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Original Research Article

In vitro Micropropagation of *Aloe vera* – Impacts of Plant Growth Regulators, Media and Type of Explants

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ABSTRACT

***Aloe vera is* an important medicinal perennial herb belonging to the family liliaceae. A method for mass propagation of *Aloe vera* by using different explants and different media with different PGR contain has been developed. Two type of explants (with and without sheath Type A and B respectively) were cultured on MS, B5 and SH media supplemented with different combination of different NAA with BA and Kin for Shoot induction. Highest rate of shoot induction observed in MS medium supplemented with 0.2 mg l^{-1} NAA and 4 mg l^{-1} BA in type A explants. Also, the highest shoot proliferation response obtained successfully by using MS medium containing 4 mg l^{-1} BA. The optimal rooting response was observed on B5 medium supplemented with 2 mg l^{-1} NAA, on which 100% of the regenerated shoots developed roots with an average of 7.8 roots per shoot within 3 weeks. The plantlets were acclimatized and transferred to greenhouse with 95% success. This *in vitro* propagation protocol should be useful for conservation as well as mass propagation of this medicinal plant.**

Key Words: Acclimatization, Callus induction, Medicinal Plant, Rooting, Shoot Regeneration

INTRODUCTION

Aloe vera, a monocotyledonous, is a member of the family liliaceae. The genus Aloe has more than 500 species but only a few are medicinally important (Deng et al., 1999). Among these, *Aloe vera* is the plant of greatest interest. Its leaves have been found to contain over 200 bioactive constituents (Waller et al., 1978). *Aloe vera* contain different bioactive matherials such as saponins, anthraquinones, mucopolysaccharides, steroids, vitamins and glucomannans (Liu et al., 2006, 2007). Due to the wide spectrum of application in human health, the products of *A. vera* have showed a strong demand in both Iran and international markets. In nature, *A. vera* is propagated through lateral buds, which is slow, expensive and low income practice (Aggarwal and Barna, 2004). Sexual reproduction by seeds due to male sterility in *Aloe vera* is almost not effective and vegetative propagation through off shoots only possible during growing seasons. Thus, there is a need to undertake its large – scale cultivation. To overcome this problem in vitro cloning and multiplication (by means of development of apical meristems as explants cultured in appropriate basal medium, for instance) in commercial scale can be a solution and further it can dissolve the time of propagation. There are different research reports on the shoot regeneration and proliferation of various species of the Aloe genus (Hosseini and Parsa, 2007; Albanyl, 2006). 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, whereas Roy and Sarkar (1991) and Natali et al. (1990) obtained shoots on medium containing 2,4-D and Kn. Richwine et al. (1995) reported the induction of shoots using zeatin. Debiasi et al. (2007) and Liao et al. (2004) studied the effects of BA, IAA and NAA on bud initiation. Applying of plant growth regulators is necessary for rooting in culture (Abrie and Staden, 2001; Feng et al., 2000; Hongzhi, 2000). However, the protocols developed for the micropropagation of Aloe species were not promising because the multiplication rate was low. The present investigation was launched, to maximize micropropagation rate of *A.vera*. by using different media, different type of explants and develop a rapid, less expensive, efficient and easy method of micropropagation of *A. vera* plants grown in Iran.

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MATERIALS AND METHODS

Plant material, Explant preparation, explant sterilization, medium culture

Healthy *Aloe vera* showing good biomass yield were collected for plant material. Shoots with young leaves were collected from the elite plants. The healthy plants, having 8–10 leaves and stem length of 5– 8 cm were selected as the source of explants. Two types of explants (with and without sheath Type A and B respectively) were used in this experiment. Both type A and B explants were washed thoroughly under running tap water for about 40-50 minutes. Then explants were disinfected by 30% sodium hypochlorite for 30 min. then washed 3-4 times with sterile distilled water to make the material free from Sodium hypochlorite. Subsequently the explants were transferred to laminar airflow cabinet and transferred to 500 ml sterilized glass bottle containing MS (Murashige and Skoog, 1962) , SH (Sckenk and Hildebrant, 1972) and B5 (Gamborg et al., 1968) medium containing various concentration of NAA alone or in combination with Kinetin (Kin) and BA for shoot induction in various concentration. For proliferation the best responding explants were transferred to B5, MS and SH media containing different concentration of BA (0, 1, 2, 4 mg/l-1) and Kin(0, 1, 2, 4 mg/l-1). B5, MS and SH media containing 3% sucrose and gelled with 0.8% agar was employed throughout the experiments. The pH of the medium was adjusted to 5.8 before autoclaving at 1.5 kg cm−2 and 121 °C for 15 min. Regenerated shoots measuring about 3-4 cm in length were transferred to rooting medium, which consisted of different medium (MS, B5 and SH) supplemented with different concentrations of naphthalene acetic acid (NAA) at 0, 0.5, 1, 1.5, 2, 2.5, 3 and 3.5mg l-1. After 3 weeks, the rooted plantlets were thoroughly washed in distilled water to remove the agar and transferred to small pots containing 1/3 Vermimiculite, 1/3 perlite, 1/3 sand (V.V). The pots placed under transparency box and maintained under 25±3 0C temperature and 70% relative humidity. After an adaptation period (4 weeks), acclimatized plantlets were transferred to greenhouse. Also, the calli were produced from the explants after 4 weeks in incubation media at the presence of NAA only in some both type of explants. The obtained calli were transferred to MS medium containing 2, 4-D (0, 0.2, 0.5 and 1mg l-1) callus for callus proliferation. After proliferation calli were transferred to shoot induction media (MS medium containing BA (0, 0.5, 1, 1.5, 2, 2.5 and 3 mg l-1)). All the cultures were grown at 25 ± 2 °C under 16 h photoperiod supplied by two with a photosynthetic photon flux density of 40 µmol m−2 s−1 provided by white fluorescent lamps. This experiment was conducted as a complete randomized design in a factorial arrangement with four replicate and each replicate contain 12 explants. Duncan's multiple range test (DNMRT) was used for comparison among treatment means.

