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IN VITRO REGENERATION OF ALOE VERA – A MEDICINALLY IMPORTANT PLANT

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ABSTRACT
Aloe vera (*Aloe barbadensis* Mill.) is a valuable medicinal plant. The present study have designated to standardize a sterilization technique and regenerate the plant. Maximum green cultures without contamination were found when treated with 0.1% $HgCl_2$ for 2 minutes. An economical small propagation technique has been developed by treating the shoot explants cultured on MS medium with different phytohormonal supplements of BAP, IBA, and Kinetin (Kn) for shoot proliferation and maturation. Shoot cultures were initiated on MS medium containing BAP 0.2 mg/L, with IBA 0.2 mg/L. Maximum shoot proliferation was achieved on medium containing BAP 1.0 mg/L with IBA 0.5 mg/L within 28 days of culture. Maximum of 5-multiplication rate of shoots was achieved with the utilization of growth regulators along with the basal MS medium. After 3 weeks, the microshoots were transferred into rooting medium for root regeneration. Hundred percent rooting of microshoots was obtained on phytohormone – free MS medium. The regenerated plants were morphologically similar to control plants. This protocol could be used for the massive *in vitro* production of the plantlets of the A. vera. In its natural state Aloe propagates vegetatively, but propagation rate is too slow and as a result, it cannot meet demand of high quality planting material for commercial purposes. Therefore micropropagation protocol is standardized for multiplication of plants.

KEYWORDS: *Aloe barbadensis* Mill., micropropagation, phytohormone, microshoots, medicinal plants, shoot tip culture.

INTRODUCTION
Aloe vera is a very important natural medicinal herb which is used worldwide in cosmetic and pharmaceutical industry. It belongs to the Liliaceae family. The leaves contain very useful secondary metabolite like anthracene derivatives, which occur either free or in the form of glycosides. A number of biological activities have been reported about substances present, such as antiseptic (saponins and anthraquinones), antitumoral (mucopolysaccharides), anti- inflammatory (steroids and salicylic acid), anti-oxidant (Vitamins) and immune regulator (glucomannans).

In nature, *Aloe vera* is propagated through lateral buds which is slow, very expensive and low income practice.[1] Propagation is primarily done by means of off shoots, which are separated carefully from mature plants and transplanted. Through conventional method of propagation it is difficult to meet the demand of pharmaceutical industry.

Tissue culture technique can be exploited for meeting the demand of this plant. The technique to tissue and organ culture is used for rapid multiplication of plants, for genetic improvement of crops, for obtaining disease-free clones and for progressive valuable germplasm.[2] Micropropagation of the plant under an efficient protocol in laboratory condition providing artificial media composing the essential nutrients and growth regulators and the transfer of the new plantlets to the field can be exploited to meet its demand.

For present studies, shoot tip explants were used for initiation of cultures. Shoot tip culture method has been more successful in herbaceous plants because of weak apical dominance and strong root generating capacities as compared to woody species.

MATERIALS AND METHODS
Modern plant tissue culture techniques are performed under aseptic conditions in HEPA filtered air provided by a laminar flow cabinet. Young and juvenile nodes and

shoots with young leaves of *Aloe vera* were collected from Herbal and Conservatory Garden of Non-wood Forest Products Division, F.R.I, Dehradun. Shoots and axillary buds and leaves were used as explant material. The work was performed in Plant Tissue Culture Lab of Biotechnology department of Dolphin (P.G.) Institute of Biomedical & Natural Sciences, Dehradun.

Sterilization of explants were performed in which axillary nodes and shoots were washed in running tap water to remove the dust particles adhering to surface. Then they were washed with surfactant Tween- 20 (3 drops per 100 ml solution) in gentle agitating for about 2-3 minutes. After that it was thoroughly washed with distilled water for 5-6 times. Now different concentration of $HgCl_2$ were allowed for surface sterilization. Surface disinfected nodal explants were inoculated onto full strength MS medium supplemented with different concentrations and combinations of plant growth regulators that are BAP and IBA.

