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Ecorestoration of *Ceropegia odorata* Hook and *C. maccannii* Ansari, Endangered Asclepiads, by Micropropagation

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ABSTRACT

An *in vitro* propagation system based on axillary shoot proliferation was developed for the ecorestoration of threatened medicinal and starchy edible tuber-producing asclepiads *Ceropegia odorata* Hook and *C. maccannii* Ansari. Shoot multiplication was induced by different concentrations of benzylamino purine (BA) and kinetin (Kin) (0.0 to 20.0 µM) added separately. In both *C. maccannii* (5.2 ± 0.8 shoots per explant) and *C. odorata* (6.1 ± 0.7 shoots per explant), maximum shoots formed when nodal segments of *in vitro*-raised shoots were cultured on full strength Murashige and Skoog (MS) basal medium fortified with 7.5 µM BA. Inclusion of indoleacetic acid (IAA) (1.0–2.5 µM), naphthaleneacetic acid (NAA) (1.0–2.5 µM) and 2,4-dichlorophenoxyacetic acid (2,4-D) (0.5–1.0 µM) in nutrient media counteracted the shoot induction effect caused by BA (7.5 µM) or Kin (7.5 µM). Shoots rooted most successfully on half-strength liquid medium fortified with 5% sucrose and 0.5 µM indolebutyric acid (IBA). Micropropagated plantlets were transplanted into soil and about 80% of the plantlets survived which were morphologically identical to donor plants. The regeneration protocol developed in this study provides a basis for germplasm conservation and utilization of these plant species.

Keywords: conservation, endemic, *in vitro* propagation, plant growth regulators, starchy tuber
Abbreviations: BA, 6-benzyladenine; **IAA**, indole-3-acetic acid; **IBA**, indole-3-butyric acid; **Kin**, kinetin; **MS**, Murashige and Skoog medium; **NAA**, 2-naphthalene acetic acid; **2,4-D**, 2,4-dichlorophenoxyacetic acid

INTRODUCTION

The genus *Ceropegia* (Asclepiadaceae) consists of about 200 species, distributed in tropical and subtropical Asia, Africa, Australia, Malaysia and the Pacific Islands (Ansari 1984; Jagtap and Singh 1999). Among these, *Ceropegia odorata* Hook. and *C. maccannii* Ansari are highly threatened annual tuberous herbs endemic to India (Lucas and Synge 1978; Nayar and Sastry 1987; Tetali and Tetali 2000; MoEF 2007). In the genus *Ceropegia*, *C. odorata* is unique; its flowers expel an intensive jasmine fragrance and have ornamental potential. Natural regeneration of these herbs is through perennial tubers but it results in the development of a single plant. The tubers of these species are starchy, edible and are also used by locals in drug preparations to cure gastric disorders and stomachache (Jain and Defillips 1991). Cross fertilization reduces seed set and the development of a new plant. Due to drought and over-utilization of wild material by the locals and animals and the lack of attempts to cultivate them, both species are rapidly disappearing from their natural habitats (Tetali and Tetali 2000; Jagtap *et al*. 2004).

Therefore, extensive *in situ* as well as *ex situ* conservation is needed for ecorestoration and utilization of these species (MoEF 2007). To date an *in vitro* multiplication protocol has only been reported in *C. bulbosa* and *C. jainii* (Patil 1998), *C. candelabrum* (Beena *et al*. 2003) and *C. sahyadrica* (Nikam and Sawant 2007).

Over the years, tissue culture has been utilized as a valuable tool not only for mass propagation of rare endangered and threatened medicinal plant species but also for their *ex situ* conservation (Pence 1999; Joshi and Dhar 2003; IAEA 2004; Anis and Faisal 2005). However, according to Sarasan *et al.* (2006), published articles on *in vitro* conservation of threatened plants in international journals are still limited. Biotechnological methods are now an essential component of plant genetic resource management and for this an efficient micropropagation protocol is a prerequisite (Pence 1999; Almeida *et al.* 2005). Since both *C. maccannii* and *C. odorata* are endemic endangered species with difficulties to propagate them by conventional methods, conservation by *in vitro* multiple shoot induction is urgently required. The present paper describes a simple and reliable micropropagation protocol for ecorestoration and utilization of these endangered species.