RESULTS AND DISCUSSION

Shoot induction, Explant type and Different Media

The overall objective of the present study has been to develop a system for the mass propagation of *Aloe vera* by using different explants and different media with different PGR contain. Manipulating the relative ratio of auxin to cytokinin have been successfully used in the current investigation. The response of the different *Aloe vera* explants on different media containing different levels and combination of cytokinins and NAA were tested. No callus or shoot was observed from both type of explants on any of the media that tested without growth regulators or media containing BA alone and Kinetin. The response of the explants became visible during first week of the culturing. After 2 weeks, the green pigment of the tissue disappeared and little swelling occurred (Fig. 1A). Highest rate of shoot induction (100%) were observed in MS medium containing NAA (0.2 mg l-1) and BA (4 mg l-1) in type A culture. This treatment show significant differences with other induction treatments. Whereas the highest responding explants rate in SH and B5 media obtained in media containing NAA (0.4 mg l-1) and BA (4 mg l-1) in type A culture (Table 1). This examination demonstrated that a combination with auxin and cytokinin is needed for optimum shoot induction. However, in some previous reports (Chaudhuri and Mukundan, 2001; Aggarwal and Barna, 2004; Liao et al., 2004; Baksha et al., 2005; Hashemabadi and Kaviani, 2008), BA alone was never used instead a combination with different auxins was tried for optimal shoot induction. Of the various concentrations of BA tested in all media and different concentration of Kin, the maximum response was observed on MS medium in the presence of 4 mg l-1 BA. Between cytokinis, BA was

effective than Kin in shoot induction. Also, the calli were produced from the explants after 4 weeks in incubation media at the presence of NAA (0.4 mg l-1) only in some both type of explants (Fig 1b). Transferring callus to MS medium containing 2, 4-D (0.2 mg l-1) produced high volume of callus (Data not shown). The calli were soft, friable, light yellow to greenish and nonorganogenic (Fig 1d). Therefore indirect shoot induction from callus was not successful. In our experiments between two explants, type A was more responsive than type B segments. For type A explants with covering of leaf sheath, the maximum number of regenerated shoots per explants was found to be 9.7 on MS medium (0.2 NAA and 4 BA) after 4 weeks, while in type B (explants without leaf sheath) the same best MS medium composition yielded only 7.2 shoots after 4 weeks (Data not shown). In most previous research studies (Natali et al., 1990; Meyer and Staden, 1991; Chaudhuri and Mukundan, 2001; Aggarwal and Barna, 2004; Baksha et al., 2005; Hosseini and Parsa, 2007), using only shoot tips for micropropagation of *Aloe vera* has been reported. Also, using leaf explants by Hosseini and Parsa (2007) and underground stems as explants by Liao et al. (2004) for micropropagation of *Aloe vera* var. chinensis are reported. Retention of leaf base around the explants proved highly significant and led to proliferation of multiple shoots. This step added to the efficacy of our micropropagation method, hence enhanced shoot induction was achieved. Kinetin and BA, when used alone could not induce shoots in both the type of explants. The explants having leaf base around them (type A) were significantly better than those without leaf base (type B). The retention of leaf base around the explant protects the buds underneath hence the explants perform much better than those without this covering.

Table 1. Effects of explant type, different medium and various concentration of NAA alone or in combination with BA on percent culture responding in shoot formation from type a and b segments of *Aloe vera*.