Sterile explants were inoculated to autoclaved MS basal medium supplemented with different combination of BAP and IBA, contained in pre sterilized clear bottles aseptically using a sterilized forceps and carefully transferred to tissue culture room, for shoot regeneration. The explants were inoculated in various concentrations and combination of BAP (0- 3 mg/L) and Kn (0-1 mg/L) at different concentrations in combination with IBA (0.5 mg/L). Media poured in culture vessels and were steam sterilized by autoclaving at 121°C and 15 psi for 15-20 min. The incubation of cultures were under controlled conditions of temperature (25±2°C), light (2000 – 2500 lux for 16 h/d provided by fluorescent tubes) and 60-70% humidity.

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

Newly formed shoots measuring 3-4 cm 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 1 mg/L). Three- five shoots per culture bottle were used and 5 replicates were used per treatment. The data were recorded after 15 days of culture.

RESULTS

In this present study nodal segments and shoots were used for micropropagation of Aloe vera. For sterilization of explants the nodal explants were washed with surfactant tween 20 further treated with 0.1% $HgCl_2$ for 2 mins, and 85% of the cultures were contamination free. It was observed that explants were very susceptible and majority of them turned brown when treated with 0.2% $HgCl_2$ for 5 mins. However in 0.3% $HgCl_2$ the survival rate of the explants got reduced when treated for 5 mins the explants turned dark brown. Young leaves were treated with 0.1% $HgCl_2$ for 2 mins and 80% of the green cultures were contamination free. It was observed that explants were very susceptible to $HgCl_2$ and majority of them turned brown when treated with 0.2% $HgCl_2$ for 5 mins and when treated with 0.3% $HgCl_2$ for 5 mins, they turned black and dead. Table 1 shows the effects of different concentrations of $HgCl_2$ on the survival of explants.

After surface sterilization of shoot tip explants, the explants were inoculated in culture bottles aseptically. For proper inoculation, the explants were transferred to large sterile glass petridish 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 (2-3 cm), explants are inoculated to culture bottles containing MS medium with (0.2 mg/L BAP and 0.2 mg/L IBA). After two weeks of observation, all explants gave axenic cultures. 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.

Table 1: Effect of different concentration of $HgCl_2$ on the survival of explants (nodes and young leaves).

S. No.	Sterilant	Concentration	Explant	Time Duration	Survival %	Explant Condition
1.	$HgCl_2$	0.1%	Node	2 mins	85	Green
				5 mins	60	Greenish brown
		0.2%	Node	2 mins	40	Greenish brown
				5 mins	20	Brown
		0.3%	Node	2 mins	15	Greenish brown
				5 mins	5	Brown
2.	$HgCl_2$	0.1%	Leaves	2 mins	80	Green
				5 mins	55	Greenish brown
		0.2%	Leaves	2 mins	40	Greenish brown
				5 mins	20	Brown
		0.3%	Leaves	2 mins	10	Dark brown
				5 mins	Dead	Black

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 BAP and Kn (in combination of IBA 0.2 mg/L) for shoot proliferation. Both BAP and Kn were found to be give the indications of shoot proliferation after 2 weeks of incubation. It was found that BAP gave better shoot proliferation than Kn. In medium containing BAP in different concentration, on an average each explant gave rise to 3- 4 shoots. Hundred percent cultures showed shoot proliferation on BAP containing medium. On medium containing Kn 1.0 mg/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.

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.

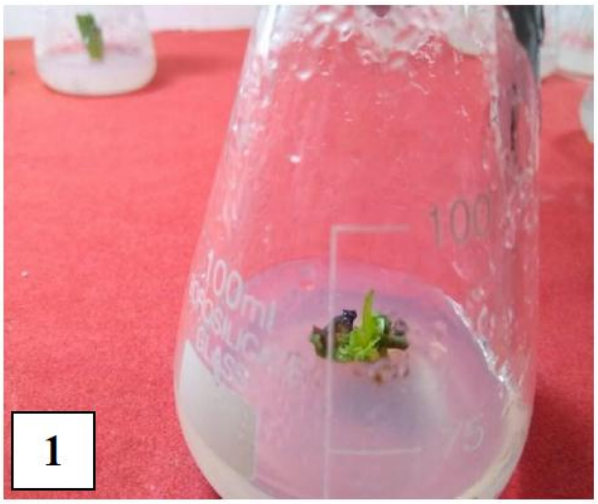
However, the response was better in hormone- free medium. After the 15 days of inoculation, rooting was 100% in hormone- free medium. The number of roots per shoot was more on hormone - free medium. In case of hormone- free medium, roots were more thick and elongated, while the roots on hormone supplemented 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.

