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In vitro morphogenesis of a medicinal plant–*Aloe Vera L.*

Mukesh Kumar*, Sumer Singh and Satyapal Singh

Department of Biotechnology, Singhania University, Rajasthan, India

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ABSTRACT

Aloe vera syn barbadensis Mill is an important medicinal plant and used world wide in drug and cosmetic industry. Although Aloe propagates vegetative in its natural state, but propagation rate is too slow to meet demand of high quality planting material for commercial cultivation. Micropropagation method for elite selection of Aloe vera by axillary branching method using shoot tip as explants was standardized. Shoot cultures were initiated on MS medium containing BA 0.2mg/L with IBA 0.2mg/L. Maximum shoot proliferation was achieved on medium containing BA 1.0mg/L with IBA 0.2 mg/L within 28 days of culture. Shoot proliferation was better in liquid medium with same composition. Citric acid also enhanced shoot proliferation. A maximum of 5-multiplication rate of shoots was achieved with citric acid (10mg/L) in the medium. Hundred percent rooting of microshoots was obtained on phytohormone– free MS medium. Regenerated plants after hardening were transferred to soil and they showed 85% survival. The regenerated plants were morphologically similar to control plants.

Key words: BA, IBA, Citric Acid, auxiliary buds and Aloe vera syn barbadensis Mill.

INTRODUCTION

Aloe vera syn barbadensis Mill. belongs to the family Liliaceae (Anonymous 1976). It is commonly called as 'Burn plant'. It is a xerophytes and can be grown even in dry lands under rain fed conditions. Aloe is a coarse looking perennial plant with a short stem, found in the semi-wild state in many parts of the country . Leaves 30-60 cm long, erect, crowded in a basal rosette, full of juice, glaucous-green, narrow –lanceolate, long-acuminate, smooth except for the spiny teeth on the margins. Scape longer than leaves, scaly, branched. Flowers yellow, in dense racemes terminating the scapes. Commercial Aloes are obtained from wild as well as cultivated plants. Propagation is primarily by means of suckers (or) offshoots, which are separated carefully from mature plants and transplanted. Medium sized suckers are chosen and carefully dugout without

damaging the parent plant at the base and can be directly planted in the field. Plants will produce a commercial crop in one year (Venkataramaiah 2003). Leaves exude a bitter liquid, which is dried and known as "bitter Aloes." They also contain a clear gel, which is a soothing skin remedy. Leaves are broken off and the clear gel is applied to the skin as a first aid for burns. *Aloe* contains cathartic anthra-glycosides and its active principle ranging from 4.5 to 25% of Aloin. These are extensively used as active ingredients in laxative, anti-obesity preparation, as a moisturizer, emollient, wound healer, in various cosmetic and pharmaceutical formulations. It is a drug as well as a cosmetic. There are about more than 40 Aloe-based formulations being marketed in the global market. Aloin portion, which forms 'Musambar', is considered as drug and other two portions i.e., chips, gel is used in cosmetic industry. The clear gel contained in the leaf is a remarkably effective healer of wounds and burns, speeding up the rate of healing and reducing the risk of infection. The yellow sap from the base of the leaf when dried is known as "bitter Aloes" (Musambar). It is a strong laxative, useful for short-term constipation.

MATERIALS AND METHODS

Explants were collected from herbal garden of Singhania university, Rajasthan. The explants Were washed thoroughly under running tap water for 30 minute then treated with a few drop of liquid detergent (Rankleen, Ranbaxy India) and Tween 20 (Himedia Laboratories, India) for 10 minutes with vigorous shaking. After washing with detergent explants were again washed with running tap water to remove any traces of detergent for 30 minutes and kept in 1% w/v solution of Bavistin (BASF India Limited) for one hour. After that explants was shifted to the 1% v/v solution of savlon (Johnson and Johnson, USA) for 1-2 minutes. After these treatments explants were taken inside the laminar hood for further sterilization. Here 2-3 sterile water washings are given. After these washings, explants were taken out and dipped in 70% ethyl alcohol for 30 seconds. After alcohol dip, explants were surface sterilized with freshly prepared 0.1% w/v aqueous solution of $Hgcl_2$ for 5 minutes. After $Hgcl_2$ treatment, explants were thoroughly washed for 3-4 times with sterile water to remove any traces of $Hgcl_2$. The explants were inoculated on MS basal nutrient medium which contained 3% Sucrose(Analytical grade, Himedia, India) and 0.8% agar (Bacteriological grade, Himedia, India).The medium is always supplement with Various Phytohormons either singly or in combination. Different hormones likes BA, IBA, kinetin, Adenine sulphate were used at different cultural times. After an initial 5.6-5.8 pH adjustment sterilization is made at 121^0C for 20 minutes at 15psi. Inoculated cultures were kept at 25 ± 2^0C under 16 hrs. Light daily (2000-2500 lux).

