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IN-VITRO MICROPROPAGATION OF THE MIRACLE PLANT *ALOE VERA* – A METHOD OF RAPID PRODUCTION

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Abstract: - *Aloe vera s*yn *barbadensis* Mill. is an important medicinal plant and used world wide in drug and cosmetic industry. Although *Aloe* propagates vegetatively 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 explant 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.

Keywords: *Aloe vera*, Micropropagation, Protocol, In-vitro, Shoot Proliferation, Culture media, Growth Hormones.

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INTRODUCTION

Aloe barbadensis Mill. belongs to the family Liliaceae. It is a xerophyte 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 (Figure 1). It is indigenous to the Eastern and Southern Africa, the Canary Island and Spain. Its species are spread to the Mediterranean basin and reached the West Indies, India, China, Pakistan in the 16th century. The plant prefers sunny weather, requires well-drained soil and can grow in nutritionally poor soil. Aloe vera has been used in traditional medicine and cosmetics since ancient times.

MORPHOLOGY

Aloe vera plant has a fibrous or woody stems. In general, stem is very short and hardly visible as it is covered by dense leaves and partially buried in the soil. But there are also several species of aloe vera in the form of a tree with a height of 3-5 m. Leaves are 30-60 cm long, erect, fleshy thick and reinforced, grayish green in colour and have a waxy coating on the surface. They are Flat on the top and rounded (convex) at the bottom crowded in a basal rosette, full of juice. Flowers are yellow, in dense racemes terminating the scapes. Commercial *Aloe*s 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.

Although *Aloe barbadenesis* propagates vegetatively in its natural state, but propagation is too slow for commercial plant production (Meyer &Staden1991). To overcome slow propagation rate, micro propagation will be a very useful technique for mass multiplication of *Aloe*. Thus, with all this in view and consideration, the current project was undertaken to standardize the necessary cultural conditions of *Aloe vera* by tissue culture techniques.

The presence of the plant growth regulators in media are necessary for shoot and root initiation (Aggarwal and Barna, 2004; Debiasi et al., 2007; Liao et al., 2004). Natali et al. (1990) showed micr- opropagation of *A. vera* L. by culturing shoot apices on medium containing 2,4-D and kinetin. Meyer and Staden (1991) reported axillary shoot formation using only IBA, whereas Roy and Sarkar (1991) and Natali et al. (1990) got shoots on medium containing 2,4-D and kinetin. Richwine et al. (1995) reported the induction of shoots using zeatin. Liao et al. (2004) studied the effects of benzyladenine (BA), α-naphtaleneacetic acid (NAA) and sucrose on bud initiation from explants.

Budhiani (2001) demonstrated that the best combination for initiation of shoot was MS medium supplemented with 0.2 mg/l BAP + 0.002 mg/l NAA. Chaudhuri and Mukundan (2001) reported that best multiplication of shoots was obtained on medium containing 10 mg/l BA + 160 mg/l Adenine Sulphate + 0.1 mg/l IBA.

Sucrose and BA were recognized the most important factors affecting the bud initiation and promoted efficient multiplication. Abrie and Staden (2001) cultured aloe plantlets on medium containing BA alone, or with combination of BA and NAA.

Rooting was performed spontaneously on bud initiation medium and the rooting rate was significantly improved in the presence of other plant growth regulators (Abrie and Staden, 2001; Feng et al., 2000; Hongzhi, 2000). In some studies, rooting was performed on MS media lacking growth regulators (Velcheva et al., 2005; Aggar- wal and Barna, 2004).

EXPERIMENTAL PROCEDURE

MATERIAL AND METHODS

The present research was carried out in Plant Tissue Culture Lab. Department of Biotechnology, Bengal College of Engineering and Technology, Durgapur during the year 2014- 2015.

Plant Material

Elite plants were healthy and free of symptoms of disease, pest problems and showed good biomass yield. Shoot with young leaves was collected from the elite plants. The extra leaves were removed and shoot was trimmed to size of 2-3 cm for further work.

Explant Sterilization

For the surface sterilization, the explants first were washed thoroughly in running tap water for 30 minutes. After that they were again washed with 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 Mercuric

chloride for 5 minutes. After Mercuric chloride treatment, explants were thoroughly washed for 3-4 times with sterile distilled water to remove any traces of Mercuric chloride.

Culture Media

The basal medium used for the culture is Murashige and Skoog medium (MS, 1962) with sucrose 3% (Analytical grade, Himedia, India) and 0.8% agar (Bacteriological grade, Himedia, India).