MATERIALS AND METHODS

Plant material, culture media and incubation conditions

Wild plants of a *C. maccannii* and *C. odorata*, collected from Western Ghats (a hotspot of biodiversity), Maharashtra, India, were grown in the botanical garden, Department of Botany, University of Pune, Pune, India. These plants (~four weeks old, vegetative stage) served as the source of explants for establishing aseptic cultures. The internode, node and leaf explants excised from the stem apex up to the 6th node were first washed thoroughly with sterilized distilled water (SDW) followed by surface sterilization with 0.1% $HgCl_2$ solution for 5-7 min. The surface sterilized explants were washed thoroughly 5X with SDW and left in it until inoculation (maximum 10 min). The exposed cut portions of the surface sterilized explants were excised by applying a fresh cut and internode (10 mm), node (10 mm) and leaf (10 mm^2) explants were inoculated on MS (Murashige and Skoog 1962) medium supplemented with 3% (w/v) sucrose, 8 g l^{-1} agar and benzylamino purine (BA), kinetin (Kin), indole-3-acetic acid (IAA), naphthalene acetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D), individually and in combination. The pH of the media was adjusted to 5.5-6.0 before autoclaving at 121°C for 15 min. The

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Original Research Paper

Table 1 Effect of cytokinins (BA and Kin) alone and in combination with auxins (IAA and NAA) on shoot multiplication in *Ceropegia maccannii* Ansari and *C. odorata* Hook.

MS + Plant Growth Regulator (µM)				*Ceropegia maccannii* Ansari		*Ceropegia odorata* Hook	
Cytokinins		Auxins		% Shoot regeneration	Mean № of shoots/explant	% Shoot regeneration	Mean № of shoots/explant
BA	Kin	IAA	NAA				
0.0	0.0	0.0	0.0	10.6 ± 0.4	0.1 ± 0.04 j	9.2 ± 0.4	0.3 ± 0.3 k
2.5				62.9 ± 0.8	1.8 ± 0.3 ef	55.9 ± 0.6	3.7 ± 0.5 d
5.0				100 ± 0.0	3.3 ± 0.7 c	100 ± 0.0	5.7 ± 0.6 b
7.5				100 ± 0.0	5.2 ± 0.8 a	100 ± 0.0	6.1 ± 0.7 a
10.0				91.7 ± 0.9	3.8 ± 0.7 b *	98.2 ± 0.9	2.9 ± 0.7 e *
	2.5			55.7 ± 0.7	1.4 ± 0.6 h	49.6 ± 0.8	1.9 ± 0.5 f
	5.0			75.4 ± 0.9	2.0 ± 0.7 d	82.1 ± 0.6	3.8 ± 0.8 d
	7.5			98.2 ± 0.8	3.1 ± 0.6 d	98.4 ± 0.7	5.0 ± 0.3 c
	10.0			97.3 ± 0.9	1.6 ± 0.8 g *	75.6 ± 0.8	1.3 ± 0.7 hi *
7.5		1.0		95.9 ± 0.7	1.9 ± 0.5 de **	98.5 ± 0.6	1.6 ± 0.6 g *
7.5		2.5		64.7 ± 0.7	1.4 ± 0.7 h **	84.2 ± 0.5	1.3 ± 0.7 hi **
7.5			1.0	65.7 ± 0.5	1.6 ± 0.7 g **	59.5 ± 0.9	1.6 ± 0.8 g *
7.5			2.5	-	- ***	44.0 ± 0.7	1.1 ± 0.6 j **
	7.5	1.0		95.6 ± 0.4	1.7 ± 0.5 fg **	93.4 ± 0.8	1.4 ± 0.7 h *
	7.5	2.5		64.7 ± 0.6	1.1 ± 0.6 i **	67.4 ± 0.6	1.2 ± 0.6 ij **
	7.5		1.0	65.6 ± 0.5	1.4 ± 0.7 h **	63.8 ± 0.6	1.3 ± 0.8 hi **
	7.5		2.5	-	- ***	-	- ***