Shoot induction:
Multiple shoot cultures were established from the aseptic, *in vitro*, plant plant leave segments and maintained in MS medium supplemented with the BA (0-1mg/L) and Kn (0-1mg/L) at different concentrations in combination with IBA (0.2 mg/L), Citric acid (0,10,100 mg/L), adenine sulphate (160 mg/L) and agar (0,0.8%) used.Each treatment of hormonal combinations consisted of 4-6 replicates. The cultures were evaluated in terms of numbers and height of shoots.

Shoot elongation and root induction:
Shoots, generally 3-4 Cm long, obtained from, shooting media, were excised individually from the parent explant and transferred to rooting media. Three types of rooting medias were used one

MS basal media without hormone and other MS basal media with hormone (IBA 1mg/L).The number of roots per shoots and their length were recorded after 15 days.

Acclimatization:

To achieve acclimation, Planlets were removed from the rooting medium ,washed througly with hot water to remove agar and then dipped in 1% w/v solution of Bavistine to prevent any fungal infection to newly developed plant. After Bavistine treatment the plantlets were carefully planted in plastic pots containing 1:1 mixture of soil and farmyard manure. After planting the plants were thoroughly watered and kept under polyhouse having 80% humidity and 31^0C temperature for ten days. In-between the ten days plants were thoroughly watered with the help of sprinkler to maintain required level of humidity. Then the plants were shifted to shade house with less humidity level and indirect sunlight. In shade house also plants were watered two times a day i.e. morning and evening to prevent wilting then plants were transferred in to the field.

RESULTS

Shoot proliferation: Explants starts to show signs of proliferation after two weeks of culturing. New buds starts to appear from the axil of leaves of shoot explants and buds develop into shoots by 4 weeks of culture. Microshoots were inoculated on MS basal medium with different concentrations and combinations of BA and Kn (in combination of IBA 0.2 mg/L) for shoot proliferation. Both BA and Kn were found to be give the indications of shoot proliferation after 2 weeks of incubation. It was found that BA gave better shoot proliferation than Kn (Table- 1).

Table-1. Effect of different combinations of cytokinins* on shoot proliferation in *Aloe vera* after four weeks of culture

Phytohormones (mg/L)	% Of explants showing shoot formation (Mean±SD) n=5	Number of shoots per explant (Mean±SD) n=10
Hormone- Free (Control)	Nil	1
BA 0.2	100 0	3.0 – 0.8
1.0	100 – 0	3.3 – 0.9
Kn 0.2	40 – 41	1.4 – 0.5
1.0	90 – 22	3.1 – 1.2

**In combination with 0.2 mg/L IBA*

In medium containing BA in different concentration, on an average each explant gave rise to 3.0-3.3 shoots (Table 1, Figure-1, 2). Hundred percent cultures showed shoot proliferation on BA containing medium. On medium containing Kn 1mg/L, only 90% cultures showed shoot proliferation. In medium containing higher concentration of Kn (1.0 mg/L) the average number of shoots per plant were 3.1±1.2. While on the other hand in medium containing less concentration of Kn (0.2mg/L) the average number of shoots per plant was 1.4±0.5. The explants which were cultured on medium without any phytohormone, failed to produce any new shoots. Adenine sulphate was also used to check whether it has any effect on shoot proliferation or not. It was observed that adenine sulphate has no significant effect on shoot proliferation in *Aloe vera*.

