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Axillary Multiplication of *Ceropegia mahabalei* Hemadri & Ansari and *Ceropegia media* (Huber) Ansari: Critically Endangered Ethnomedicinal Herbs of Western Ghats, Maharashtra State of India

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ABSTRACT

A rapid micropropagation system was developed for critically endangered ethnomedicinal herbs *Ceropegia mahabalei* Hemadri & Ansari and *C. media* (Huber) Ansari. For shoot multiplication nodal, internodal and leaf explants were cultured on Murashige and Skoog (MS) medium supplemented with different concentrations of cytokinins [(6-Benzyladenine: BA or Kinetin: Kin (0.0-10.0 µM)] alone and in combination with auxins [(α-naphthaleneacetic acid: NAA; indole-3-acetic acid: IAA; 2,4-dichlorophenoxyacetic acid: 2,4-D (0.5-1.5 µM)]. Maximum number of shoots were produced on subculture of nodal explants of *C. mahabalei* and *C. media* on MS medium supplemented with 5.00 µM BA. The type and age of explant and addition of auxins (NAA, IAA or 2,4-D: 0.5-1.5 µM) in the medium influenced shoot multiplication. The best medium for rooting of shoots of both the species was liquid MS medium containing 1.0 µM NAA and 4% sucrose. Well rooted plantlets were transplanted into soil and about 88% of the plantlets survived well upon transferred to natural conditions. The plantlets were morphologically identical to the parental plants. This work may be helpful for *in vitro* propagation, *ex situ* conservation and genetic manipulation of these species.

Keywords: Asclepiadaceae, conservation, critically endangered, micropropagation, Western Ghats
Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; **BA**, 6-benzyladenine; **IAA**, indole-3-acetic acid; **IBA**, indole-3-buyric acid; **Kin**, kinetin; **MS**, Murashige and Skoog; **NAA**, α-naphthalene acetic acid

INTRODUCTION

Ceropegia L. (Asclepiadaceae, subfamily Asclepiadoideae, tribe Stapelieae) is a tropical genus containing more than 200 species. The Indian subcontinent is represented by 48 species (Malpure *et al*. 2006) and most of them are endemic to Western Ghats an important biodiversity hotspot considered as center for the genus (Malpure *et al*. 2006; Surveswaran *et al*. 2009). To satisfy the needs of overgrowing population, the land of Western Ghats is used for agricultural expansion, plantations and infrastructural projects. At the same time intensive harvesting of forest products has resulted in loss of forests, grasslands and habitat for several plant and animals (Davidar *et al*. 2007). The huge raw material of medicinal plants is met from their wild populations. This threatens the survival of many rare species (Natesh 1999) Presently, 2180 plant species are recorded as endemic and some of them are categorized as rare, endangered and threatened in Western Ghats (Myers *et al*. 2000).

The loss of germplasm with such ease is not affordable and needs to apply conservation strategies. The conservation strategies through biosphere reserves, sanctuaries and national parks can preserve the breeding system, but this alone is insufficient for their sustained use (Krishanan *et al*. 2011). Tissue culture can be seen as one of the valuable tool in conservation of rare, endangered and threatened species. Particularly axillary bud induction and proliferation is a well established concept in tissue culture and widely used technique for micropropagation (Joshi and Dhar 2003; Bapat *et al*. 2008). The technique may provide complementary conservation and ecorestoration options for plant species with limited reproductive capacity or which cannot be propagated by traditional propagation method (Joshi and Dhar 2003; Anis and Faisal 2005). It is also the prerequisite for genetic transformation and applications of other biotechnological approaches. *In vitro* propagation through organogenesis and somatic embryogenesis has been reported in some medicinal plants of the Asclepiadaceae (Reddy *et al*. 1998; Sreekumar *et al*. 2000; Gangaprasad *et al*. 2005; Thomas and Shankar 2009; Ugraiah *et al*. 2010) including some species of *Ceropegia* (Patil 1998; Beena *et al*. 2003; Nikam and Sawant 2007; Nikam *et al*. 2008; Chandore *et al*. 2010; Chavan *et al*. 2011).

