J. Plant Biotechnology (2006) Vol. 8 (1), pp. 27~35

Journal of Plant Biotechnology

High Frequency Regeneration of Plantlets from Seedling Explants of *Asteracantha longifolia* (L.) NEES

Ramya Ranjan Mishra, Motilal Behera, Deep Ratan Kumar, Jogeswar Panigrahi*

Plant Tissue Culture laboratory, Division of Biotechnology, Majhighariani Institute of Technology and Science, Sriram Vihar, Rayagada-765017 Orissa, India

Abstract

Plantlet regeneration in *Asteracantha longifolia* (L.) Nees (Acanthaceae), a medicinal herb has been achieved from seedling explants on basal MS medium. Three different seedling explants including node, internode and leaf segments were used. Of these three explant, leaf explants gave better response for both callus mediated organogenesis and direct multiple shoot induction. Number of explants showing differentiation of shoot buds was higher on MS media supplemented with BA compared to kinetin. MS medium fortified with BA (2.0 mgl^{-1}) and NAA (0.5 mgl^{-1}) was found to be most suitable for both callus mediated organogenesis and elongation of shoots. The elongated shoots were successfully rooted on MS medium fortified with NAA or IBA. Among them 0.1 mgl^{-1} NAA or 0.2 mgl^{-1} IBA provides better response for rhizogenesis. Regenerated plantlets were successfully established in soil where 85.4% of them developed into morphologically normal and fertile plants. RAPD profiling using four decamer primers confirmed the genetic uniformity of the regenerated plantlets and substantiated the efficacy and suitability of this protocol for *in vitro* propagation of *A. longifolia*.

Key words: Callus, genetic fidelity, organogenesis, RAPD, shoot bud differentiation

Introduction

Asteracantha longifolia (L.) Nees syn. *Hygrophila auriculata* (Schum) Heine (Family-Acanthaceae), an important important medicinal herb is described in ayurvedic literature Asteracantha longifolia (L.) Nees syn. *Hygrophila auriculata* (Schum) Heine (Family-Acanthaceae), an as Ikshura, Ikshugandha, Talmakhan and Kokilaks-hya important inedicinal hurb is described in ayurvedic literature. The plant parts including leaves, inflorescences, seeds, roots and ashes have been extensively used in traditional system of medicine for various ailments like diuretics, jaundice, diopsy, rheumatism, hepatic obstructions and dissolution of gallstones, kidney stones, liver dysfunction and diseases of urino-genital tracts (Singh et al. 2003; Mishra 1985; Shailajan et al. 2005). The plant is known to possess antitumor (Ahmed et al. 2001, Majumdar et al. 1997), hypoglycaemic (Fernando et al. 1991), antibacterial (Vlietnick et al. 1995), hepatoprotective (Singh and Handa 1995; Hewawasam et al. 2003) and anti-nociceptive activity (Shanmugasundaram and Venkataramana 2005). The plant has been used in several ayurvedic preparations-Lukol, Speman, Confindo etc. by Himalaya Health Care Pvt. Ltd. (www. himalayahealthcare.com), Breastone by Vedic Biolabs Pvt. Ltd. (www.vedicbiolabs. com), Biogest and Rasanagugul by Trihealth Care, Kerala Ayurveda Pharmacy division (www. oilbath.com) and Microlactin by Victoria Health Care Ltd. (www.victoriahealth. com).

A. longifolia is a marshy, hispid herb, growing in the banks of fresh or stagnant water ditches and swampy grounds, mixed with marshy grasses and sedges. The requirement of *A. longifolia* is presently obtained from the natural populations. This extensive utilization has positive threat for the plant, to the extent of being extinct. Hence, an immediate need for accessing the natural population leading to rapid multiplication of this drug yielding plant has become imperative. Tissue culture technology can provide an alternative way to substantiate the rapid multiplication of elite clone and germplasm conservation (Vasil 1988). However, in tissue

* Corresponding author, E-mail: jogesh_ck@yahoo.co.in
Received Nov 29, 2005; Accepted Mar 15, 2006

culture genetic variability often arises as a manifestation of epigenetic influence or changes in the genome of differentiating somatic cells induced by tissue culture conditions (Larkin and Scowcroft 1981; Muller et al. 1990). Therefore, genetic stability of *in-vitro* regenerated plantlets has always been assessed during standardization of an efficient *in-vitro* propagation protocol. Molecular markers based on either gene or gene products are quite stable, uniform, quick, reliable and reproducible. Randomly amplified polymorphic DNA (RAPD) based detection of genetic polymorphism (Williams et al. 1990) is a kind of molecular marker based on gene and it has been successfully utilized in describing the genetic variability of *in-vitro* regenerated plantlets in several species (Rival et al. 1998; Rout et al. 1998; Zucchi et al. 2002; Pontaroli and Camadro 2005; Soniya et al. 2005)

Therefore, the present study has been initiated to standardize a simple protocol for *in-vitro* propagation of this important medicinal herb *A. longifolia* from seedling explants and to access the genetic fidelity of *in-vitro* regenerated plantlets using RAPD markers.