FIG-1, Microshoot inoculated on MS medium with IBA0.2mg/L+BA 1.0mg/L

FIG-2, Shoot Proliferation after 4 weeks of culture on medium containing BA 1.0mg/L + IBA 0.2mg/L

Table-2. Effect of Adenine sulphate on shoot proliferation in *Aloe vera* after Four weeks of culture.

Adenine sulphate (mg/L)	% of explants Showing shoot formation (Mean±SD) n=4	Number of shoots per explant (Mean±SD) n=8
0 (Control)	100 ±0	3.1 ± 0.8
160	75 ± 29	3.1 ± 1.8

In combination with BA 0.2mg/L andIBA0.2mg/L

In both the cases i.e. with and without adenine sulphate the average number of shoots per plant was 3.1 (Table-2). The percentage of explant showing shoot proliferation was also lower than control i.e. 75±29.It was observed that citric acid aided in the increased shoot proliferation. The average number of shoots in medium with 10 mg/L citric acid was 5.0±1.9 (Table 3). All explants showed shoot formation response in all the experiments while on the medium lacking citric acid, the average number of shoots was 3.3±0.9. Higher concentration of citric acid (100mg/L) was found to be less promotive.

Table-3. Effect of citric acid* on shoot proliferation in *Aloe vera* after 4 Weeks of culture.

Citric acid (mg/L)	% of explants showing shoot formation (Mean±SD) n=5	No. Of shoots per explants (Mean±SD) n=10
0	00 ±0	3.3 ±0.9
10	100 ± 0	5.0 ± 1.9
100	100 ± 0	4.2 ± 2.3

**In combination with BA 1.0mg/L and IBA0.2mg/L*

To check whether there is any difference between solid and liquid medium on shoot proliferation in *Aloe vera*, both solid and liquid medium were tested. It was observed that in liquid medium shoot proliferation was better. The average number of shoots on liquid medium were 4.80±2.5

while on solid medium average number of shoots were 4.08±2.0 (Table 4). Growth of cultures was fast in case of liquid medium than the solid medium.

Table-4, Effect of liquid and solid medium on shoot proliferation in *Aloe vera* after 4 weeks of culture.

Media*	%Percentage of explants showing shoot proliferation (Mean ± SD) n=4	No. Of shoots per explants (Mean ± SD) n=8
Solid	91±17	4.08±2.0
Liquid	91±17	4.80±2.5

Rooting of Microshoots

Three to four centimetres long shoots were excised individually from the proliferated shoot clumps and cultured on rooting medium. The shoots inoculated on hormone – free (medium lacking IBA) and IBA supplemented medium showed rooting response within a week of inoculation. However, the response was better in hormone- free medium. After the 15 days of inoculation, rooting was 100% in hormone- free medium (Table-5, Figure-3, 4). The number of roots per shoot was 2.8+0.5 on hormone - free medium.

FIG-3, Microshoots showing rooting after 15 days of culture

FIG-4. A rooted microshoot

Table-5. Effect of IBA on root induction in Aloe vera after 15 days of culture.

IBA (mg/L)	Percentage of microshoots showing root formation (Mean± SD) n=5	No. Of roots premicroshoot (Mean ± SD) n=10
0	100 ±0	2.8 ± 0.5
1	90 ±22	1.7 ± 1.1

In case of hormone- free medium, roots were more thick and elongated, while the roots on hormone supplemented medium were thin and less elongated. There was no difference in colour of roots. In both the cases colour of roots was ceramist yellow. In both the cases roots were without any branches and normal in appearance. In hormone- free medium average number of

roots per plant was 2.8 –0.5 and on hormone supplemented medium average number of roots per plant was1.7–1.1. To check the effect of solid and liquid medium on root induction so that rooting response can be improved or/and cost of plants produced could be reduced, Microshoots were inoculated on both the media. The microshoots inoculated on solid medium showed better rooting response.

Table-6. Effect of solid and liquid medium on root induction in *Aloe vera* after 15 days of culture.

