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CALLUS CULTURE AND MICROPROPAGATION OF *CEROPEGIA SAHYADRICA* ANS. AND KULK.: AN EDIBLE STARCHY TUBEROUS RARE ASCLEPIAD

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SUMMARY

Micropropagation by axillary shoot multiplication of nodal explants of *Ceropegia sahyadrica* Ans. & Kulk. was achieved. The highest mean number of shoots per nodal explants (6.1±0.6) was obtained on Murashige and Skoog (MS) medium supplemented with 10mM BAP. Mature indehiscent follicle was the suitable source to obtain the aseptic seedlings and explants. For morphogenetic study, 195 combinations of BAP, KN, IAA, NAA and 2,4-D in the range of 0 to 22 mM were fortified in the MS medium. Extensive callus proliferation took place on MS +1.0 mM 2,4-D +5.0 mM BAP. The calli could be maintained on the parent medium over a period of 18 months. Rooting of shoots was favored by addition of 6.0 mg^{-1} spermine and 5 % sucrose. The hardened plantlets remained stunted after transfer in the month of November to May. These stunted plantlets and fresh plantlets grew normally during the month of June to October and completed their life cycle similar to wild plants. The micropropagation protocol can be successfully utilized for conservation and domestication of the *Ceropegia sahyadrica* Ans. and Kulk.

***Key words*: Axillary shoot multiplication, callus, *Ceropegia sahyadrica*, growth regulator**

INTRODUCTION

Ceropegia sahyadrica Ans. & Kulk. (Asclepiadaceae) is an erect annual herb grown widely in the Western Ghats, (Maharashtra, India) and is under the rare category (Ansari and Kulkarni 1971, Lucas and Synge 1978, Nayar and Sastry 1987, IUCN 1994, Jagtap and Singh 1999 and Tetali and Tetali 2000). The tubers are edible and a rich source of carbohydrates. The tubers and the leaves of the species are used by the localites to satisfy their hunger and in the traditional drug preparations on diseases like diarrhoea and dysentery. Natural obligation for cross-pollination coupled with low seed setting makes it unsuitable for propagation through seeds. Though the tuber is perennial, in one growth season it produces only a single plant. The natural populations are rapidly decreasing due to consumption of tuber by humans and animals. Therefore, there is an urgent need for the conservation and domestication of this species.

In vitro techniques offer the powerful tools for germplasm conservation, multiplication and introduction of new crop. It also facilitate the engineering and selection of elite superior genotypes and serve as a vehicle for in-depth investigation of physiological and biochemical processes (Smith 1994, Altman and Loberant 1998). However, for application of *in vitro* techniques, an efficient micropropagation protocol is the most important pre-requisite. The successful use of tissue culture technique for propagation has been reported in *Ceropegia jainii* Ans. & Kulk., *Ceropegia bulbosa*

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Roxb and *Ceropegia bulbosa var lushii* Hook (Patil 1998) and *Ceropegia candelabrum* L. (Beena *et al*. 2003). However, to date, there are no reports on the micropropagation of *Ceropegia sahyadrica* Ans. & Kulk., though it is an important edible tuberous asclepiad. Here we describe a protocol for callus culture and *in vitro* multiplication of *Ceropegia sahyadrica* Ans. & Kulk., which may be helpful in the conservation, domestication and improvement of this species.

MATERIALS AND METHODS

Initiation of aseptic cultures and culture conditions: The node, internode, leaf and the green mature follicle explants of *Ceropegia sahyadrica* Ans. and Kulk, were obtained from the wild plants growing in the Western Ghats of India. The explants were washed thoroughly with sterilized tap water and then surface sterilized with 0.1% (w/v) mercuric chloride ($HgCl_2$) for seven minutes. The surface sterilized explants were washed thoroughly (eight times) with the sterile distilled water. The node (10 mm), internode (10 mm) and leaf explants (10 mm^2), after giving the fresh cut, were cultured on MS medium (Murashige and Skoog, 1962) fortified with different concentrations (0.0-22.0 µM) of cytokinins (BAP, KN) alone or in combination with the auxins (IAA, NAA and 2,4-D).

Seeds from the surface sterilized follicles were aseptically germinated on MS basal medium containing 3% sucrose (w/v) and gelled with 0.8% agar (w/v). The root, hypocotyl and cotyledon explants were excised from 5-7 days old seedling and cultured on MS medium fortified with auxins and cytokinins.

