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Original Article

EFFECT OF GROWTH REGULATORS ON *IN VITRO* PROPAGATION OF *ALOE BARBADENSIS* MILL. AND ASSESSMENT OF GENETIC FIDELITY THROUGH RAPD MARKERS

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Abstract: A successful protocol was developed for mass propagation of Aloe barbadensis Mill., an important medicinal plant. Multiple shoots were induced in apical and axillary meristems on Murashige and Skoog (1962) medium supplemented with 1.0 – 2.0 mg/l 6-benzylaminopurine (BAP), 0.25 -0.5 mg/l Kinetin and 3% sucrose. Inclusion of 0.5 mg/l 1-napthaleneacetic acid (NAA) enhances the rate of shoot elongation and multiplication. The rate of multiplication was higher when the cultures were incubated under continuous light rather than the 16h photoperiod. Rooting was readily achieved upon transferring the microshoots onto ½ strength MS basal semi-solid medium supplemented with 0.5 mg/l NAA or IBA after 8 - 10 days of culture. Micropropagated plantlets were acclimatized and successfully grown in soil mixture. About 90% of micropropagated plantlets were hardened in the polyhouse and successfully established in the soil. Random ampliûed polymorphic DNA marker was used to detect the variability among the micropropagated plants developed through in vitro. The results showed that there was no polymorphism among the micropropagated plants. This study will help for propagation of quality planting material of Aloe barbadensis for commercialization.

Key words: Growth regulators, *Aloe barbadensis*.

INTRODUCTION

Aloe barbadensis is belongs to family Liliaceae and very important indigenous medicinal herb. It is a perennial succulent plant growing up to 1.5 meters 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 [1]. The demand of this plant is very high and mostly used in pharmaceutical and cosmetic industry [2,3]. 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 [4]. The plant contains the antioxidant vitamins (A, C and F), niacin, riboflavin and folic acid and is used as a laxative, antihelminthic and uterine stimulant. An unplanned exploitation by the ever growing human population has resulted in the rapid depletion of plant resources particularly the economically important plants. Pharmaceutical companies largely depend upon materials procured from naturally occurring stands raising concern about possible extinction and providing justification for development of *in vitro* techniques for mass propagation of *Aloe barbadensis*. 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 [5]. Preservation of genetic stability in germplasm collections and micropropagation of elite plants is of the utmost importance and propagation of plants through apical or axillary meristem culture allows recovery of genetically stable and true to type progeny. Preliminary study on *in vitro* micropropagation of *Aloe barbadensis* by using meristems have been standardized by the various researchers [6,7]. However, there is urgent need to develop efficient protocol on rapid propagation and genetically uniformity. 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 techniques have been developed to assess the genetic purity of *in vitro* raised plants but molecular marker techniques presents a most powerful and reliable methods. Among the various molecular markers, random ampliûed polymorphic DNA (RAPD) analysis is the simplest and quickest method used for assessment of genetic stability with no obvious phenotypic alternations [8]. Based on above facts the present investigation was to standardize the efficient protocol on *in vitro* clonal propagation of *Aloe barbadensis* by using apical and axillary meristems and to assess the genetic stability by using RAPD markers.

MATERIALS AND METHODS

Plant material and explant source: Actively growing young shoots of *Aloe barbadensis* were collected from greenhouse grown plants and washed with 2% (v/v) detergent 'Teepol' (Qualigen, India) and rinsed several times with running tap water. The explants were surface sterilized in 0.1 % (w/v) aqueous mercuric chloride solution for 20 min followed by four washings with sterile distilled water. The apical and axillary meristems (~ 2.0 mm) were isolated and used as explants.