Materials and Methods

Plant materials and explant preparation

Seeds of *A. longifolia* were collected from paddy fields of Narayanapatna, Orissa and the plants were maintained in the experimental garden of the Division of Biotechnology, MITS, Rayagada, Orissa, India. Seeds washed with 5% (v/v) Teepol solution for 2 min were surface sterilized with 70% alcohol for 45 sec followed by 5 min of soaking in 0.1% (w/v) $HgCl_2$ (E-Merck, India). The sterilized seeds were washed thoroughly with sterilized double distilled water (ddH_2O). They were germinated aseptically on a medium containing salts and vitamins according to Murashige and Skoog (1962), 3% sucrose (w/v) and 0.8% (w/v) agar in dark. Leaves, nodes and internodes were excised from 10-12 days old seedlings and were used for *in-vitro* culture studies.

Culture medium and conditions

The culture medium was that of MS with 100 mgl^{-1} Meso-inositol (Hi-media, India) and 3% (w/v) Sucrose (Qualigen, India). The medium was augmented with different concentrations and combinations of N^6- Benzyladenine (BA), Kinetin (Kn) and α-Napthalene acetic acid (NAA) (Hi-media, India). The pH of the medium was adjusted to 5.8 prior to gelling it with 0.8% Agar-agar (bacteriological grade, Hi-media, India). All the media contained in culture vessels were autoclaved at 104 kpa and 121°C for 20 min. One explant (~25 mm^2) was implanted in each tube and cultures were incubated at 25±1°C, 60-70% relative humidity and 16 hr photoperiod of 35 $Em^{-2}s^{-1}$ irradiance level provided by cool white fluorescent tubes (Philips, India).

Effect of phytohormones for multiple shoot induction and shoot elongation

The experiment was designed to study the effect of different concentration and combination of BA, Kn and NAA on shoot bud regeneration and multiple shoot induction. Three types of explants such as leaf, node and internode were used. After eight week of culture, the number of shoot buds or shoot initials per explant was counted. Each treatment was replicated five times using five explants for each treatment.

Rooting of the elongated shoots and acclimatization

Elongated shoots with 5-6 leaves (>3 cm long) were excised *in-vitro* from all treatments and were transferred to half strength MS medium containing 0.1 to 0.5 mgl^{-1} of NAA and (Indole-3-Butyric Acid) IBA individually. Cultures were incubated as described previously. Plantlets with well-developed roots were transferred to plastic cups containing autoclaved sand and soil (1:1) and maintained in same environmental condition for one week. They were watered regularly with $1/10^{th}$ strength MS liquid medium. Subsequently, they were transferred to earthen pots containing coarse sand, compost and garden soil and kept in shade for two weeks before transferring to the experimental garden.

Observation and statistical analysis

Visual observations were made every week and data on explant response, number of shoot buds/ shoots per explant and number of roots per shoot were recorded at the end of 8^{th} week. Each phytohormone treatment of five replicated tubes and was repeated thrice, and the standard error was calculated. Data on shoot bud regeneration, multiple shoot production and rooting were statistically analyzed using a completely randomized block design and means were evaluated at $p < 0.05$ level of significance using Duncan's multiple range test (Harter 1960). For this SPSS V 8.0.1 software was used with parameters - one

way ANOVA and homogeneity of variance.

RAPD Marker analysis

Isolation of genomic DNA

Genomic DNA was isolated from fresh and young leaf samples (~1.2 g) by using SDS method (Dellaporta et al. 1983) with few modifications. The crude DNA was purified using RNAse treatment (75 µg of RNAse for 1.5 ml of crude DNA) and Proteinase treatment (100 µg proteinase-K for 1.5 ml of crude DNA) followed by two washes with Phenol: Chloroform: Isoamyl alcohol (25:24:1 v/v/v) and subsequently three washes of chloroform: isoamyl alcohol (24:1 v/v). The upper aqueous phase was separated after centrifugation and mixed with $1/10^{th}$ volume of 3M Sodium Acetate. DNA was precipitated by adding 2.5 volume of chilled ethanol, pelleted, dried in vacuum and dissolved in 10 mM Tris/1mM EDTA ($T_{10}E_1$) buffer. DNA concentration and purity was measured by using UV-Vis spectrophotometer (UV 1601, Shimadzu, Japan) with $T_{10}E_1$ buffer (pH 8.0) as blank. For further confirmation the quantification of DNA was accomplished by analyzing the purified DNA on 0.8% agarose gel along with diluted uncut lambda DNA as standard. DNA is diluted to concentration of 20ng/µl using $T_{10}E_1$ buffer.