The culture medium contained 3% sucrose (w/v) and it was gelled with 0.8 % (w/v) agar- agar. Before gelling with agar, pH of the medium was adjusted to 5.6-5.8. The gelled medium was dispensed into culture tubes, plugged with non- absorbent cotton and then autoclaved at 121°C for 15-18 minutes under 15-lb/ square inch pressure. The cultures were kept in the culture room maintained at 25 ±2°C and illuminated with 9h d^{-1}photoperiod with 30-38 mmol m^{-2} s^{-1} Spectral flux photon (SFP). Subculturing was done after every 28-30 days. Cultures were grown in 25x150 mm culture tubes or in 300 ml glass bottles.

Rooting of shoots and establishment of plants in soil: For root induction, excised micro shoots (4-6 cm) were cultured on full strength as well as half strength, semisolid or liquid basal MS salts supplemented with various concentrations of sucrose (1, 3, 5, 8, & 10%) or polyamine spermine (4, 6, 10 mg^{-1}) or various concentrations of IAA or NAA or IBA (0.0-5.0 mM) and 3% (w/v) sucrose. The rooted plantlets were washed thoroughly and planted directly in the pots filled with garden soil and transferred to the field conditions. In the second set of experiments, the potted plants were placed under glasshouse conditions (Temperature 27±5°C, humidity 70±10% and day light) for one week and then transferred to the field conditions.

Data analysis: The experiments had minimum 35 replicates and each experiments was repeated thrice. Data were analyzed by analysis of variance (ANOVA) to detect significant differences between means. Means differing significantly were compared using Duncan's (1955) multiple range test (DMRT) at the 5 % probability level. Variability in data has been expressed otherwise as mean ± standard error.

RESULTS AND DISCUSSION

Callogenesis: Callus formation was observed in all the explants (root, hypocotyls, cotyledons, node, internode and leaf) on MS media with BAP (18mM); KN (18mM); IAA (7.5-12mM); NAA (5-12 mM) and 2,4-D (0.25-5 mM), either alone or in combination. However, conspicuous callus mass was obtained on the MS + 1.0 mM 2,4-D + 5.0 mM BAP; MS + 1.0 mM 2,4-D + 5.0 mM KN; MS + 10.0 mM IAA + 5.0 mM BAP; MS + 10.0 mM IAA + 5.0 mM KN; MS + 7.5 mM NAA + 2.0 M BAP; MS + 7.5 mM NAA + 2.0mM KN; MS + 2.0mM 2,4-D; MS +10.0 mM IAA and MS+ 7.5 mM NAA media and were therefore chosen for subsequent subcultures.

The texture and type of callus depended on the concentration and type of growth regulator. The callus formed in conjunction with IAA and NAA occasionally showed formation of fine hairy mass from the surface of calli while the calli produced on medium containing 2,4-D were soft, pale yellowish and non-morphogenic (Fig.1B). Fresh as well as 2-18 month old calli when