Table 2: Shoot induction frequency of explants on MS medium with different concentrations of plant growth regulators.

Media	Total no. of explants	No. of explants showing shoots	Shoot induction percentage
MSB_1	10	8	80
MSB_2	10	6	60
MSB_3	10	4	40
$MSB_{0.5}I_1$	10	4	40
$MSB_{0.5}I_2$	10	6	60
$MSB_{0.5}I_3$	10	4	40
$MSB_1I_{0.5}$	10	9	90
$MSB_2I_{0.5}$	10	8	80
$MSB_3I_{0.5}$	10	6	60

Table 3: Root induction of explants on MS medium with different concentrations of plant growth regulators.

Media	Total no. of explants	No. of explants showing roots	Root induction percentage
MS	10	7	70
MSI_1	10	4	40
MSI_2	10	2	20
MSI_3	10	3	30
$MSI_1Kn_{0.5}$	10	4	40
MSI_2Kn_1	10	3	30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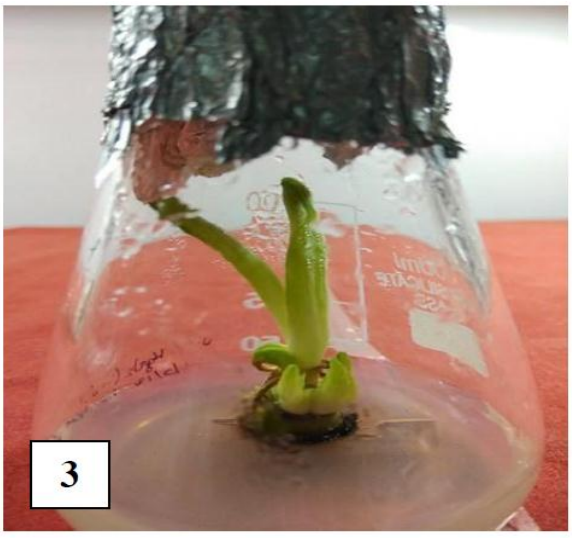
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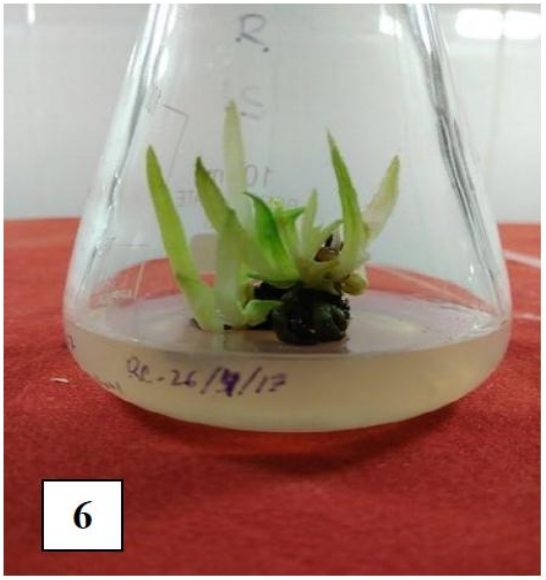
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Figure: Successful *in vitro* culture of *Aloe Barbadensis* Mill. from shoot tip explants. 1-2: Shoot induction and proliferation on MS medium supplemented by BAP and IBA. 3-6: Multiple shoot formation on MS medium supplemented by BAP and IBA. 7: Root proliferation on MS medium supplemented by BAP and Kn.

