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In vitro organogenesis in *Aloe barbadensis* Mill.: An aloin A rich plant

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ABSTRACT

Efficient plant regeneration in *Aloe barbadensis* Mill. was achieved using callus derived from shoot meristem. Organogenesis was maximum on MS medium supplemented with 2.5 mg/l BA, 0.25 mg/l IAA. Though sucrose (2%) showed maximum shoot bud regeneration capacity, D-glucose had an intermediatory effect, however, fructose and maltose had no effect at all. Of the conditions tested shoot bud regeneration was highest (82.8) under 12 h illumination. The regenerative potential was stable up to 12 sub-cultures. The rate of shoot bud regeneration was dependent on the concentration of hormones in the nutrient media. Regenerated shoots rooted on half-strength MS medium containing 0.1 mg/l IAA and 1% sucrose. The *in vitro* derived plantlets were hardened in the nethouse with 75% light and successfully established in soil.

Key words: Organogenesis, photoperiod, tissue culture, aloe, sugars.

INTRODUCTION

The genus *Aloe* belonging to family Liliaceae comprised of about 30 species of herbs is widely found in tropical and sub-tropical regions of the world. *Aloe barbadensis* has been widely used in cosmetics, skin lotions or medicine for thousand of years in different parts of the world. It is a rich source of Aloin that is used as catharatic agent in many countries and also products containing aloin have been in market in major western countries (Joshi, 6). The leaf gel or polysaccharides have known to possess important biological properties such as anti-inflammatory, antibacterial, antitumour, antiallergic, infected wound healing by immunoenhancement and general tonic effect. The demand for aloe product either as fresh gel or as formulations is increasing day by day; this calls for an urgent need to mass propagate the species as its natural propagation is rather slow for commercial purposes (Groenewald *et al*., 4).

Clonal multiplication of *A. barbadensis* through multiple shoot induction has been reported by several workers (Aggarwal and Barna, 1; Marfori and Malasa, 7; Samantaray and Maiti, 12). A possible alternative to the conventional approach in crop improvement programmes is to utilize emerging biotechnologies such as somatic hybridization in and/or recombinant DNA technology. However, application of either of technique is dependent on the availability of a regenerative i.e. embryogenic or organogenic cell culture procedure (Gray, 3; Gui *et al*., 5; Roy and Sarkar, 10).

Regeneration of shoot buds from callus derived from different explants require a balance of minerals, growth regulators, carbohydrates and photoperiod. Since no systematic work has been carried out on these factors affecting organogenesis in this species, the present communication deals with the media composition and culture conditions necessary for plant regeneration *via* organogenesis in *A. barbadensis*.

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MATERIALS AND METHODS

Small suckers (9-10 cm) of *Aloe barbadensis* Mill., high in aloin-A content (data not shown) were collected from the field gene bank of Directorate of Medicinal and Aromatic Plants, Gujarat, India. The suckers with apical meristem, after removing the mature leaves, were washed with 2% (v/v) aqueous solution of 'Teepol' (Qualigens, India) for 20 min., followed by rinsing with running tap water and surface sterilized in 0.1% (w/v) mercuric chloride solution and then rinsed several times in sterile distilled water. Thereafter, the shoot tips (1-2 cm) with 1 or 2 leaf primordia were excised as explants.

The shoot meristems were placed on Murashige and Skoog (MS) basal salts (Murashige and Skoog, 8) supplemented with the cytokinin [6-benzyladenine (BA)] and auxins Indole-3-acetic acid (IAA), α-napthalene acetic acid (NAA), indole-3-butyric acid (IBA), and 2,4-dichloroacetic acid (2,4-D)]. The mineral salts and growth regulators containing media were properly gelled with 0.6% (w/v) agar-agar (Qualigens, India). The pH of the media were adjusted to 5.8 using 0.1N NaOH or KOH or 0.1N HCl before autoclaving at 121°C and 104 kpa for 15 min.

