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Manish Kumar
Krishi Vigyan Kendra (ICAR-NRRI-CRURRS), Koderma, Jharkhand, India

Binit Kumar
Krishi Vigyan Kendra (ICAR-NRRI-CRURRS), Koderma, Jharkhand, India

Chanchila Kumari
Krishi Vigyan Kendra (ICAR-NRRI-CRURRS), Koderma, Jharkhand, India

Namit Kumar Upadhyay
College of Biotechnology, Birsa Agricultural University, Ranchi, Jharkhand, India

Correspondence
Manish Kumar
Krishi Vigyan Kendra (ICAR-NRRI-CRURRS), Koderma, Jharkhand, India

In vitro morphogenesis of *Aloe vera*: The "Medicinal Wonder" Plant

Manish Kumar, Binit Kumar, Chanchila Kumari and Namit Kumar Upadhyay

Abstract
Aloe vera "cactus," actually a desert succulent, has been touted as one of the miracle plants. It is a complex plant containing numerous biologically active substances. The present study was focused on to minimize a fast and efficient protocol to propagate *Aloe vera* by tissue culture technique. Murashige and Skoog (MS) basal supplemented with 6-Benzyl Amino Purine (13.5 µM) and α-Napthalene Acetic Acid (8.0 µM) concentration showed best performance as compared to BAP alone (13.3 µM) for bud breaking, and two different combinations of BAP (13.3 µM) + Adenine sulphate (61.8 µM) and BAP (13.5 µM) + NAA (8.0 µM) in MS basal medium showed maximum shoot regeneration and development. The combination of NAA of 10.7 µM with BAP of 8.8 µM recorded the highest percentage (90%) of callus initiation. The best rooting was observed with NAA at 16.1 µM with BAP at 10.7 µM than Indole Acetic Acid (IAA) at 16.1 µM alone.

Keywords: Murashige and Skoog (MS), 6-Benzyl Amino Purine (BAP), α-Napthalene Acetic Acid (NAA) & Indole Acetic Acid (IAA)

Introduction

Aloe vera has long been known for its curative medicinal properties (Hirat and Suga, 1983) [14]. *Aloe barbadensis* Miller (also known as *Aloe vera*), a member of the family Liliacae, is cultivated for its thick fleshy leaves from which many substances are obtained. There are approximately 325 species of aloe (Klein and Penneys, 1988 [16] However, not all of them are useful in healing / cosmetic sources (fulton, 1990). It is propagated vegetatively and growth rate is very slow (Gui, Y.I *et al*., 1990). The demand of this plant is very high and mostly used in pharmaceutical and cosmetic industry (Aggarwal, D. *et al*. 2004 and Chaudhary A.K. *et al*.) [1, 3]. It is widely used in ayurvedic system of medicine against skin disease. *Aloe* leaves contain gel like substances which are very effective in wounds, burn and other diseases (Jayakrishna, C. *et al*., 2011) [15]. It is this gel that contains the plant's nutrients and healing properties (Klein and Penneys, 1988) [16]. First, it's a tremendous source of nutrients. *Aloe vera* contains over 200 nutritional compounds, including 200 nutritional compounds, including 20 minerals, 18 amino acids, 12 vitamins and including vitamin B12. However, *Aloe vera* actually helps the body cleanse itself so that it can better absorb those nutrients. In this way the cells of the intestinal wall can absorb essential food nutrients much more efficiently (Lorenzetti, 1964). The aloe "cactus," actually a desert succulent, has been touted as one of the "miracle" plants. *Aloe vera* is a complex plant containing many biologically active substances (Klein and Penneys, 1988) [16]. It has been reported that glycoprotein extracted from *Aloe vera* has a strong anti-inflammatory response (Davis, 1988; Davis *et al.,* 1991; Shelton, 1991) [8, 6] and antiallergic reactions (Ro *et al*., 1998), and that the polysaccharides, especially mannose-6-phosphate, in *Aloe vera* have strong wound healing activity and an anti-inflammatory response (Davis *et al.*, 1994a) [10]. It has also been reported that sterols extracted from *Aloe vera* have good anti-inflammatory activity. This study deals with the mucilagenous leaf-gel of *Aloe vera* which is well reputed for its therapeutically effect on inflammatory-based disorders (Hart *et al*., 1989) [12]. Using anion-exchange and gel permeation chromatography a highly active polysaccharide fraction was isolated, that is present in the gel in various chain lengths. The data obtained tend to suggest that gibberellins or a gibberellins-like substance is an active anti-inflammatory component in *Aloe vera* (Davis and Maro, 1989) [9]. Immunomodulatory compounds (which are functionally distinct) have been also characterized in detail in *Aloe vera* (Hart *et al*., 1989) [12].