Ceropegia mahabalei Hemadri & Ansari (Gauti Kharpudi) an erect, tuberous herb, stem hairy, leaves linear-lanceolate, sub-sessile, inflorescence cymes uni-flowered, flowering in August to September (Jagtap and Singh 1999). *Ceropegia media* (Huber) Ansari (Medi Kharchudi) a slender, tuberous twiner, stem glabrous, leaves linear-lanceolate, cymes few flowered. Both species of *Ceropegia* are endemic to Western Ghats region of Maharashtra state of India (Jagtap and Singh 1999). Tubers are used by the tribal's for the treatment of many diseases especially diarrhea and dysentery (Kirtikar and Basu 1935). The beautiful flowers of these species are of great interest and are under risk due to excessive collection by enthusiasts. Natural regeneration of both species is through perennial tubers, which sprouts in next rainy season and developed single plant. Seed setting and development of new plants through seeds is poor may be due to reciprocal pollination and availability of limited

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number of plants. In addition uncertain raining in the region disturbs the developmental synchronicity to regenerate natural populations. Due to loss of its habitat through overgrazing by domestic animals and use of roasted tuber as starchy food for humans, both the species *C. mahabalei* (Threat E:13883; IUCN 1978; Nayar and Sastry 1988; Singh *et al*. 2001; BSI 2002) and *C. media* are critically endangered (Singh *et al*. 2001). In view of its utility and restricted distribution, extensive *in situ* as well as *ex situ* conservation is needed for restoration and exploitation. In the present investigation, we describe an efficient micropropagation protocol for *C. mahabalei* and *C. media*.

MATERIALS AND METHODS

Explant source, culture conditions and shoot regeneration

The plants of *C. mahabalei* and *C. media* collected from natural habitats of Junnar Tehsil area, Maharashtra, India were established at Botanic Garden, Department of Botany, University of Pune and used as a source of explants. The voucher specimen of *C. mahabalei* (CERMAP1) and *C. media* (SGGCM5) were deposited and authenticated at Botanical Survey of India (BSI), Western Circle, Pune, Maharashtra, India.

Nodal, internodal and leaf explants from a one-month-old plant were washed thoroughly under tap water for 10 min followed by 5 min with 0.1% (v/v) aqueous solution of Tween-20 and rinsed 5 times with sterile distilled water (SDW). Then surface sterilized with 0.1 % (w/v) aqueous $HgCl_2$ solution for 5 min. After rinsing with SDW 8 times, they were inoculated in culture tubes containing 15 ml of agar-solidified MS (Murashige and Skoog 1962) medium and MS fortified with various concentrations (0.0-10.0 µM) of cytokinins (6-benzyladenine: BA or kinetin: Kin) alone and in combination with auxins [α-naphthaleneacetic acid: NAA; indole-3-acetic acid: IAA or 2,4-dichlorophenoxyacetic acid: 2,4-D (0.5-1.5 µM)]. The pH of medium was adjusted to 5.8 prior to autoclaving at 121°C for 15 min. The cultures were incubated at 25 ± 2°C under 60 ± 10% relative humidity and 8 h photoperiod with 40 µmol m^{-2} s^{-1} photon flux density provided by white fluorescent tubes.

To know the influence of position of explant and physiological age of plant, the nodal explants from basal, middle and apical portion of stem of 1-, 2- and 3-month-old plants were cultured on shoot regeneration medium (MS + 5.0 µM BA) and observed for multiple shoot regeneration.

Maintenance and proliferation of shoot cultures

To promote proliferation, regenerated shoots were excised and remaining portion of mother nodal explants were transferred onto fresh shoot regenerative medium supplemented with 3% sucrose, 5.0 µM BA at 3-week intervals. The cultures were incubated under same conditions as for shoot initiation. The mother explants were maintained and proliferated by subculturing at 3-week intervals over a period of 14 months.

Induction of rooting and acclimatization

The shoots (3-4 cm) were excised from 5-week-old cultures grown on MS medium containing 5.0 µM BA and transferred to liquid MS medium supplemented with 3% of sucrose and different concentrations of NAA, IAA or indole-3-butyric acid, IBA (0.0-2.5 µM). Shoots were also transferred to liquid MS medium containing various concentrations of sucrose (1-5%) alone and in combination of selected concentrations of sucrose (3-5%) and NAA or IAA (0.5-1.5 µM). One excised shoot was cultured in each tube (25 × 150 mm) containing 15 ml medium.

Rooted shoots were thoroughly washed with tap water in tray to remove the medium and planted in earthen pots (28 cm diameter × 30 cm height) containing a well watered mixture of garden soil and sand in the ratio of 1:1 (w/w). The potted plantlets were covered with perforated plastic glass, maintained in shade net house (temperature 25 ± 5°C; humidity 80 ± 10%; maximum light 200 µmol m^{-2} s^{-1}) for two weeks acclimatization and then transferred to field conditions (temperature 18–32°C; humidity 55–80%; maximum light 400 µmol m^{-2} s^{-1}). For the first two weeks daily 10 ml watering was made near the plantlets in the morning (8.00 am) and evening (6.00 pm). Later watering (one liter) was made at 2-day intervals. Percentage of survival was recorded two months after transfer.

