Indian Journal of Biotechnology
Vol 7, January 2008, pp 129-132

Micropropagation of *Ceropegia hirsute* Wt. & Arn.—A starchy tuberous asclepid

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Received 19 December 2006; revised 30 April 2007; accepted 21 May 2007

A protocol is described for micropropagation of the starchy, tuberous, herbaceous twinner, *Ceropegia hirsute* Wt. & Arm. by *in vitro* culture of nodal segments. Of the cytokinins (BAP and Kn) and auxins (IAA, NAA, 2,4-D) evaluated as supplements individually and in combinations to Murashige and Skoog (MS) medium, BAP (7.5 µ*M*) was the most effective in inducing axillary multiple shoots (5.7±0.7 shoots/culture). Root were induced to *in vitro* derived shoots on half-strength MS medium supplemented with IAA (2 µ*M*) and sucrose (5%). *in vitro* plants were acclimatized and were successfully established in the soil.

Keywords*:* BAP, *Ceropegia hirsute*, micropropagation, nodal explants

Introduction

The genus *Ceropegia* (Family: Asclepiadaceae) contains about 200 species of upright or twinning herbs with swollen tuber and fusiform roots, and is distributed worldwide[1,2]. However, most of the species of *Ceropegia* have a restricted distribution only in certain regions. These species are placed under the category of rare, endangered, vulnerable, extinct and threatened plants[3-5]. *Ceropegia hirsute* Wt. & Arn., a rare herbaceous twinner species, is endemic to India[2,6]. This species is of economic importance[2] due to its starchy edible tubers with medicinal value. Because of sparse distribution and compelled cross-pollination, seed setting in *C. hirsute* is very poor. Severe habitat destruction coupled with over-collection has resulted in severe depletion of the population of the species. Few studies have only been conducted on *in vitro* propagation of *C. jainii*[7] Ansari & Kulk. and *C. candelabrum* L.[8]. Therefore, there is a need to protect the genetic resource of *C. hirsute* by establishing *ex situ* conserved germplasm.

In the present study, a protocol has been established for *in vitro* micropropagation of *C. hirsute* through optimization of cytokinins and auxins and then transfer of plants to the field condition.

Materials and Methods

Plant Material

The plants of *C. hirsute* were collected from a naturalized population in Western Ghats (near Pune) Maharashtra, India. For periodic harvest of explants, stocks were maintained in the Botanic Garden, Department of Botany, University of Pune, Pune. Various plant parts, viz. leaf, internode and node, were tested for culture initiation. The explants were thoroughly washed with sterile distilled water and then surface-sterilized with an aqueous solution of 0.1% $HgCl_2$ for 4-5 min and rinsed 5-6 times with sterile distilled water. After removal of exposed cut surface, the explants were inoculated horizontally on the medium. Nodes, internodes and leaves were isolated from 4-wk-old *in vitro* raised shoots and cultured on nutrient media.

Shoot Multiplication

Initially, media were prepared using MS (Murashige and Skoog) salts[9] containing 3% sucrose and gelled with agar (0.8%). The auxins and cytokinins were incorporated in the media in the range of 0.5-22 µ*M* and the *p*H was adjusted to 5.8. The media were then sterilized for 15 min at 121°C and 1.05 kg/cm^2 pressure in an autoclave. For evaluation of the abilities of different basal media to support the multiple shoot induction, B_5 (Gamborg *et al*[10]), SH (Schenk and Hildebrandt[11]) and WPM (Lloyd and McCown's woody plant medium[12]) were supplemented with 7.5 µ*M* BAP. The *in vitro* cultures were maintained at 25±2ºC and illuminated for 9 h with fluorescent light (18-24 µmole m^{-2}s^{-1}) followed by 15 h dark period.

Rooting of Shoots and Acclimatization

In vitro multiple shoots (4-5 cm) were inoculated for rooting on liquid and agar solidified, half and

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full-strength MS medium supplemented with IAA, NAA, IBA (0.5-5.0 µ*M*) and sucrose (1-8%) individually or in combination. Rooted shoots with 3-4 pairs of leaves and 1-2 roots were washed to remove the adhering agar and medium, and then transferred to pots containing moist garden soil. The experiments for acclimatization were conducted wherein the first set of plants were exposed to natural condition from the first day; while in the another set, the plants were first transferred to glass house with 60±10% relative humidity, natural light and temperature 25±8°C. After every wk, 5 potted plantlets from the glasshouse were transferred to natural conditions and their growth was compared to that of wild plants.

