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THE STUDY OF IN VITRO MICROPROPAGATION IN ALOE VERA (L.) BURM. F. – BARBADOS ALOE

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ABSTRACT
The study of micro propagation in Aloe vera and the investigation of developed a dependable protocol for large scale clonal multiplication of Aloe vera under the in vitro conditions. The Standardize optimum conditions for micropropagation of Aloe vera through a tissue culture. The Multiplication of shoots was found best on MS medium in combination of BA 2.0 mg/l, KN 0.5 mg/l and NAA 0.2 mg/l and the other good combinations of growth regulators in proliferating shoot were BA 2.0 mg/l and KN 0.2 mg/l (95.30%), followed by BA 2.0 mg/l (90.91%). MS medium containing BA and NAA was found to be the best medium in Aloe micropropagation Establishment of sterile axenic shoot culture from elite germplasm. Multiplication and elongation of micro shoots, Root formation and Hardening of multiplied shoots.

INTRODUCTION
Aloe vera is belongs to the family of Liliaceae (Anonymous 1985). Itis an evergreen perennial, drought-resisting, succulent plant commonly called as 'Burn plant'. It is a xerophyte and can be grown even in dry lands under rain fed conditions. *Aloe* is a course 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, and 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 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). Plant tissue culture forms the backbone of plant biotechnology, i.e. micropropagation, induction of soma clones, somatic hybridization, cryopreservation and regeneration of transgenic plants.

The first successful plant tissue culture & cell culture was accomplished by "Gottlied Haber land" near the turn of 20th century, when he reported the callus of leaf mesophyll tissue & hair cells (Steward 1968, Krikorian & Borquan 1969). Hanning (1904) cultured nearly matured embryos excised from seeds of several species of crucifers. Kolte (1922) reported the growth of isolated root tips on a medium consisting primarily of inorganic salts. At the same time and quite independently, Robbins (1922) reported a similar success with the root tips & stem tips. An important breakthrough, for continuously growing root tip cultures, came from White (1934).

MATERIAL AND METHODS
Selection And Isolation Of Explant
Elite plants were healthy and free of symptoms of disease, pest problems and showed good biomass yield. We collected the plant growing from the green house of the institute. Shoot with young leaves were collected from the elite mother plants. The extra leaves were removed and shoot was trimmed to size of 2-3 cm for further work. The best explants for micropropagation of *A. vera* are shoot tip and axillary bud (Meyer and Staden, 1991). Also, the presence of the plant growth regulators is necessary for this purpose (Aggarwal and Barna, 2004; Debiasi et al., 2007; Liao et al., 2004).

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

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IJESMR
International Journal OF Engineering Sciences & Management Research

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 1-5 minutes. After Mercuric chloride treatment, explants were thoroughly washed for 3-4 times with sterile water to remove any traces of Mercuric chloride. The sterilized explants were than inoculated on the prepared medium in the laminar air flow chamber. Inoculated cultures were incubated in the culture or growth room at controlled environment of the temperature of 25 +/- 4^0c of 16 hours photoperiod of 2000 lux and dark condition.

Shoot Induction Experiment

The effect of season, age of explant and the effect of various cytokinins on initiation of shoots were studied simultaneously. For these studies the sterile apical and axillary buds were inoculated on Murashige and Skoog (1962) basal medium supplemented with cytokinins like BAP , in the concentration of (0.5-2.0) mg/l alone or in combination with other cytokinins of each, containing sucrose 30 g and gelled with agar 4 g/l. In addition auxins like or NAA (0.1-1.0 mg/l) were used for promoting the shoot induction.

Medium Used In Shoot Initiation

Medium 1	**MS + 0.5 mg/l BAP**
Medium 2	**MS + 1.0 mg/l BAP**
Medium 3	**MS + 1.0 mg/l BAP + 0.5 Kn**
Medium 4	**MS + 2.0 mg/l BAP**
Medium 5	**MS + 0.5 mg/l BAP + 0.5 mg/l NAA**
Medium 6	**MS + 1.0 mg/l BAP + 0.5 mg/l NAA**

Number of experiments was carried out for initiation of shoot from axillary and apical meristem. The measurement of growth was taken by the percentage of buds showing response, number of shoots initiated per explants, shoot length and callus formation.

