Plant regeneration from leaf explants of *Aloe barbadensis* Mill. and genetic fidelity assessment through DNA markers

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Abstract An efficient plant regeneration protocol was developed from leaf explants of *Aloe barbadensis* Mill on Murashige and Skoog's (MS) medium supplemented with 2.0 mg/l 6-benzyladenine (BA) or Kinetin (Kn), 0.25–0.5 mg/l NAA (1-naphthalene acetic acid) and 3 % (w/v) sucrose within 4 weeks of culture. The maximum number of shoot buds were obtained on MS medium supplemented with 2.0 mg/l BA, 0.5 mg/l NAA, 40 mg/l Ads (adenine sulphate) within 4–6 weeks of subculture. Inclusion of 0.25–0.50 mg/l gibberellic acid into the medium, the shoot buds became elongated. Repeated subculture on regeneration medium induces higher rate of shoot regeneration. The root induction from excised microshoots was achieved on half-strength MS medium supplemented with 0.25–1.0 mg/l NAA or indole-3-butyric acid (IBA) and 2 % (w/v) sucrose. Maximum percentage of rooting was achieved on medium having 0.5 mg/l NAA with 3 % (w/v) sucrose. About 80 % of in vitro raised plantlets were hardened in the greenhouse and successfully established in the soil. Both Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeat (ISSR) markers were used to detect the variability among the regenerated plants developed in vitro. The results showed that there was no polymorphism among the regenerated plantlets. This study will help for propagation of quality planting material of *Aloe barbadensis* for commercialization.

Keywords Shoot bud regeneration · Genetic stability · Medicinal plant · RAPD · ISSR marker

Introduction

Aloe barbadensis (family Liliaceae) is a very important indigenous medicinal herb. It is a perennial succulent xerophyte and growing up to 1.5 m in height, with a strong root. The leaves are whitish green on both sides and bear spiny teeth on the margins. It is propagated vegetatively and growth rate is very slow (Gui et al. 1990). This plants are mostly used in pharmaceutical and cosmetic industry (Aggarwal and Bama 2004; Chaudhary et al. 2011). It is widely used in ayurvedic system of medicine against skin disease. *Aloe* leaves contain gel like substances which are very effective in wounds, burn and other diseases (Jayakrishna et al. 2011). The plant contains the antioxidant vitamins (A, C and F), niacin, riboflavin and folic acid and used as a laxative, antihelminthic and uterine stimulant (Kathi and Victoria 1999). Indiscriminate over exploitation coupled with insufficient attempts for replenishment of wild stock have contributed to its threatened status requiring scientific efforts for conservation and commercial cultivation. Due to low seed viability, germination rate, limited availability of raw material with high quality and slow vegetative growth, tissue culture technique is an alternate solution for conservation of those valuable medicinal plants (Rout et al. 2008; Bantawa et al. 2011; Swarna and Ravindhran 2012). Though tissue culture is recognized as one of the key areas of biotechnology for large scale propagation and conservation, but it is severely hindered due to somaclonal variations. In vitro studies of *Aloe barbadensis* by using meristems have also been reported (Natali et al. 1990; Meyer and Staden 1991; Baksha et al. 2005; Hashemabadi and Kaviani 2010). There was no report on direct shoot bud regeneration from leaf explants of *Aloe*

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barbadensis. Optimization of hormonal treatment, periodic monitoring of the genetic stability is utmost important for commercial utilization of true-to-type plants of the desired genotype. Several molecular techniques were used to assess the genetic purity of in vitro raised plants regenerated through in vitro. Among the various molecular markers, random amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSR) analysis are the simplest and quickest methods used for assessment of genetic stability (Mohanty et al. 2011). Based on above facts the present investigation was carried out with the following objectives: i) standardization of protocol for direct plant regeneration from leaf explants of *Aloe barbadensis*, ii) assessment of genetic stability of the in vitro raised plants using molecular markers.