Culture medium and condition: The meristem (apical and axillary) was placed on semi-solid basal MS medium [9] supplemented with different concentrations and combinations of 6-benzylaminopurine (BAP: 0.0, 0.5, 1.0, 1.5 and 2.0 mg/l), kinetin (Kn: 0.0, 0.5, 1.0, 1.5 and 2.0 mg/l) and 1-napthalene acetic acid (NAA : 0.0, 0.25 and 0.5 mg/l) for shoot proliferation and multiplication. The pH of the media was adjusted to 5.8 using 0.1N NaOH or 0.1N HCl before autoclaving. Routinely, 25 ml of the molten medium was dispensed into culture tubes (25 x 150 mm), plugged with non-absorbent cotton wrapped in one layer of cheese cloth and sterilized at 121 ºC and 1.06 Kg/cm^2 pressure for 15 min. The cultures were maintained at 25 ± 2 ºC either under 16 h photoperiod or continuous light (55 mmol $m^{-2}s^{-1}$) from cool, white fluorescent lamps. The cultures were maintained by regular subcultures at 4-week intervals on fresh medium with the same compositions.

Induction of rooting and acclimatization: For root induction, excised microshoots were transferred to ½ strength MS basal medium supplemented with different concentrations of NAA or IBA (0.0, 0.1, 0.25 and 0.5 mg/l) and 2% (w/v) sucrose. One excised shoot was placed in each tube (25 x 150 mm) having 15 ml of the culture media. All the cultures were incubated at 25 ± 2 ºC under 16h photoperiod with cool, white fluorescent lamps. Rooted micro-propagules were thoroughly washed to remove the adhering gel and planted in 2.5 cm earthen pots containing a sterile mixture of sand: soil and cow-dung manure in the ratio of 1:1:1 (v/v) and kept in the greenhouse for acclimatization.

Observation of cultures and presentation of results: Twenty cultures were used per treatment and each experiment was repeated at least three times. The data pertaining to mean percentage of cultures showing response, number of shoots/culture and mean percentage of rooting were statistically analysed by the Post-Hoc Multiple Comparison test [10]. Between the treatments, the average figures followed by the same letters were not significantly different at $P < 0.05$ level.

DNA isolation and quantiûcation: Genomic DNA was extracted by the CTAB method of Doyle and Doyle [11] with some modiûcations. Leaf tissue (1.0 g) derived from *in vitro*-raised plantlets of *Aloe barbadensis* was ground in liquid nitrogen to a ûne powder with a chilled mortar and pestle and added to preheated, modiûed CTAB buffer [3% w/v CTAB, 2M NaCl, 50 mM EDTA, 100 mM Tris-HCl (pH 8), 0.2% (v/v) 2-mercaptoethanol]. Extraction with chloroform: isoamyl alcohol (24:1) was performed twice. RNase A (ûnal concentration 10 mg/l000 µl) was added, followed by incubation at 37^{0}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 (5mg/10ml). 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).