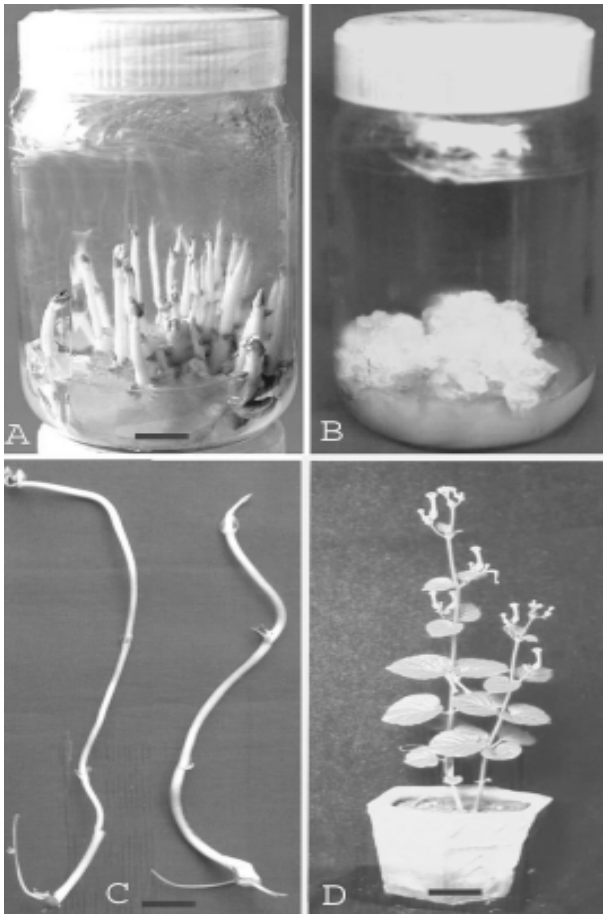

Fig. 1. (A-D). Callus culture, *in vitro* propagation and establishment of *Ceropegia sahyadrica*
(A) Callus derived from nodal explant on MS + 1µM 2,4-D + 5µM BAP (- = 5 mm), (B). Multiple shoots from nodal explant on MS + 10.0µM BAP (- = 5 mm), (C). Root development in a regenerated shoot on MS + 5% sucrose + 6.0 mg/l spermine (- = 10 mm), (D). *In vitro* generated plant established in soil (- = 100 mm).

transferred onto regeneration media failed to differentiate shoots under all growth regulator treatments. On the contrary, Patil (1998) reported that the shoot pieces of *Ceropegia jainii*, and *C. bulbosa* produced callus on MS medium supplemented with 9.05 mM 2, 4-D. This callus showed somatic embryogenesis after transfer to 0.5 MS medium containing 2.2 mM BAP; while in *Ceropegia candelabrum*, after transfer on to the MS medium containing 2,4-D, friable callus derived from leaf and internode explants underwent somatic embryogenesis (Beena and Martin 2003). This might be due to either genotypic differences and/or the methodology used for induction of callus and differentiation.

Multiple shoot induction: The choice of explant in *Ceropegia sahyadrica* was the most critical feature in induction of caulogenesis. For establishment of the micropropagation system, the cotyledonary node from the seedlings was the most suitable explant. However, in *Ceropegia jainii* and *C. bulbosa* (Patil 1998) and *C. candelabrum* (Beena *et al.* 2003), the nodal explants responded better for shoot induction Attempts to regenerate adventitious shoots from roots, hypocotyls and cotyledons from aseptically grown seedlings were unsuccessful. Excessive fungal contamination of explants isolated from field grown stock prevented the establishment of cultures.

On MS medium fortified with 10 mM BAP, the multiple shoot regeneration occurred during the second week of initiation of culture from the cotyledonary node. The regenerated shoots were fast growing, healthy and attained 6±0.2 cm height over a period of four weeks. The nodal explants derived from *in vitro* differentiated shoots which originated from the cotyledonary node showed sprouting of axillary buds on the fifth to seventh day of inoculation on MS + BAP (0.0-18 mM) or KN (0.0-18 mM) (Table 1). Maximum number of explants producing shoots (100%) and mean number of shoots (6.1±0.6) per explant occurred on MS + 10 mM BAP (Table 1, Fig 1A). Less number of shoots per explant was noticed on media fortified with similar concentration of KN (Table 1).

At lower concentrations of BAP (0.5-5 mM), the number of shoots per explant was less while at its optimum concentration (10mM), the shoot number per explant increased. The differentiated shoots were healthy, elongated and with very small leaves. Higher concentrations of BAP caused callusing from the cut surface of the explants and inhibited the regeneration of shoots and their subsequent growth. The effect of BAP/ KN on shoot regeneration was counteracted by the addition of IAA/NAA, which was evident by the reduction of percent frequency of number of explants producing shoots and the mean number of shoots per nodal explant (Table 1). However, it elicited callus induction and proliferation.

Our observations indicated that BAP was more effective than KN for *de novo* shoot bud formation in

Table1. Effect of cytokinins (BAP, Kin) alone and in combinations with auxins (IAA, NAA) on shoot multiplication in *Ceropegia sahyadrica* Ans.& Kulk.

