TISSUE CULTURE OF A MEDICINAL PLANT-*ALOE VERA* L.

A

DISSERTATION

By

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for the award of the degree of
Masters of Science in Biotechnology

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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.

CANDIDATE'S DECLARATION

I hereby declare that the work presented in the dissertation entitled, **Tissue culture of a medicinal plant-*Aloe vera*** in partial fulfillment of the requirement for the award of the degree of Masters in Biotechnology, Department of Biotechnology and Environmental Sciences, Thapar Institute of Engineering and Technology, Patiala; is an authentic record of my own work done during the period of five months from January 2003 to May 2003,under the supervision of Dr. Kuldeep S. Barna, Research scientist, **TIFAC-CORE(Center of Relevance and Excellence) in Agro and Industrial Biotechnology,**Thapar Institute of Engineering and Technology (TIET),Patiala. I have not submitted the matter embodied in this dissertation for the award of any other degree or diploma.

Place: Patiala
Date: Diwakar Aggarwal

This is to certify that the above statement made by the candidate is correct and true to the best of our knowledge.

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(DIWAKAR AGGARWAL)

CONTENTS

INTRODUCTION

Aloe vera syn *barbadensis* Mill. belongs to the family Liliaceae (Anonymous 1985). It is commonly called as 'Burn plant'. 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). 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 *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. Plants will produce a commercial crop in one year (Venkataramaiah 2003). Leaves exude a bitter liquid, which is dried and known as "bitter *Aloe*s." 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

FIG-1, Elite Plant Material of *Aloe vera* Growing in the experimental plot.

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 *Aloe*s" (Musambar). It is a strong laxative, useful for short-term constipation.

Key Constituents:

Anthraquinones (aloin, *Aloe* - emodin), Resins, Tannins, Polysaccharides etc. The principal constituent of aloin is a water-soluble crystalline glycoside barbaloin [10(1)-deoxyglucosyl *Aloe*-emodin anthrone, $C_{21}H_{22}O_9.H_2O$, m.p.148-149^{0}C(anhyd.)].

Key Actions:

It heals wounds, is emollient and stimulates secretions of bile. *Aloe* is laxative. Gels of different strengths, oil extracts, leaf concentrates, and powders are available. South African *Aloe* gives quality gel. The processed gel is used in different preparations such as1. Drugs 2. Cosmetics 3. Soft drinks 4.Food Preparations 5. Moisturizers 6. Healthcare products etc.

Traditional & Current Uses

*Beauty treatment***:** *Aloe vera* has a long history as a skin lotion. Cleopatra is said to have attributed her beauty to it.

Western remedy: In the West, *Aloe vera* first became popular in 1950s when its ability to heal burns, in particular radiation burns, was discovered.

First aid: *Aloe vera* is an excellent first aid to keep in the home for burns, scrapes, scalds, and sunburn. A broken off leaf releases soothing gel, which may be applied to the affected part.

Skin conditioner: The gel is useful for almost any skin condition that needs soothing and astringing and will help varicose veins to some degree.

Ulcers: The protective and healing effect of *Aloe vera* also works internally and the gel can be used for peptic ulcers and irritable bowel syndrome.

Laxative: The bitter yellow liquid in the leaves (bitter *Aloe*s) contains anthraquinones, which are strongly laxative. They cause the colon to contract, generally producing a bowel movement 8-12 hours after consumption. At low doses, the bitter properties of the herb stimulate the digestion. At higher doses, bitter *Aloe*s is laxative and purgative.

Minor burns & Sunburn: Apply *Aloe-vera* gel to the affected area as needed.

St*retch marks:* Rub *Aloe vera* gel over the affected area.

Warts: Apply the gel directly to the wart 2-3 times a day for up to 3 months.

Weeping skin: Apply *Aloe*-vera gel to the affected area 2-4 times a day.

Wounds: Cleanse the wound with the gel and cover with a dressing soaked in gel.