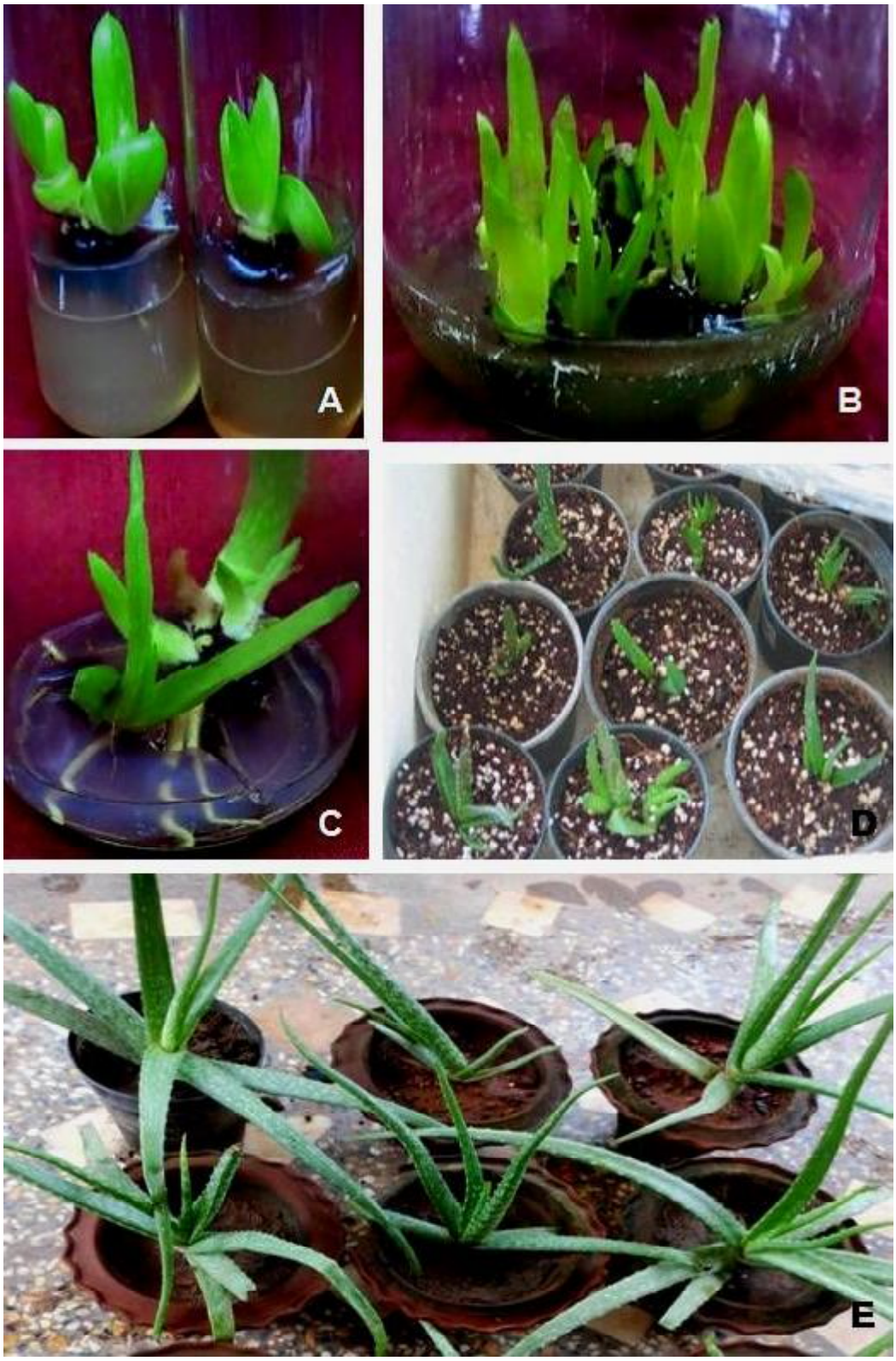
Randomly ampliûed polymorphic DNA analysis: Initial optimization of polymerase chain reaction (PCR) was done for random ampliûed polymorphic DNA (RAPD) assay, including concentration of template DNA, primer, $MgCl_2$, number of PCR cycle and above annealing temperature. Twenty-ûve microliter reaction mixture for RAPD assay contained 20 ng template DNA, 100 mM each DNTPs, 15 ng of decanucleotide primers (M/S operon technology, Inc., Almeda, CA) (OPN, OPC, OPD series) , 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 ampliûcation was performed in a thermal cycler (PeQlab, Germany) programed as follows: preliminary activation at 94 ^{0}C for 5 min followed by 45 cycles of 1 min at 94 ^{0}C, 1 min at annealing temperature (37 ^{0}C) and 1 min at 72 ^{0}C, with a ûnal extension at 72^{0}C for 10 min. Ampliûcation products were visualized in 1% (w/v) agarose gel for RAPD electrophoresis in 1% TAE prestained with ethidium bromide. The 3.0-kb plus ladder (M/S Bangalore Genei, India) was run in each gel as size marker. Gel photographs were scanned through Gel Doc System (UVITEC, UK) and the ampliûcation products size were evaluated using the software Quantity One (Bio-Rad, USA).