Plant Growth regulators		Nodal explants which form shoots %	Numberof shoots/ node Mean ± SE		Shoot length cms
Cytokinins BAP (mM)	Auxins				
0	-	35.1±0.4^{b}	0.3±0.2^{a}		3.1±0.9^{b}
0.5	-	35.4±0.4^{b}	0.3±0.2^{a}		4.1±0.9^{c}
1.0	-	40.0±0.4^{c}	0.4± 0.3^{a}		5.0±0.8^{d}
2.0	-	85.6±0.4^{f}	1.9± 0.5^{ab}		5.3±0.8^{d}
5.0	-	92.6±0.4^{g}	3.2±0.6^{c}		5.6.±0.6^{d}
7.5	-	98.1±0^{g}	3.7±0.5^{c}		5.9±0.1^{d}
10.0	-	100±0^{h}	6.1±0.6^{e}		6.0±0.2^{e}
12.5	-	85.4±0.4^{f}	4.9±0.6^{cd}	CS	3.9±0.6^{b}
18.0	-	28.6±0.4^{a}	0.3±0.3^{a}	CS	0.9±0.6^{a}
22.0	-	*	*		*
	(IAA)				
10.0	1	92.7±0.3^{g}	5.1±1.0^{de}	C	5.8±0.2^{d}
10.0	2	57.6±0.4^{d}	2.1±0.7^{bc}	C	5.3±0.1^{d}
10.0	5	-	-	EC	
	(NAA)				
10.0	1	78.3±0.3^{e}	5±0.7^{dc}	C	5.3±0.2^{d}
10.0	2	-	-	EC	
Kin (mM)					
0.5	-	31.6±0.4^{a}	0.2±0.2^{a}		3.9±0.8^{b}
1.0	-	34.6±0.3^{b}	0.3±0.3^{a}		4.0±1^{c}
2.0	-	78.4±0.4^{e}	1.5± 0.5^{ab}		4.5±0.7^{c}
5.0	-	92.4±0.4^{g}	3.1±0.8^{c}		5.0±0.6^{d}
7.5	-	97.2±0^{g}	3.6±0.3^{c}		5.5±0.2^{d}
10.0	-	100±0^{h}	4.9±0.4^{cd}		5.9±0.3^{d}
12.5	-	85.6±0.4^{f}	4.0±0.3^{cd}	CS	3.8±0.6^{b}
18.0	-	28.4±0.4^{a}	0.4±0.3^{a}	CS	0.8±0.6^{a}
22.0	-	*	*		*
	(IAA)				
10.0	1	92.6±0.4^{g}	4.2±0.3^{cd}	C	5.7±0.2^{d}
10.0	2	57.7±0.3^{d}	2.6±1.3 ^{bc}	C	5.4±0.1^{d}
10.0	5	-	-	EC	
	(NAA)				
10.0	1	85.7±0.3^{f}	4.0 ±0.3^{cd}	C	5.2±0.2^{d}
10.0	2	-	-	EC	

The values represent the mean ± SE. Values followed by same letter were not significantly different at 5% level (DMRT). CS= Callus mediated shoots. * = Explants did not respond for callus and regeneration of shoot buds. C= Explants produced callus. EC = Explants produced extensive callus.

Ceropegia sahyadrica (Table 1). These results are in consonant with the multiple shoot induction in *Ceropegia jainii* and *C. bulbosa* Roxb (Patil 1998). In contrast to this, a synergistic effect of BAP in combination with an auxin has been reported in *Ceropegia candelabrum* (Beena *et al.* 2003) and other Asclepiad viz. *Holostemma annularae* (Sudha *et al.* 1998), *Hemidesmus indicus* (Sreekumar *et al.* 2000), *Holostemma ada–kodien* (Martin 2002) and *Leptadaenia reticulata* (Arya *et al.* 2003). The endogenous levels of growth regulators in the members of Asclepiadaceae might be responsible for the observed variations in the response and growth regulator requirement for *in vitro* shoot regeneration.