The study revealed that the hypoglycemic effect of aloes and its bitter principle may be mediated through stimulating synthesis and / or release of insulin from the beta-cells of Langerhans. *Aloe vera* does not affect cutaneous erythema and blood flow following ultraviolet B exposure (Crowell *et al.*, 1989) [4]. Pharmaceutical companies largely depend upon materials procured from naturally occurring stands raising concern about possible extinction and providing justification for development of *in vitro* techniques for mass propagation of *Aloe barbadensis*. Due to low seed viability, germination rate, limited availability of raw material with high quality and slow vegetative growth, tissue culture technique is an alternate solution for conservation of those valuable medicinal plants (Swarna, J. *et al*., 2012). The explant is grown aseptically in growth medium under control environmental conditions. Micro propagation has great potential to capture genetic gain and to obtain uniform crop which is difficult under the naturally occurring *in-vivo* regime. Preliminary study on *in vitro* micropropagation of *Aloe barbadensis* by using meristems have been standardized by the various researchers (Baksha, R., *et al*., 2005) [2]. However, there is urgent need to develop efficient protocol on rapid propagation and genetically uniformity.

Materials and Methods

Plant material and explant source: Actively growing young shoots of *Aloe barbadensis* were collected from greenhouse grown plants and washed with 2% (v/v) detergent 'Teepol' (Qualigen, India) and rinsed several times with running tap water. The ex-plant were surface sterilized in 0.1 % (w/v) aqueous mercuric chloride solution for 20 min followed by four washings with sterile distilled water. The apical and axillary meristems (~ 2.0 mm) were isolated and used as explants.

Culture conditions: Each experiment for the *Aloe vera* was set up with 5 replications and repeated twice. The cultures were grown under 3000 lux light from Phillips fluorescent day tube for 16 hours light and 8 hours dark period. The ambient temperature was maintained at 25 ± 2 ^{0}C and the relative humidity was adjusted to approximately 55%.

Culture medium and condition: The meristem (apical and axillary) was placed on semi-solid basal MS medium [9] supplemented with different concentrations and combinations of 6- benzylaminopurine (BAP: 0.0, 0.5, 1.0, 1.5 and 2.0 mg/l), kinetin (Kn: 0.0, 0.5, 1.0, 1.5 and 2.0 mg/l) and 1-napthalene acetic acid (NAA: 0.0, 0.25 and 0.5 mg/l) for shoot proliferation and multiplication. The pH of the media was adjusted to 5.8 using 0.1N NaOH or 0.1N HCl before autoclaving. Routinely,

25 ml of the molten medium was dispensed into culture tubes (25 x 150 mm), plugged with non- absorbent cotton wrapped in one layer of cheese cloth and sterilized at 121 ºC and 1.06 Kg/cm2pressure for 15 min. The cultures were maintained at 25 ± 2 ºC either under 16 h photoperiod or continuous light (55 mmol m-2s-1) from cool, white fluorescent lamps. The cultures were maintained by regular subcultures at 4-weekintervals on fresh medium with the same compositions.