Callus induction medium (25 ml) and shoot bud regeneration media (80 ml) were prepared in culture tubes (25 mm × 150 mm) and 250 ml graduated flasks (Borosil, India) respectively plugged with non-absorbent cotton. The thermo-labile auxin (IAA) and carbohydrate (glucose and fructose) were sterilized with 0.45 µm micro-filter (Nalgene, USA) and added to autoclaved medium.

All cultures for callus induction and shoot regeneration were incubated under a light intensity 61 $\mu Em^{-2}s^{-1}$ from cool, white fluorescent lamps (Philips, India) at 25 ± 2^{0}C. Incubation of callus was done under a 16/8 h light and dark photoperiod. For organogenesis, 500 ± 20 mg of fresh callus were incubated on the media containing different levels and combinations of cytokinins (Kn, BA, adenine sulphate) and auxin (IAA) under 16 and 24 h photoperiods. The different regeneration media employed were M1 – ½MS + 0.5 mg/l BA + 0.25 mg/l IAA; M2 - ½MS + 1.0 mg/l BA + 0.5 mg/l IAA; M3- ½MS + 1.5 mg/l BA + 0.25 mg/l IAA; M4- ½MS + 2.5 mg/l BA + 0.25 mg/l IAA and M5- ½MS + 3.0 mg/l BA + 0.25 mg/l IAA.

In a second experiment, 4-week-old callus were sub-cultured on organogenesis medium (½ MS + 2.5 mg/l BA , 0.25 mg/l IAA) with different types of carbohydrate (3% w/v). In a third and fourth experiments, different carbohydrates and sucrose concentrations were added to the same organogenesis medium for shoot bud regeneration. In a fifth experiment, the effect of different sub-cultures at 4-week interval on shoot bud regeneration was studied keeping media and incubation conditions constant. Sub-culture for all the experiments was done at interval of 4 weeks. A propagation profile was prepared for a total of 24 sub-cultures.

Twenty cultures were used per treatment and each experiment was repeated at least three times. All cultures were examined periodically and visual observations of any morphological changes were recorded. The data on percentage of culture showing response, number of shoots/ culture, percentage of rooting and number of roots /shoot were statically analysed for arithmetic mean and standard error (SE). Regenerated shoots (~ 1.0-2.0 cm) were excised from the parent cultures and cultured on semi-solid half-strength MS basal medium supplemented with different concentrations of IAA, IBA and NAA along with 1% (w/v) sucrose for root induction. Shoots which formed roots were removed from the culture tubes, washed gently under running tap water and planted in (6" × 6" inch) earthen pots containing a mixture of sand and soil (1:1; v/v). The plantlets were kept in a nethouse with 75% intermittent light for acclimatization before field transfer.

RESULTS AND DISCUSSION

Calli were initiated on the half-strength MS basal medium supplemented with different concentrations of BA or Kn (0.5-1.0 mg/l) in combination with 2,4-D (2.0-4.0 mg/l) within 15 to 20 days of inoculation of explant derived from the shoot meristems. The initial calli developed in the base of the explant were pale green in colour. Callus growth was maximum in half-strength MS media containing 0.5 mg/l BA, 2.5 mg/l 2,4-D, 20 mg/l ascorbic acid and 3% (w/v) sucrose (Fig. 1a).

Among the regeneration media tested, the half-strength MS basal medium containing BA (2.5 mg/l) and IAA (0.1 mg/l) produced highly organized structures which subsequently developed high frequency of shoot buds (96.2%) (Table 1; Fig. 1B). Our studies were consistent with earlier reports indicating high cytokinins in combination with low auxins help in shoot bud differentiation in monocot (Rout and Das, 9). Clusters of slender shoots with shoot elongation were relatively high at 0.5 mg/l GA_3 which is known to promote shoot primordia development (Geekiyanga *et al.*, 2) but its effect depends on maximum shoot differentiation and shoot elongation.