RAPD Analysis

For RAPD analysis PCR amplification of 30 ng genomic DNA was carried out using four standard decamer oligonucleotide primers (Operon Tech., Alameda, CA, USA) as described by Williams et al. (1990). The Primers were OPA01, OPA02, OPA03 and OPA04.

Each amplification reaction mix of 25 µl contained the 30 ng template DNA, 2.5 µl of 10 X assay buffer (100 mM Tris.Cl, pH 8.3; 0.5 M KCl; 0.1% Gelatin), 2.0 mM $MgCl_2$, 200 µM each of the dNTPs, 20ng primer, 1.0 U Taq DNA polymerase (Bangalore Genei Pvt. Ltd., Bangalore, India). The amplification was performed in a thermal cycler (TC-312, Techne, UK) programmed for initial denaturation of 5.0 min. at 94°C; 45 cycles of 2.0 min denaturation at 94°C, 1.0 min annealing at 37°C and 2.0 min elongation at 72°C; and final elongation step at 72°C for 5.0 min.

The PCR products were separated on 1.4% agarose gel containing ethidium bromide solution (0.5 µg/ml of gel solutions) in submarine electrophoresis apparatus (Biotech, Yercaud) using TAE (40 mM Tris acetate; 2mM EDTA) buffer at constant 50 V for about 4 hour. A gel loading buffer (20% Sucrose; 0.1 M EDTA, 1.0% SDS; 0.25% Bromophenol blue; 0.25% xylene cyanol) was used as tracking dye. Amplified DNA fragments were visualized by UV transilluminator (M-15, UVP, Upland, CA 91786, USA) and photographed/ documented using photostation compact (Lark Innovatives, India). The size of the amplicons was determined using Lambda DNA double digest, λ-EH, (Bangalore Genei Pvt. Ltd., Bangalore, India) as standard and TOTAL LAB SOLUTIONS-V 2003.02 software. To test the reproducibility the reactions were repeated twice. Each amplicon was considered as unit character and the data were organized into 0-1 matrix and analysed using SPSS V 8.0.1 software.

Results and Discussion

Three different plant parts (Leaf, Internode and Node) derived from *in-vitro* raised plants of *A. longifolia* were excised and implanted in culture media to study the regenerative potentiality of the vegetative parts. For this MS medium was variously supplemented with cytokinins and combination of cytokinins and auxins and the results are enumerated below:

Effects of Phytohormones on shoot regeneration

Cytokinins

The effect of cytokinin is known to be critical in shoot organogenesis (Skoog and Miller 1957). Therefore, we compared the response of three different seedling explants to various concentrations of BA and Kn. Direct shoot bud differentiation was observed as protrusions at the cut ends after two weeks of culture initiation (Fig.1a). By the end of 5^{th} week, these protruded structures developed into shoot buds without intervening callus phase and 40-50% of them were flanked by green leaves (Fig.1b). By the end of 8^{th} week well developed multiple shoots were initiated from all three explants. The frequency of shoot formation was influenced by the type of the explant, choice of the cytokinin and its dosage. Explants implanted on MS medium without any cytokinin did not respond. The highest frequency of shoot formation was observed from leaf explants followed by node and internode explants (Table1). A maximum of 11-12 shoots were obtained from leaf explants on MS with 1.5 mgl^{-1}BA (Fig. 1c). Similarly, a maximum of 8-9 shoots were developed on MS medium with 2.0 mgl^{-1} Kn (Fig. 1d). Of the two cytokinins used, BA was more effective than Kn in inducing multiple shoots in all three explants, but the dose and response varied according to each individual explant. The regeneration frequency increased with increase in the concentration of cytokinins and 1.5 mgl^{-1} BA was found to

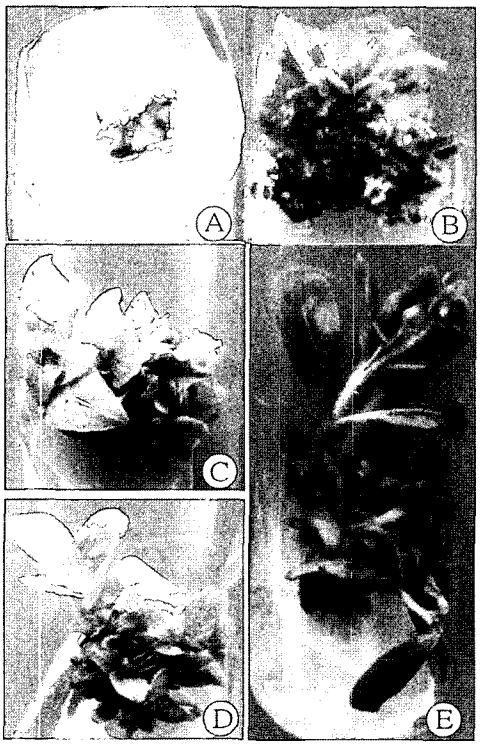

Figure 1. Plant regeneration in *Asteracantha longifolia* through direct organogenesis. Protuberances at the cut end of leaf explant (A), Multiple shoot initials after 5th week on MS + 1.5 mgl^{-1}BA(B), Well developed multiple shoots after 6th week from leaf explants on MS + 1.5 mgl^{-1}BA (C) and on MS + 2.0 mgl^{-1}Kn (D), Elongation of multiple shoots developed from leaf explants after 8th week on MS + 1.5 mgl^{-1} BA (E).