Induction of rooting and acclimatization: For root induction, excised microshoots were transferred to MS basal medium supplemented with different concentrations of NAA or IBA (0.0, 0.1, 0.25 and 0.5 mg/l) and 2% (w/v) sucrose. One excised shoot was placed in each tube (25 x 150 mm) having 15 ml of the culture media. All the cultures were incubated at 25 ± 2 ºC under 16 h photoperiod with cool, white fluorescent lamps. Rooted micro- propagules were thoroughly washed to remove the adhering gel and planted in 2.5 cm earthen pots containing a sterile mixture of sand: soil and cow- dung manure in the ratio of 1:1:1 (v/v) and kept in the greenhouse for acclimatization.

Observation of cultures and presentation of results: Twenty cultures were used per treatment and each experiment was repeated at least three times. The data pertaining to mean percentage of cultures showing response, number of shoots/culture and mean percentage of rooting were statistically analyzed by the Post-Hoc Multiple Comparison test [10]. Between the treatments, the average figures followed by the same letters were not significantly different at $P < 0.05$ level.

Growth Index Value

The Growth Index Rate (G.I.) of *Aloe vera* was measured at 15 days interval, starting from 15th days up to 90th day.

$$\text{Growth Index (G.I.)} = \frac{\text{Mean final Fresh weight} - \text{Mean initial Fresh weight}}{\text{Mean initial fresh weight}} \times 100$$

Statistical analysis

The significance of data observed from different treatments was analyzed statistically and calculate the S.Em, C.D. as per the formula given below

$$\text{S. Em} = \sqrt{\frac{\text{Variance due to error}}{r}}$$

Where S. Em =Standard Error mean

r =number of replications

Critical Difference (CD)

In order to compare mean of variance due to treatments, Critical Difference was calculated as follows:

CD = S. Em x t at 5% or 1% with error d.f.

Where S.Em is the Standard Error of Difference of the treatments to be compared and thus,

$$\text{(i) S.Em} = \sqrt{\frac{\text{2x Error mean sum of square}}{\text{No of replications}}}$$

(ii) t at 5% or 1% is the tabular value

Results and Discussion

The explants

The result obtained with respect to the suitability of ex-plants used in the micro propagation of *Aloe vera* showed the similar findings of Meyer & Staden, 1994. They obtained callus of *Aloe vera* from different ex-plants *viz*. leaves, stem and shoot tips (Richwine, *et al.,* 2001, Aggarwal and Barna, 2004) [1] and have reported multiple shoot production, using shoot tip of *Aloe vera* as ex-plant.

Survival percentage

The survival percentage of ex-plant showed in table-1 under both conditions *viz*. sub-culture and without sub-culture. It was observed that younger ex-plants (one to three weeks older) exude comparatively more phenolic compounds than the older ex-plants (four to seven weeks older). It ultimately

affects the survival percentage of inoculated ex-planting media containing different hormonal combinations. The higher exudation of ex-plant showed lower survival percentage than the lower exudates ex-plant. The survival percentage (85%) was found to be best in the four-week old ex-plant after the sub culturing.

Bud Breaking & Shoot Formation.

Bud breaking and shoot formation were observed in different phytohormonal combinations of BAP, BAP + NAA and BAP + ADS, the maximum percentage of bud breaking and shoot formation per ex-plant were observed in the medium containing BAP (13.3 µM) + ADS (61.82 µM) and BAP (13.3 µM) + NAA (8.0 µM) respectively (Table 2; Fig. 2a & 2b).