Statistical Analysis

The experiments were set up in a completely randomized design with minimum 35 replicates per treatment (unless otherwise mentioned separately) and repeated at least three times. Data were analyzed by analysis of variance (ANOVA) to detect significant differences between means. Means were compared using Duncan's Multiple Range Test (DMRT)[13].

Results and Discussion

A single shoot was developed with an average shoot length of 4.0±0.7 cm within 4 wk from about 80% nodal explants cultured on MS medium without any growth regulator supplement (Table 1). The explants cultured on MS+1.0 µ*M* BAP showed sprouting of axillary bud and elongation of single shoot within 2 wk. Additional shoots (2-3 shoots/node) developed adjacent to this primary axillary shoot within 3rd wk of culture. The shoots attained a mean height of 4.1±1.4 cm and possessed 2-3 pairs of leaves in 4 wk of culture. On MS medium supplemented with BAP (2.0-7.5 µ*M*), per cent shoot development and number of shoots per explant increased with the increase in the concentration of BP; maximum 5.7±0.7 shoots per explant (Table 1, Fig. 1a). Further, higher concentration of BAP (12.5, 18 and 22 µ*M*) resulted in swelling at the cut ends and yellowing of shoots.

Table 1—Effect of cytokinins (BAP, Kn) alone and in combinations with auxins (IAA, NAA) on shoot multiplication in *C. hirsute*

Plant growth regulators		% nodal explants formed shoots	No. of shoots/ node (mean±SE)	Shoot length (cm)
Cytokinins (µ*M*)	Auxins (µ*M*)			
BAP				
0	-	80.7±0.3	0.9±0.4^{a}	4.0±0.7^{b}
1.0	-	92.7±0.3	1.7±0.6^{ab}	4.1±1.4^{e}
2.0	-	98.4±0.4	3±0.5^{cd}	4.2±0.4^{e}
5.0	-	100±0.0	4.7±0.8^{de}	5.4±0.5^{f}
7.5	-	100±0.0	5.7±0.7^{e}	6.0±0.5^{g}
10.0	-	95.7±0.3	4.9±0.8^{de} CS	3.6±1.9^{e}
12.5	-	34.7±0.3	1.4±1.1^{ab} CS	2.8±1.1^{c}
18.0	-	18.4±0.4	0.1±0.4^{a} CS	0.8±1.1^{a}
22.0	-	*	*	*
	IAA			
7.5	1	85.4±0.4	3.7±0.8^{bcd} C	5.6±0.2^{f}
7.5	2	57.6±0.4	2.2±1.1^{ab} C	5.3±0.1f
7.5	5	-	- EC	
7.5	NAA			
7.5	1	50.6±0.4	1.8±1.0^{a} C	5.0±0.2^{f}
7.5	2	-	-	-
Kn				
0.5	-	90.6±0.4	0.8±0.4^{a}	3.9±1.3^{c}
1.0	-	91.4±0.6	1.4±0.7^{ab}	4.1±1.4^{d}
2.0	-	97.7±0.3	2.7±1.2^{c}	4.2±0.5^{e}
5.0	-	100±0.0	4.4±1.1^{de}	5.3±0.5^{f}
7.5	-	100±0.0	5.4±0.6^{e}	5.5±0.5f
10.0	-	93.6±0.6	3.0±0.5^{cd} CS	3.4±1.9^{d}
12.5	-	48.6±0.4	0.7±0.9^{a} CS	2.6±1.0^{c}
18.0	-	14.6±0.4	0.2±0.6^{a} CS	0.7±0.9^{a}
22.0	-	*	*	*
	IAA			
7.5	1	85.3±0.4	3.2±0.9^{bcd} C	5.3±0.2^{f}
7.5	2	57.6±0.4	2.1±1.1^{ab} C	5.0±0.1^{f}
7.5	5	-	- EC	
	NAA			
7.5	1	50.7±0.4	1.6±1.0^{a} C	5.0±0.2^{f}
7.5	2	-	- EC	

The values represent the mean±SE calculated on three independent experiments, each based on minimum 35 replicates. Values followed by same letter were not significantly different at 5% level (DMRT).