RESULTS

Meristem proliferation and multiplication: Meristem proliferation was initiated from apical and axillary explants of *Aloe barbadensis* within 8-10 days of inoculation onto MS basal medium supplemented with BAP, Kn and NAA. Of the different cytokinins tested, BAP was the most effective for shoot proliferation and multiplication. The maximum shoot proliferation and multiplication was observed both in apical and axillary meristems cultured on MS medium supplemented with 1.5 - 2.0 mg/l BAP and 0.25 – 0.5 mg/l Kn within 4 weeks of culture under 16h photoperiod (Table 1). The apical and axillary shoots proliferated and elongated to 1.5 -2.0 cm within 4 weeks of culture (Fig. 1A). There was no sign of shoot proliferation when explants were cultured in media devoid of cytokinin. At higher concentrations of BAP, the rate of shoot proliferation declined. Inclusion of NAA (0.5 mg/l) in the culture medium help in proliferation and multiplication of shoot. The maximum rate of shoot elongation and proliferation was achieved on MS medium supplemented with 2.0 mg/l BA + 0.5 mg/l NAA. Prolonged culture on the proliferation and multiplication media resulted in the blackening of the basal ends of the developing shoots. There were differences among the treatments for both the percentage of cultures with multiple shoots and the mean number of shoots/culture. The apical meristem produced more number of shoots (4.82) in 4 weeks of culture (Fig. 1B) (Table 1). The study also revealed that the continuous light (24h) was more conducive to higher rate of shoot multiplication than 16h photoperiod (Table 2). The highest percentage of cultures with multiple shoots (82.4) was observed on media containing 2.0 mg/l BAP and 0.5 mg/l NAA when the cultures were incubated in the continuous light for 4 weeks. The frequency of multiple shoots per culture varied from 1.94 to 5.23 in case of the 16h photoperiod and 2.06 to 6.0 in case of the continuous light incubation (Table 2). The rate of multiplication was high and stable up to 8th subculture and declined in subsequent subcultures.

Induction of rooting from microshoots: Elongated shoots (1-2 cm long) were rooted on ½ strength MS basal medium supplemented with various concentrations of either NAA or IBA (Table 3). The rooting in the microshoots was inhibited in the medium devoid of growth regulator. Root initiation took place within 8 - 10 days of transfer to ½ MS basal medium supplemented with 0.5 mg/l NAA or IBA. However, optimal rooting (75.6%) and growth of microshoots were observed on medium containing 0.5 mg/l NAA with 2% (w/v) sucrose (Fig. 1C). The rooting ability was reduced with the increase in the concentration of NAA in the culture medium. The percentage of shoots forming roots and days to rooting significantly varied with different concentrations of NAA or IBA.

Acclimatization and field establishment: Rooted plantlets grown in vitro were washed thoroughly to remove the adhering gel, transplanted to 2.5cm earthen sterile pots containing perlite for 2 weeks



Figs. 1A-E: Micropropagation of *Aloe barbadensis*. A. Proliferation of shoot from apical meristems on MS medium supplemented with 2.0 mg/l BA, 0.5 mg/l NAA and 3 % sucrose. (Bar = 5 mm). **B**. Multiple shoots from apical meristems on MS medium supplemented with 2.0 mg/l BA, 0.5 mg/l NAA and 3 % sucrose after 4 weeks of culture. (Bar = 10 mm). **C.** Rooting in the in vitro derived shoots after 10 days of culture on ½ MS medium supplemented with 0.5 mg/l NAA and 2 % (w/v) sucrose. (Bar = 10 mm). **D.** *In vitro* raised plantlets transferred to perlite for acclimatization (Bar = 50 mm). **E.** *In vitro* raised plantlets established in soil (Bar= 1.0 cm).