Table 1. Effect of different cytokinins (BA and Kn) on development of multiple shoots of *A. longifolia* from three different explants (Leaf, Internode and Node) after 8 weeks*

Media code	Plant growth regulator conc. (mg/l)		Explant Response in term of No. of shoot buds/ initials(Mean ± S.E.)		
	BA	Kn	Leaf	Internode	Node
T_0	Control		0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
			(Protrusion)	(Swelling)	(Swelling)
T_1	0.5	-	9.2 ± 0.2^{h}	0.4 ± 0.24^{ab}	0.0 ± 0.0^{a}
					(Organogenic callus)
T_2	1.0	-	10.8 ± 0.2^{i}	1.2 ± 0.2^{cd}	1.6 ± 0.24^{cd}
T_3	1.5	-	11.2 ± 0.2^{i}	2.4 ± 0.24^{f}	2.6 ± 0.24^{e}
T_4	2.0	-	6.2 ± 0.2^{e}	6.6 ± 0.24^{i}	5.8 ± 0.2^{g}
T_5	2.5	-	3.8 ± 0.2^{d}	6.0 ± 0.32^{h}	6.2 ± 0.2^{g}
T_6	3.0	-	2.8 ± 0.2^{c}	2.0 ± 0.0^{ef}	3.4 ± 0.24^{f}
T_7	4.0	-	2.6 ± 0.24^{bc}	2.0 ± 0.0^{ef}	2.8 ± 0.2^{ef}
T_8	5.0.	-	2.6 ± 0.24^{bc}	1.6 ± 0.24^{de}	2.4 ± 0.24^{e}
T_9	-	0.5	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
T_{10}	-	1.0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
			(Organogenic callus)	(Organogenic callus)	(Organogenic callus)
T_{11}	-	1.5	6.8 ± 0.37^{f}	0.8 ± 0.2^{bc}	1.0 ± 0.32^{bc}
T_{12}	-	2.0	8.6 ± 0.24^{g}	1.4 ± 0.24^{cde}	0.4 ± 0.24^{ab}
T_{13}	-	2.5	5.8 ± 0.2^{e}	3.4 ± 0.24^{g}	2.4 ± 0.24^{e}
T_{14}	-	3.0	3.4 ± 0.24^{d}	2.0 ± 0.0^{ef}	2.4 ± 0.4^{e}
T_{15}	-	4.0	2.0 ± 0.0^{b}	1.8 ± 0.2^{def}	2.0 ± 0.55^{de}
T_{16}	-	5.0	2.0 ± 0.32^{b}	1.2 ± 0.37^{cd}	1.4 ± 0.24^{cd}

*Means within a column having the same letter are not statistically significant ($p<0.05$) according to Duncan's multiple range test (SPSS V 8.0.1)