Data analysis

A completely randomized design (CRD) was used in all experiments. In each experiment 21 replicates were used and repeated at least three times. Data were subjected to analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) at the 5% probability level.

RESULTS AND DISCUSSION

Shoot regeneration

The nodal cultures were initiated on MS basal medium supplemented with various concentrations of BA and Kin (0.0-10.0 µM). The first visible sign of axillary shoot initiation were observed 12-14 days after culture incubation, with swelling at cut end of explants. The nodal explants, when inoculated on MS basal medium without growth regulators, showed single shoot proliferation in 29 and 21% of explants in *C. mahabalei* and *C. media*, respectively (**Figs. 1, 3**).

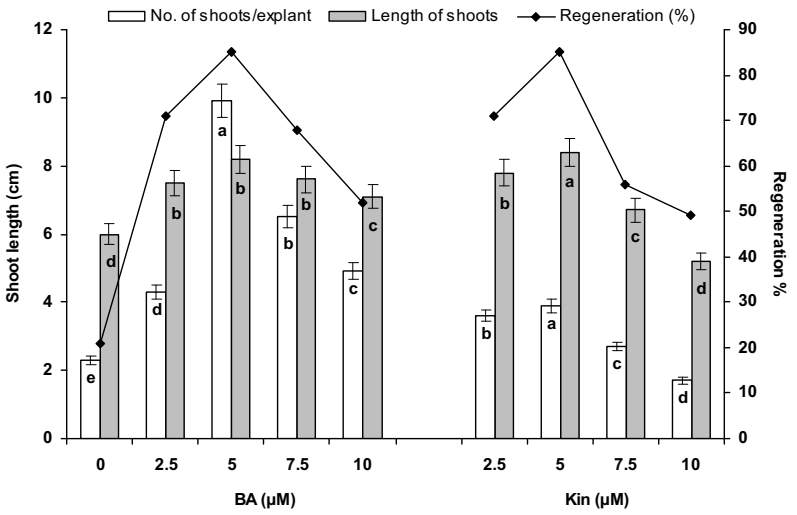

Fig. 1 Effect of BA and Kin on shoot regeneration in nodal explants of *C. media*. The data represent mean ± SE followed by same letter within columns are not significantly different by DMRT at the 5% probability level. DMRT was applied to BA and Kin separately for each parameter.

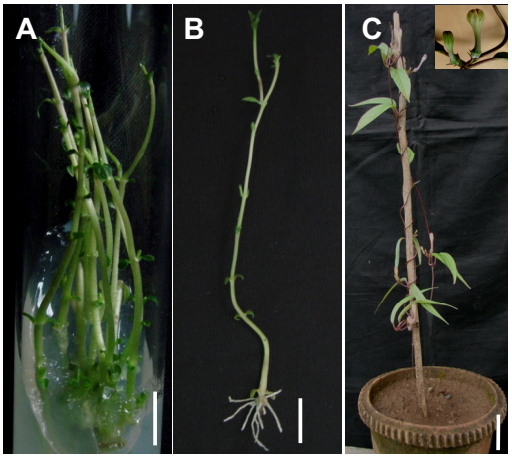

Fig. 2 Micropropagation of *Ceropegia media*. (**A**) Multiple shoot regeneration from nodal explant on MS medium containing 5.0 µM BA (bar = 1.0 cm). (**B**) Rooting of *in vitro* regenerated shoots on MS medium with 1.0 µM NAA and 4% sucrose (bar = 1.0 cm). (**C**) Plant acclimatized to natural condition (bar = 10.0 cm).