Rooting of shoots: Different media were used for *in vitro* rooting and the results are presented in Table 2. Rooting of *in vitro* regenerated shoots (4-6 cm) was achieved within 7-14 days by the incorporation of IAA, NAA, or IBA in the MS medium containing 3% sucrose. Similar results were obtained by addition of increased concentration of sucrose (5%) in the medium without growth regulators. The induction of swelling and callusing at the base of the shoots grown on the media containing 5.0 mM IAA or IBA and 2.0 and 5.0mM NAA was observed. Slight improvement in the percent frequency of rooted shoots (68%) was achieved by addition of 5% sucrose together with IAA (2.0 mM) (Table 2). Addition of polyamine spermine (6 mg^{-1}) along with optimum concentration of sucrose (5%) enhanced the percentage of shoots producing roots by 20 % over the use of sucrose alone and in combination with IAA.In all these media, 1-3 healthy, elongated roots were developed (Fig. 1C). Earlier reports on poplar shoots (Hausmen *et al.* 1994, 1995 a,b) , pear micro-cuttings (Baraldi *et al.* 1995) and spindle tree (Bonneau, *et al.* 1995) and the results of the present investigation suggest the positive role of polyamine spermine in the induction of roots. Full strength liquid medium and half strength agarified medium with IAA, NAA, IBA, spermine and increased concentration of sucrose (5%) responded differently for rooting of shoots. Rooting of multiplied shoots in *Ceropegia candelabrum* (Beena *et al.* 2003) was achieved on half strength MS medium with 1.5 mg^{-1} NAA and 0.49 mM IBA.

Table 2. Rooting response of the shoots of *Ceropegia sahyadrica* Ans.& Kulk. with auxins (IAA. NAA, IBA), sucrose and spermine in half and full strength agarified and liquid MS medium.

Medium	Shoots producing roots (%)
AHS-MS	37.1±0.5^{b}
AFS–MS	29.3±0.6^{a}
LHS–MS	48.3±0.4^{d}
LFS-MS	41.4±0.4^{c}
AHS-MS +2.0µM IAA	54.7±0.3^{e}
AHS-MS +1.0µM NAA	43.4±0.4^{d}
AHS-MS +2.0µM IBA	50.4±0.4^{d}
LHS-MS +2.0µM IAA	62.3±0.3^{f}
LHS-MS +1.0µM NAA	50.4±0.4^{d}
LHS-MS +2.0µM IBA	56.6±0.4^{e}
LHS-MS+Sucrose (%)	
1	32.4±0.4^{b}
3	42.4±0.4^{d}
4	46.3±0.3^{d}
5	65.1±0.5^{f}
6	40.3±0.5^{bc}
7	20.3±0.3^{b}
8	10.3±0.4^{a}
LHS-MS+5%Sucrose+ IAA	
0.5	36.1±0.4^{c}
1.0	51.3±0.6^{e}
2.0	68±0.4^{f}
LHS-MS+5%Sucrose+ NAA	
0.5	35.1±0.5^{b}
1.5	63.1±0.5^{f}
LHS-MS+5%Sucrose+ IBA	
0.5	18.3±0.3^{a}
1.0	40.4±0.4^{c}
2.0	63.4±0.4^{f}
LHS-MS+3%Sucrose+Spermine	
4	49±0.4^{c}
6	64±0.4^{f}
10	-
LHS-MS+5%Sucrose+ Spermine	
4	54.3±0.6^{d}
6	88.6±0.4^{h}
10	-

Results are mean of three replicates. Each replicate is of minimum 35 cultures. Values followed by same letter were not significantly different at 5% level (DMRT). AHS= Agarified half strength, AFS=Agarified full strength, LHS=Liquid half strength, LFS=Liquid full strength.

Hardening: The well-rooted plantlets (4-6 cm) at the age of four weeks were transferred to the earthen pots filled with garden soil. These were grown for one week in the glass house and when transferred to the field conditions in the month of June, July, August, September or October grew vigorously and completed their life cycle by flowering at the end of November similar to the wild plants (Fig 1D). However, when the plantlets were transferred to the field condition in the month of November to May by following the same procedure, the plants attained a height of only 18-22 cm with 4-7 nodes and remained stunted until the month of June. These stunted plantlets grew vigorously up to the month of October-November, then flowered and completed their life cycle. The plantlets eventually died when they were directly transferred to the natural conditions in winter (November-February) and summer (March - June). However, when they were transferred directly to the field conditions during the rainy season (July- September); the plantlets grew well and completed their life cycle. Beena *et al.* (2003) reported that the plantlets of *Ceropegia candelabrum* grew well when directly transferred to the pots filled with mixer of solirite and sand (1:1) for 15 days and then transferred subsequently to the field conditions.

In conclusion, the protocol with a high rate of multiplication can be successfully utilized for conservation, micropropagation and domestication of *Ceropegia sahyadrica* Ans. and Kulk. Further, improving it by genetic engineering with other major crops viz. potato and sweet potato may be possible.

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