Out of the 22 amino acids required for the human body, 8 essential amino acids are found in *Aloe vera*. Vitamins namely A, B1, B2, B6, B12, C and E, which the human body cannot prepare by itself, are available in *Aloe vera*. Daily intake of *Aloe vera* Gel free from aloin - a yellow sap, is certainly good for health and improves natural immunity. The research studies conducted on *Aloe vera* plant

have revealed that through strengthening the T-lymphocyte cells of the blood, it is able to heal the wounds and improve immunity (Lee & Kim, 2000).

Fresh leaf juice mixed with little turmeric power is best external application for wounds, burns etc. Tribals and villagers who feed goats and cattle in forest areas usually apply *Aloe vera* Juice externally to the scalp (head skin) in extreme summer to protect the body from the effects of radiation and sunstroke. It is an ideal, safe, proven anti-oxidant useful to prevent aging process and can be safely used right from infants to extremely aged groups irrespective of sex, age differentiation. *Aloe vera* gel bestowed with very powerful immunomodulatory effect can be used in Aids, TB, and Cancer patients to overcome the symptoms and to prolong their life span. It is the natural healer and safe cosmetic for all age groups of men and women. It protects the skin, and improves complexion. It is a natural moisturizer.

The technique of tissue and organ culture is used for rapid multiplication of plants, for genetic improvement of crops, for obtaining disease- free clones and for preserving valuable germplasm (Bhojwani & Razdan1992). One of the major applications of plant tissue culture is micropropagation or rapid multiplication. Compare to conventional propagation, micropropagation has the advantage of allowing rapid propagation in limited time and space.

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.

Current studies aim at following objectives

Standardize optimum conditions for

- **Establishment of axenic shoot culture from elite germplasm**
- **Multiplication of microshoots,**
- **Rooting of micro shoots,**
- **Hardening and transfer of plants to soil.**

REVIEW OF LITRATURE

Plant tissue culture forms the backbone of plant biotechnology, i.e. micropropagation, induction of somaclones, somatic hybridization, cryopreservation and regeneration of transgenic plants. Plant Tissue culture is an essential component of Plant Biotechnology. Plant Cell and tissue culture has already contributed significantly to crop improvement and has great potential for the future (Kumar and Kumar, 1996). Research efforts in plant cell and tissue culture have increased dramatically worldwide in recent years including efforts in developing nations. Plant cell and tissue culture is defined as the capability to regenerate and propagate plants from single cells, tissues and organs under sterile and controlled environmental conditions (Murashige & Skoog, 1974). In India, Tissue Culture research began nearly four decades ago with the first report on production of test tube fertilization (Kanta *et al.*1962). Tissue culture techniques are now being widely applied for improvement of field crop, forest, and horticulture and plantation crop for increased agricultural and forestry production. Today tissue culture technology is being exploited mainly for large-scale production or micropropagation of elite planting material with desirable characteristics. This technology has now been commercialized globally and has contributed significantly towards the enhanced production of high quality planting material. Recently, emphasis has been on genetic transformation, especially for (1) increased production of secondary metabolites, (2) production of alkaloids, pharmaceutics, nematocidal compounds, and also some novel compounds not found in the whole plant, () regeneration of plant resistant to herbicides,

diseases, and pests, (3) scale up of cultures in bioreactors, (4) plants with different morphological traits, and (5) transgenic plants for the production of vaccines etc. These developments have far-reaching implications in the improvement of medicinal plants as well. (Bajaj, 1998)

Micropropagation has been useful for the rapid initial release of new varieties prior to multiplication by conventional methods, e.g. pineapple (Drew, 1980) and strawberry (Smith and Drew, 1990). Micropropagation is also used to promote germplasm storage for maintenance of disease-free stock in controlled environmental conditions (Withers, 1980) and in long -term via cryopreservation (Kartha *et al.* 1980). The *in vitro* vegetative propagation has important benefits to produce stable lines in plants that have no named varieties e.g., Annona spp. (Bridg, 1993), Australian dioecious papaw genotypes (Drew, 1988; Drew, 1992) where traditional plant breeding has failed. It has also a great potential for the propagation of important crops like: *Cassava* spp., *Phaseolus* spp., *Solanum* spp., (Roca and Mroginski, 1991). The micropropagation of elite or selected plants showed good results, which benefit the agriculture, horticulture and forestry (Conger, 1981; Drew, 1997). Worldwide there is much interest to promote the development of an in vitro technology that permits the propagation and breeding of commercial valuable woody, semi woody, ornamental, basic food, industrial and medicinal plants. Which species are in danger of extinction should receive a priority in terms of germplasm conservation (Conger, 1981; Deberg and Zimmerman, 1990; Drew, 1997).