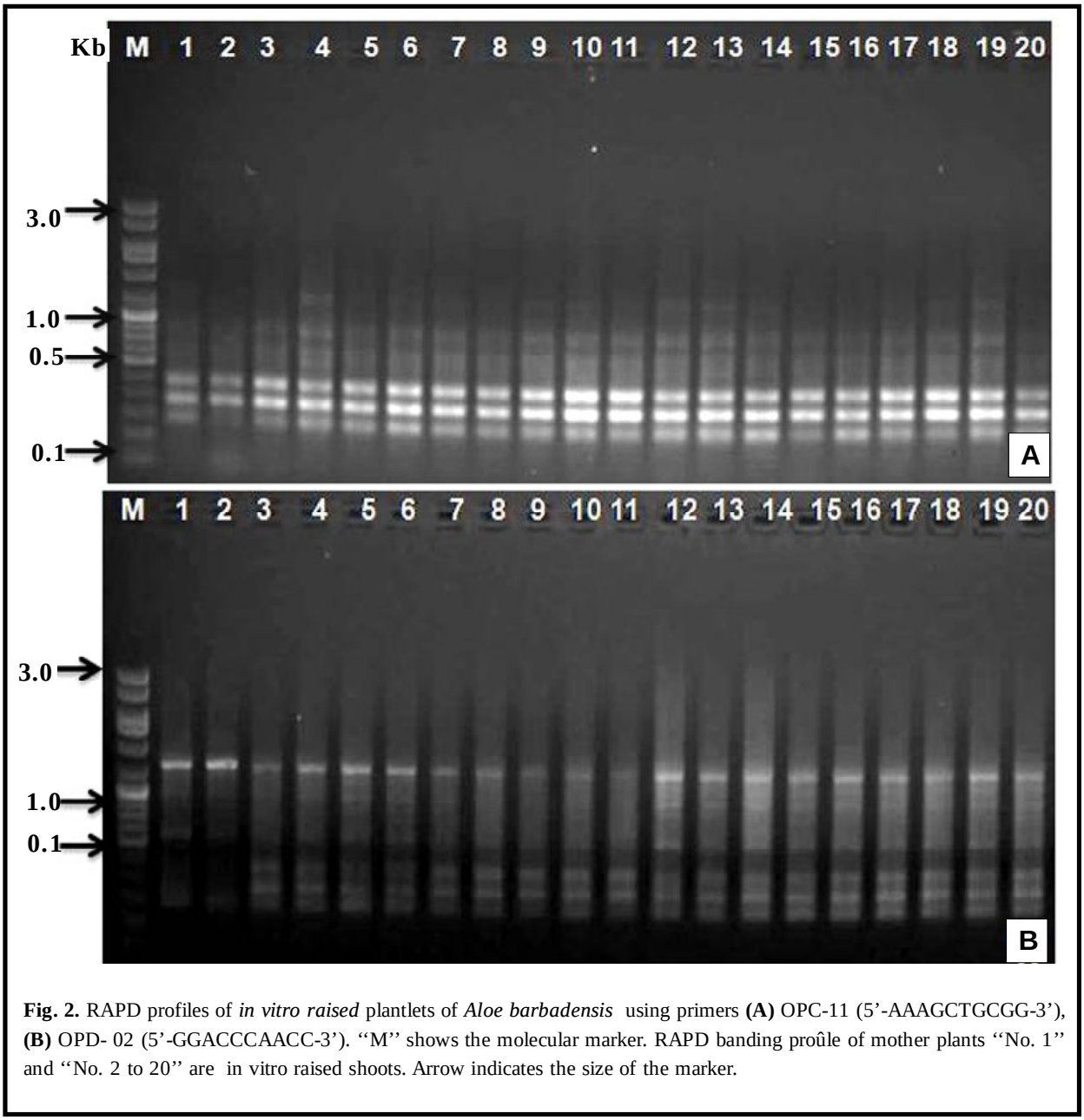


Fig. 2. RAPD profiles of *in vitro raised* plantlets of *Aloe barbadensis* using primers **(A)** OPC-11 (5'-AAAGCTGCGG-3'), **(B)** OPD- 02 (5'-GGACCCAACC-3'). ''M'' shows the molecular marker. RAPD banding proûle of mother plants ''No. 1'' and ''No. 2 to 20'' are in vitro raised shoots. Arrow indicates the size of the marker.