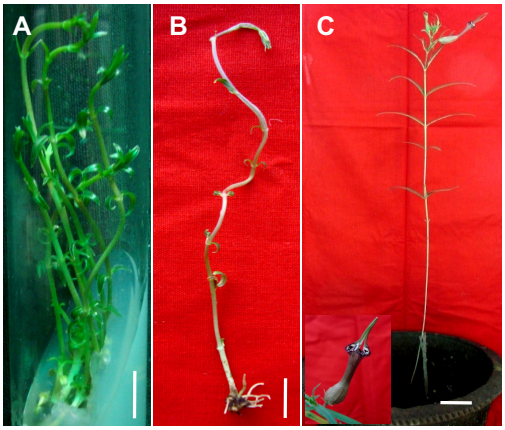

Fig. 4 *In vitro* propagation of *Ceropegia mahabalei*. (**A**) Shoot proliferation from a nodal explant on MS medium containing 5.0 µM BA (bar = 1.0 cm). (**B**) Rooting of *in vitro* derived shoot on MS medium containing 1.0 µM NAA and 4% sucrose (bar = 1.0 cm). (**C**) *In vitro* raised plantlet grown in pot (bar = 5.0 cm).

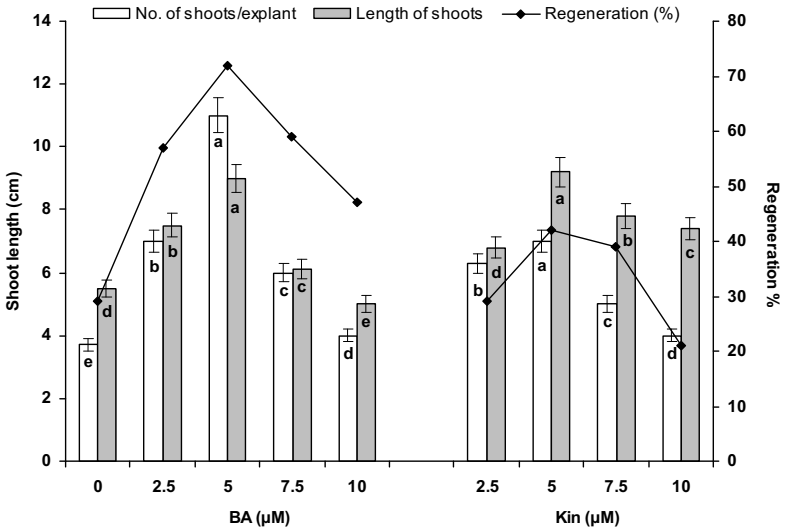

Fig. 3 Effect of BA and Kin on shoot regeneration in nodal explants of *C. mahabalei*. The data represent mean ± SE followed by same letter within columns are not significantly different by DMRT at the 5% probability level. DMRT was applied to BA and Kin separately for each parameter.

Incorporation of cytokinins in the medium greatly promoted the induction of axillary shoot proliferation from nodal explants. The percent frequency of explants responding for shoot regeneration and mean number of shoots per explant increased with increasing concentrations of cytokinins (BA or Kin) up to 5.0 µM (**Figs. 1, 3**).

Of the two cytokinins tested, BA was found to be superior than Kin for shoot formation from nodal explants of *C. mahabalei* and *C. media*. The percent frequency of explant responding for multiple shoot formation (85%), and mean number of shoots/nodal explant (11.0 ± 0.5) has maximum on MS medium fortified with 5.0 µM BA in *C. media* (**Figs. 1, 2A**). Similarly, the incorporation of 5.0 µM BA in MS medium was best for multiple shoot formation in nodal explant of *C. mahabalei*. However, the percent frequency of explants responding to shoot formation (72%) and mean number of shoots/explant (9.9 ± 1.4) (**Figs. 3, 4A**) were less compare to that of *C. media*. Nodal segments are preferred explants for micropropagation due to the presence of pre-existing meristems, which can be easily developed into shoots while maintaining clonal fidelity (Ahuja 1993; Frabetti *et al*. 2009). Cytokinins have the property of bud breaking and induction of multiple shoots (Murashige 1974; Sugla *et al*. 2007). In this investigation, the concentration and the type of cytokinins, position of explants along the stem, age of the plant and genotype used had profound effect on shoot formation in nodal explant of *C. mahabalei* and *C. media*.

The percentage of explants responding for multiple shoot formation (64%) and mean number of shoots/explant (7.0 ± 1.0) in *C. media* declined when Kin (5.0 µM) was substituted for BA in MS medium. Explants took 18-21 days for shoot formation on a Kin-containing medium compared to a BA-fortified medium. Significant differences were not observed in explants responding for multiple shoot formation in *C. media* on Kin- or BA-containing medium. However, in *C. mahabalei* there was significant difference in the average number of shoots/explant (9.9 ± 1.4) on BA-fortified medium compared to Kin-containing medium (3.9 ± 1.0 shoots/explant). Incorporation of different concentrations of Kin (2.5-10.0 µM) to medium fortified with optimum concentrations of BA (5.0 µM) or *vice versa* failed to improve the number of multiple shoots (data not shown). The percentage of explants responding to shoot formation and average number of shoots/explant declined on medium containing optimum concentrations of BA and Kin (5.0 µM) together with different concentrations of auxins IAA, NAA and 2,4-D (0.5-1.5 µM). The presence of 2,4-D drastically affected the capacity of shoot formation (**Table 1**). A similar counteracting effect of auxins on cytokinins (BA or

Table 1 Effect of cytokinins (BA, Kin) in combination with auxins (NAA, IAA) on shoot regeneration in *Ceropegia* spp. nodal explants.