Aloe vera have been cultured in vitro by various researchers like Sanchez *et al.* (1988), Natali *et al.* (1990), Meyer & Staden (1991), Roy & Sarkar (1991), Corneanu *et al.,* (1994). Richwine *et al.* (1995), Abrie & staden (2001), Chaudhuri & Mukandan (2001).

Sanchez *et al.* (1988) performed micropropagation from shoot meristems. They found that *in vitro* culture of *Aloe barbadensis* is very difficult for both callus induction and plant regeneration. A DNA microdensitometric study was performed on different organs *of A.barbadensis* and during *in vitro* culture of different explants.

Natali *et. al.* (1990) reported a rapid and highly effective plant micropropagation from vegetative meristems. Micropropagation was achieved by culturing shoot apices on medium containing 2,4-D and Kn within 15-30 days. High morphogenetic ability was maintained by transferring explants on media containing 2,4-D and 6-benzylaminopurine. (Natali *et.al.* 1990),

*Aloe*s have been cultured *in vitro* with 2,4-D and cytokinins in the growth medium (Groenewald *et al.* 1975, Natali *et al* 1990). Axillary bud development and adventitious bud formation was obtained with decapitated shoot explants of *Aloe barbadensis* Mill. Maximal bud growth and rooting of shoots was obtained on a modified medium of Murashige and Skoog supplemented with IBA. The optimal temperature for bud growth and development was 25^0C. Bud growth was slowed at 10^0c (Meyer & Staden, 1991).

Aloe has also cultured for their high medicinal and ornamental value (Vij *et al.*, 1980). Little work has been done on callus culture of *Aloe* species because establishment of primary cultures is difficult owing to the secretion of the phenolic substances by explant. There is only one report on callus formation and plant regeneration from seed calli of *Aloe pretoriensis* (Groenewald *et al.,* 1975). Roy and Sarkar (1991) report the rapid propagation by the formation of shoots from calli of *Aloe vera*. They induced the callus formation in stem segments from young axillary shoots grown on the underground rhizomatous stem. Polyvinylpyrrolidone was used to reduce the secretion of phenolic substances from the explant. Modified MS media with 2,4-D and Kinetin was used for callus induction. (Roy & Sarkar, 1991).

Micropropagation of *Aloe* was carried out by culturing fragments from axillary shoots on MS medium without growth regulators (the presence of which was found to inhibit the first stage of development). For the second stage of development involving the newly formed plantlets, the presence of a magnetic fluid in the culture medium stimulated secondary shoot production, general plant development and rhizogenesis (Corneanu *et al.,* 1994).

Richwine *et. al*. (1995) reported the induction of shoot cultures of *Aloe, Gasteria* and *Haworthia* species from immature inflorescence. Shoots were initiated on a modified MS medium containing zeatin and later maintained on medium containing Zeatin and BA.

A rapid propagation protocol was established for the highly endangered *A. polyphylla*. Seed was germinated *in vitro* on MS medium with or without sucrose. Plantlets were cultured on medium containing benzyl adenine (BA) only, or a combination of BA and NAA. After initial problems with browning, the explants rapidly formed axillary and adventitious buds (Abrie & staden, 2001).

Chaudhuri and Mukandan (2001), also reported that in *Aloe vera* formation of multiple shoots *in vitro* was a function of cytokinin and auxin concentrations. The presence of only cytokinin or auxin resulted in formation of callus or roots. Best multiplication of shoots was obtained on medium containing BA + Adenine sulphate + IAA.