Media*	Liquid Percentage of microshoots showing root formation (Mean±SD)	No. of roots per microshoot (Mean ± SD) n=10
Solid	100±0	2.7±1.2
Liquid	18±20	0.2±0.5

** MS hormone- free medium.*

Hundred percent shoot showed rooting and the mean number of roots per shoots was 2.7±1.2 (Table 6) .On the other hand in liquid medium, only 18%–20 microshoots gave rooting. The shoots inoculated on liquid medium were failed to give any further rooting response even after 3-4 weeks of inoculation (data not shown).

Hardening of plantlets:

After 15 days of culture of microshoots on rooting medium, which resulted in the sufficient rooting of shoots, the plantlets were transplanted to plastic pots containing garden soil and Farmyard manure (1:1) for their hardening. For first ten- days the plantlets were kept in polyhouse. To maintain the appropriate humidity level (80%), plants were thoroughly watered with the help of manual sprinkler every 2 hours the temperature of playhouse was maintained at 31^0 C with humidity level of nearly 80%.

Table-7. Survival rate of plantlets of Aloe vera at different stages of Hardening.

Stage of transplantation	Number of plants transplanted	Percentage of survival
Poly House(1^{st}stage)	13	85
Shade House(2^{nd}stage)	11	82

**Plantlets transferred to shade house after 10 days growth on poly house.*

Plantlets that were transferred to the plastic pots in polyhouse showed good percentage of survival of 85% (Table 7, Figure 5). After keeping plantlets for initial ten days in playhouse, the plantlets were transferred to shade house under less humidity and temperature controlled conditions and indirect sunlight. In shade house, these plants showed percentage of 82% survival (Table 7). In shade house plants started to elongate and leaves also start to thicken. In shade house plants were watered two times a day i.e. morning and evening. Among the survived plants, some plants showed the symptoms of leap tip necrosis during shade house conditions. But this does not hamper the overall growth of the plants. Plants with these symptoms were also growing well.

FIG-5, Fully Hardened Plants growing in Open Shade.

DISCUSSION

For shoot proliferation, growth regulators especially cytokinins (Lane 1979, Stolz 1979, Bhojwani 1980, Garland & Stolz 1981) are one of the most important factors affecting the response. A range of cytokinins (Kinetin, BA, 2-ip and zeatin) has been used in micropropagation work (Bhojwani and Razdan 1992). Murashige (1974) and Hussy (1978) described 2-ip as more effective than either BA or kinetin. A number such as blueberry (Cohen 1980) and garlic (Bhojwani 1980) were successfully multiplied by using 2-ip. But a wider survey of the existing literature suggests that BA is the most reliable and useful cytokinin. A number of plants has been were successfully multiplied on medium containing BA. In white clover (Bhojwani 1981) and hybrid willow (Bhojwani 1980), chickpea (Barna & Wakhlu 1994). Nair et al (1979), and Iresine lendenii (Sebastin & Barna 2003) BA is the most effective cytokinin for the shoot tip, meristem and bud culture. At higher levels cytokinins tends to induce adventitious bud formation (McComb, 1978; Zimmerman and Broome, 1980) .In the present study also, shoot proliferation occurred only in the presence of cytokinin. Among the cytokinins tested, BA proved to me more effective. This is in contrast to earlier reports in *Aloe vera* by Meyer and Staden (1991) and Natali et al (1990) in *Aloe vera*. These researchers reported that better proliferation occurred on medium containing Kn instead of BA in *Aloe vera.* This difference may be due to difference in the genotype of plant used. Abrie and Staden (2001) Chaudhuri and Mukandhan (200) also reported use of BA in shoot proliferation of *Aloe polyphylla* and *Aloe vera* respectively.

Adenine sulphate was also used to check its effect on shoot proliferation. In our case, adenine sulphate did not improve shoot proliferation in *Aloe vera.* But earlier adenine sulphate was used for shoot proliferation in *Aloe vera* by Chaudhuri & Mukandan (2001).

