of various concentrations of auxins on *in vitro* root induction of *Ceropegia fantastica* Sedgw.

Growth regulators (mg l <sup>-1</sup> )			Roots/shoot (mean ± SE)
IBA	IAA	NAA	
Growth regulator free (MS)			00
0.5 <sup>#</sup>			3.4 ± 0.30**
1.0 <sup>#</sup>			9.0 ± 0.78**
1.5 <sup>#</sup>			5.2 ± 0.57**
2.0 <sup>#</sup>			2.1 ± 0.48**
2.5 <sup>#</sup>			0.7 ± 0.21*
3.0 <sup>#</sup>			0.8 ± 0.32*
1.0	1.0		0.5 ± 0.16*
	0.5		0.7 ± 0.21*
	1.0		1.0 ± 0.21*
	2.0		0.6 ± 0.16*
	3.0		0.4 ± 0.16*
0.5		0.5	0.5 ± 0.16*
1.0		1.0	0.3 ± 0.15*
1.0		0.5	0.9 ± 0.23*
0.5		1.0	0.6 ± 0.22*

<sup>#</sup>Medium without CaCl<sub>2</sub>.

The values represent mean ± SE of 10 replicates per treatment and all the experiments were repeated thrice. Values are significantly different \**P* < 0.05 and \*\**P* < 0.01 level when compared by Dunnett multiple comparisons test using one way ANOVA.

without the basal callus. The effect of 1 mg l<sup>-1</sup> IBA was found to be superior to IAA, exhibiting an average of nine rootlets (Figure 1 b).

The rooted plantlets were first hardened in the culture room in the pots with a mixture of soil and coco-pit (1 : 1) supplemented with 1/2 strength MS basal medium without sucrose, Gelrite and growth regulators for two weeks (Figure 1 e) which showed 90% success. After two weeks, plants were transferred in the polyhouse and irrigated with water for four weeks and successfully established in the pots at 65% success rate (Figure 1 f). The hardened plants were transplanted in their natural habitat in Western Ghats at 18 different localities.

An efficient and reproducible protocol developed for *in vitro* propagation of *C. fantastica* (Figure 1) has been demonstrated in the present report. This regeneration protocol will help in restoration and conservation of other rare and threatened plants. Micropropagation approach could be a viable option in domestication and commercial cultivation of *C. fantastica*. It is the first step towards its conservation and recovery. Probably, it is the first case of restoration of critically endangered, herbaceous plant species in its natural habitat by using biotechnological tools.

1. Surveswaran, S., Kamble, M. Y., Yadav, S. R. and Sun, M., Molecular phylogeny of *Ceropegia* (Asclepiadoideae, Apocynaceae) from Indian Western Ghats. *Plant Syst. Evol.*, 2009, **281**, 51–63.
2. Jagtap, A. and Singh, N. P., *Fascicle of Flora of India*, BSI, Calcutta, 1999, pp. 211–241.
3. Malpure, N. V., Kamble, M. Y. and Yadav, S. R., *Curr. Sci.*, 2006, **91**, 1140–1142.
4. Yadav, S. R., Flytrap flowers of Western Ghats. *Hornbill*, 1996, **1**, 1–7.
5. Kirtikar, K. R. and Basu, B. D., *Indian Medicinal Plants*, Bishen Singh Mahendrapal Singh, Dehradun, 1935, vol. 3, p. 1638.
6. Mabberley, D. J., *The Plant Book*, Cambridge University Press, Cambridge, 2001, p. 143.
7. Anon., *Ceropegia* Linn. (Asclepiadaceae). In *The Wealth of India*, CSIR, New Delhi, 1992, vol. 3, pp. 448–449.
8. Yadav, S. R., Sardesai, M. M. and Gaikwad, S. P., *J. Bombay Nat. Hist. Soc.*, 2004, **101**, 141–143.
9. Bapat, V. A., Yadav, S. R. and Dixit, G. B., Rescue of endangered plants through biotechnological applications. *Natl. Acad. Sci. Lett.*, 2008, **31**, 201–210.
10. Murashige, T. and Skoog, F., A revised medium for growth and bioassays with tobacco tissue culture. *Physiol. Plant.*, 1962, **15**, 473–497.
11. Patil, V. M., Micropropagation studies in *Ceropegia* spp. *In Vitro Cell Dev. Biol. Plant.*, 1996, **34**, 240–243.

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