MS + PGR (µM)					*C. mahabalei*			*C. media*		
Cytokinins		Auxins			% of explants responding for shoot regeneration	No. of shoots/explant	Length of shoots (cm)	% of explants responding for shoot regeneration	No. of shoots/explant	Length of shoots (cm)
BA	Kin	NAA	IAA	2,4-D						
0.0	0.0	0.0	0.0	0.0	29	3.7 ± 2.0	5.5 ± 0.2	21	2.3 ± 1.0	6.0 ± 1.0
5.0	-	0.5	-	-	50	4.1 ± 0.3	7.4 ± 0.3	40	2.9 ± 0.3	6.3 ± 0.5
5.0	-	1.0	-	-	61	5.1 ± 0.3	8.3 ± 0.3	48	3.6 ± 0.3	7.2 ± 0.3
5.0	-	1.5	-	-	52	3.8 ± 0.4	7.2 ± 0.6	34	2.6 ± 0.5*	5.8 ± 0.6
5.0	-	-	0.5	-	43	4.1 ± 0.3	7.1 ± 0.3	38	2.6 ± 0.4	6.3 ± 0.5
5.0	-	-	1.0	-	48	4.5 ± 0.3*	7.9 ± 0.2	41	3.3 ± 0.4*	6.9 ± 0.2
5.0	-	-	1.5	-	39	3.2 ± 0.2**	6.8 ± 0.4	-	-***	-
5.0	-	-	-	0.5	-	-***	-	-	-***	-
5.0	-	-	-	1.0	-	-***	-	-	-***	-
5.0	-	-	-	1.5	-	-***	-	-	-***	-
-	5.0	0.5	-	-	46	3.9 ± 0.2	6.8 ± 0.5	32	2.3 ± 0.6	5.8 ± 0.5
-	5.0	1.0	-	-	59	4.9 ± 0.2	7.3 ± 0.6	35	2.8 ± 0.4	6.1 ± 0.4
-	5.0	1.5	-	-	48	3.8 ± 0.5*	6.7 ± 0.5	29	1.9 ± 0.3*	4.5 ± 0.4
-	5.0	-	0.5	-	39	3.5 ± 0.3*	6.5 ± 0.3	27	1.6 ± 0.3*	4.2 ± 0.3
-	5.0	-	1.0	-	45	4.1 ± 0.2**	5.8 ± 0.2	31	2.9 ± 0.5**	5.5 ± 0.5
-	5.0	-	1.5	-	-	-***	-	-	-***	-
	5.0	-	-	0.5	-	-***	-	-	-***	-
	5.0	-	-	1.0	-	-***	-	-	-***	-
	5.0	-	-	1.5	-	-***	-	-	-***	-

The values represent Mean ± SE followed by same letter within columns are not significantly different by DMRT at the 5% probability level. *= Shoot regeneration associated with callus formation; **= Shoot regeneration associated with extensive callus formation; ***= No shoot regeneration but extensive callus formation

Kin) during shoot formation was observed in *Cucurbita* interspecific hybrids (Sarowar *et al*. 2003), *Lagenaria siceraria* (Han *et al*. 2004), *Ceropegia odorata* and *C. maccannii* (Nikam *et al*. 2008) and *Uraria picta* (Ahire *et al*. 2011). In contrast to this synergistic effect of BA in combination with an auxin has been demonstrated in some asclepiad medicinal plants: *Gymnema sylvestre* (Reddy *et al*. 1998), *Holostemma annulare* (Sudha *et al*. 1998), *Hemidesmus indicus* (Sreekumar *et al*. 2000), *Holostemma adakodien* (Martin 2002), *Leptadenia reticulata* (Arya *et al*. 2003), *Ceropegia candelabrum* (Beena *et al*. 2003).