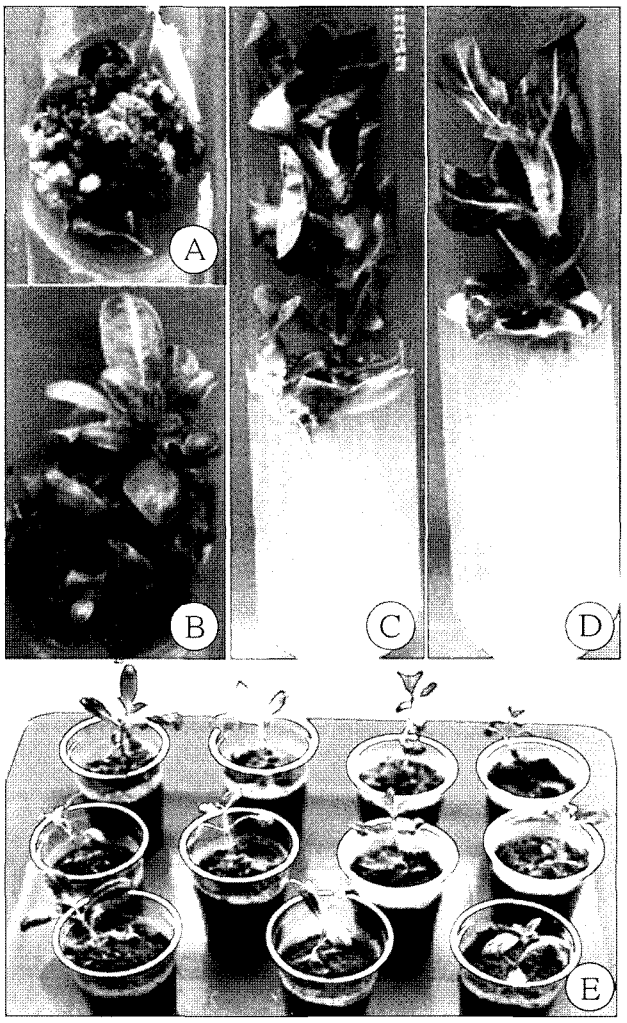

Figure 2. Plant regeneration in *Asteracantha longifolia* through callus mediated organogenesis. Greenish, hard, compact calli developed after 5th week on MS + 2.0 mgl^{-1} BA + 0.5 mgl^{-1} NAA (A), Multiple shoot bud regeneration from the callus after 8th week (B), Elongated shoot showing profuse rooting on MS + 0.1 mgl^{-1} NAA (C) or MS+ 0.2 mgl^{-1}IBA (D), A set of regenerated plantlets in plastic cups containing sand and soil mixture(E)

be optimal for maximum frequency of shoot bud formation from leaf explant. With BA concentrations above 1.5 mgl^{-1}, the frequency of shoot bud regeneration decreased drastically. The multiple shoots, obtained on various concentrations of BA, elongated on the same media resulting in numerous shoots (Fig. 1e). However, the elongation required more time in comparison to multiple shoot induction. Similar observations were also observed by Mishra et al. (2005) for the leaf explants of *A. longifolia*. Therefore, BA is more effective cytokinin for shoot organogenesis in *A. longifolia*, indicating cytokinin specificity for multiple shoot induction. Similar results were also obtained in *Atropa belladonna* (Benjamin et al. 1987), *Picrorhiza kurroa* (Lal and Ahuja 1996) and medicinal Yam (Borthakur and Singh 2002).

Auxin-cytokinin combinations

The induction of callus growth and subsequent differentiation and organogenesis is accomplished by the differential application of cytokinins and auxins to the culture medium. In the present experiment the medium was supplemented with various combinations of cytokinin (BA) and auxin (NAA) to study the regeneration frequency and elongation of shoots (Table.2). The highest frequency of shoot bud regeneration was obtained from leaf explants followed by internode and node explants. The implanted explants gave rise greenish compact callus by the end of 5th week (Fig. 2a). By the end of 8th week, the best result in terms of maximum number of healthy shoot buds/ tube was obtained in MS medium with BA (2.0 mgl^{-1}) and NAA (0.5 mgl^{-1}) from leaf explants (Fig. 2b). Multiple shoot buds were proliferated and elongated in the same medium. Similarly, plant regeneration were also observed from leaf callus of *Solanum laciniatum* and *Echinacea pallida* on medium supplemented with BA and NAA (Okslar et al. 2002 ; Koroch et al. 2003). Azad et al. (2004) studied the effects of explant type on organogenesis in a medicinal tree *Phellodendron amurense* and reported that better response was obtained with hypocotyl explant, followed by cotyledon, leaf and internode explants. Again they have studied the response of various cytokinins and auxins on multiple shoot induction and found that MS medium supplemented with 0.9 μM BA and 0.5 μM NAA was most suitable. Temjensangba and Deb (2005) induced multiple shoots from the leaf explants of *Cleisostoma racimeferum* on MS medium supplemented with various combinations of BA and NAA, and reported that BA induced multiple shoots alone while BA in combination with NAA induced organogenic calluses. Anis and Faisal (2005) standardized *in-vitro* mass multiplication of *Psoralea corylifolia* from leaf and nodal explants on MS medium supplemented with various combinations of BA and NAA. Nodal explants are superior to the leaf explants in response to multiple shoot induction. The most of multiple shoots were produced on MS medium supplemented with 5.0 μM BA and 0.5 μM NAA, suggesting that cytokinin : auxin ratio is important for this response. This led to the conclusion that *in-vitro* organogenesis requires a critical amount of cytokinin and auxin in addition to macro/micro-nutrient composition of media. Cytokinin at its high concentration stimulates development of meristems and at its optimal

Table 2. Effect of different plant growth regulators on multiple shoot bud regeneration and elongation shoots derived from different explants of *A. longifolia* after eight weeks*

