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In-vitro Propagation of *Aloe vera* (L.) Burm. *f*

Sharmistha Gupta[1], Pankaj K. Sahu[2*], Devki L. Sen[3] and Priyanka Pandey[2]

[1]West Bengal State Council of Science & Technology, DSTGo WB, Kolkata, West Bengal, India.
[2]Department of Botany, Dr. C.V. Raman University, Bilaspur, (C.G.) India.
[3]Department of Botany, Government Science College, Raipur, (C.G.) India.

Authors' contributions

This work was carried out in collaboration between all authors. Authors SG and PKS designed the study, performed the analysis of study, wrote the protocol, and wrote the first draft of the manuscript. Author PKS improved the manuscript and Authors DLS and PP managed the Literature searches. All authors read and approved the final manuscript.

Short Research Article

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ABSTRACT

Aim: *In vitro* culture is used for commercial production and is achieved in aseptic condition using different concentration and combination of Plant Growth Regulators (PGR).
Material and Methods: In present work studied the effects of different plant growth regulator singly or in combination in tissue culture of *Aloe vera* (L.) Burm. *f*. belongs to the family *Liliaceae* or *Asphodelaceae*, used in ayurveda as well as pharmaceutical industry. Murashige and Skoog (MS) media with different combinations and concentration of growth promoters i.e. Auxin (Indole-3-butyric acid (IBA), α-Naphthalene acetic acid (NAA) and Indole-3-acetic acid (IAA) and cytokinin (Benzyl Amino Purine (BAP) were used in this study. Surface sterilization was standardised with $HgCl_2$ with various concentration and time.
Observation and Result: The development of callus type was observed in the MS media supplemented with BAP for best regeneration, IBA for root formation and NAA, was found the best media for root formation (0.5mg/l) were seen to grow onwards from the tenth day of culture and 90% of root formation took place within a span of 3-4 weeks for maximum callus induction. The maximum number of root & shoot produced is 4.8±0.53 with average length of 3.5±0.35cm.

**Corresponding author: Email: sahu.pankaj1@gmail.com;*

Discussion: The present work deals with *in vitro* plant growth of *Aloe vera* through tissue culture for propagation and *ex situ* conservation. Regenerated plants after acclimatization were transferred to soil and they showed 85% survival. The culture response was maximum in apical buds (100%) and minimum time required is 9.67 days in the same media. The average length of shoots per culture was 4.0±0.16cm. In present study among the three types of auxins, NAA was found to be the best for root induction.

Keywords: Aloe vera; micropropagation; MS medium; in vitro; plant growth regulator.

1. INTRODUCTION

Aloe vera (L.) Burm. *f*. (Ghritakumari) is perennial herb is used for cosmetic, burn and medicinal application and it remains for us to introduce it to ourselves and thank the nature for its never-ending gift belongs to family *Liliaceae* or *Asphodelaceae* [1]. *Aloe vera* (L.) Burm. *f*. had been cultured *in vitro* by various researchers by [2-8]. Natural propagation of *Aloe vera* is primarily by means of axillary shoots and it is rather a slow way of multiplication to meet the growing demand and the presence of male sterility is also a barrier in rapid propagation [3]. In India, Tissue culture research began nearly four decades ago with the first report on production of test tube fertilization. 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 [9]. Tissue culture methods have also been employed to study the basic aspects of plant growth, metabolism, differentiation and morphogenesis and provide ideal opportunity to manipulate these processes. Advancement in tissue cultural methodology led many recalcitrant plants amenable to *in vitro* regeneration and to the development of haploids, somatic hybrids and pathogen free plants.

1.1 Morphology of *Aloe vera*

Aloe vera (L.) Burm. *f*. syn. *Aloe barbadensis* Miller, succulent perennial herb with triangular, sessile stem, shallow root system, fleshy, serrated leaves, arranged in rosette having 30-50 cm length and 10 cm breadth at the base; colour pea-green. The bright yellow tubular flowers, length 25-35cm, axillary spike and stamens are frequently projected beyond the perianth tube and fruits contain many seeds [10]. Leaves have three layers; the outer most layers consist of 15-20 cells thick protective layer synthesizing carbohydrates and proteins [11]. The juice that is originated from cells of the pericycle and adjacent leaf parenchyma, flowing spontaneously from the cut leaf get dried with or without the aid of heat and get solidified should not be confused with *Aloe vera* gel which is also the colourless mucilaginous gel that is obtained from the parenchymatous leaf cells [12].