Therefore, BA at 5.00 µM was found to be best for multiple shoot regeneration in both *C. media* and *C. mahabalei* (**Figs. 1, 2A, 3, 4A**). Similar results were reported earlier for shoot regeneration in *Ceropegia bulbosa* var. *bulbosa*, *C. bulbosa* var. *lushii*, *C. jainii* (Patil 1998), *C. candelabrum* (Beena *et al*. 2003); *C. sahyadrica* (Nikam and Sawant 2007), *C. odorata* and *C. maccannii* (Nikam *et al*. 2008), *C. fantastica* (Chandore *et al*. 2010), *C. attenuata* (Chavan *et al*. 2011) and in other asclepiads *viz*. *Hemidesmus indicus* (Patnaik and Debata 1996; Sreekumar *et al*. 2000); *Gymnema sylvestre* and *G. elegans* (Komalavalli and Rao 2000); *Tylophora indica* (Reddy *et al*. 1998); *Decalepis arrayalpathra* (Gangaprasad *et al*. 2005); *Sarcostemma brevistigma* (Thomas and Shankar 2009); *Marsdenia brunoniana* (Ugraiah *et al*. 2010) where BA alone was most effective in multiplication of axillary shoots. BA was reported to be highly effective in axillary shoot proliferation from nodal explants of several other medicinal plants including *Chlorophytum borivilianum* (Dave *et al*. 2003), *Orthosiphon stamineus* (Lee and Chan 2004), *Swertia chirayita* (Joshi and Dhawan 2007), *Pongamia pinnata* (Sugla *et al*. 2007) and *Uraria picta* (Ahire *et al*. 2011). However, *Ceropegia juncea* was exceptional where Kin was more effective for induction of caulogenesis (Nikam and Savant 2009) and BA in combination with NAA and ascorbic acid was best for maximum multiple shoot formation in *Tylophora indica* (Faisal *et al*. 2007); in *Huernia hystrix* maximum shoot induction was observed on MS medium containing BA together with NAA (Amoo *et al*. 2009). Multiple shoot induction efficiency was increased on ascorbic acid supplemented medium along with KN and IBA in *Hoya wightii* (Lakshmi *et al*. 2010). The results revealed that some asclepiads, including most *Ceropegia* spp. respond best to multiple shoot induction on medium containing BA while only *C. juncea* and a few other asclepiads produced maximum shoot regeneration on medium fortified with Kin as well as BA and Kin together with auxins, These differences might be due to interactions in endogenous growth regulators and exogenously applied auxins and cytokinins in asclepiads.

After excising the regenerated shoots on medium fortified with 5.0 µM BA, half of the original explants were subcultured onto medium containing BA at 5.00 µM and observed for shoot proliferation. Average number of shoots/explant in *C. media* (11.0 ± 0.5) and *C. mahabalei* (9.9 ± 1.4) on this medium was increased on subculture at three-week intervals. These results are in accordance with earlier reports on asclepiad where subculture of original explants on parental shoot regeneration medium increased the shoot proliferation in *Hemidesmus indicus* (Sreekumar *et al*. 2000), *Gymnema sylvestre* (Komalavalli and Rao 2000) and *Holostema adakodien* (Martin 2002). Multiplication of plants through the sequential subculture of nodal segments has been achieved for a large number of plant species (Frabetti *et al*. 2009).

After 5 weeks of inoculation of explants, the mean length of shoots was measured. No significant differences were observed in the length of regenerated shoots on MS medium containing BA or Kin up to 7.5 µM (**Figs. 1, 3**). However, shoot elongation was less on media containing higher concentrations of BA (10.0 µM) (**Figs. 1, 3**). Similar observations were recorded in *Mucuna pruriens* (Faisal *et al*. 2006), *Pseudarthria viscida* (Cheruvathur and Thomas 2011), and *Momordica balsamina* (Thakur *et al*. 2011).

The physiological age of the explant is an important factor determining the morphogenetic response (Ammirato 1986). Young meristematic tissue is generally more responsive to *in vitro* culture treatments than mature differentiated tissue (Bhojwani and Razdan 1996). In the present investigation, the nodal, internodal and leaf explants from 1-, 2- and 3-months-old plants on excision were categorized as basal, middle and apical were incubated on MS containing 5.0 µM BA. No significant differences were observed for shoot formation in basal, middle and apical nodal explants of 1- and 2-month-old shoots. However, decline in shoot regeneration response was observed in the explants of 2- and 3-month-old plants. The internodal and leaf explants did not respond to shoot regeneration.