Media code	Plant growth regulator conc. (mg/l)		Explant Response in term of no. of shoot initials (Mean ± S.E.)		
	BA	NAA	Leaf	Internode	Node
M_1	Control		0.0±0.0[a]	0.0±0.00[a]	0.0±0.0[a] (Swelling)
M_2	0.5	-	9.6±0.24[f]	1.4±0.4[b]	1.6±0.24[b]
M_3	1.0	-	10.4±0.24[fg]	2.4±0.24[cd]	2.6±0.24[cd]
M_4	1.5	-	11.2±0.2[g]	6.4±0.24[g]	5.8±0.2[h]
M_5	2.0	-	3.4±0.24[c]	7.6±0.24[ij]	6.2±0.2[hi]
M_6	2.5	-	3.8±0.2[c]	2.0±0.0[bc]	3.4±0.24[ef]
M_7	0.5	0.2	3.8±0.2[c]	0.0±0.0[a]	2.4±0.24[c]
M_8	1.0	0.2	3.4±0.24[c]	2.0±0.0[bc]	4.0±0.0[fg]
M_9	1.5	0.2	9.6±0.24[f]	4.4±0.24[f]	4.6±0.24[g]
M_{10}	2.0	0.2	17.4±0.24[h]	6.8±0.2[ghi]	11.6±0.4[m]
M_{11}	2.5	0.2	4.8±0.37[d]	6.6±0.4[gh]	10.0±0.32[kl]
M_{12}	0.5	0.5	10.8±0.4[g]	3.2±0.2[de]	4.2±0.2[g]
M_{13}	1.0	0.5	18.2±0.2[hi]	6.4±0.24[g]	7.6±0.24[j]
M_{14}	1.5	0.5	19.0±0.32[i]	11.4±0.24[k]	10.6±0.24[l]
M_{15}	2.0	0.5	22.8±0.37[j]	14.4±0.24[l]	13.0±0.32[m]
M_{16}	2.5	0.5	10.8±0.49[g]	7.4±0.4[hj]	9.4±0.24[k]
M_{17}	0.5	1.0	7.6±0.4[e]	4.8±0.37[f]	4.2±0.2[g]
M_{18}	1.0	1.0	6.8±0.37[e]	3.4±0.4[e]	3.2±0.2[de]
M_{19}	1.5	1.0	10.4±0.24[fg]	8.4±0.4[aj]	6.8±0.38[i]
M_{20}	2.0	1.0	2.2±0.44[b]	6.4±0.4[g]	4.0±0.32[fg]
M_{21}	2.5	1.0	0.0±0.0[a] (callus)	4.8±0.4[f]	4.2±0.2[g]

*Means within a column having the same letter are not statistically significant ($p<0.05$) according to Duncan's multiple range test (SPSS V 8.0.1).

concentration promotes shoot proliferations. However, the inclusion of low concentration of auxin(s) along with cytokinin(s) triggers the rate of shoot proliferations. The differential response of explants in different species may be attributed to its varying tissue composition.

Rooting of Shoots

Elongated and well developed shoots (>3 cm long) were excised from the shoot clumps and transferred to MS medium augmented with various concentration of NAA and IBA alone for root initiation. Rooting occurred in three weeks in all treatments. The frequency of rooting varied with its origin from the excised explant, auxin type and auxin concentration. Among the shoots derived from various explants used, those from node explants responded favourably for root initiation; maximum percentage of rooting was observed in all responses to the two auxins (Table.3). The frequency of rooting per shoot was significantly different among the treatments. The percentage of rooting increased with increase in the concentration of auxin. All kinds of shoots showed highest rooting response in either treatment- MS with 0.1 mgl^{-1} NAA (*Fig. 2c*) or 0.2 mgl^{-1} IBA (*Fig. 2d*).

Acclimatization and Field transfer

After four weeks, the rooted plantlets were transferred into plastic pots containing autoclaved sand and soil mixture (1:1) and were maintained in the culture room for two weeks (Fig. 2e), and then transferred to shade and then to field conditions. The survival rate is 85.4%. The regenerated plantlets did not show any variation in morphology or growth characteristics when compared with the respective donor parents.

Table 3. Influence of two auxins on rooting of *in-vitro* derived shoots of *A. longifolia* after 3 weeks*

Media code	Plant growth regulator conc. (mg/l)		No. of roots developed from the shoot developed from different explants (Mean ± S.E.)		
	NAA	IBA	Leaf	Internode	Node
R_1	Control		10.8±0.58[a]	9.8±0.37[a]	10.2±0.58[a]
R_2	0.1	-	32.4±1.96[d]	30.2±1.02[c]	42.6±0.93[d]
R_3	0.2	-	24.8±1.16[c]	30.0±0.84[c]	48.8±1.39[e]
R_4	0.5	-	20.4±0.24[b]	28.8±1.53[c]	39.2±0.58[c]
R_5	-	0.1	9.8±0.49[a]	7.4±0.6[a]	9.4±0.51[a]
R_6	-	0.2	27.8±2.52[c]	34.4±0.75[d]	40.2±1.36[cd]
R_7	-	0.5	20.6±0.4[b]	20.6±0.98[b]	28.2±0.24[b]

*Means within a column having the same letter are not statistically significant ($p<0.05$) according to Duncan's Multiple range test (SPSS V 8.0.1)