		PGR	BA (mg/l^{-1})				Kin (mg/l^{-1})			
Medium	Explant type	NAA (mg/l^{-1})	0	1	2	4	0	1	2	4
MS	A	0	0 h	0 h	0 h	0 h	0 h	0 h	0 h	0 h
		0.2	0 h	56 d	58 d	100 a	0 h	43 e	48 de	46 e
		0.4	0 h	60 d	72 c	77 c	0 h	54 d	68 cd	45 e
	B	0	0 h	0 h	0 h	0 h	0 h	0 h	0 h	0 h
		0.2	0 h	88 b	77 c	90 b	0 h	45 e	25 f	12 gh
		0.4	0 h	81 c	90 b	88 b	0 h	63 d	32 ef	14 fg
SH	A	0	0 h†	0 h	0 h	0 h	0 h	0 h	0 h	0 h
		0.2	0 h	0 h	10 g	18 fg	0 h	66 d	88 b	59 d
		0.4	0 h	0 h	14 fg	25 f	0 h	58 d	68 cd	58 d
	B	0	0 h	0 h	0 h	0 h	0 h	0 h	0 h	0 h
		0.2	0 h	0 h	25 f	50 de	0 h	51 de	41 e	24 f
		0.4	0 h	5 gh	25 f	66 cd	0 h	45 e	34 ef	25 f
B5	A	0	0 h	0 h	0 h	0 h	0 h	0 h	0 h	0 h
		0.2	0 h	55 d	58 d	76 c	0 h	43 e	50 de	48 e
		0.4	0 h	68 d	72 c	77 c	0 h	55 d	65 cd	47 e
	B	0	0 h	0 h	0 h	0 h	0 h	0 h	0 h	0 h
		0.2	0 h	88 b	77c	91 b	0 h	46 e	24 f	12 gh
		0.4	0 h	82 c	90 b	89 b	0 h	65 d	32 ef	14 fg

BA, 6-benzyladenine**;** MS, Murashige and Skoog medium; Kin, Kinetin; NAA, Naphthaleneacetic acid; SH, Sckenk and Hildebrant medium, B5, Gamborg medium.Cultures were evaluated after at 27 days of culture Means within a column followed by the same letters are not significantly different by new Duncan's multiple range test ($P > 0.05$)

Shoot Proliferation

For shoot proliferation, generally the growth regulators like cytokinins influence the process seriously. Variety of cytokinins (Kinetin, BA, 2-ip and zeatin) has been used in micropropagation of aloe by some researchers. Wider survey of the existing literature suggests 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. Abrie and Staden (2001) Chaudhuri, Mukandhan (2001) and Aggarwal and Barna, (2004) also reported use of BA in shoot proliferation of Aloe polyphylla and *A.vera* respectively. At higher levels of cytokinins tend to induce adventitious bud formation also. In agreement with these concepts in the present study also, shoot proliferation

occurred only in the presence of cytokinin with particular reference to BA. However, it is in contrast to earlier reports in *Aloe vera* by Meyer and Staden (1991) and Natali et. al. (1990). Where in they reported that better proliferation occurred on medium containing Kn instead of BA. It may be due to the genotypic variation. Other studies indicate that BA is more efficient than Kin for shoot proliferation in A. vera (Velcheva et al., 2005; Debiasi et al., 2007). According to the literature, BA is better than other cytokinins for shoot initiation and proliferation. Velchera et al. (2005) concluded that efficient shoot initiation was observed in media supplemented with BA. However, ZR or 2iPR containing media gave only a moderate response while inclusion of CPPU and Kin in the media gave very low or no initiation, respectively. Debiasi et al. (2007) also reported the best multiplication on medium containing BA and IAA. Shoot initiation was more pronounced in MS medium contain 0.2 mg/l-1 NAA and 4 mg/l-1 BA (Fig. 1c). A maximum number of shoots per explant was achieved in MS medium with and 4 mg/l-1 BA (Table 2). Other previous reports on the micropropagation of *Aloe vera* using more than one type of media for initiation and multiplication are available. In the present study a simple two step protocol was established using MS with BA and NAA for shoot initiation, BA for multiplication and NAA for rooting in *Aloe vera*. This protocol could be used for the massive in vitro production of the plantlets of the *Aloe vera*.

Table 2. Effects of different medium and various concentrations of BA and Kin on shoot proliferationation rate of *Aloe vera*.

	BA (mg/l^{-1})				Kin (mg/l^{-1})			
Medium	0	1	2	4	0	1	2	4
MS	0 a†	1.2 a	6.5 a	11.2 a	0 a†	2.1 a	6.5 a	8.2 a
SH	0 a	0 a	4.7 a	5.9 b	0 a	1 a	7.1 a	5.5 b
B5	0 a	0 a	5.5 a	6.1 b	0 a	0 a	5.5 a	6.7 b

BA, 6-benzyladenine**;** MS, Murashige and Skoog medium; Kin, Kinetin; SH, Sckenk and Hildebrant medium, B5, Gamborg medium.Cultures were evaluated after at 27 days of culture Means within a column followed by the same letters are not significantly different by new Duncan's multiple range test ($P > 0.05$).