Multiplication Of Shoots

After one- two week of initiating experiment the bud start responding by bud break and initiation of shoot after 10 to 20 days of culture, initiated shoots were separated and transferred to different multiplication medium containing different high concentration of growth hormones. Number of experiments was carried out to maximize the rapid multiplication of shoots. These include use of high concentration of cytokinins, BAP (1.0-3.0 mg/l), KN (1.0-2.0 mg/l) as compared to induction medium.

Different Media Used For Multiplication

Medium 1	**MS + 1.0 mg/l BAP + 0.5 mg/l Kn**
Medium 2	**MS + 2.0 mg/l BAP**
Medium 3	**MS + 1.0 mg/l BAP +0.5 mg/l NAA (liquid)**
Medium 4	**MS + 1.0 mg/l BAP +0.5 mg/l NAA (Solid)**
Medium 5	**MS+ 2.0 BAP + 0.5 NAA**

The measurement was taken on the basis of % age of shoot response, number of multiple shoot developed, shoot length and callus formed from each ten replicates.

Experiment For Root Induction

Regeneration multiple shoots were separated in different rooting medium.
Different media for root induction

Medium 1	**MS+ 0.5 mg/l NAA**
Medium 2	**MS+ 1.0 mg/l NAA**
Medium 3	**MS+ 1.5 mg/l NAA**
Medium 4	**MS+ 2.0 mg/l NAA**
Medium 1	**MS + 0.5 mg/l IAA**
Medium 2	**MS + 1.0 mg/l IAA**
Medium 3	**MS + 1.5 mg/l IAA**

ISSN 2349-6193
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IJESMR
International Journal OF Engineering Sciences & Management Research

Medium 4 **MS + 2.0 mg/l IAA**
Medium 1 **MS+ 10g/l Sucrose**
Medium 2 **MS+ 1.0 mg/l NAA + 20g Sucrose**
Medium 3 **MS +200 mg activated charcoal**

Results and Observations

Shoot Initiation

The shoot tip explants of *aloe vera* were surface sterilized by 0.1% mercuric chloride solution for 1 to 10 minutes; by different treatment of sterilization 90-95 % of explants were sterilized when treated for 4-5 minutes. After surface sterilization of shoot tip explants, the explants were inoculated in culture bottles aseptically. 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 0.5 -1.0 mg/l BAP and 0.5 mg/l NAA.

Multiplication Of Shoots

In order to optimize a suitable medium for mass multiplication of shoots of *aloe vera* from a single initiated region, the highest number of shoots was observed in the medium containing higher concentration of BAP (1.0-2.0 mg/l).

Root Induction Experiment

The shoot of 2-3cm shows 80-90% rooting in the medium containing activated charcoal and NAA. About 2-4 thin long roots were developed which increase with the age of culture. Root length was 2-8cm.

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%)

TABLE-1: EFFECT OF GROWTH REGULATORS ON SHOOT INDUCTION OF ALOE VERA.

S.No	Medium + Growth hormones mg/l	%age of shoot induction	No. of shoots per culture	Average shoot length in cm.	Callussing
BAP					
1.	MS+0.5 mg/l BAP	70%	1-2	1-2	-
2.	MS+1.0mg/l BAP	75%	1-3	2 cm	-
3.	MS+1.5mg/l BAP	70%	1-2	2cm	-
4.	MS+2.0mg/l BAP	60%	1-2	1-2	-
BAP + KN					
1.	MS+0.5 KN	40%	1	1-2	+
2.	MS+1.0 KN	20%	1	0.5	+
3.	MS+ 2.0KN	10%	1	0.3	++
BAP + NAA					

ISSN 2349-6193
Impact Factor (PIF): 2.243

IJESMR
International Journal OF Engineering Sciences & Management Research

1.	MS+0.5 BAP + 0.5 NAA	40%	1	1	--
2.	MS+1.0 BAP + 0.5 NAA	70%	2	2	--
3.	MS+2.0 BAP+0.5 NAA	60%	1-2	1	-

Table no. 1. Red color line are show effects of Growth Harmon Shoot Induction

Table 2: EFFECT OF DIFFERENT CONCENTRATIONS OF BAP AND NAA ON SHOOT MULTIPLICATIONS IN ALOE VERA L. AFTER 4 AND 8 WEEKS OF CULTURE.