Materials and methods

In vitro culture

The leaf explants were collected from greenhouse grown plants and surface sterilized with 0.1 % (w/v) mercuric chloride (HgCl_2) for 20 min followed by washing with sterile distilled water in thrice, thereafter the explants were trimmed into small pieces ($\sim 0.5 \text{ cm}^2$) and aseptically cultured into agar-gelled basal Murashige and Skoog (1962) medium and pH was adjusted to 8.0 using 0.1 N NaOH and HCl. The media was solidified with 0.7 % (w/v) agar and was sterilized for 15 min at 121 °C. Different concentrations of 6-benzylaminopurine (BAP: 0–3.0 mg/l), kinetin (Kn: 0–3.0 mg/l), 2,4-dichlorophenoxyacetic acid (2,4-D: 0.25–0.5 mg/l), 1-naphthalene acetic acid (NAA: 0.25–0.5 mg/l, indole-3-butyric acid (IBA: 0.25–0.5 mg/l) and Adenine sulfate (Ads: 25–40 mg/l) were used singly or in combination for shoot bud regeneration from leaf explants followed by shoot elongation. The culture was maintained at temperature of 25 ± 2 °C, 16 h photoperiod with 3000 lux of light intensity. The culture was maintained by sub-culturing at an interval of 4 weeks. The in vitro regenerated elongated shoots (3–4 cm) were excised aseptically and implanted on half strength MS medium with and without growth regulators (NAA or IBA : 0–1.0 mg/l) and 2 % (w/v) sucrose for root induction. Rooted plantlets were thoroughly washed and planted in plastic pots containing soilrite for 15–17 days and subsequently transferred to soil mixture of soil, sand and dry cow-dung manure (1:1:1, v/v/v) kept in the greenhouse for growth. Water was sprayed at every two days interval. All the experiments were repeated three times with 15 replicates. All the cultures were examined periodically, and the data were analyzed statistically by the Duncan's multiple range test (Harter 1960) at $P < 0.05$ for each experiment. Healthy and young leaves of *Aloe barbadensis* were collected both from in vitro grown plantlets and in vivo grown mother plant in every 6 months interval for molecular analysis.

DNA isolation and quantification

To study the genetic uniformity, genomic DNA was extracted from young leaves of in vitro regenerated plants as well as mother plants. Young leaves (1 g) were collected and brought to the laboratory for isolation of DNA by using modified CTAB method (Doyle and Doyle 1990). The modified CTAB buffer [3 % w/v CTAB, 2 M NaCl, 50 mM EDTA, 100 mM Tris-HCl (pH 8), 0.2 % (v/v) 2-mercaptoethanol] was preheated at 65 °C for 1 h and the leaf powder was poured into pre-heated buffer and incubated for 2 h. Further, the extraction with chloroform: isoamyl alcohol (24:1) was performed twice. RNase A (final concentration 10 mg/1000 μl) was added, followed by incubation at 37 °C for 60 min. Final centrifugation at 5000 g for 5 min was performed to remove any impurities and suspended in TE buffer (10 mM Tris-HCl pH 8.0 and 0.1 mM EDTA pH 8.0). DNA was examined by gel electrophoresis in 0.8 % (w/v) agarose in Tris-acetate-EDTA (TAE) buffer prestained with ethidium bromide (5 mg/10 ml). Electrophoresis of DNA was performed at 50 V for 45 min and DNA was visualized with a UV transilluminator. The concentration of DNA was estimated by comparing it with an uncut lambda DNA marker (MBI, Fermentas, Richlands B.C., Old). Twenty RAPD (kits A, B, C, K and N from Operon Technologies, Alameda, CA) primers and 15 synthesized ISSR (M/S EMERK Bioscience, India) primers were used for DNA profiling.

Molecular analysis

Initial optimization of polymerase chain reaction (PCR) was done for Random Amplified Polymorphic DNA (RAPD) assay, including concentration of template DNA, primer, MgCl_2 , number of PCR cycle and above annealing temperature. Twenty-five microliter reaction mixture for RAPD assay contained 20 ng template DNA, 100 mM each dNTPs, 15 ng of decanucleotide primers (M/S Operon Technology, Inc., Alameda, CA), 1 μl Taq buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl, 0.01 % gelatin) and 0.5 U Taq DNA polymerase (M/S EMERK Bioscience, India). A total of 1.5 mM MgCl_2 was used in the case of RAPD. DNA amplification was performed in a thermal cycler (Peqlab, Germany) with preliminary activation at 94 °C for 5 min followed by 45 cycles of 1 min at 94 °C, 1 min at annealing temperature (37 °C) and 1 min at 72 °C, with a final extension at 72 °C for 10 min. In case of ISSR, the annealing temperature was varied from 38 to 57 °C as per the primers tested. Amplification products were visualized in 1 % (w/v) agarose gel for RAPD & ISSR electrophoresis in 1 % TAE prestained with ethidium bromide. The 1.0-kb plus ladder (M/S Bangalore Genei, India) was run in each gel as size marker. Gel photographs were scanned through Gel Documentation System (UVITEC, UK) and the

amplification products size were evaluated using the software Quantity One (Bio-Rad, USA). Both RAPD and ISSR markers were scored visually and recorded as the presence (1) or absence (0) of bands. The data were entered into a binary data matrix as discrete variables. All the PCR reactions were repeated twice.