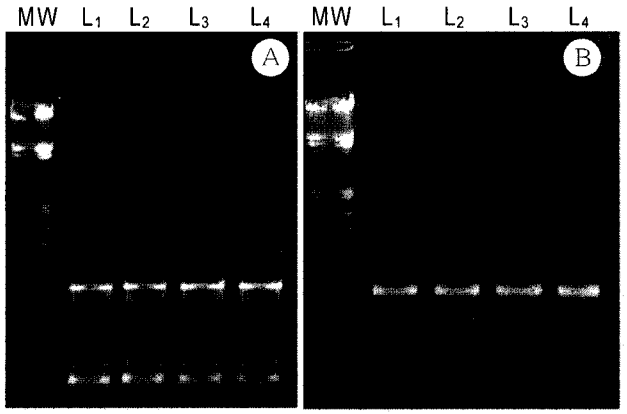

Figure 3. Electrophoretic pattern of PCR amplified DNA fragments of the donor plant (L1) and three *in vitro* regenerants (L2-L4) using primer OPA02 (A) and OPA04 (B).

RAPD analysis to test genetic fidelity

To test the genetic fidelity of *in-vitro* regenerated plantlets randomly amplified polymorphic DNA (RAPD) markers had been used. For this purpose the genomic DNA isolated from three *in-vitro* regenerated plantlets developed from three different explants (leaf, node and internode) and one donor plant. Four decamer primers (OPA1, OPA2, OPA3 and OPA4) had been used to regenerate RAPD markers. All four decamer primers produced scoreable RAPD bands. The analysis revealed quite homogenous banding pattern. Amplification with OPA1 generated four amplicons with molecular weight 1456 bp to 541 bp and all the samples exhibited similar profile. Similarly, OPA2, OPA3 and OPA4 generated four (1757 to 246 bp), five (1255 to 256 bp) and four (767 to 119bp) amplicons respectively and were present uniformly in all four samples including the donor plant and three *in vitro* regenerants. Figure.3 shows a representative picture of the electrophoretic pattern of PCR amplified DNA fragments of the donor plant and three *in vitro* regenerants using primer OPA02 and OPA04. The proximity matrix analysis of all four samples resulted in a single cluster having proximity matrix coefficient 1.0. The results indicated that this *in-vitro* regeneration protocol of *A. longifolia* has no effect on its genomic organization. Genetic uniformity of *in-vitro* regenerated plantlets of *Drosera* species was also assessed by RAPD analysis (Ewaojkowska and Kawiak 2004) and a similar kind of observations were also made in several species including oil palm (Rival et al. 1998), ginger (Rout et al. 1998), sugarcane (Zuchhi et al. 2002) and garden asparagus (Pontaroli and Camadro 2005), tomato (Soniya et al. 2005). This substantiates the utility of RAPD marker to test the genetic fidelity of *in- vitro* regenerated plantlets.

The present study could be of enormous significance, since data on direct shoot bud induction and multiple shoot production of *A. longifolia* is not available. This efficient and reliable plant regeneration system via direct and indirect organogenesis can be potentially utilized for its *ex-situ* conservation as well as to meet the growing demand of *A. longifolia* plants for herbal preparations.

Acknowledgements

The authors are grateful to the Department of Science and Technology (DST), Govt. of India for the financial assistance through the SERC FAST TRACK scheme (SR/FT/L-164/2004) to the senior author (JP). Thanks are due to the Chairman and the Director, MITS for providing necessary facilities.

References

Ahmed S, Rahman A, Mathur M, Athar M, Sultana S (2001) Anti-tumor promoting activity of *Asteracantha longifolia* as experimental hepatocarcinogenesis in rats. Food Chem and Toxicol 39: 19-28

Anis M, Faisal M (2005) *In vitro* regeneration and mass multiplication of *Psoralea corylifolia*-An endangered medicinal plant. Indian J Biotechnol 4: 261-264

Azad MAK, Yokota S, Yahara S, Yoshizawa N (2004) Effects of explant type and growth regulators on organogenesis in a medicinal tree, *Phellodendron amurense* Rupr. Asian J Plant Sci 3: 522-528

Benjamin BD, Roja P, Heble MR, Chadha MS (1987) Multiple shoot cultures of *Atropa belladonna*: Effect of physiochemical factors on growth and alkaloid formation. J Plant Nutr 12: 129-135

Borthakur M, Singh RS (2002) Direct plantlet regeneration from male inflorescences of medicinal yam (*Dioscorea floribunda* Mart. & Gal.). In Vitro Cell Dev Biol-Plant 38: 183-185

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA mini preparation: Version II. Plant Mol Biol Rep 1: 19-21

Ewaojkowska, Kawiak A (2004) Application of RAPD in the determination of genetic fidelity in micropropagated *Drosera* Plantlets. In Vitro Cell Dev Biol-Plant 40: 592-595

Fernando MR, Nalinie WSMD, Thabrew MI, Ariyananda PL, Karunanayake EH (1991) Effect of *Artocarpus heterophyllus* and *Astearacantha longifolia* on glucose tolerance in normal human subjects and in maturity onset diabetic patients. J Ethanopharmacol 31: 277-282.