MATERIALS AND METHODS

Plant material:

Elite plants were healthy and free of symptoms of disease, pest problems and showed good biomass yield. We collected the plant growing in the experimental plot of TIFAC- CORE building. 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 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). Growth hormones,

Composition of basal MS medium

MACRO SALT	(mg/L)
KNO_3	1900
NH_4NO_3	1650
$MgSO_4.7H_2O$	370
$CaCl_2.2H_2O$	440
KH_2PO_4	170
MICRO SALTS	
$MnSO_4.H_2O$	22.3
$ZnSO_4.7H_2O$	8.6
H_3BO_3	6.2
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_20$	0.025
Na_2Fe-EDTA	37.24

ADDITIVES	mg/L
Thiamine HCl	0.1
Nicotinic acid	0.5
Pyridoxine HCl	0.5
Glycine	2.0
Myo-inositol	100
Sucrose	30000

6-benzylaminopurine (BA), 3-Indolebutyric acid (IBA), kinetin (Kn), Adenine sulphate were added to the basal medium either singly or in various combinations.

The concentrated stock solutions of all the ingredients were prepared and stored under refrigeration. To prepare stock solution of micro salts, all the micro salts in required quantities were dissolved in one liter of distilled water and used as stock solution. Like wise stock solutions of all other ingredients are also prepared and kept under refrigeration. Similarly stock solutions of growth hormones were also prepared. Cytokinins were dissolved in few drops of acidic solutions (1N HCl) and Auxins were dissolved in few drops of basic solutions (1N KOH), after dissolving final volume is made up with the help of distilled water and kept at 4^0C. (Growth regulators are from Sigma, USA).

The medium was prepared by adding required quantities of all the ingredients in the conical flask. After adding all the ingredients in required amounts, the final

volume is made up with the help of distilled water. pH of the medium is adjusted to 5.8 by using 1N KOH or 1N HCl (Cyberscan 510,Eutech Instruments, Singapore). After adjusting the pH, agar (Himedia Labs Limited, India) is added to the medium at the rate of 0.8% w/v for solidification of the medium. For preparing liquid medium (wherever used) agar was not added to the medium. After pouring media (50-ml/300ml bottle), bottles are tightly capped and labeled properly. After that media is autoclaved (Equitron, Medica Instruments. India) at 121^0C for 20 minutes at 15psi.

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 are 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 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, 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 microshoots:

Newly formed shoots measuring 3-4cm in length 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). 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± 1^0C).

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 polyhouse having 80% humidity and 31^{o}C 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 (if any).

DNA isolation:

For the DNA isolation, we use CTAB plant DNA extraction kit provided by GENEI, Bangalore. The protocol and solutions used in the kit are as fallow and as per manufacturer instructions.

Solutions of CTAB DNA isolation kit

Solution A (CTAB extraction buffer)

CTAB	2% (w/v)
Tris-HCl	10 mM (pH 8.0)
EDTA	20 mM (pH 8.0)
NaCl	1.4 M

Solution B (Extraction buffer)

Tris-HCl	100 mM (pH 8.0)
EDTA	100 mM (pH 8.0)
NaCl	250 mM

Solution C (CTAB Precipitation solution)

CTAB	1% (w/v)
Tris-HCl	50 mM (pH 8.0)
EDTA	10 mM (pH 8.0)

Solution D (High salt TE buffer)

Tris-HCl	10 mM (pH 8.0)
EDTA	0.1 mM (pH 8.0)
NaCl	1.0 M

Procedure:

The young and soft leaves (~1.2 g) was pulverized to a fine powder in the presence of liquid nitrogen and was distributed equally to three microfuge tubes (2.0 ml capacity). Immediately 0.9 ml of solution A + 2% (v/v) â-mercaptoethanol

(at 65ºC) was mixed to the tissue to wet it thoroughly. The tubes were incubated at 65• C for 1 hr. with occcasional mixing, after every 15 min.

Equal volume of chloroform: iso-amyl alcohol (24:1) was added to it. The solution was mixed well by inversion for 7 min and then centrifuged at 8000 rpm for 5 minutes.