1.2 Chemical Constituents

Aloe vera has marvellous medicinal properties due to nutritional ingredients in *Aloe vera*. The ten main chemical constituents of *Aloe vera* include: amino acids, anthraquinones, enzymes, minerals, vitamins, lignins, monosaccharide, polysaccharides, salicylic acid, saponins, and sterols [1]. *Aloe* has complex chemical ingredients, from literature; there are Aloe has over 100 secondary complex metabolites in leaf [13].

2. MATERIALS AND METHODS

The targeted species for the present experiment is *Aloe vera* (L.) Burm. *f*. Explants were collected from the Green house of Botanic garden, Plant Biotechnology Unit at Banabitan Complex Salt Lake and research work was done at Laboratory of West Bengal State Council of Science and Technology, Kolkata.

2.1 *In vitro* Propagation

Tissue culture Murashige and Skoog (MS) medium was prepared from the stock solutions & vitamins etc. along with 3% sucrose in some amount of distilled water (dH_2O) details given below. Final volume of the medium was made up to 1 litre after adjusting the pH to 5.7. For solidification, 0.8% (w/v) agar was added. The media were then dispensed in culture tubes and sterilized by autoclaving at 15 psi at 121ºC for 15 minutes. The media were stored in aseptic condition for 48 hours and used after screening for contamination.

Profuse leaves with nodal part were collected from plants already maintained in the green house of botanic garden. After trimming the leaves were cut into pieces (2.5-3.0cm) each with a single node. Profuse leaves tips with one node were collected and fully expanded leaves were removed and both segments were thoroughly washed under running tap water to remove soil and superficial contaminants. They were then dipped in 5% of Teepol (liquid detergent) for 10min. The explants were then washed with distilled water (dH_2O). Further steps of surface sterilization were carried out in the laminar airflow cabinet where they were treated with 0.05-0.1% $HgCl_2$ for 5-15min., followed by a quick rinse in 70% ethanol. Thorough rinsing with sterile dH_2O for 4-5 times was done to remove all traces of the chemicals. Explants were finally prepared by trimming them to 1-1.5cm, which are now ready for inoculation.

2.2 Leaf Explants

Leaves from healthy plants of *Aloe vera* (L.) Burm. *f*. were collected from mature plants and washed thoroughly under running tap water followed by a dip in 5% Teepol (liquid detergent) for 5min. The leaves were washed clean of any traces of detergent prior to transfer to laminar flow cabinet. Further sterilization was done with 0.05-0.4% $HgCl_2$ for 5-15min., followed by a quick dip in 70% ethanol for 1min. and then washed thoroughly with sterile dH_2O 3-4 times to remove all traces of chemicals. The leaves were placed over sterile blotting paper for soaking the excess water from the surface. With the help of a sterile blade, the leaves were then cut into rectangular sections of 5mm by 5mm with the midrib intact and placed on the medium with the dorsal side down.

During the process the glass wares were soaked in commercial detergent for 1 hr and then the traces of detergents were removed thoroughly by washing with tap water and then rinsed with distilled water (dH_2O). To recycle the glass wares that had contaminant, the glassware was autoclaved directly without opening the cotton plug to destroy the microbial contaminants and then washed thoroughly. The glass wares were then allowed to dry in hot air oven at 100ºC. The forceps and scalpels were first rubbed with cotton wool dipped in absolute alcohol and then wrapped with aluminum foil. Petri dishes along with blotting papers were put in plastic packets. Three different explants were tested, *viz.* apical bud, nodal bud and leaf to observe the better totipotency and response. The culture tubes, flasks, pipettes and other accessories were properly cleaned before use. The basal medium in

which the explants exhibit regeneration was further supplemented with various concentrations and combinations of growth regulators viz., cytokinin and auxin, depending on the type of experiments carried out to study their effects on *in vitro* response of different explants. Basal media devoid of growth regulators was used as control for all the tissue culture experiments. All these accessories were autoclaved at 15 psi at 121ºC for 30min. and kept in culture room.