Results and discussion

Among the three cytokinins tested, BA was effective for shoot bud development than Kn and Ads. The shoot buds initials were observed within 3–4 weeks of culture on MS medium supplemented with 2.0 mg/l BA (Fig. 1a). Callusing was not developed in the basal end of the explants. Combination of BA + NAA favours more effective for shoot bud proliferation than the Kn + NAA. Inclusion of 25 to 40 mg/l Ads in the

culture medium enhances the rate of shoot bud proliferation (Table 1). The inclusion of gibberelic acid (0.25–0.50 mg/l) in the culture medium showed the maximum elongation of shoots. The percentage of shoot bud regeneration was maximum (55.6 %) within 4–6 weeks of culture on MS medium fortified with 2.0 mg/l BA, 0.5 mg/l NAA, 40 mg/l adenine sulfate and 3 % sucrose (Table 1; Fig. 1b & c). There was no sign of meristematic shoot bud proliferation when leaf explants were cultured in media without cytokinin. Further, increase in the concentration of either BA or Kn alone or in combination had no effect on the rate of shoot bud development or multiplication. Budhiani (2001) reported that the best shoot bud initiation and proliferation from meristems on MS medium supplemented with 0.2–2.0 mg/l BA + 0.002 mg/l NAA. Liao et al (2004) achieved shoot multiplication from shoot tip explants by using MS medium supplemented with 2.0 mg/l BA + 0.3 mg/l NAA. The present study indicated that

Fig. 1 In vitro plant regeneration of *Aloe barbadensis*. **a** Proliferation of leaf explant of *Aloe barbadensis*. **b & c** Development of shoot buds from leaf explants (arrows) on MS medium supplemented with 2.0 mg/l BA, 0.25 mg/l NAA and 40 mg/l Ads after 4 weeks (**b**) and 6 weeks (**c**) of subculture. **d** Induction of roots from microshoots on half strength MS medium supplemented with 0.5 mg/l NAA and 2 % sucrose after 3 weeks of culture. **e** In vitro raised rooted shoots grown in the sand: soil: cow dung in the ratio 1: 1: 1(v/v/v) after 1 month of transfer to greenhouse

Table 1 Effect of different concentrations of cytokinins and auxins on shoot bud regeneration from leaf explants of *Aloe barbadensis* after 4 weeks (A) and 6 weeks (B) of subculture