The upper aqueous layer was recovered, to which, 1/10th volume (100 ì l) of 65• C solution B was added and this solution was mixed well by inversion for 5 min. This solution was extracted in the same way with equal volume of chloroform: iso-amyl alcohol (24:1) and the upper aqueous layer were recovered. To this solution, 1 volume (900 ì l) of solution C was added. The solution was mixed well and was incubated at 65• C for ½ hr. This solution was centrifuged for 5 min. at 5000 rpm. The pellets were resuspended in ~500 ì l of solution D by intermittent incubation at 65• C. 300 ì l of isopropanol was added to each, mixed well and this was kept at 4• C for 15 min. This solution was centrifuged at full speed (rpm 15000) for 15 min., when the pellets were washed with 80% ethanol. The pellets were air-dried and were finally resuspended in 60 ì l of TE buffer and store at –20 ^{o}C.

AGAROSE GEL ELECTROPHORESIS:

Gel Loading Buffer:

Sucrose	35% (w/v)
EDTA	50.0 mM (pH 8.0)
Bromophenol blue	0.2% (w/v)

TBE BUFFER (5X):

Tris base	54 g
Boric acid	28 g
EDTA	3.8 g

The pH of the buffer was set at 8.0

Horizontal agarose gel electrophoresis was performed using standard methods. 0.7% agarose gel was made in 0.5X TBE buffer to which ethidium bromide dye was added and was casted in a gel tray. The DNA samples were loaded after mixing well with the gel loading buffer containing tracking dye bromophenol blue and electrophoresis was carried out at 65 V till the tracking dye covered 2/3rd of the gel length. Finally, the DNA bands were visualized under UV light.

Statistical analysis:

All experiments were repeated once and data presented is mean of two values. Means and standard deviation were calculated by using software Graph Pad Prism 2.01.

Photography: Photos were taken with Ashai pentex camera using 400ASA color film.

RESULTS

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. 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.

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.

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

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 Kn (1.0 mg/L) the average number of shoots per plant