DISCUSSION

The use of field grown plants as direct sources of explants for the production of 'clean' *in vitro* plantlets, presents a major challenge. This is because the surface on the plant carries a wide range of microbial contaminants. To avoid this source of infection, the explants must be thoroughly sterilized before inoculating them onto the nutrient medium. In the present study, 0.1% mercuric chloride solution for 2 min proved to be efficient in controlling contamination on explants. Surface disinfected explants showed best results of shoot proliferation on MS medium supplemented with 1.0 mg/L BAP. For shoot proliferation, growth.[3][4][5][6] regulators especially cytokinins are one of the most important factors affecting the response. A range of cytokinins (Kn, BAP, 2-ip and Zeatin) has been used in micropropagation work.[2][7] A number such as blueberry and garlic[5] were successfully multiplied by using 2-ip. But a wider survey of the existing literature suggests that BAP is the most reliable and useful cytokinin. A number of plants has been successfully multiplied on medium containing BAP. It is the most effective cytokinin for the shoot tip, meristem and bud culture. At higher levels cytokinins tends to induce adventitious bud formation.[8] In the present study also, shoot proliferation occurred only in the presence of cytokinin.

Rooting response of microshoots is reported to be controlled by growth regulators in the medium,[2] basal salt composition,[6][9][10] genotype[11] as well as cultural conditions.[12] For most of the species auxin is required to induce rooting. NAA and IBA are most commonly used for root induction.[2] By the use of IBA many plants such as *Lycoperscicon esculemtum*,[13] *Hedychium roxburgii*[14] 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 done earlier.[1][15][16]

In Aloe vera induction of roots in hormone medium also reported for some other plants like *Gasteria and Haworthia.* 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. These plants are grown under controlled conditions. Under these conditions the leaves of plants develop cuticle and its photosynthetic system starts functioning.

CONCLUSION

Aloe vera is an indigenous 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 explants, shoot proliferation, rooting of micro shoots.

Aloe is extensively used internally as a laxative, antihelminthic, haemorrhoid remedy, and uterine stimulant (menstrual regulator); in combination with

licorice root, to treat eczema or psoriasis. The plant contains the important antioxidant vitamins (A, C and F), B1 (thiamine), niacin, B2 (riboflavin), B12, choline and folic acid. Regeneration of Aloe vera in nature (*in- vivo*) is too slow and insufficient to meet the industrial demand. Also they experienced a slow increase due to limited availability of raw material with high quality. Therefore, there is a need to develop suitable and alternative method for traditional propagation like *in- vitro* propagation for rapid plant production. Micropropagation is performed by using stem and lateral pieces of Aloe vera. However; source of explants, size, age, genotype, media composition, culture conditions and phenolic content of explants and media discoloration greatly affect shoot regeneration from different genotypes of the same species. The prime objective of this study was to carry out the alternative protocol for rapid *in vitro* propagation of this medicinally important Aloe vera.

In the present study, 0.1% mercuric chloride ($HgCl_2$) proved to be efficient in controlling contamination of nodal explants. The young leaves were treated with 0.1% $HgCl_2$ for 2 mins. 80% respectively of the green cultures were contamination free. Then the present study shoot proliferation was best observed on medium supplemented with 1.0 mg/L BAP. It was also observed improvement in shoot multiplication. The different concentrations of BAP 1.0-3.0 mg/L in medium along with Kn (0.5 mg/L) and also different concentration of IBA (1.0-3.0 mg/L) along with BAP (0.5 mg/L) in the medium. Best shooting response was observed on medium containing 0.5 mg/L Kn + 1.0 mg/L BAP (shoot induction frequency 0.9). The maximum amount of root proliferation was observed in hormone free MS basal media and less amount of roots were achieved in MS medium supplemented with IBA (1.0 mg/L). Regenerated plants were found to be morphologically similar to the mother / control plant.

It could be concluded that the tissue culture protocol used in this study could successfully be applied for mass propagation of Aloe vera. Therefore, development of a rapid clonal multiplication of the medicinal important plant Aloe vera has become imperative in order to reduce existing pressure of natural population and supply constant plant material for pharmaceutical industry.

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