CS=callus mediated shoots, *=Explant did not respond for callus and regeneration of shoot buds, C=Explant produced callus, EC=Explants produced extensive callus.

Media with Kn (0.5-22 µ*M*) were less effective than BAP for multiple shoot induction (Table 1). The present findings are in agreement with previous reports on *C. bulbosa* and *C. jainii* claiming BAP alone is sufficient to induce axillary shoot multiplication from nodal segments[7]. The auxins (0.1-5 µ*M* IAA & NAA) supplemented media had an inhibitory effect on bud sprouting and shoot multiplication (Table 1), but promoted the callus induction and its proliferation. Our results are consonant with the results in *C bulbosa* and *C. jainii*[7], and with another asclepiad *Leptadenia*[14]. On the other hand, a synergistic effect of a range of growth regulators in combinations with BAP for shoot multiplication is well documented for members of

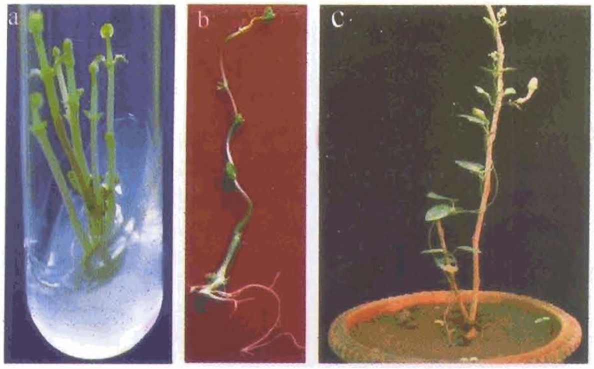

Fig. 1—*In vitro* propagation of *C. hirsute*: a. Multiple shoots formed from nodal explant on MS+7.5 μ*M* BAP; b. Rooting of shoots on MS+5% Sucrose+2 μ*M* IAA; c. Well established plant after transplanting into pots in rainy season.

Asclepiadaceae, viz. *C. candelabrum*[8], *Holostema annulare*[15], *H. ada-kodien*[16] and *Hemidesmus indicus*[17].

MS, B_5, SH and WPM media supplemented with 7.5 μ*M* BAP had variable effects on multiple shoot induction in nodal explant (Table 2). The maximum number of shoots was recorded on MS and WPM media, while the number of shoots per explant was low on B_5 and SH media. On the other hand, the differences in shoot length on various media were insignificant. These differences and similarities might be due to their basal salt formulation, particularly the concentrations of ammonium nitrate in the MS (20.6 m*M*), WPM (4.9 m*M*), B_5 (1.0 m*M*) and SH (2.6 m*M*) media[12,18]. The root, internode and leaf explants did not show shoot regeneration on media containing auxins and cytokinins, individually or in combinations.

Half strength or full-strength MS medium without growth regulator induced one or two roots in about 22-35% of the excised shoots (Table 3). Percentage of shoots producing roots was increased in liquid as well as on agar solidified half-strength and full-strength MS medium supplemented individually with IAA (0.1-5.0 μ*M*), IBA (0.1-5.0 μ*M*) and NAA (0.1-2.0 μ*M*) and sucrose (1-8%). In general, for the induction of root, liquid half-strength MS with above supplements was superior to the agar solidified half-strength as well as full-strength and liquid full-strength MS medium (Table 3). Of the three auxins tested, IAA and IBA were equally effective in inducing roots. More or less similar results were obtained on the medium with 5% sucrose (Table 3). Similarly, the half-strength MS with IBA was effective for inducing roots in shoots of *C. candelabrum*[8].