Similarly, in *Hemidesmus indicus*, a decline in response of shoot regeneration of node along the basipetal gradient was noticed and the most mature 8th node was least efficient to produce shoots (Sreekumar *et al*. 2000). Contrasting

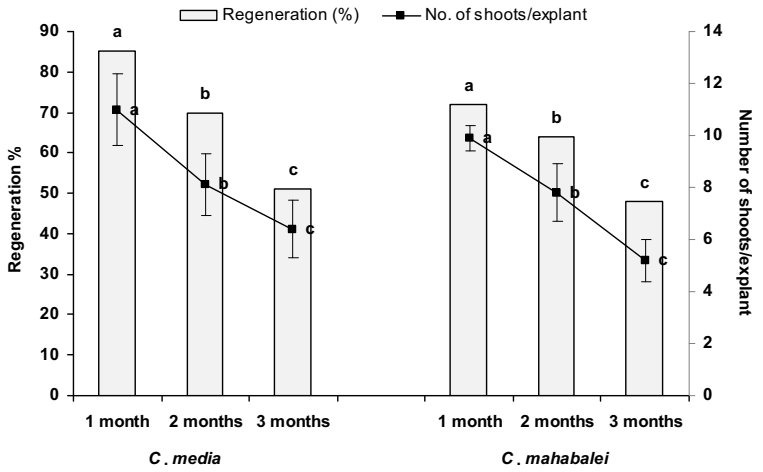

Fig. 5 Effect of age of explant on shoot regeneration on MS medium supplemented with 5.0 µM BA. The data represent mean ± SE followed by same letter within columns are not significantly different by DMRT at the 5% probability level.

Table 2 Effect of liquid MS medium with different concentrations of sucrose together with IAA or NAA on root formation in *in vitro* raised shoots of *Ceropegia* spp..

Sucrose (%)	NAA (µM)	IAA (µM)	*C. media*			*C. mahabalei*		
			Shoots that produce roots (%)	No. of roots/shoot (mean ± SE)	Days to rooting	Shoots that produce roots (%)	No. of roots/shoot (mean ± SE)	Days to rooting
0	0	0	17	1.9 ± 0.6^{i}	23.1	14	1.6 ± 0.4^{j}	24.0
3	0.5	-	39	2.1 ± 0.4^{h}	23.3	24	2.9 ± 0.3^{efg}	24.2
3	1.0	-	50	2.7 ± 0.7^{fgh}	20.9	38	3.5 ± 0.4^{ef}	21.7
3	1.5	-	41	2.5 ± 0.5^{efgh}*	23.6	31	3.2 ± 0.4^{de}*	24.7
4	0.5	-	53	3.2 ± 0.4^{defg}	20.7	68	4.9 ± 0.5^{b}	21.8
4	1.0	-	69	6.5 ± 0.5^{b}	14.6	80	6.1 ± 0.4^{a}	15.1
4	1.5	-	65	5.4 ± 0.4^{a}*	19.4	74	5.7 ± 0.4^{a}*	21.4
5	0.5	-	51	5.0 ± 0.2^{b}	21.3	47	4.2 ± 0.4^{cd}	22.5
5	1.0	-	61	5.7 ± 0.4^{b}	20.3	61	4.4 ± 0.5^{bc}*	22.6
5	1.5	-	56	4.1 ± 0.4^{e}*	22.4	52	4.2 ± 0.4^{bcd}	24.2
3	-	0.5	21	2.9 ± 0.3^{efg}*	21.4	19	2.8 ± 0.5^{efgh}*	23.6
3	-	1.0	29	3.4 ± 0.4^{cde}*	21.3	23	3.3 ± 0.6^{e}*	23.1
3	-	1.5	36	2.7 ± 0.5^{efgh}**	22.5	31	2.5 ± 0.5^{fghi}**	24.7
4	-	0.5	24	3.2 ± 0.3^{defg}**	23.4	22	2.4 ± 0.4^{ghi}**	23.3
4	-	1.0	31	3.7 ± 0.5^{cd}**	22.1	29	3.1 ± 0.4^{efg}**	23.1
4	-	1.5	36	3.2 ± 0.5^{def}**	24.5	31	2.8 ± 0.5^{efgh}**	25.8
5	-	0.5	21	2.1 ± 0.4^{h}**	26.2	17	1.8 ± 0.6^{i}**	26.1
5	-	1.0	26	2.7 ± 0.4^{efgh}**	25.2	23	2.4 ± 0.5^{ghi}**	25.2
5	-	1.5	29	2.4 ± 0.5^{fh}**	24.3	28	2.1 ± 0.4^{hi}**	23.1