S.No	Medium + Phytohormones (mg/l)	Explants showing shoot multiplication (%)	Average number of shoot per single explant (after 4 weeks	Average number shoot per single explant (after 8 weeks)	Callus sing
1	MS+0.5 mg/l BAP + 0.5 Kn	50%	2-4	2-3	-
2	MS + 2.0 BAP + 0.5 NAA	50%	1-2	2 -3	-
3	MS + 1.0 mg/l BAP +0.5 mg/l NAA (liquid)	100%	6-8	2-5	+
4	MS + 1.0 mg/l BAP +0.5 mg/l NAA (Solid)	90%	4-5	3-5	+
5	MS+1.0 BAP+1.0KN	70%	3-4	1-2	+
6	MS+1.0 BAP+2.0 KN	60%	4-9	1-2	++

	Low Growth
	Medium Growth
	Higher the Growth

ISSN 2349-6193
Impact Factor (PIF): 2.243

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International Journal OF Engineering Sciences & Management Research

TABLE 3: EFFECT OF GROWTH REGULATORS, ACTIVATED CHARCOL & LOW SUGAR CON. + MS MEDIA ON ROOT INDUCTION IN ALOE VERA.

MS medium + Growth Regulators mg l-1	No of days for rooting	% of Rooting	Root Morphology	Root length / explants (cm)
NAA				
0.5	10-12	42	Thin, short	2.3
1.0	10-12	80	Thin, short	2.5
1.5	10-15	30	Thin, short	3.9
2.0	9-10	40	Thin, long	3.2
IAA				
0.5	8-9	10	Thin, short	2.0
1.0	10-12	10	Thin, long	4.0
1.5	11-12	12	Thin, short	3.3
2.0	12-14	30	Thin, short	2.8
ACTIVATED CHARCOAL				
MS+100 mg activated charcoal	10-20	80	Thin, short	2.5
MS+200 mg activated charcoal	10-11	90	Thin, long	3.5
LOW SUGAR CONCENTRATION IN MS MEDIUM				
MS+ 10g/l sucrose	10-12	60	Thin, short	2.7
MS+ 1.0 mg/l NAA+20g Sucrose	10-12	70	Thin, short	3.2

TABLE-4.SURVIVAL RATE OF PLANTLETS OF ALOE VERA AT DIFFERENT STAGES OF HARDENING.

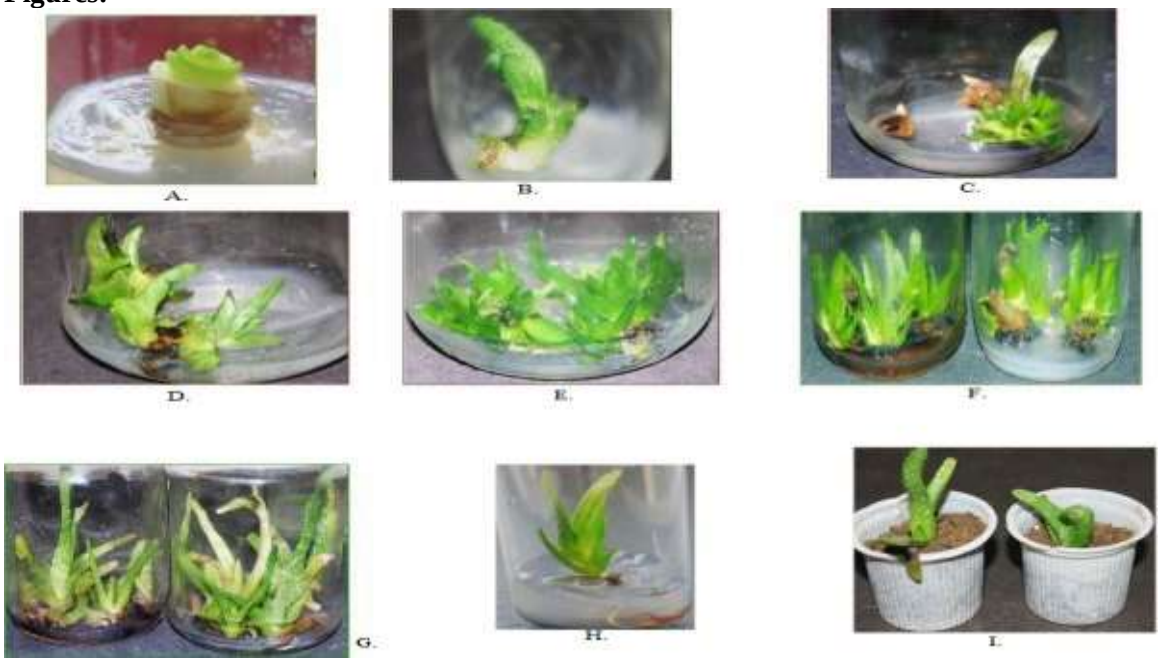
Stage of transplantation	Number of plants transplanted	Percentage of Survival
Poly House (1st stage)	20	90%
Shade House* (2nd stage)	*18*	*85%*