Table 2—Effect of basal media salt formulation fortified with 7.5 μ*M* BAP on multiple shoot induction in nodal explants of *C. hirsute* after 5 wk of culture

Medium	% nodal explants formed shoots	No. of shoots/node (mean±SE)	Shoot length (cm)
MS	100	5.7±0.7^{b}	6.2±0.6
B5	100	4.1±0.4^{a}	5.8±0.3
SH	100	4.0±0.3^{a}	5.8±0.5
WPM	100	5.4±0.2^{b}	5.9±0.8

The values represent the mean ± SE calculated on three independent experiments, each based on minimum 35 replicates. Values followed by same letter were not significantly different at 5% level (DMRT).

Table 3—Rooting response of shoots of *C. hirsute* after 5 wk of culture

Medium	% shoots producing roots	No. of roots/shoot (mean±SE)
AHS-MS	22.6±0.4	1.4±0.6^{ab}
AFS-MS	24.4±0.4	1.1±0.5^{a}
LHS-MS	32.6±0.4	2.2±0.7^{c}
LFS-MS	35.4±0.4	1.6±0.7^{ab}
AHS-MS +2.0 μ*M* IAA	66.3±0.3	2.4±0.8^{c}
AHS-MS +1.0 μ*M* NAA	58.4±0.4	1.2±0.6^{b}
AHS-MS +2.0 μ*M* IBA	62.4±0.4	1.2±0.3^{b}
LHS-MS + 2.0 μ*M* IAA	74.6±0.4	2.8±0.5^{c}
LHA-MS +1.0 μ*M* NAA	65.8±0.4	1.4±0.5^{bc}
LHS-MS +2.0 μ*M* IBA	74.3±0.4	1.4±0.5^{bc}
AFS-MS+ Sucrose (%)		
1	15.1±0.5	0.9±0.5^{ab}
3	24.1±0.5	0.7±0.6^{ab}
4	48.0±0.4	1.5±1.0^{b}
5	70.4±0.4	2.6±1.3^{d}
6	48.3±0.3	2.3±1.4^{c}
7	32.1±0.5	1.9±0.2^{b}
8	28.1±0.5	0.1±0.4^{a}
LHS-MS + IAA (μ*M*) +5% Sucrose		
0.5	45.1±0.5	0.4-±0.4^{a}
1.0	78.0±0.3	1.9-±0.9^{b}
2.0	88.6±0.4	3.2-±1.2^{d}
LHS-MS + NAA (μ*M*) + 5% Sucrose		
0.5	44.4±0.4	0.7-±0.5^{ab}
1.5	64.3±0.4	1.4-±0.6^{b}
LHS-MS + IBA (μ*M*) +5% Sucrose		
0.5	18.3±0.7	
1.0	78.3±0.3	0.3-±0.4^{a}
2.0	55.4±0.4	1.8-±0.8^{b}

Results are mean of three replicate, each replicate is of minimum 25 cultures.

Values followed by same letter were not significantly different at 5% level (DMRT).

At 5 μ*M* IAA, IBA and 2.0, 5.0 μ*M* NAA, shoots show callusing at the base.

AHS=Agar solidified half-strength, AFS=Agar solidified full-strength, LHS=Liquid half-strength, LFS=Liquid full-strength.

Among the combination of auxin (IAA, NAA, IBA) and 5% sucrose, the best rooting response (88%) was obtained in half-strength liquid MS+2.0 μ*M* IAA+5% sucrose within 2 wk of culture (Table 3). Each shoot produced 2-3 white non-hairy roots (Fig. 1b). In rainy season, (June to September 2003, 2004 and 2005), 100% rooted shoots survived when transferred directly to soil and exposed to natural conditions. They grew well, developed underground tuber and showed morphological characters similar to wild plants (Fig. 1c). However, in winter and summer season, initial 1 wk glasshouse hardening was necessary before transfer to the natural conditions.

In conclusion, present study reports an efficient tissue culture system for micropropagation of starchy tuberous asclepiad C. *hirsute via* organogenesis. The shoot regeneration was direct from nodal explants, which has been crucial for employing micropropagation technique for plant regeneration and conservation as it ensures genetic stability.

Acknowledgement

We thank the authorities of PES' Modern College, Pune and Department of Botany, University of Pune, Pune for the necessary facilities. The financial support provided by the University Grants Commission (UGC), New Delhi under DRS-SAP-PHASE-II and ASSIST, and Department of Science and Technology, Government of India, New Delhi under FIST Programme is also gratefully acknowledged.

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