Values are mean ± SE. Different letters indicate significant differences at P=0.05 in each column.
* = Callus-mediated shoot formation
** = Explant produced more callus
*** = Only extensive callus formation

nodal explants from 5 weeks-old *in vitro*-raised shoots were excised and inoculated in 25 × 150 mm borosilicate culture tubes (single explant per tube) containing MS basal medium supplemented with 7.5 µM BA. All cultures were incubated at 25 ± 2°C with 8/16 h photoperiod provided by cool white fluorescent tubes (50–80 µmole $m^{-2}s^{-1}$). Tissue culture stocks of *C. odorata* and *C. maccannii* were maintained with regular sub-culturing at 28 day intervals to fresh micropropagation medium.

MS salts, sucrose and agar (extra pure grade) were obtained from HiMedia Ltd., India. Vitamins and growth regulators were from Sigma Chem, USA.

Rooting and acclimatization of plantlets

The regenerated elongated shoots (4-5 cm) obtained on MS basal medium supplemented with 7.5 µM BA were excised and transferred to agar-solidified or liquid rooting MS media containing either IAA, NAA or IBA and sucrose (3 to 5%). After 5 weeks, rooting was evaluated and expressed in terms of rooting frequency, and root number per plantlet. Plants with a well-developed root system (1–2 cm long) and shoots (7–9 cm long) were removed from the rooting medium. The base of the shoot with a root system was washed with tap water to remove the media constituents and planted in pre-watered earthen pots filled with garden soil. The shoots were covered with a glass beaker to maintain high humidity (90–100%) for two days. Humidity was then decreased gradually by lifting and keeping the beaker 2 cm above the soil, for one week. Later, in the subsequent week the beaker was removed frequently and in the third week potted plantlets were transferred to natural conditions (temperature approximately 22–34°C, light intensity approximately 300 µmol m^{-2} s^{-1}). The phenotypic characters of *in vitro*-raised potted plants were observed.

Experimental design and statistical analysis

There were five explants per bottle (300 ml) or single explant per test tube (25 × 150 mm) and the treatments were arranged in a completely randomized design (CRD) with 10 replicates per treatment. Data were recorded at 5-week intervals: number of explants responding to shoot formation and number of axillary shoots per explant. Data were analyzed for significant differences using ANOVA and Duncan's Multiple Range Test (DMRT) (Duncan 1955).

RESULTS AND DISCUSSION

Shoot multiplication

Results of our preliminary study showed that nodal explants from tuber-derived glasshouse-grown plants of both *Ceropegia* species formed 1–2 shoots on MS medium containing 10.0 µM BA. The rate of multiple shoot formation increased in the case of nodal explants obtained from five week-old *in vitro*-raised shoots. Of the two cytokinins tested, BA yielded the maximum number of shoots from single nodal explants in *C. odorata* (6.1 ± 0.7 shoots per explant) (**Fig. 1A**) and in *C. maccannii* Hook (5.2 ± 0.8 shoots per explants) (**Fig. 2A**) at 7.5 µM BA in the medium (**Table 1**). MS fortified with 7.5 µM BA induced multiple shoot formation in 100% of the nodal explants within 2–3 weeks of culture. The addition of a similar concentration of Kin also induced shoot bud multiplication in about 98% of nodal explants but the number of shoots per explant was less than BA-fortified medium (**Table 1**). Similar results were reported earlier for shoot regeneration in *C. bulbosa*, *C. jainii* (Patil 1998) and *C. sahyadrica* (Nikam and Sawant 2007) where BA alone was the most effective in multiplication of axillary shoots.

In order to improve shoot multiplication in nodal explants, different auxins were combined in the range of 1.0–2.5 µM with the optimized BA or Kin concentration in MS medium. At lower and medium concentration of auxins IAA and NAA, number of shoots declined, shoot length was suppressed and callusing was induced at the cut surface of the explants. Whereas with 0.5 to 1.0 µM 2,4-D only callus development was observed. However, a promoting effect of BA in combination with an auxin has been demonstrated in *C. candelabrum* (Beena *et al.* 2003) and in other members of Asclepiadaceae viz., *Holostemon annulare* (Sudha *et al*. 1998), *Hemidesmus indicus* (Sreekumar *et al*. 2000) and *Holostemon adakodien* (Martin 2002). These results suggested higher endogenous levels of auxin in *C. maccannii* Ansari and *C. odorata* Hook and needs exogenous supply of cytokinins in the medium for induction of multiple shoots from nodal explants.