Harter HL (1960) Critical values for Duncan's multiple range test. Biometric 16: 671-685

Hewawasam RP, Jayatilaka KAPW, Pathirana C, Mudduwa LKB(2003) Protective Effect of *Astercantha longifolia* extract in mouse liver injury induced by carbon tetrachloride and paracetamol. J Pharmacy Pharmacol 55: 1413-1418

Koroch AR, Kapteyn J, Juliani HR, Simon JE (2003) *In-vitro* regeneration of *Echinacea pallida* from leaf explants. In Vitro Cell Dev Biol-Plant 39: 415-418

Lal N, Ahuja PS (1996) Plantlet regeneration from callus in *Picrorhiza kurroa* Royal ex Benth. An endangered medicinal plant. Plant Tissue Cult 6: 127-134

Larkin PJ, Scowcroft WR (1981) Somaclonal variation-a novel source of variability from cell cultures for plant improvement. Theor Appl Genet 60:197-214

Majumdar UK, Gupta M, Maiti S, Mukherjee D (1997) Antitumor activity of *Hygrophila spinosa* in Ehrlich ascites carcinoma and sarcoma-180 induced mice. Indian J Exp Biol 35: 473-477

Mishra L (1985) Anubhut Yogamala and Sahaja Chikishya, Raj Edition, Puri, Orissa, India. pp 128

Mishra RR, Behera M, Panigrahi J (2005) *In vitro* multiplication of *Asteracantha longifolia* (L.) Nees- A medicinal herb. In: National Seminar on Medicinal and Aromatic Plants-Biodiversity, Conservation, Cultivation and Processing, IGAU, Raipur, India, pp189-190

Muller E, Brown PTH, Hartke S, Lorz H (1990) DNA variation in tissue culture derived rice plants. Theor Appl Genet 80: 673-679

Murashige T, Skoog F (1962) A Revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plantarum 15: 473-497

Okslar V, Strukeij B, Kreft S, Bohanec B, Zel J (2002) Micropropagation and hairy root culture of *Solanum laciniatum*. In Vitro Cell Dev Biol-Plant 38: 352-357

Pontaroli AC, Camadro EL (2005) Somaclonal variation in *Asparagus officinalis* plants regenerated by organogenesis from long-term callus cultures. Genet Mol Biol 28: 423-430

Rival A, Bertrand L, Beule T, Trouslot P, Lashermes P (1998) Suitability of RAPD analysis for the detection of somaclonal variants in oil palm. (*Elapis guineensis jacq*) Plant Breed 117: 73-76

Rout GR, Das P, Goel S, Raina SN (1998) Determination of genetic stability of micropropagated plants of ginger using Random Amplified Polymorphic DNA (RAPD) markers. Bot Bull Acad Sin 39: 23-27

Shailajan S, Chandra N, Sane RT, Menon SK (2005) Effect of *Asteracantha longifolia* Nees. against CCl_4 induced liver dysfunction in rat. Indian J Exp Biol 43: 68-75

Shanmugasundaram P, Venkataraman S (2005) Anti-nociceptive activity of *Hygrophila auriculata* (Schum) Heine. African J of Trad Compl Alt Medicines 2: 62-69

Singh A, Handa SS (1995) Hepatoprotective activity of *Apium graveolens* and *Hygrophilla auriculata* against paracetamol and thioacetamide in toxication in rats. J Ethnopharmacol 49: 119-126

Singh U, Wadhwani AM, Johri BM (2003) Dictionary of Economics Plants in India, IACR, New Delhi, India, pp. 104

Skoog F, Miller CO (1957) Chemical regulation of growth and organ formation in plant tissue culture *in vitro*. Symp Soc Exp Biol 11: 118-131

Soniya EV, Banerjee NS, Das MR (2005) Genetic analysis of somaclonal variation among callus-derived plants of tomato. Curr Sci 80: 1213-1215

Temjensangba, Deb CR (2005) Regeneration of plantlets from *in vitro* raised leaf explants of *Cleisostoma racimeferum* Lindl. Indian J Exp Biol 43: 377-381

Vasil IK (1988) Progress in the regeneration and genetic manipulation in crops. Biotechnol 6: 397-402

Vlietnick AJ, Vanhoof L, Totte J, Lasure A, Vanden Berghe D, Rwangabo PC, Mvukiyumwami J (1995) Screening of hundred Rwandese medicinal plants for antimicrobial and antiviral properties. J Ethanopharmacol 46: 31-47

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic marker. Nucleic Acids Res 18: 6531-6535

Zucchi MI, Arizono H, Morais VA, Helena M, Fungaro P, Vieira MLC (2002) Genetic instability of sugarcane plants derived from meristem cultures. Genet Mol Biol 25: 91-96