2.3 Hormonal Stock Solutions

Cytokinin: BAP (6-benzyl amino purine), TDZ (Thidiazuron) and Kinetin (6-furfuryl amino purine) stock solutions were prepared by dissolving 5 mg each of plant growth regulator in a few drops of 1N NaOH and stirred gently. The volume was made up to 10 ml by adding dH_2O. They were stored at 4ºC. *Auxins:* IAA (Indole-3-acetic acid), NAA (α-Naphthalene acetic acid), 2, 4-D (2, 4-Dichloro phenoxy acetic acid), IBA (Indole-3-butyric acid) were used singly or combination with Benzyl Amino Purine (BAP). The stock solutions of the above plant growth regulators were made by dissolving 5 mg each of the above plant growth regulators in a few drops of 1 N NaOH. The volume was made up to 10ml by adding sterile dH_2O. The stocks were stored at 4ºC.

2.4 Inoculation, Incubation and Subculture

The media used was Murashige and Skoog and various plant growth regulator combinations and concentrations were used to select the best media suitable for each explant. Laminar airflow cabinet was properly cleaned with absolute alcohol. Forceps and scalpels already sterilized by autoclaving were properly flamed once again to avoid the risk of contamination during inoculation. The explants already prepared were placed on media in culture tubes and flasks. The cultures were kept at an incubation temperature of 25±2ºC and light 45 micro (µ) $m^{-2}s^{-1}$ for 16h per day using fluorescent lights. Transfer of the inoculated materials into fresh media with same composition as the parent or into media with a different composition was done depending on the purpose of the study, regularly at an interval of 15-30 days. The interval at which subculture was done was sometimes a factor of the type of explants, stage of growth and growth rate.

3. OBSERVATION

3.1 Shoot Tip/Nodal Explants

The most suitable combination of growth regulator by recording the multiple shoot induction and the number and growth of shoots produced per explants at the end of subculture. For micro propagation, each treatment had 10 replications and was repeated thrice.

3.2 Leaf Explants

Leaf growth was observed in respect to callus induction and callus growth and whether any organogenesis or embryogenesis occurred or not. Days to swelling of leaves, days to callus initiation, colour and texture of callus and regeneration capacity were recorded for each treatment, amount of the callus has been denoted by '+' or '-' symbol.

3.3 Rooting of Shoots Produced *In vitro*

Shoots produced from nodal segments were transferred by separating the multiple shoots into flask containing MS media with different concentrations of IBA. The optimum concentration for root initiation and development was considered to be the one which took least time to initiate root [Figs. 1-3].

Fig. 1. Profuse leaves induced from callus

Fig. 2. *In vitro* germination of plantlets

Fig. 3. Multiplication of seedling

3.4 Acclimatisation of Plantlets

Development of maximum number of healthy roots of the *in vitro* raised shoots in the rooting medium for 4-5 weeks. Hardening of rooted plants was carried out in pots containing 1:2 (v/v) mixture of sterile sand and soil in the green house at 25±2ºC under 2000lux light intensity provided by white fluorescent lamps for 16h photoperiod. During the first week of hardening period, regenerated plants were covered by perforated polythene sheets for maintaining high humidity and irrigated with sterile distilled water, followed by irrigation with tap water in the second week. After an additional 2-3 weeks of incubation hardened plants were transferred to the field.

4. RESULTS AND DISCUSSION

This is an effort to develop an efficient *in vitro* tissue culture protocol to obtain maximum plantlet regeneration that has a tremendous importance in *ex situ* conservation of biodiversity. The formulation of the best nutrient media, selection of right explants and role of plant growth regulators either in single or in combination is a prerequisite for exploitation of tissue culture technology in *ex situ* conservation strategies. Keeping this information in mind, a series of experimentation has been carried out on a medicinally important plant, *Aloe vera* (L.) Burm. *f*. Tissue culture technologies have a tremendous impact on the *ex situ* conservation of medicinal plants and *in vitro* maintenance of germplasm which provides an excellent means of mediating international germplasm exchange.

Surface sterilization was standardized with $HgCl_2$ at varying concentrations and time duration. Among the different concentrations so far tested the best concentration were 0.1% $HgCl_2$ (w/v) for 10 min. This combination was found to give best results (80% successful sterilization) in both apical and nodal explants. Further washing was made in both the explants in 70% ethyl alcohol after $HgCl_2$ treatment for protection from fungal contamination. Finally the explants were washed at least 3 to 5 times with sterile distilled water for 5 min each. Standardization of selection procedure of *Aloe vera* (L.) Burm. *f*. was done in two different concentration (0.05) and (0.1) of $HgCl_2$ with regular interval of 5, 7, 10, 12 & 15 days. The total 10 inoculation were done for each concentration and observed the percentage of fresh & healthy *in vitro* callus formation and was point out in 0.1 concentration of HgCl2 after 10 days was observed maximum germination up to 80-85%.