Callus initiation and proliferation were better under 16h photoperiod than the continuous photoperiod, whereas the frequency of regeneration was higher under a 12 h photoperiod than under continuous light (24 h) photoperiod (Table 2). The rate of shoot bud regeneration were inhibited in both 12 h photoperiod and continuous light with a high concentration of IAA (>0.5 mg/l). The effect of light on *in vitro* morphogenesis has been reported (Samantaray *et al*., 11). Interactions between light intensity and plant growth regulators on shoot bud regeneration have been well demonstrated in *Trema orientalis* (Samantaray *et al*., 13). Among the carbohydrates tested, sucrose (2%) promoted the highest number of shoot buds per culture (Fig. 1C). Incorporation of D-glucose to the media considerably

Table 1. Effect of different concentrations of BA and IAA on shoot bud regeneration in calluses derived from leaf-base of *A. barbadensis* after 4-week of sub-culture.

Medium	Percent of cultures regenerated	Average number of shoot buds/culture
½ MS + 0.5 mg/l BA + 0.25 mg/l IAA	34.5 ± 0.62	26.4 ± 1.51
½ MS + 1.0 mg/l BA + 0.5 mg/l IAA	71.3 ± 0.36	43.2 ± 0.97
½ MS + 1.5 mg/l BA + 0.25 mg/l IAA	89.4 ± 0.91	52.3 ± 1.26
½ MS + 2.5 mg/l BA + 0.25 mg/l IAA	96.2 ± 0.25	46.3 ± 0.84
½ MS + 3.0 mg/l BA + 0.25 mg/l IAA	46.2 ± 0.91	31.1 ± 0.97

Table 2. Effect of photoperiod on shoot bud regeneration of *A. barbadensis* after 4-week of culture.

Medium	Photoperiod (h)	Regeneration (%)	No. of shoot buds/culture
½ MS + 0.5 mg/l BA + 0.25 mg/l IAA	12/12	32.1 ± 0.228	4.6 ± 0.33
½ MS + 1.0 mg/l BA + 0.5 mg/l IAA	12/12	50.8 ± 0.740	10.4 ± 0.68
½ MS + 1.5 mg/l BA + 0.25 mg/l IAA	12/12	72.6 ± 0.468	18.6 ± 0.414
½ MS + 2.5 mg/l BA + 0.25 mg/l IAA	12/12	82.8 ± 0.575	26.2 ± 0.620
½ MS + 3.0 mg/l BA + 0.25 mg/l IAA	12/12	56.7 ± 0.942	15.2 ± 0.413

lowered down the frequency of shoot bud development, whereas complete inhibition of shoot bud initiation was observed in presence of fructose and maltose (Table 3). Conjugation of sucrose and growth regulators make a compound of sugar alcohols that could be transported quickly to the cellular system and simultaneously help in maintaining the protein stability in the cell (Wareing and Philips, 15). Considerable variation in shoot bud regeneration was noted when the concentration of sucrose varied in the basal medium. The highest average number of shoot buds per culture was obtained at 2 % sucrose (Table 4). While 4 % showed stunted growth; 7% level enhanced the formation of poly phenols and inhibited the shoot growth.

There was a gradual increase in the shoot production rate till 12th sub-culture. The rate of shoot production (37.0) was stable at 12th sub-culture without loss of regenerative ability (Table 5; Fig. 1D) showing 37.0 shoots per culture. Sauer *et al.* (14) reported that the

Table 3. Effect of different concentrations of sucrose on shoot bud regeneration of *A. barbadensis.*

MS (1/2)+ BA (2.5 mg/l) + IAA (0.25 mg/ l)	Percent of cultures regenerated	Average No. of shoot buds/culture
1%	34.5 ± 0.62	8.4 ± 1.51
2%	89.4 ± 0.91	23.2 ± 0.97
3%	81.3 ± 0.36	14.3 ± 0.84
4%	26.2 ± 0.25	9.3 ± 0.84

Table 4. Effect of different types of carbohydrates supplemented with ½ MS + 2.5 mg/ l BA + 0.25 mg /l IAA on shoot bud regeneration of *A. barbadensis.*