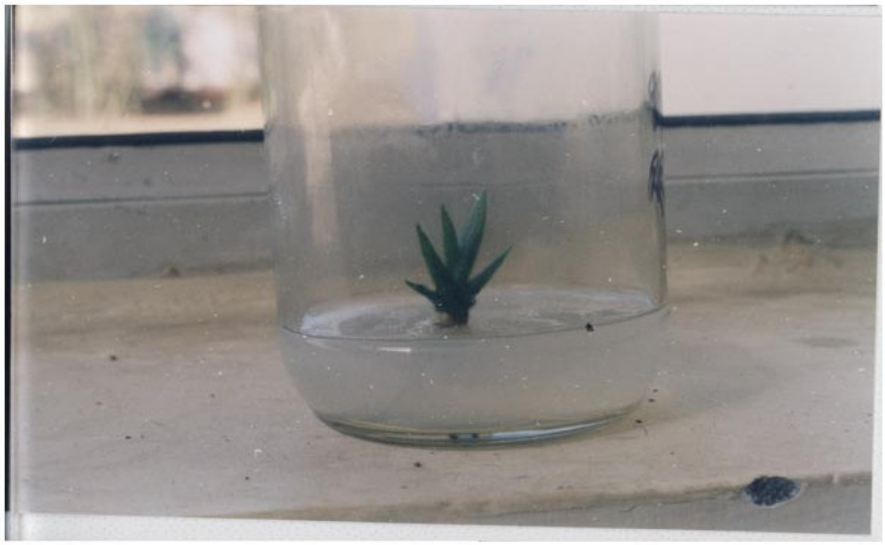

FIG-2, Microshoot inoculated on MS medium with IBA0.2mg/L+BA 1.0mg/L

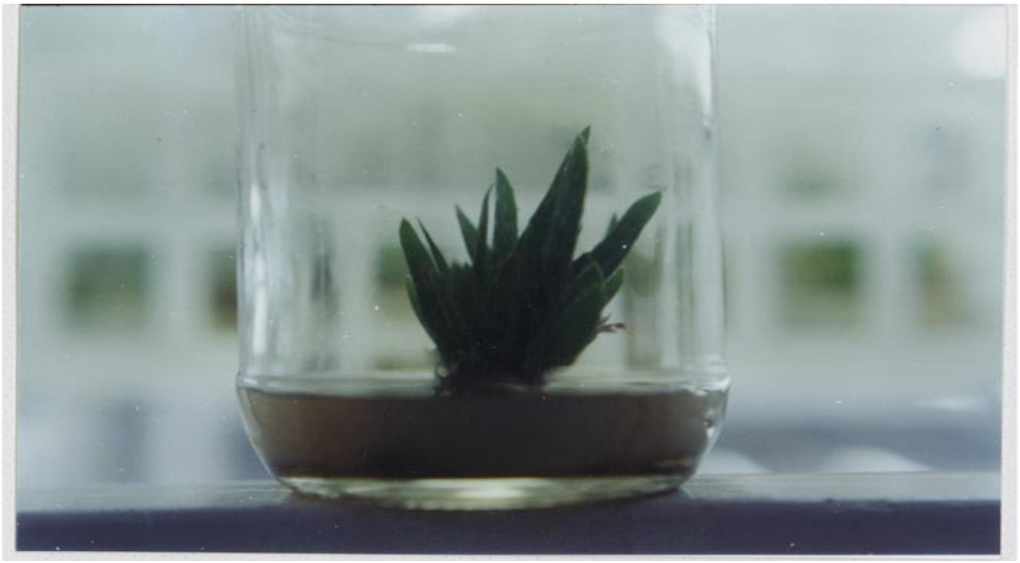

FIG-3,Shoot Proliferation after 4 weeks of culture on medium containing BA1.0mg/L + IBA0.2mg/L

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*.

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 0.2 mg/L and IBA 0.2 mg/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 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 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 explant (Mean ±SD) n=8
Solid	91±17	4.08±2.0
Liquid	91±17	4.80±2.5

*Containing BA 1.0mg/L and IBA 0.2 mg/L

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-5, Figure-4, 5). The number of roots per shoot was 2.8+0.5 on hormone - free medium.

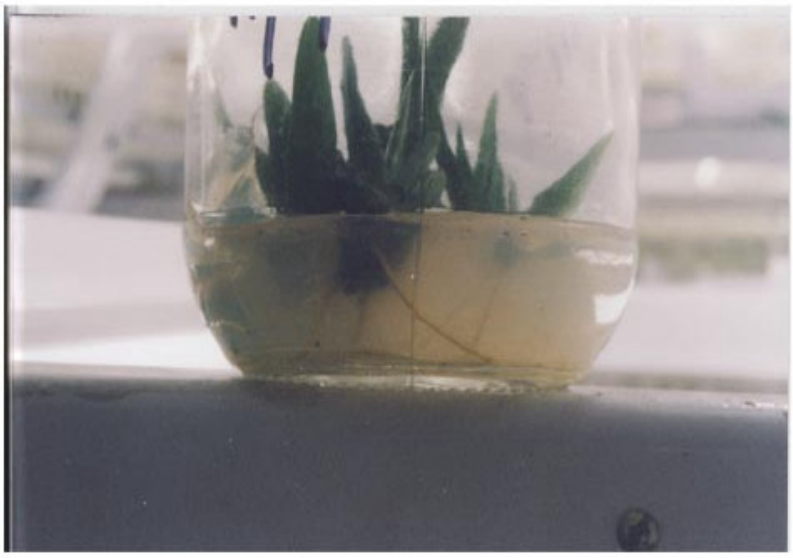

FIG-4, Microshoots showing rooting after 15 days of culture.

FIG-5. 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 per microshoot (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 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-6. Effect of solid and liquid medium on root induction in *Aloe vera* after 15 days of culture.

Media*	Percentage of microshoots showing root formation (Mean±SD) n=5	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 polyhouse was maintained at 31°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 (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 7,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. Plants with these symptoms were also growing well.

FIG-6, Fully Hardened Plants growing in Open Shade.

Morphology of the regenerated plants:

All the plants produced were morphologically similar to the mother/control plants. To find any possible (if any) somaclonal variation had occurred at molecular/DNA level, genomic DNA of control plants and tissue-cultured plants were isolated. So, the DNA isolated will be further used to find out any possible somaclonal variation. Agarose gel electrophoresis showed that all these samples were about 23kb, when compared with the molecular size marker (Figure-8). Further work with the isolated is under progress.