4.1 Effect of Diverse Explants in Governing *In vitro* Culture Response

Various explants such as apical, nodal segments and leaves were tested for understanding *in vitro* response in the nutrient media. Among the 3 explants, the apical bud explants gave the best results and were used for further experiments. The Table 1 shows a comparative studies of the three explants used for *Aloe vera* (L.) Burm. *f*. The percentage of culture response, the number of days required for the cultures to establish and the plantlet formation in culture medium was noted in all the explants (shoot & root). Interestingly the nodal explants gave rise to multiple shoots whereas the apical buds directly develop a single shoot rarely two shoots whereas the leaf explants gave little callus without formation of any shoot. The percentage of culture response was maximum in apical buds (100%) and minimum time required is 9.67 days in the same media.

4.2 Effect of PGR on Shoot Multiplication

In different sets of experiments, auxin and cytokinin were tested at different concentrations and their effects in shoot initiation and multiple shoot production. Shoot tip explants were cultured on MS supplemented with various concentrations and combinations of BAP, NAA and BAP alone for induction of adventitious. The best and rapid *in vitro* formation micro shoots/roots through the callus phase were observed on MS supplemented with 2mg/l BAP + 0.5 mg/l NAA. This treatment yielded highest number (75) of regenerated shoots with ten shoots per culture. The average length of shoots per culture was 4.0±0.16 cm. The apical bud explants initially produced three - five shoots within three to four weeks after inoculation. Subculture in the same medium yielded a cluster of eight to ten shoots per explants (Table 1).

Table 1. Percentage of explants response and days required for first bud break in *Aloe vera*

Explants	% of response	No of days required for bud break
Apical bud	100.0±0.00	9.67±0.88
Nodal bud	96.66±3.33	14.33±1.20
Leaf	0.00	0.00

Abrie and Staden [7], Chaudhuri and Mukundan [8], reported the use of BA in shoot proliferation of *Aloe polyphylla Schonl.ex Pillans* and *Aloe vera* (L.) Burm. *f*. respectively. Shoot groups were segregated into pieces, each of which was sub-cultured individually on the same medium. After the 4th subculture the shoot multiplication rate remained constant. On the other hand, *in vitro* formation of shoot buds was found low (50%) on a medium containing 0.5mg/l BAP and 0.5mg/l NAA and the number of shoots per explants was 3.2± 0.81, while in a medium containing BAP alone the number of adventitious shoots found was comparatively lower.

Further increase in the concentration of BAP had no effect on the number of multiple shoots. At the highest concentration (4.0mg/l) of BAP and concentration (2.0mg/l) of BAP combination with (0.5mg/l) or without NAA did not increase shoot proliferation (Table 2). The results reported in this work confirm that some plant species have enough levels of endogenous plant growth regulator s and do not require any extra amount of exogenous growth regulators for their regeneration [14]. BA alone in concentration 2.0mg/l has been also found effective for shoot proliferation in *Aloe vera* [15]. Tissue culture can accelerate propagating of *Aloe* and improve its partial economic traits [16-17]. The lateral buds developed into shoots 10-15 days after inoculation. Liao et al., [18] reported that a combination of BAP and NAA enhanced the multiple shoot proliferation from shoot tip explants of *Aloe barbadensis* Miller. Sanchez et al., [2] 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 micro-densitometric study was performed on different organs *of Aloe barbadensis* and during *in vitro* culture of different explants.

4.3 Effect of PGR on Rooting of Microshoots

Table 2 shows root formation was induced in *in vitro* regenerated shoots by culturing them on half strength of MS supplemented with 0.5 to 1.5mg/l of any of the three plant growth regulators IBA, NAA or IAA. Root formation was not observed when shoots were cultured on

a medium lacking auxin. Among the three types of auxins NAA was found to be the best for root induction. MS medium containing BA and NAA was found to be the best medium in Aloe micropropagation [18-19]. It was also reported that the highest shoot multiplication in *Aloe vera* was found in MS medium containing BA 1.0mg/l and IBA 0.2mg/l [20]. Liao et al., [18] showed the same results in MS supplemented with 2.0 mg/l BAP, 0.3mg/l NAA and 0.6 mg/l PVP. In contrast, the best shoot multiplication medium reported [8].