Prolific callus was produced from all the explants cultured on media supplemented with 18.0 µM BA while a higher concentration of BA (22.0 µM) suppressed shoot regeneration and callus formation. The auxins IAA and NAA (1.0–2.5 µM) induced callus and rooting.

Internode and leaf explants from tuber-derived plant

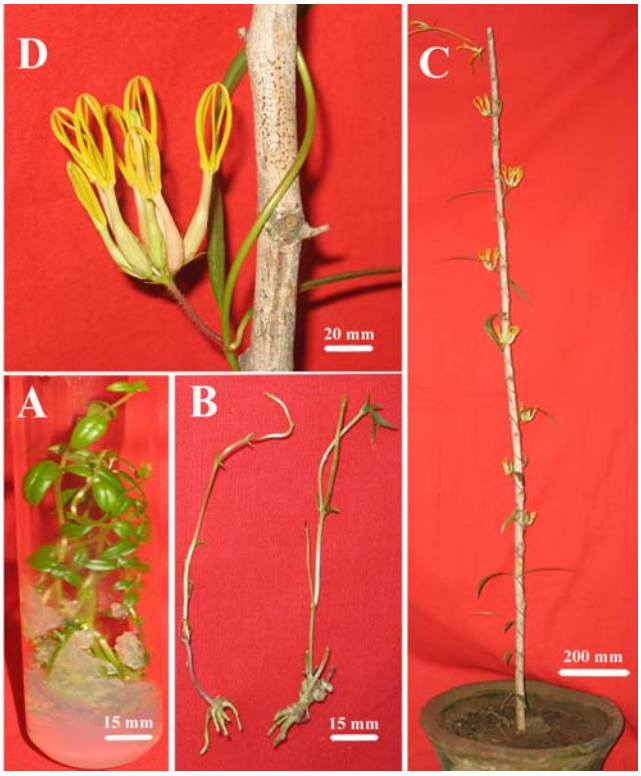

Fig. 1 ***Ceropegia odorata*** **Ansari.** (**A**) Axillary bud proliferation on MS medium containing 7.5 µM BA. (**B**) Rooted shoot grown on ½ MS + 0.5 µM IBA + 5% sucrose. (**C**) Transplanted flowered plantlet. (**D**) Flowering twig.

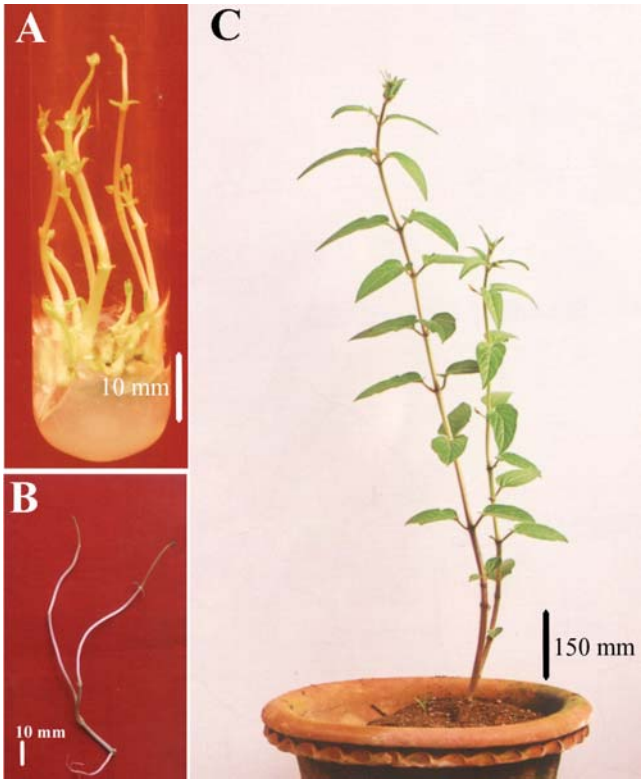

Fig. 2 ***Ceropegia maccannii*** **Hook.** (**A**) Axillary bud proliferation on MS medium containing 7.5 µM BA. (**B**) Rooted shoot grown on ½ MS + 0.5 µM IBA + 5% sucrose. (**C**) Transplanted flowered plantlet.