Inoculation of Explants

After sterilization of explants, explants were inoculated in culture bottles aseptically. For inoculation explants were transferred to large sterile glass petriplate or glass plate with the help of sterile forceps under strict aseptic conditions. Here the explants were further trimmed and extra outer leaves were removed to make them in suitable sizes. Trimming and leaves were removed with sterile scalpel blade. After cutting explants into suitable size (2-3cm), explants were transferred to culture bottles containing MS medium with 0.2mg/L BA and 0.2 mg/L IBA. After vertically inoculating the explants in culture bottle the mouth of bottle is quick flamed and bottles are tightly capped and mouths of the bottles was properly sealed with klin film to avoid entry of external air. After proper labeling clearly mentioning media code, date of inoculation etc. the bottles was transferred to growth room.

Shoot Culture Initiation

After surface sterilization of shoot tip explants, the explants were inoculated in culture bottles aseptically. For inoculation, explants were transferred to large sterile glass petriplate or glass plate with the help of sterile forceps under strict aseptic conditions. Here the explants were further trimmed and extra outer leaves were removed to make them in suitable sizes. Trimming was done with sterile scalpel blade. After cutting explants into suitable size (2-3cm), explants are inoculated to culture bottles containing MS medium with 0.2 mg/L BA and 0.2 mg/L IBA.

Shoot Proliferation

For shoot proliferation, 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 (0.8%) used. After 28 days of culture period of the explants with newly form shoots were taken out under strict aseptic conditions and were excised from the parent plant with help of sterile scalpel blade and sterile forceps and inoculated into new bottles containing solid and liquid MS basal medium with different set of growth hormones as mentioned earlier. Two

shoots per culture bottle were used and 4-6 replicates per treatment were also used. Data were recorded after 28 days of culture and only shoots greater than 2cm were considered for taking data. Every possible care has been taken to prevent any further contamination.

Rooting of Micro Shoots

Newly formed shoots measuring 3-4cm in length were excised individually from the parent explant and transferred to rooting media. Three types of rooting media were used one MS basal media without hormone and other MS basal media with hormone (IBA 1mg/L). Here also we used both liquid as well as solid mediums. Three- five shoots per culture bottle were used and 5 replicates were used per treatment. Data were recorded after 15 days of culture.

Culture Conditions

All cultures were incubated under 16 hr photoperiod with light intensity of 2000- 2500 lux (Provided by Polylux XL, GE Britain, 36W and temperature of 25± 10ºC).

Acclimatization

After 15 days of culture on rooting media, the plantlets were shifted to plastic pots for their hardening prior to final transfer to soil to natural conditions. For hardening of plants, plants with newly formed roots were taken out from the culture bottles with the help of forceps with utmost care to prevent any damage to newly formed roots and dipped in warm water not hot to remove the any traces of solidified agar media. After removing media, plants were dipped in 1% w/v solution of Bavistine to prevent any fungal infection to newly developed plants. 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 poly house having 80% humidity and 31ºC 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.

RESULTS

Shoot Culture Initiation

After two weeks of observation, all explants gave axenic cultures. Plants were free from both fungal as well as bacterial contamination. 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. After successful initiation of the culture (28 day culturing), newly formed shoots were excised individually from the proliferated explant and further cultured on the same medium to increase the number of shoots for further work.

Figure 1: Callus Formation and Shoot Regeneration in *Aloe vera.* Callus Induction was Developed in MS Media with Kn (0.5 mg/L) and 2,4-D (0.5mg/L)

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 BA and Kinetin (in combination with IBA 0.2 mg/L for shoot proliferation. Both BA and Kinetin were found to give indications of shoot proliferation after 2 weeks of incubation.

Table 1: Effect of Different Combinations of Cytokinins* on Shoot Proliferation in *Aloe vera* After 4 Weeks of Culture

Phytohormones (mg/L)	Percentage of Explants Showing Shoot Formation (Mean ± SD) n=5	Number of Shoots Per Explant (Mean ± SD)
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

It was found that BA gave better shoot proliferation than Kn (Table- 1). In medium containing BA in different concentration, on an average each explant gave rise to 3.0- 3.3 shoots (Table 1, Figure-2, 3). 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 Kinetin (1mg/L), the average number of shoots per plant was 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.