The values represent Mean ± SE followed by same letter within columns are not significantly different by DMRT at the 5% probability level. * = Swelling and callusing at base of the shoot followed by rooting; ** = Swelling and extensive callusing at base of the shoot followed by rooting

results were reported by Komalavalli and Rao (1997) in *Gymnema sylvestre*, where node positions 3 and 4 were found to be more responsive than from distal and proximal nodes. It suggest that in members of the Asclepiadaceae, including *Ceropegia*, that the choice of explant is of cardinal importance and makes an absolute difference between success and failure in inducing shoot regeneration *in vitro*. These results agree with the fact that the response of axillary shoot proliferation is genotype dependent (Vijayan *et al*. 2000; Bhau and Wakhlu 2001; Cao *et al*. 2007). Therefore, from a practical point of view, it is of great importance to find protocols that are appropriate for several genotypes (Ryynänen and Häggman 2001).

The findings showed that nodal explants from one-month-old plant perform better than 2- and 3-month-old plants for shoot proliferation (**Fig. 5**). However, the performance for shoot proliferation was declined in basal nodal explants compare to middle and apical explants of 2- and 3-month-old explants. This physiological condition may be related to the fact that, in one month old plants axillary meristem of nodal explants are in active form while the activity was declined with increase in age of the plants.

Rooting of shoots and acclimatization

The best rooting (64.2%) with no intervening swelling and callus was observed within two weeks of transfer to medium containing 1.0 µM NAA with 4% sucrose and culture tubes covered from bottom with black paper (**Table 2; Figs. 2B, 4B**). The application of black paper was advantageous for root formation. The percentage of shoots that formed roots and the number of roots per shoot varied significantly on medium free of growth regulator but with different concentrations of sucrose (1-5%) and on medium containing different concentrations of IAA, NAA and IBA or different combinations of selected sucrose concentrations (3-5%) and IAA or NAA (**Table 2**). Root formation was, however, slow and low on medium without growth regulator and with low concentrations of IAA or NAA (0.5 µM). Prominent swelling followed by callusing and no root formation was observed at higher concentrations of NAA (more than 1.0 µM).

Incorporation of higher concentrations of sucrose (> 5%) causes prominent swelling in the stem portion under the medium; 18% of the shoots showed root formation from the swollen stem base. In the present investigation, rooting of shoots was also influenced by sucrose concentrations (**Table 2**). These results correspond with the reports on *C*.

sahyadrica where maximum frequency of rooting was observed on MS medium containing 5% sucrose and 6.0 mg l^{-1} spermine (Nikam and Sawant 2007). Patil (1998) also reported the rooting of shoots of *C. bulbosa* var. *bulbosa*, *C. bulbosa* var. *lushii* and *C. jainii* on 0.25 MS medium supplemented with 60 g l^{-1} sucrose and 0.5 mg l^{-1} IBA. The rooting was significantly affected by concentration of MS nutrient medium, various concentrations of auxins (IAA, NAA and IBA) and sucrose in *C. odorata* and *C. maccannii* (Nikam *et al*. 2008).

A crucial aspect of *in vitro* propagation is to prepare regenerated plants that are capable of surviving outside the sterile and protected *in vitro* environment. In the present study, the shoots of *C. media* and *C. mahabalei* (**Fig. 2C, 4C**) rooted on MS containing 1.0 µM NAA with 4% sucrose without swelling and callusing showed maximum (88%) survival rate. About 88% survived two months after transfer. The acclimatized plants exhibited normal growth and flowering but rarely set the fruits (**Fig. 2C, 4C**). No apparent phenotypic differences were seen between regenerated from nodal explant and tuber derived control plants.

Results of the present study and earlier reports on *Ceropegia* spp. such as *C. bulbosa* and *C. jainii* (Patil 1998), *C. candelabrum* (Beena *et al*. 2003), *C. sahyadrica* (Nikam and Sawant 2007) and *C. odorata* and *C. maccannii* (Nikam *et al*. 2008) suggest that rooting of *in vitro* regenerated shoots of *Ceropegia* is slightly difficult but acclimatization of the plantlets to the field conditions is easier.

CONCLUSION

Protocol describe in the present study can be useful for conservation and propagation of the important critically endangered medicinal herbs *Ceropegia media* (Huber) Ansari and *Ceropegia mahabalei* Hemadri & Ansari and possibly lead to the synthesis and extraction of active metabolites from the tuber sources.

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