Table 2 Rooting response of the shoots of *C. odorata* Hook and *C. maccannii* Ansari with different auxins in liquid MS medium

Liquid MS + auxin (µM)	***Ceropegia odorata* Hook**		***Ceropegia maccannii* Ansari**	
	% Rooting	**Mean № roots/shoot**	**% Rooting**	**Mean № roots/shoot**
MS + 3% sucrose				
0.0	-	-	-	-
0.25 NAA	9.9 ± 0.8	1.1 ± 0.06 o	11.3 ± 0.8	1.3 ± 0.07 kl
0.5 NAA	41.6 ± 0.9	1.8 ± 0.07 ef	36.1 ± 0.6	1.7 ± 0.05 j
1.0 NAA	39.1 ± 0.7	1.7 ± 0.06 kl	34.6 ± 0.8	1.4 ± 0.09 k
0.25 IAA	11.3 ± 0.8	1.2 ± 0.07 o	13.6 ± 0.7	1.1 ± 0.06 m
0.5 IAA	40.6 ± 0.6	1.6 ± 0.09 lm	40.2 ± 0.8	2.3 ± 0.09 fg
1.0 IAA	37.3 ± 0.7	1.1 ± 0.04 o	37.7 ± 0.8	1.9 ± 0.06 i
0.25 IBA	17.6 ± 0.7	1.4 ± 0.07 n	18.8 ± 0.6	1.2 ± 0.06 lm
0.5 IBA	49.2 ± 0.6	3.5 ± 0.06 b	48.4 ± 0.9	2.4 ± 0.05 f
1.0 IBA	55.1 ± 0.9	1.8 ± 0.04 jk	42.1 ± 0.9	2.1 ± 0.08 h
MS + 5% sucrose				
0.25 NAA	15.2 ± 0.6	1.2 ± 0.07 o	14.5 ± 0.9	1.3 ± 0.05 kl
0.5 NAA	43.7 ± 0.7	1.8 ± 0.06 jk	40.6 ± 0.5	2.3 ± 0.05 fg
1.0 NAA	41.1 ± 0.6	1.5 ± 0.06 mn	38.5 ± 0.6	2.1 ± 0.06 h
0.25 IAA	17.3 ± 0.7	1.4 ± 0.07 n	16.9 ± 0.7	1.3 ± 0.06 kl
0.5 IAA	46.6 ± 0.8	2.2 ± 0.09 fg	44.6 ± 0.6	2.4 ± 0.09 f
1.0 IAA	44.8 ± 0.8	2.1 ± 0.06 gh	41.7 ± 0.3	2.1 ± 0.04 h
0.25 IBA	18.9 ± 0.8	1.6 ± 0.07 lm	18.9 ± 0.7	1.6 ± 0.06 j
0.5 IBA	55.9 ± 0.7	2.0 ± 0.08 hi	52.7 ± 0.3	2.9 ± 0.04 e
1.0 IBA	42.1 ± 0.7	2.4 ± 0.06 de	43.1 ± 0.9	2.2 ± 0.08 gh
½ MS + 5% sucrose				
0.25 NAA	16.1 ± 0.7	1.6 ± 0.07 lm	14.9 ± 0.7	1.4 ± 0.06 k
0.5 NAA	45.4 ± 0.9	1.9 ± 0.04 ij	51.2 ± 0.8	3.5 ± 0.06 b
1.0 NAA	47.2 ± 0.6	2.5 ± 0.09 d	45.4 ± 0.9	3.4 ± 0.07 bc
0.25 IAA	18.3 ± 0.7	1.7 ± 0.07 kl	19.1 ± 0.8	1.7 ± 0.08 j
0.5 IAA	58.3 ± 0.5	2.9 ± 0.05 c	55.6 ± 0.5	3.4 ± 0.07 bc
1.0 IAA	48.7 ± 0.7	2.3 ± 0.07 ef	53.6 ± 0.8	3.2 ± 0.08 d
0.25 IBA	19.7 ± 0.6	1.9 ± 0.07 ij	20.1 ± 0.7	1.9 ± 0.07 i
0.5 IBA	67.9 ± 0.7	4.3 ± 0.08 a	58.6 ± 0.9	9.6 ± 0.05 a
1.0 IBA	57.7 ± 0.8	3.6 ± 0.05 b	52.4 ± 0.9	3.3 ± 0.06 cd