MS + Growth regulators (mg/l)		Percent of cultures with multiple shoots (Mean ± S.E.)*	Number of shoots/ explant (Mean ± S.E.)*
BAP	Kn		
0	0	0	0
0.50	0	32.6 ± 0.4 a	2.10 ± 0.2 b
1.00	0	45.2 ± 0.5 d	2.21 ± 0.4 b
2.00	0	58.6 ± 0.7 g	3.12 ± 0.5 e
0	0.50	38.7 ± 0.3 b	1.72 ± 0.5 a
0	1.00	42.2 ± 0.5 c	2.27 ± 0.4 c
0	2.00	48.7 ± 0.4 e	2.80 ± 0.3 d
1.00	0.25	52.8 ± 0.5 f	2.26 ± 0.2 c
1.50	0.50	51.7 ± 0.4 f	3.16 ± 0.4 e
2.00	0.5	68.3 ± 0.6 h	4.82 ± 0.7 f
1.00	1.0	39.4 ± 0.4 b	2.86 ± 0.6 d

Table 1. Effect of cytokinins on shoot multiplication of *Aloe barbadensis* after 4 weeks of culture under 16h photoperiod. * Mean of 20 cultures per treatment; repeated thrice. a-h Means having the same letter in a column were not significantly different by Post-Hoc Multiple Comparison test $P < 0.05$ level

Table. 2. Effect of photoperiod on shoot multiplication of *Aloe barbadensis* cultured on MS medium supplemented with various concentrations of BAP, kinetin and 0.5 mg/l NAA after 4 weeks of culture. * Mean of 20 cultures per treatment; repeated thrice. a-j Means having the same letter in a column were not significantly different by Post-Hoc Multiple Comparison test $P < 0.05$ level.

Growth regulators (mg/l)			Percent of cultures with multiple shoots (Mean ± S.E.)*	Number of shoots/ explant (Mean ± S.E.)*
BA	Kn	16 hr Photoperiod		
0	0		0	0
0.50	0		23.4 ± 0.3 a	1.94 ± 0.4 a
1.00	0		38.2 ± 0.6 c	2.23 ± 0.5 b
0	0.5		36.5 ± 0.4 b	2.36 ± 0.3b,c
0	1.00		41.5 ± 0.7 d	2.66 ± 0.5 d
0.5	0.5		51.2 ± 0.5 g	4.42 ± 0.3 h
1.0	0.5		52.4 ± 0.3 h	4.18 ± 0.7 h
2.0	0.5		68.2 ± 0.4 i	5.23 ± 0.6 i
		Continuous light		
0	0		0	0
0.5	0		23.1 ± 0.5 a	2.06 ± 0.4a,b
1.00	0		42.2 ± 0.4 d	2.91 ± 0.2 e
0	0.5		39.6 ± 0.3 c	2.82 ± 0.4e
0	1.0		43.4 ± 0.7 d	3.27 ± 0.5f
0.5	0.5		45.4 ± 0.5 e	3.62 ± 0.6g
1.0	0		50.3 ± 0.4 f	4.32 ± 0.3h
2.0	0		82.4 ± 0.6 j	6.00 ± 0.4 j

Table 3: Effect of NAA and IBA on rooting from excised shoots of *Aloe barbadensis* cultured on ½ strength MS basal medium supplemented with 2% (w/v) sucrose. * Mean of 15 cultures per treatment; repeated thrice.

MS + Growth regulators (mg/l)		Percentage of shoot rooted (Mean ± S.E)*	Days to rooting
IBA	NAA		
0	0	0	0
0.1	0	48.4 ± 0.6	11-12
0.25	0	52.8 ± 0.3	12-13
0.50	0	36.4 ± 0.4	13-14
0	0.1	60.3 ± 0.6	11- 12
0	0.25	52.8 ± 0.7	12 -13
0	0.50	75.6 ± 0.4	12 -13

and subsequently transferred to garden soil, sand and cow-dung at the ratio of 2: 1: 1 (v/v). About 90% of the rooted plantlets established in the greenhouse within 3-4 weeks of transfer (Fig.1D). The plant grew well and attained 6 -8 cm height within 8 weeks of transfer (E). The acclimatized plants were established in the field condition and grew normally without morphological variation.