MS + growth regulators (mg/l)					Percent of organogenic calli development (Mean $\pm$ S.E)*		Av. No. of shoot buds per culture (Mean $\pm$ S.E)*	
BAP	Kn	Ads	NAA	2,4-D	A	B	A	B
0	0	0	0	0	0	0	0	0
1.0	0	0	0	0	8.36 $\pm$ 0.7 a	12.6 $\pm$ 0.8 a	1.3 $\pm$ 0.5 a	2.2 $\pm$ 0.3 b
1.5	0	0	0	0	12.5 $\pm$ 0.8 b	18.5 $\pm$ 0.7 c	1.6 $\pm$ 0.6 c	2.1 $\pm$ 0.5 b
2.0	0	0	0	0	20.2 $\pm$ 0.6 e	24.2 $\pm$ 0.8 e	1.4 $\pm$ 0.4 b	2.3 $\pm$ 0.4 b
2.5	0	0	0	0	20.8 $\pm$ 0.8 e	26.8 $\pm$ 0.4 f	1.4 $\pm$ 0.6 b	2.6 $\pm$ 0.7 b, c
3.0	0	0	0	0	20.5 $\pm$ 0.9 e	21.6 $\pm$ 0.7 d	2.3 $\pm$ 0.8 d	3.2 $\pm$ 0.2 c, d
0	1.0	0	0	0	11.8 $\pm$ 0.6 b	14.2 $\pm$ 0.8 b	1.8 $\pm$ 0.4 c	2.4 $\pm$ 0.3 b
0	2.0	0	0	0	12.6 $\pm$ 0.9 b	17.6 $\pm$ 0.5 c	2.2 $\pm$ 0.4 d	2.9 $\pm$ 0.6 c
0	3.0	0	0	0	14.4 $\pm$ 0.5 c	18.8 $\pm$ 0.4 c	2.4 $\pm$ 0.6 d	2.5 $\pm$ 0.4 b
2.0	0	25.0	0.25	0	30.2 $\pm$ 0.8 f	32.2 $\pm$ 0.7 g	3.5 $\pm$ 0.5 e	3.8 $\pm$ 0.2 e
2.0	0	40.0	0.25	0	42.7 $\pm$ 1.0 g	45.5 $\pm$ 0.9 h	3.6 $\pm$ 0.3 e	4.6 $\pm$ 0.5 f
0	2.0	25.0	0.25	0	20.6 $\pm$ 0.8 e	26.8 $\pm$ 1.0 f	2.8 $\pm$ 0.5 d	3.4 $\pm$ 0.2 d
0	2.0	40.0	0.25	0	22.2 $\pm$ 0.7 e	26.6 $\pm$ 0.8 f	3.2 $\pm$ 0.7 e	3.4 $\pm$ 0.3 d
2.0	0	25.0	0.50	0	52.8 $\pm$ 1.2 h	62.7 $\pm$ 1.0 i	6.8 $\pm$ 0.6 f	8.0 $\pm$ 0.6 g
2.0	0	40.0	0.50	0	55.6 $\pm$ 1.0 i	74.2 $\pm$ 0.8 j	8.7 $\pm$ 0.3 g	10.7 $\pm$ 0.4 h
2.0	0	25.0	0	0.25	12.2 $\pm$ 0.7 b	12.8 $\pm$ 0.6 a	1.2 $\pm$ 0.5 a	1.6 $\pm$ 0.5 a
2.0	0	40.0	0	0.50	18.8 $\pm$ 0.4 d	20.2 $\pm$ 0.5 d	1.7 $\pm$ 0.4 c	2.5 $\pm$ 0.3 b

*Means of 15 replicates/treatment; repeated thrice

Mean followed by different letters are significantly different at the 5 % level

the inclusion of NAA along with BA and Ads improved the higher rate of shoot bud proliferation from leaf explants. Addition of GA₃ (0.25–0.5 mg/l) into the culture medium enhanced the rate of shoot elongation as compared to BA or BA + Ads. Hashemabadi and Kaviani (2008) achieved the maximum number of multiple shoots (9.67) per explant on medium supplemented with 0.5 mg/l BA + 0.05 mg/l NAA. In contrast, the present findings showed that the direct shoot bud regeneration from leaf explant was achieved on medium containing 2.0 mg/l BA, 0.5 mg/l NAA, 40 mg/l Ads and a maximum 10.7 shoots per culture within 4–6 weeks of first subculture (Table 1). The rate of shoot bud proliferation declined on medium containing higher concentrations of NAA or BA or Kn. The rate of shoot bud proliferation ability was maintained up to 8th subculture period on MS medium supplemented with 2.0 mg/l BA, 0.5 mg/l NAA and 40 mg/l Ads by regular subculture at every 4 weeks. This might be due to a balance between the endogenous and exogenous growth regulators and the ionic concentrations of nutrient salts (Rout 2002).

Elongated shoots (3–4 cm long) were excised from parent culture and transfer onto half-strength basal MS medium with or without growth regulators. Initiations of root from microshoots were delayed in the medium without auxin. Inclusion of NAA or IBA (0.25–0.5 mg/l) with 2 % sucrose induced the rooting within 3 weeks of culture. The optimal

percentage of rooting (76 %) and growth of microshoots were observed on medium having 1/2 strength MS medium supplemented with 0.5 mg/l NAA (Table 2; Fig. 1d). The percentage of shoots forming roots and the number of roots per shoot were significantly varied with different concentrations

Table 2 Effect of different concentrations of NAA and IBA on root induction from excised shoots of *Aloe barbadensis* after 3 weeks of culture

½ MS + auxin concentrations (mg/l)		Days to rooting	Percent of rooting (Mean $\pm$ SE)*
IBA	NAA		
0	0	0	0
0.25	0	20–21	28.42 $\pm$ 2.2 a
0.50	0	18–19	42.24 $\pm$ 1.4 c
1.0	0	18–19	40.12 $\pm$ 1.3+ c
0	0.25	17–18	52.20 $\pm$ 1.2 e
0	0.50	15–16	76.04 $\pm$ 2.3 f
0	1.00	18–19	46.30 $\pm$ 1.2+ d
0.25	0.50	20–21	40.16 $\pm$ 1.3+ c
0.50	0.25	20–21	36.22 $\pm$ 1.1+ b