Values are mean ± SE. Different letters indicate significant differences at P=0.05 in each column.
The higher concentration (3.0-10.0 µM) IAA or NAA cause only swelling and callusing at the base of the shoot.

grown in a glasshouse as well as from five weeks-old *in vitro*-raised shoot failed to respond to shoot regeneration on any of the PGR-supplemented MS media. Callus formation was restricted to the cut ends on internode and leaf explants. The lack of shoot regeneration potential was also reported in root, internode and leaf explants when cultured on MS medium fortified with BA, Kin, IAA, NAA and 2,4-D alone and in combination in *C. candelabrum* (Beena *et al*. 2003), *C. bulbosa* (Patil 1998) and *C. sahyadrica* (Nikam and Sawant 2007).

Rooting

For rooting of shoots, half-strength liquid MS medium was superior to agar-solidified medium, and was effective after the addition of auxins (IAA, NAA, IBA and sucrose). No rooting was observed on half- and full-strength liquid and agar-solidified MS medium without PGRs even after 5–6 weeks of culture.

The inclusion of IAA, NAA and IBA in the medium induced the formation of roots, but the difference was significant (**Table 2**). The level of IAA, NAA, and IBA higher than 2.5 µM resulted in swelling and callus formation at the shoot base. Shoots grown on half-strength liquid MS medium supplemented with 0.5 µM IBA and 5% sucrose formed roots at the highest frequency in *C. odorata* and *C. maccannii* (**Table 2, Fig 2B**). These results correspond with the reports on *C. candelabrum* (Beena *et al*. 2003) and *C. bulbosa* (Patil 1998). The maximum frequency of rooting was observed in *C. sahyadrica* on MS medium containing 5% sucrose and 6.0 mg l^{-1} spermine (Nikam and Sawant 2007). It was observed that rooting was significantly affected by the concentration of MS nutrient medium, various concentrations of auxins (IAA, NAA and IBA) and sucrose in *Ceropegia* species.

Acclimatization

A crucial aspect of micropropagation is to achieve regenerated plants that are capable of surviving outside the aseptic and controlled *in vitro* environment (Aracama *et al.* 2006). In the present *in vitro* propagation system about 80% of plantlets of both *Ceropegia* species survived and grew considerably well when initially transferred to a glasshouse for three weeks and then to natural conditions (temperature approximately 22–34°C, light intensity approximately 300 µmol m^{-2} s^{-1}). However, plantlets exposed directly to natural conditions did not survive. All plantlets of both species retrieved from these cultures were morphologically normal, flowered similar to the parental plant and produced single tuber similar to the naturally grown plants.

The results of our study and earlier reports on *C. candelabrum* (Beena *et al*. 2003), *C. bulbosa* and *C. jainii* (Patil 1998) and *C. sahyadrica* (Nikam and Sawant 2007) suggest that rooting is slightly difficult although the acclimatization of *in vitro*-raised plantlets to field conditions is easier in *Ceropegia.* This might be due to the genetic and physiological makeup of the species to tolerate drought conditions and smaller leaf size at the initial stage of development.

This protocol can serve as a very efficient method for the conservation, ecorestoration and utilization of *C. odorata* Hook (an endangered, flower-producing plant with a unique fragrance) and *C. maccannii* Ansari (an endangered medicinal plant).

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