The effect of Bud breaking (%) and number of shoot multiplication were also studied by using the different concentration of BAP (1.11 µM to 13.3 µM) with respect to time. It was recorded after six weeks of culturing with BAP (13.3 µM) and NAA of (8.0 µM) on the ex-plant of *Aloe vera* in MS medium. Maximum number of shoots (86%) was obtained in 13.3 µM BAP and 61.8 µM $AdSO_4$ concentration. Similar findings were reported by Natali, et, al. (1999) in *Aloe vera,* Aggarwal and Barna (2004) [1] and Das & Rout (1991) [5] in *Dendrocalamus giganteus* and *Dendrocalamus strictus.*

Induction of rooting from microshoot

Half strength MS basal medium supplemented with various concentrations of either NAA or IAA was used for root formation. Root initiation was started within 8 - 10 days after transferring the shooted ex-plant in half strength MS basal media supplemented with 0.5 mg/l NAA or IAA. However, optimal rooting (75.6%) and growth of microshoots were observed on medium containing 0.5 mg/l NAA with 2% (w/v) sucrose.

Callus Response

For callus formation NAA and BAP were used in single and combination. It was stared after 21 to 30 days of inoculation of leaves. The best callus was found after four weeks in BAP (13.3 µM) + NAA (10.7 µM). The role of phytohormons could be more prominent for callus induction if a well suited carbon source is selected medium. Studies show that basic culture medium and carbon sources effect the callus formation and morphogenesis considerably. Similar results can be found in studies conducted by other worker. Meyer & Staden, (1996) obtained soft and brown callus of *Aloe vera* from the leaf ex-plant in MS medium supplemented with 10.7 µM NAA + 4.4 µM BAP. Aggarwal and Barna (2004) [1] could successfully initiate callus of *Aloe vera* from leaves in MS medium supplemented with 0.1 mg/l BAP and 0.01 mg/l NAA.

Root Formation

Root formation started after three and four weeks of inoculation of shootlets in rooting media which contained higher percentage of Auxin as compared to Cytokinin. The maximum percentage of root formation was observed in NAA (16.1 µM) + BAP (10.7 µM) at six week after inoculation in rooting media. The number of roots observed per ex-plant varied from 3.0-10.0 after four weeks, and 5.0-15.0 after six weeks, similar findings were also reported by Sivaram and Mukundan, 2003 that rooting of the *in vitro*-derived shoots could be achieved following subculture onto auxin-containing medium. Low concentration of IAA (1.3 µM) produced less amount of roots (3.5). With further increase in the concentration of IAA, increase in the percentage of shootlets showing rooting was obtained (16.1 µM IAA produced 12.5 root, per shootlets after 6weeks). The minimum number of roots was observed in 1.3 µM IAA and maximum percentage (90.5 %) was observed in 16.1 µM of IAA after 6 weeks. The present investigation is substantiated by the other work Saxena and Bhojwani (1994) [19].

Acclimatization and field establishment

After the emerging of root the plants were transferred into mist chamber house in sterile pots that was filled with cocopit and sand (1:1) for 15 to 20 day there after they were transferred into net house for 20 to 25 days. The plants were ready to cultivate in the field condition. 80% of survival was recorded from pot to field condition.

Table1: Effect of age, phenolic exudation and sub-culturing on survival percentage of explants.

Age of ex-plant weeks	Degree of phenolic exudation	Survival % (without sub culturing)	Survival % (with sub culturing at each 15 days interval)
01	++++	22.25	42.00
02	+++	34.75	64.75
03	+++	41.75	75.50
04	++	64.25	85.25
05	++	52.50	76.50
06	+	47.25	57.50
07	+	41.25	51.00
SEm		0.866	0.626
CD1%		3.3	2.38

Table 2: Effect of BAP, ADS and NAA concentrations on bud breaking and shoot formation.

BAP	0.0	1.3	2.2	5.5	8.8	11.1	13.3	NAA
(µM)								
0.0	-	+	+	++	++	++ +	++ ++	
1.3	-	+	++	++	++	++ +	++ ++	
2.6	-	-	+	++	++ +	++ ++	++ ++	
5.4	-	-	-	++	++	++ ++	++ ++ +	
8.0	-	-	-	+	++	++ +	++ ++ +	
10.7	-	-	-	-	+	++ +	++ +	
16.1	-	-	-	-	-	+	++	
Adenine Sulphate (ADS)								
24.72	-	+	+	++	++ +	++ +	++ +	
61.87	-	+	++	++ +	++ ++	++ ++	++ ++ ++	

Table 3: Effect of BAP on Bud breaking percentage and shoot multiplication with respect to time.