Sugar (2%)	Percent of cultures regenerated	Average number of shoot buds/culture
Sucrose	88.5 ± 0.71	23.2 ± 0.58
Glucose	70.3 ± 0.58	17.3 ± 0.26
Fructose	32.8 ± 0.32	5.4 ± 0.12
Maltose	28.2 ± 0.27	3.2 ± 0.09

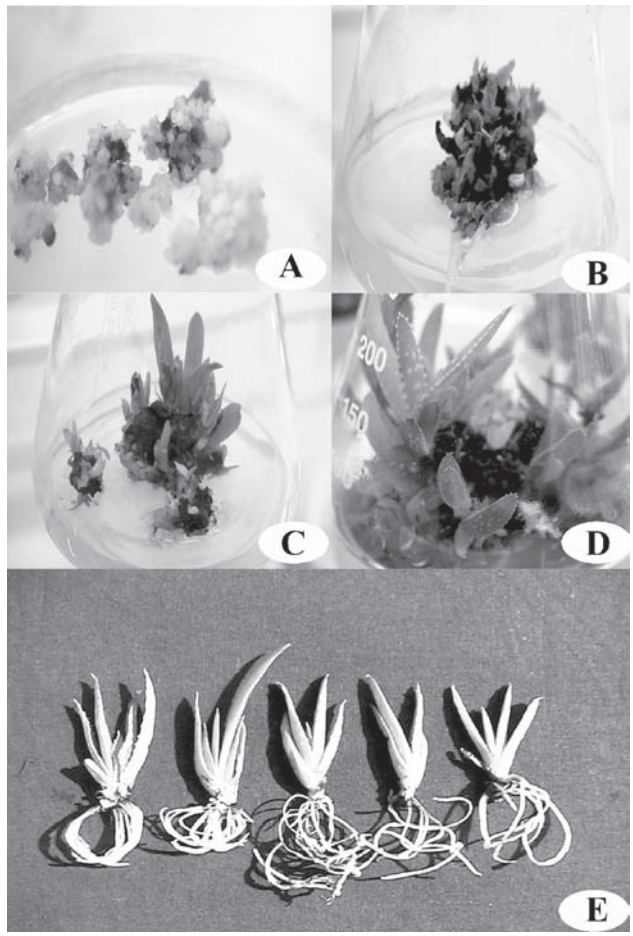

Fig. 1. A. Callus growth in half-strength MS + 0.5 mg/l BA + 2.5 mg/l 2,4-D + 20 mg/l ascorbic acid after 6-week of culture; B. Shoot bud differentiation from callus grown on half-strength MS + 2.5 mg /l BA + 0.25 mg/l IAA; C. Effect of 2% sucrose on shoot bud regeneration cultured on half-strength MS + 2.5 mg /l BA + 0.25 mg/l IAA; D. Shoot bud regeneration at 12th subculture; E. Root induction cultured on half-strength MS + 0.1 mg/l IAA + 1% sucrose after 7-8 days of culture.

Table 5. Effect of subculture in the shoot bud regeneration media ½ MS + 2.5 mg/ l BA + 0.25 mg/l IAA + 2% sucrose under 12-h of *A. barbadensis*.

Age of culture (week)	Number of shoot buds/culture*
2nd	16.07 ± 0.8
4th	20.2 ± 0.97
6th	25.3 ± 1.26
8th	30.3 ± 0.84
10th	33.1 ± 0.97
12th	37.0 ± 0.85

*Mean ± S.E. of 20 cultures per treatment in three repeated experiments

shoot multiplication rates over the successive sub-cultures were related to a time dependent adaptation of the explants to the *in vitro* conditions. In case of shoot elongation the elongation rates in the primary cultures and the first 8 sub-cultures were same (approx. 4-5 cm) and then the growth in height was limited up to 2-3 cm after 8th sub-culture.