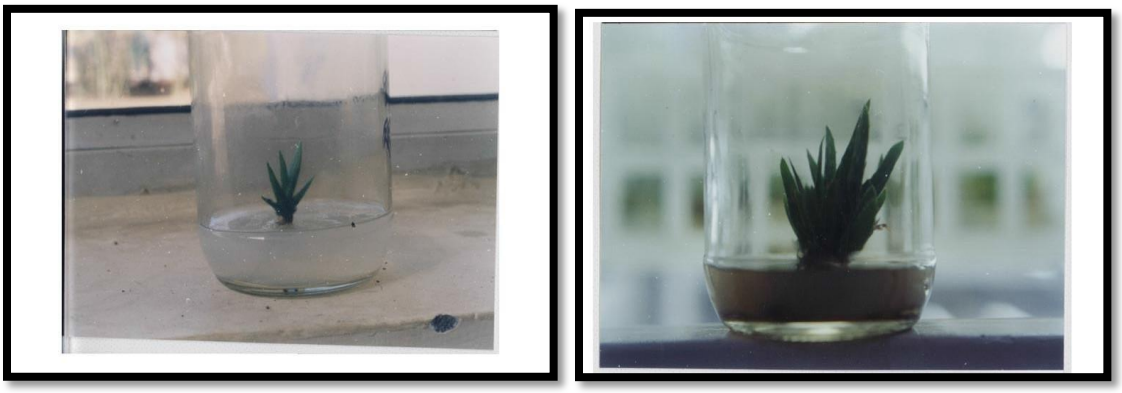

Figure 2: (a) Microshoot Inoculated on MS Medium with IBA0.2mg/L+BA 1.0mg/L (b) Shoot Proliferation After 4 Weeks of Culture on Medium Containing BA1.0mg/L + IBA0.2mg/L

Effect of Citric Acid on Shoot Proliferation in *Aloe vera*

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 2). 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 2: Effect of Citric Acid* on Shoot Proliferation in *Aloe vera* After 4 Weeks of Culture

Citric Acid (mg/L)	Percentage of Explants Showing Shoot Formation (Mean ± SD) n=5	Number of Shoots Per Explant (Mean ± SD) n=10
0	100 ± 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 was 4.80±2.5 while on solid medium average number of shoots was 4.08±2.0 (Table 3). Growth of cultures was fast in case of liquid medium than solid medium.

Table 3: Effect of Liquid and Solid Medium on Shoot Proliferation in *Aloe vera* After 4 Weeks of Culture

Media with BA (1 mg/L+ IBA 0.2 mg/L)	Percentage of Explants Showing Shoot Proliferation (Mean ± SD) n=4	Number of Shoots Per Explant (Mean ± SD) n=8
Solid	91 ± 17	4.08 ± 2.0
Liquid	91 ± 17	4.80 ± 2.5

Rooting of Microshoots

Three to four centimeters 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-4, Figure-4, 5). The number of roots per shoot was 2.8± 0.5 on hormone free medium.

Table 4: 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	Number of Roots Per Microshoot (Mean ± SD) n=10
0	100 ± 0	2.8 ± 0.5
1	90 ± 22	1.7 ± 1.1

Figure 3 : (a) Microshoots Showing Rooting After 15 Days of Culture (b) A Rooted Microshoot

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 creamish 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 5: Effect of Solid and Liquid Medium on Root Induction in *Aloe vera* After 15 Days of Culture

Media (Hormone Free)	Percentage of Microshoots Showing Root Formation (Mean ± SD) n=5	Number of Roots Per Microshoot (Mean ± SD) n=10
Solid	100 ± 0	2.7 ± 1.2
Liquid	18 ± 20	0.2 ± 0.5

Hundred percent shoot showed rooting and the mean number of roots per shoots was 2.7+1.2(Table 4). 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.

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 polyhouse was maintained at 31ºC with humidity level of nearly 80%.

Figure 4: Shoots Transferred to Plastic Cups with Sterile Sand for Hardening

Figure 5: Acclimatization of Rooted Plantlets

Figure 6: Hardened Plants in Pots in Shade House

Table 6: Survival Rate of Plantlets of *Aloe vera* at Different Stages of Hardening

Stage of Transplantation	Number of Plants Transplanted	Percentage of Survival
Poly House (1st stage)	13	85
Shade House* (2nd stage)	11	82

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

Plantlets that were transferred to the plastic pots in polyhouse showed good percentage of survival of 85% (Table 6, Figure 6). After keeping plantlets for initial ten days in polyhouse, 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.

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 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 *A.vera* respectively.

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).

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.

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:

- Surface sterilization with $HgCl_2$ (0.1% for 5-minutes) with 70% alcohol dip was best for the surface sterilization of the explants.
- For the initiation of the culture, MS medium with BA 0.2 mg/L with IBA 0.2 mg//L was used.

- Best shoot proliferation was achieved on MS medium containing BA 1mg/L with IBA 0.2 mg/L.
- Liquid medium with same composition was found to be better than solid medium for shoot proliferation.
- Addition of 10mg/L citric acid in the medium aided in the enhanced shoot proliferation. Citric acid in higher concentration (100mg/L) was found to be less effective.
- Hundred percent shoot showed rooting response on hormone-free medium.
- In liquid medium rooting response was found to very poor.
- Regenerated plantlets, 85% survival during polyhouse conditions and 82% during shade house stage of hardening.
- Regenerated plants were found to be morphologically similar to the mother/control plant.

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