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

ISSN 2349-6193
Impact Factor (PIF): 2.243

IJESMR
International Journal OF Engineering Sciences & Management Research

Figures:-



Figures: - A. Shoot Induction, **B.** Initiated Shoots, **C.** Multiplication of Shoots, **D.** Average number of shoot multiplication after 4 weeks, **E.** Average number of shoot multiplication after 8 weeks, **F.** Number of shoot multiplication in MS+1.0mg/l BAP+1.0mg/l KN, **G.** Number of shoot multiplication inMS + 1.0 mg/l BAP +0.5 mg/l NAA, **H.** Root induction(MS+1.0 mg/l NAA+20g Sucrose), **I.**Hardened plant(Plantlets were transplanted to plastic pots containing garden soil and Farmyard manure (1:1) for their hardening.)

DISCUSSION

Aloe vera L. is a medicinal plant. Regeneration of *A. vera* in nature *(in vivo)* is too slow and insufficient to meet the industry demand. Therefore, it is necessary to use *in vitro* propagation for rapid plant production.The best explants for micropropagation of *A. vera* are shoot tip and axillary bud (Meyer and Staden., 1991). Also, the presence of the plant growth regulators is necessary for this purpose (Aggarwal and Barna., 2004), (Debiasi et al., 2007), (Liao et al., 2004), (Meyer and Staden., 1991) reported axillary shoot formation using IBA, where as Roy and Sarkar., (1991) and Natali et al., (1990) obtained shoots on medium containing 2,4-D and Kn. for the young plants survival (Natali et al., 1990; Hirimburegama and Gamage, 1995). Genetic transformation and cloning requires a highly efficient system for regeneration of *A. vera*(Campestrini et al., 2006; Velcheva et al., 2005).

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(Bhojwani1980), chickpea (Barna&Wakhlu 1994). Nair et al (1979), and *Iresinelendenii*(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

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IJESMR
International Journal OF Engineering Sciences & Management Research

of *Aloe polyphylla*and *A.vera* respectively. 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. The explants began to show the signs of shoot proliferation after two weeks of culturing. All explants gave aseptic cultures. Plants were free from both fungal as well as bacterial contamination. After successful initiation of the culture (4-5 weeks culturing), newly formed shoots were excised individually from the proliferated explant. Multiplication of shoots was found best on MS medium in combination of BA 2.0 mg/l, KIN 0.5 mg/l and NAA 0.2 mg/l. (Table 1 and Figure 1) and the emergence of shoots took place in 2 weeks. In this media composition, the percentage of shoot proliferation and the number of shoots per explant was 98.96 and 15.39, respectably (Table 1). But the length of the longest shoot (4.92 cm) was maximum in MS medium supplemented with BA 2.0 mg/l and NAA 0.5 mg/l. These variations were also reported by other authors (Islam, 2001; Hossain et al., 1991). The other good combinations of growth regulators in proliferating shoot were BA 2.0 mg/l and KIN 0.2 mg/l (95.30%), followed by BA 2.0 mg/l (90.91%). MS medium containing BA and NAA was found to be the best medium in Aloe micropropagation (Wenping et al., 2004; Liao, 2004). It was also reported that the highest shoot multiplication in *Aloe vera*was found in MS medium containing BA 1.0 mg/l and IBA 0.2 mg/l (Aggarwal and Barna, 2004).

CONCLUSION

Aloe vera is a xerophytic medicinal plant of considerable importance. It is widely used in cosmetic and drug industry and its demand are 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 HgCl2 (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 BAP 0.5-1.0 mg/L with NAA 0.5 mg/l was used.
3. Best shoot proliferation was achieved on MS medium containing BAP1.0mg/l with NAA 0.5mg/L. Liquid medium with same composition was found to be better than solid medium for shoot proliferation.

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