Citric acid also helped in the enhanced shoot proliferation in *Aloe vera* in the present study. Keeping in mind the cost factor of agar, liquid medium containing was also used for the shoot proliferation in *Aloe vera.* In the present study liquid medium was found to be better for shoot proliferation in *Aloe vera.* Use of liquid medium considerably reduces the cost of producing plants for the commercial purposes. Rooting response of microshoots is reported to be controlled by growth regulators in the medium (Bhojwani & Razdan 1992), basal salt composition (Garland and Stoltz.1981, Zimmerman and Broome.1981, Skirvin & Chu 1979), genotype (Rines & McCoy 1981) as well as cultural conditions (Murashige 1977). For most of the species auxin is required to induce rooting. NAA and IBA are most commonly used for root induction (Bhojwani and Razdan 1992). By the use of IBA many plants such as *Lycoperscicon esculemtum* (Sibi

1982), *Hedychium roxburgii* (Tripathi & Bitaillion 1985), *carnation* (Werker & Leshem 1987) gave *in-vitro* rooting. For the purpose induction of roots hormone-Free and IBA supplemented medium were used in the present study. But rooting was observed better in hormone – free medium. These kinds of observations were also earlier by Sanchez et.al (1988), Meyer & Staden (1991), and Richwine et.al. (1995) in *Aloe vera*. Richwine et.al. (1995) also reported induction of roots in hormone-medium for some other plants like *Gasteria* and *Haworthia*. Many other plants such as *straw berry* (Boxus 1974), *Narcissus* (Seabrook et.al. 1976), *Gladiolus* (Hussy 1979)) and *Rose* (Barna & Wakhlu 1995) was rooted successfully rooted on hormone-free medium. Decrease in number of roots in IBA supplemented medium may be due to super optimal concentration of IBA in the medium. By keeping in mind the cost factor, liquid medium was tried for the induction of roots. But rooting was very poor in liquid medium in present study. Hardening of tissue culture plants is the most crucial step in micropropagation. The plants produced are very soft to face ambient environmental conditions.

(Bhojwani & Razdan 1992). These plants are grown under controlled conditions. Under these conditions the leaves of plants develop cuticle and its photosynthetic system starts functioning. The most crucial stage is during first 10 days in polyhouse. During the 2ndhardening stage, mortality is lower as the plants are comparatively hardened during first hardening stage or during the first 10 days in polyhouse. In the present study, rooted plantlets were transferred from culture bottles to plastic cups in mixture of 1:1 ratio of soil: FYM for their hardening prior to their final transfer to the soil, showed good percentage of survival (85%) in both polyhouse and shade house. In shade house also plants showed 82% survival rate. The growth and elongation of the plants were less in poly house whereas in shade house growth of the plants was better and they also start to elongate in shade house. The leaves also start to thicken in shade house.

CONCLUSION

Aloe vera syn barbadensis Mill. is a *xerophytic* medicinal plant of considerable importance. It is widely used in cosmetic and drug industry and its demand is increasing day by day. Due to widespread male sterility it propagates only through vegetative mode of reproduction. But its propagation rate is very slow to meet commercial demand of high quality planting material for its commercial cultivation. So keeping this thing in mind, micropropagation work is carried out on this plant. The objectives of the present study was to standardize optimum conditions for establishment of axenic culture from elite germplasm, shoot proliferation, rooting of micro shoots, hardening and transfer of plants to soil.

The conclusions Drawn from this study are,

1. Surface sterilization with $HgCl_2$ (0.1% for 5-minutes) with 70% alcohol dip was best for the surface sterilization of the explants.
2. For the initiation of the culture,MS medium with BA 0.2 mg/L with IBA 0.2 mg//L was used.
3. Best shoot proliferation was achieved on MS medium containing BA1.0mg/L with IBA 0.2mg/L.
4. Liquid medium with same composition was found to be better than solid medium for shoot proliferation.
5. Adenine sulphate did not promote shoot proliferation in the present study.

6. Addition of 10mg/Lcitric acid in the medium aided in the enhanced shoot proliferation. Citric acid in higher concentration (100mg/L) was found to be less effective.
7. Hundred percent shoot showed rooting response on hormone -free medium.
8. In liquid medium rooting response was found to very poor.
9. Regenerated plantlets, 85% survival during polyhouse conditions and 82% during shade house stage of hardening.
10. Regenerated plants were found to be morphologically similar to the mother/control Plant.

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