A total of 20 plantlets including mother plant were selected and used for molecular analysis. The quality of *in vitro*-derived plantlets was screened with decamer primers that have showed monomorphic among the plantlets. The banding pattern of PCR-ampliûed product from micropropagated plantlets was found to be monomorphic for most of the primer tested. The identical RAPD banding pattern of in vitro-raised plantlets and their mother control plant is shown in Fig. 2. The size of the monomorphic DNA fragments produced by OPC-11 and OPD-02 primers ranged from 100 to 1000 bp and 100 to 1500 bp, respectively (Fig. 2A-B). The number of monomorphic DNA fragments was 5 in the case of primer OPC-11. Most of the primers showed identical DNA proûles as compared with original mother plant.

DISCUSSION

The present study showed that it was possible to explore the morphogenetic potential of *Aloe barbadensis* by application of growth regulators and light condition. Cytokinin helps for induction and multiplication of shoots derived from apical and axillary meristems. The regulatory action of cytokinin and apical dominance helped the in vitro shoot induction and multiplication was well documented [12]. The maximum shoot induction and multiplication was observed both in apical and axillary meristems cultured on MS medium supplemented with 2.0 mg/ l BAP and 0.5 mg/l NAA within 4 weeks of culture under a continuous light. At higher concentrations of BAP, the rate of shoot proliferation declined. The

axillary meristems produced more number of shoots than the apical meristems. Similar results were reported in *Psoralea corylifolia* [13], *Picrorhiza kurroa* [14] and *Plumbago zeylanica* [15]. Mao et al. [16] reported that BAP proved superior to other cytokinins for multiple shoot induction of *Clerodendrum colebrookianum*. Our results demonstrated that the inclusion of NAA in the culture medium help higher rate of shoot multiplication. The results are consistent with earlier reports indicating cytokinins and auxins affect shoot multiplication in other plants using shoot tip or axillary bud explants [15,17,18]. The results showed that the number of shoots per culture was increased in continuous light than 16h photoperiod. The interaction of photoperiod and plant growth regulators have significant effect on shoot morphogenesis as reported earlier [19]. The results also imply that there were differences among the treatments for both the percentage of culture developing multiple shoots and the mean numbers of shoots per culture. The variation of response resulted due to the varying concentrations of growth regulators used in the medium and light condition as reported earlier in *Lavandula latifolia* [20] and *Zingiber officinale* [21]. The rate of multiplication was high and stable up to the 8th subculture and declined in subsequent subcultures. This might be due to the balancing of the endogenous and exogenous growth regulators and the ionic concentration of nutrient salts as reported earlier in other plants [21]. The elongated shoots were rooted the maximum in ½ strength MS basal salts supplemented with 0.5 mg/l NAA with 2% sucrose. The rooting ability was reduced with the increase in the concentration of NAA in the medium. The percentage of shoots forming roots and days to rooting significantly varied with different concentrations of NAA or IBA. Similar observations were made in *Plumbago rosea* [22] and *Psoralea corylifolia* [13]. The rooted plantlets were established in the field and grew normally.

There are many factors like length of culture periods, genotype and nature of explant, which could impudence the stability of the tissue cultured plants. So assessment of genetic stability of in vitro regenerated plantlets is highly signiûcant for further studies. Genetic integrity by molecular analysis has been reported earlier in many medicinal plant species [8,23]. The banding pattern of PCR-ampliûed product from micropropagated plantlets was found to be monomorphic for most of the primer tested. The number of monomorphic DNA fragments was 5 in the case of primer OPC-11. Most of the primers showed identical DNA proûles as compared with original mother plant. Harirah and Khalid [24] used 18 arbitrary decamer primers to study the clonal ûdelity of *Musa acuminata* Cv. Berangan. They found that all the micropropagated plants were monomorphic. No variation was detected among theregenerated plants of *Plumbago rosea* and *Plumbago zeylanica* by using RAPD markers [25].

In conclusion, an attempt was made to develop an *in vitro* protocol for mass multiplication of *Aloe barbadensis* by manipulating the nutrient salts, growth regulators and culture conditions. The pattern of morphogenesis on various phytohormonal regimes largely confirm to those reported in other plant species [14, 26]. This investigation may be useful for conservation of economic plant species.

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