Table-8. Quantification of the isolated DNA by UV spectrophotometeric methods

SAMPLE	Yield (ìg/ìl)
1.Normal plant (control)	0.50
2.Normal plant (control)	0.65
3 Normal plant (control)	0.65
4.Regenerated plant (without roots)	1.62
5.Regenerated plant (with roots)	2.15
6. Regenerated plant (with roots)	1.00

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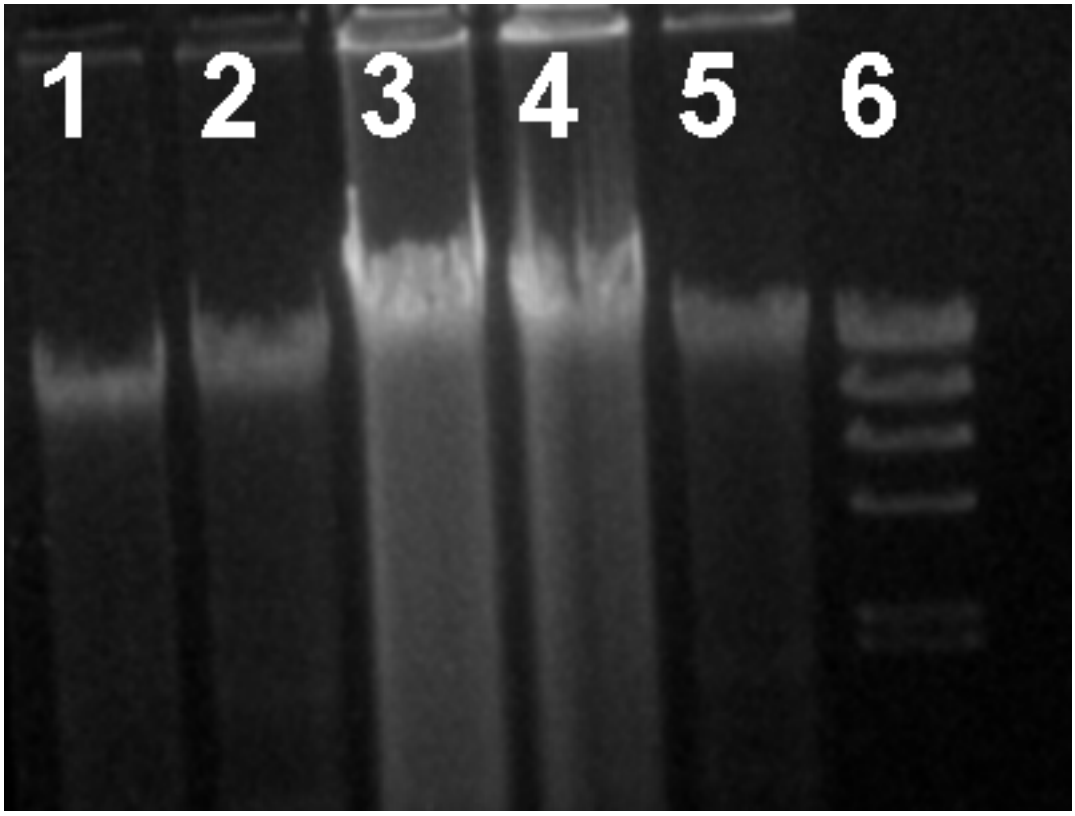

Lane 1: Normal plant genomic DNA; Lane 2: Normal plant genomic DNA; Lane 3: Tissue cultured plant genomic DNA; Lane 4: Tissue cultured plant genomic DNA; Lane 5: Normal plant genomic DNA; Lane 6:λ-*Hind* ^{222} DNA

FIG-7, Gel Profile of *Aloe vera* DNA.

DISCUSION

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 *A.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 suparoptimal 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 2^{nd} hardening 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. For the identification of any possible somaclones, in addition to their comparison with in terms of morphology we planned to do some genetic analysis also. For this purpose we isolated DNA from both normal as well as plants regenerated through tissue culture. But due to lack of time we were able to complete only first portion of the work.

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 promoted 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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