BAP	Bud Breaking (%)	Bud Breaking (%)	Bud Breaking (%)
(µM)	(2 –weeks)	(2 –weeks)	(2 –weeks)
1.11	11.00	20.75	51.00
2.22	15.50	34.00	60.50
4.44	21.00	34.00	64.00
5.55	25.50	50.50	65.50
11.1	35.50	61.00	71.00
13.3	41.00	64.50	74.50
SEmean	0.473	0.55	0.485
CD1%	2.425	2.75	2.42

Table 4: Effect of BAP and NAA on Bud breaking percentage and shoot multiplication with respect to time.

NAA (µM)	0.0	1.3	2.6	5.4	8.8	10.7	16.1	BAP
0.0	-	+	+	++	++	+++	+++	
1.1	+	+++	+	+	++	+++	+++	
2.2	+	+	∞∞∞∞∞∞	+	++	+++	+++	
4.4	+	+	++	∞∞∞∞∞∞	++	+++	+++	
5.6	++	++	++	++	++	∞∞∞	+++	
8.8	++	++	++	++	+++	∞∞∞	+++	

Table 5: Effects of (NAA +BAP) combinations of phytohormones on the formation of roots.

NAA+BAP (µM)	(%) Root initiation (4 weeks)	(%) Root initiation (6 weeks)	Number of Roots per Explant (4 weeks)	Number of Roots per Explant (6 weeks)
2.6+1.11	30.75	55.50	3.50	5.25
5.3+ 2.22	40.50	60.75	4.50	5.75
8.0+2.2	51.00	65.00	5.50	7.00
8.0+4.44	51.50	65.50	5.75	7.50
10.7+2.22	60.75	70.50	6.75	7.00
10.7+4.44	61.75	71.50	7.50	7.50
13.3+4.44	65.50	75.00	8.50	10.5
13.3+8.88	65.50	80.00	8.75	11.25
16.1+4.44	75.50	82.00	9.25	11.50
16.1+8.88	77.50	84.00	10.50	13.50
16.1+10.7	80.75	85.50	10.50	15.0
SE-mean	0.370	0.670	0.40	0.416
CD%	1.11	2.00	1.12	1.24

Table 6: Effects of (NAA +BAP) combinations of phytohormones on the formation of callus formation.

BAP(µM)	0.0	1.1	2.2	4.4	8.8	11.1	13.3	NAA
0.0	_	+	+	+	++	+++	++	
1.3	+	++++	+++	++	++	+	+	
2.6	+	+++	****	+++	++	++	++	
5.4	++	++	++	*****	++++	++	+++	
8.0	++	++	++	+++	+++++	+++++	+++	
10.7	++	++	+++	+++	******	++++	++++	
16.1	++	++	+++	++++	++++	++++	****	

* = callusing + = curling

Fig 1: Mother plant of *Aloe vera*

Fig 3: Shoot tip of *Aloe vera* showing shoot multiplication in BAP +NAA.

Fig 2: Shoot tip of *Aloe vera* showing bud breaking

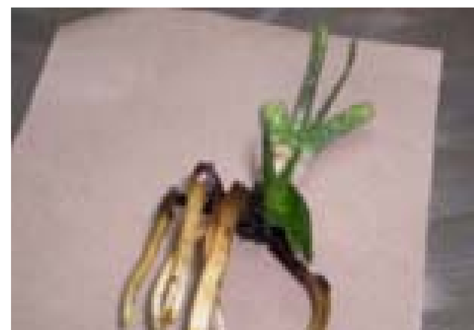

Fig 4: Shoot tip of *Aloe vera* showing root multiplication in BAP +NAA.

Fig 5: Callus in MS medium.

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