Rooting

The shoots measuring about 3–4 cm in height were transferred to different medium supplemented with various concentrations of NAA for rooting. Most of shoots were rooted (Fig. 1E) in various medium and in various concentration of NAA. The optimal medium for rooting was B5 contained 2 mg l-1 NAA, on which 100% of the regenerated shoots developed roots with an average number of 7.8 roots per shoots within 21 days. Root induction was quick in all the auxin tried and root primordia were observed in 10-14 days. Roots induced by a high concentration of NAA were thicker and shorter, without branches; thus, the survival rate of transplanted plantlets was lower. Also, highest root length and minimum number of root per shoot were observed in plant growth regulator free medium (Table 3). Some researchers reported rooting in hormone-free medium (Natali et al., 1990; Aggarwal and Barana, 2004) while some others showed the presence of plant growth regulators is necessary (Abrie and Staden, 2001; Meyer and Staden, 1991; Velcheva et al., 2005). In the present study, rooting percentage was improved in the presence of low concentrations of NAA. Our findings support those of Liao et al. (2004) and Budhiani (2001). In the previous studies, Aggarwal and Barna (2004) as well as Barna and Walkhlu (1994) reported rooting (100%) in *A. vera* and rose in hormone-free medium. Our results do not support these observations. The current study revealed that there is a negative correlation between rooting and BA concentration in the medium. This supports the results obtained by Velcheva et al. (2005) and Dubois and Vries (1996). The rooted plantlets were hardened in plastic pots containing a mixture of 1/3 Vermimiculite, 1/3 perlite, 1/3 sand (V.V) covered with transparent plastic and after 4 weeks of hardening, the plants were transferred to greenhouse (Fig. 1f). The result of acclimatization showed that 95% of plantlets survived to grow under greenhouse conditions and were morphologically similar to mother plants. A mixture of light soil with good drainage is suitable for acclimatization of this plant. Researchers have proposed a mixture of soil and sand (1:1) or soil, sand and perlite or vermiculite (1:1:1) for hardening of *A. vera* (Hirimburegama and Gamage, 1995; Natali et al., 1990). We have observed no phenotypic variations in the regenerated acclimatized plants and they behaved normally.

Table 3. *In vitro* rooting of shoots on MS medium fortified with different medium and different concentration of NAA.

NAA concentration (mg l^{-1})	Mean root length (mm)			No. of roots/shoot		
	MS	B5	SH	MS	B5	SH
0	37.1 a[†]	44 a	44.3 a	1.3 e	1.1 e	1.1 e
0.5	27.2 b	35.3 b	33.7 b	4.2 c	4.1 c	3.9 c
1	23 c	16.3 c	15.7 c	4.5 bc	4.7 bc	4.8 bc
1.5	22.7 c	15 c	11.3 d	5.1 b	5.1 b	5.2 b
2	18 d	15.7 c	12.3 d	7.7 a	8.1 a	7.4 a
2.5	11.9 e	9.7 d	10.3 d	5.3 b	5.3 b	5 b
3	10 e	8.7 d	10 d	2 d	2.3 d	2.1 d
3.5	0 f	0 e	0 e	0 e	0 e	0 e

MS, Murashige and Skoog medium; NAA, Naphthaleneacetic acid; SH, Sckenk and Hildebrant medium, B5, Gamborg medium. Culture period 21 days. Means within a column followed by the same letters are not significantly different by new Duncan's multiple range test ($P > 0.05$).

Figure 1. Effects of different treatments on *in vitro* culture of *Aloe Vera*, (a) shoot initiation of Aloe vera after 2 weeks, (b) Callus initiated in MS medium containing NAA alone without any potential for regeneration. (c) The emergence of large number of shoots from the segment on MS medium supplemented with 4 mg l-1 BA and 0.2 mg l-1 NAA after 4 weeks. (d) The soft, friable, light yellow to greenish and nonorganogenic callus in MS medium containing 2, 4-D (0.2 mg l^{-1}). (e) Rooting of a regenerated shoot in B5 medium supplemented with 1.5 mg l-1 NAA 21 days after culture. (f) two-week-old acclimatized plants growing in greenhouse

CONCLUSIONS

In conclusion, type and concentration of plant growth regulators, present in the media during shoot initiation, shoot proliferation and plant rooting, played a significant role in plant regeneration depending on the plant genotype and type of explant. This project indicated that micropropagation can be a useful tool for proliferation of *A. vera*.

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