Table 2. Effects of MS medium supplemented with different concentrations of BAP and in combinations of NAA for root number and root length in *Aloe vera*

MS media supplemented with Plant Growth Regulator (mg/l)	% of explants of Roots	Number of Root per explants	Mean length of Root (cm)
BAP			
0.5	20	2.1±0.31	1.0±0.10
1.0	25	2.2±0.25	1.8±0.22
2.0	30	3.0±0.52	2.0±0.51
4.0	25	2.8±0.50	2.4±0.50
BAP+NAA			
0.5+0.5	50	3.1±0.81	2.8±0.31
1.0+0.5	60	4.4±0.52	3.0±0.50
1.5+0.5	70	9.5±0.50	3.7±0.16
2.0+0.5	65	4.3±0.28	2.9±0.52

MS supplemented with 10mg/l BAP + 160mg/l Ads + 0.1mg/l IBA. *In vitro* technique offers a possibility to solve these problems. Several reports have been noticed rapid *in vitro* propagation of *Aloe vera* [4,20-24]. In a medium with 0.5 mg/l of NAA, roots began to grow from the tenth day of the culture and 95% of root formation was achieved within 23-28 days. The highest number of roots per shoot being 4.8±0.53 with an average length of 3.5±0.35 cm. Quality of the roots was found to be poor in a medium containing higher concentration of (1.0 to 1.5 mg/l) auxins. Low dosage of NAA (0.5 mg/l) was found optimal, may be due to the genotypic and explants specificity (Table 3).

Table 3. Effects of MS medium supplemented with different concentrations of IAA, NAA and IBA for shoot number and shoot length in *Aloe vera*

MS media supplemented with Plant Growth Regulator (mg/l)	% of Explants of Shoots	Number of Shoots per explants	Mean length of Shoot (cm)
IAA			
0.5	35	2.0±0.15	1.0±0.21
1.0	40	2.2±0.35	1.4±0.30
1.5	33	1.6±0.55	1.2±0.55
IBA			
0.5	66	2.4±0.22	1.5±0.21
1.0	70	2.8±0.41	1.8±0.51
1.5	73	3.0±0.51	2.2±0.24
NAA			
0.5	95	4.7±0.53	3.5±0.35
1.0	90	2.8±0.30	2.4±0.21
1.5	80	2.6±0.26	2.2±0.51

4.4 Acclimatization of Plantlets

Plants which were transferred directly to the field did not survive. The plantlets with well-developed roots were transferred to polythene bags and the acclimatised plants were finally transferred to soil with 70% survival rate. The protocol described in this paper is recommended for high frequency regeneration as well as for conservation of this important medicinal plant. For *in vitro* conservation using tissue culture methods is the only reliable, long term means of preservation. Diverse parameters of Cytokinins (BA, Kinetin) has been used in micropropagation reported [25]. Shoots (8-9cm long) with sufficient rooting were transferred to ½ strength MS liquid medium with filter paper raft support for hardening, for two weeks. These well developed rooted plantlets were then transferred to sterile vermiculite and covered with poly bags for 2 weeks to retain moisture [Fig. 4]. They were irrigated with ¼ strength MS medium without sucrose and kept less than 16 hr light and 25±2ºC. Further they were transferred to soil and normal growth of the potted plants was kept in room condition for 1 week.

Fig. 4. Hardening of *Aloe vera*

After one month they were transferred to large pots containing soil and moved to agro shed. Hardening response was as good as 85%. According to observation of vast literature suggest that BA is the most reliable and useful cytokinin for shooting in higher plants. Many workers succeeded in their attempts for shoot proliferation by using BA [26].

5. CONCLUSION

The regenerated plants did not show detectable morphological variation with the donor plants. The modern biotechnology is being increasingly applied for species diversity characterization and undoubtedly they have a major role in assisting plant conservation programmes. Conservation of Biodiversity as rare medicinal plant and some rare tree species through micro propagation is considered fundamental and provided priority in all sectors of global development. Thus utilizing the biotechnological approaches towards the improvement of *in situ* and *ex situ* conservation programmes are becoming vital for rare medicinal plants.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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