*15 micro-shoots/treatment; repeated thrice

+ -callusing at the basal end. Mean followed by different letters are significantly different at the 5 % level

of NAA or IBA (Table 2). Either increase or decrease in the auxin concentrations in the culture medium did not show any positive effect on root induction from microshoots of *Aloe barbadensis*. Hashemabadi and Kaviani (2008) reported that the maximum percentage of rooting was observed in medium having 0.5 mg/l BA + 0.5 mg/l NAA. Further, they reported that the rooting percentage was enhanced on MS medium supplemented with 1.0 mg/l NAA (Hashemabadi and Kaviani 2010). The roots became elongated on medium supplemented with 1.0 mg/l IBA and 1.0 mg/l NAA. The present results indicate that the percentage of rooting and number of roots per shoot were much higher than the previous report by other researchers. In vitro-raised rooted plantlets were transferred to soilrite for 15 to 17 days and subsequently transferred to polypots containing soil, sand and cow-dung manure at the ratio of 1:1:1 (v/v). Water was sprayed at every two days interval. Excess water will damage the plants. All the regenerated plantlets were kept in the greenhouse with 65 % relative humidity. About 80 % of the plantlets established within 4–6 weeks of transfer (Fig. 1e). The plants grew well and attained 4–5 cm height within 2 months of transfer. The acclimatized plants exhibited normal growth and no sign of morphological variation was noticed.

Assessment of genetic uniformity of in vitro-raised plants

There are many factors like length of culture periods, genotype and nature of explants, which could influence the stability of the tissue cultured plants (Vendrame et al. 1999; Premvaranon et al. 2011). So assessment of genetic stability of in vitro regenerated plantlets is highly significantly for commercialization. However, the reliability and efficiency of molecular markers are frequently questioned. A better analysis of genetic stability of plantlets can be made by using a combination of two types of markers which amplify different regions of the genome (Martins et al. 2004). Genetic integrity by molecular analysis has been reported earlier in many medicinal plant species (Rout et al. 1998; Mohanty et al. 2011). A total of 24 plantlets were analyzed taking 12 plantlets from each culture period (4th sub-culture & 6th subculture rooted microshoots). The quality of in vitro-derived regenerates was screened with 20 random primers and 15 synthesized ISSR primers and showed monomorphic among the regenerated plantlets. The banding pattern of regenerated plantlets was found to be monomorphic for most of the primer tested. The size of the monomorphic fragments produced by RAPD primers and ISSR primers ranged from 200 to 1500 bp, respectively (Fig. 2a–c & a–b). The number of monomorphic DNA fragments was 5 in the case of RAPD and 9 in case of ISSR primers. Harirah and Khalid (2006) used 18 arbitrary decamer primers to study the clonal fidelity of *Musa acuminata* Cv. Berangan. They found that all the regenerated plants were monomorphic. No polymorphism was detected

Fig. 2 RAPD profiles of in vitro regenerated plants of *Aloe barbadensis* using primers **a** OPC 11, **b** OPK 16, **c** OPN 11. “M” shows the molecular marker. RAPD banding profile of mother plants “No. 1” and “No. 2 to 24” is regenerated plants. Arrow indicates the size of the marker

among theregenerated plants of *Plumbago rosea* and *Plumbago zeylanica* by using RAPD markers (Rout 2002). In some cases, regeneration process is prone to somatic variation resulting in off-types as in the case of *Populus*

Fig. 3 ISSR profiles of in vitro regenerated plants of *Aloe barbadensis* using synthesized primers AM-4 (AAG)₃CC (**a**) and AM-8 (AGC)₃GA (**b**). “M” shows the molecular marker. ISSR profile of mother plants “No. 1” and “No. 2 to 24” are regenerated plants. Arrow indicates the size of the marker

tremuloides (Rahman and Rajora 2001). Senapati et al. (2013) used both RAPD and ISSR markers and showed no polymorphism among the micropropagated plantlets of *Celastrus paniculatus* (Fig. 3).

This investigation concludes that the protocol described here is reproducible for large scale propagation and conservation of this valuable medicinal plant true to type. Both RAPD and ISSR markers system were effective in confirming the genetic fidelity of plantlets regenerated directly from leaf explants. This investigation would provide an effective strategy and great potential for improvement using biotechnological approaches and production of secondary metabolites.

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