Microshoots were rooted on half-strength MS medium supplemented either IAA or IBA with 1% (w/v) sucrose within 10 days of culture. A high percentage (99%) of shoots rooted in half-strength MS medium fortified with 0.1 mg/l IAA (Table 6). Roots emerged in 7- 8 days and well developed in to a good root system within 10 days of culture without an intervening callus (Fig. 1E). Thereafter, the plantlets were transferred to pots containing sterilized soil: sand (1:1; v/v), which were acclimatized in a nethouse having 75% light (Fig. 2A & B) and 98 percent plants survived after 3 weeks

Table 6. Effect of different concentrations of auxins (IAA, IBA and NAA) on rooting from excised shoots of *A. barbadensis* cultured on MS basal salts supplemented with 1% (w/v) sucrose.

Auxin	Conc. (mg/l)	Rooted shoots (%)	Time to rooting (days)
IAA	0.05	0	0
	0.10	98.6 ± 0.6	7-8
	0.25	72.8 ± 0.7	8-9
	0.50	50.6 ± 0.3	10
IBA	0.05	0	0
	0.10	42.6 ± 0.6	10
	0.25	30.8 ± 0.4	12
	0.50	24.6 ± 0.2	14
NAA	0.05	0	0
	0.10	32.8 ± 0.4	14
	0.25	24.6 ± 0.3	14
	0.50	18.5 ± 0.6	16

Mean ± SE of 15 cultures per treatment in three repeated experiments.

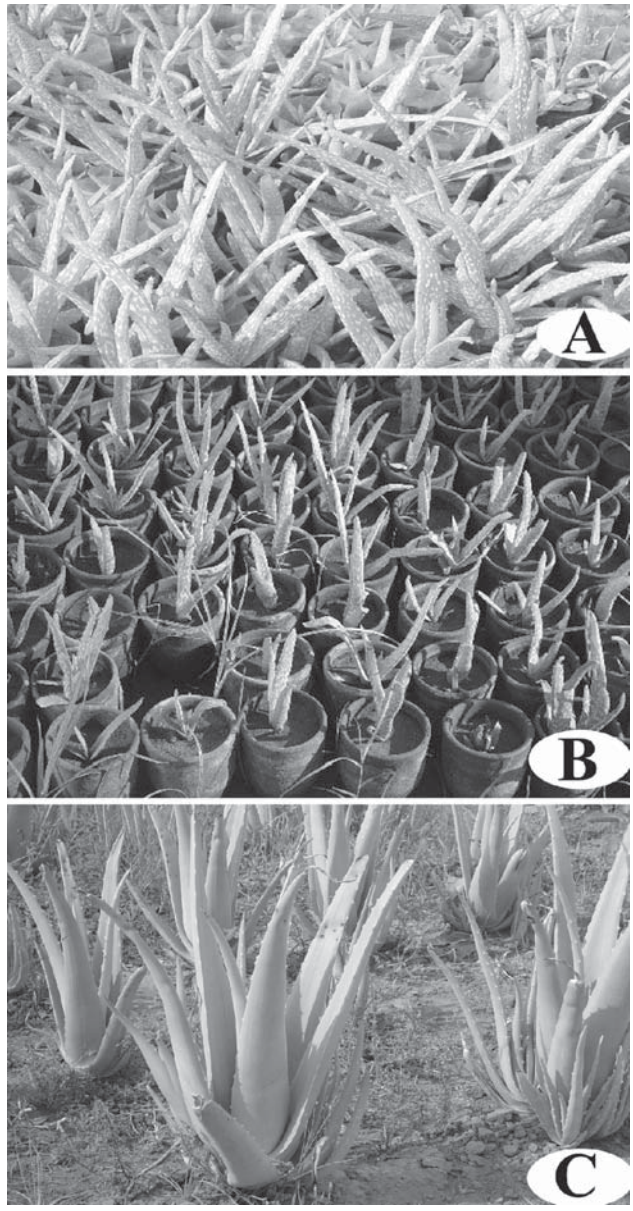

Fig. 2. A & B. Plantlets acclimatized in the greenhouse; C. Plants established in the field.

of transfer to the field. The plants grew luxuriantly and no morphological variation was noticed (Fig. 2C).

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