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In vitro proliferation of an important medicinal plant Aloe- A method for rapid production

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Abstract

The extracts of succulent leaves of *Aloe vera* L., have wide application in medicinal and cosmetic industries. Currently, production of aloe leaves is insufficient to meet the industrial demand. So, it seems necessary to use *in vitro* propagation for rapid plant production of this plant. In this study, shoot tips of *A. vera* L., was cultured in Murashige and Skoog (MS). Application of ascorbic acid at 200 mg l^{-1} along with 200 mg l^{-1} citric acid, without active charcoal, significantly improved the shoot proliferation. Furthermore, the plantlets length was increased by application of active charcoal and decreased when supplemented by ascorbic acid. The effect of carbon sources on shoot proliferation showed that sucrose is slightly better than other carbon sources. Explants were cultured on medium containing different concentrations of benzyladenine (BA), Indol-3-butyric acid (IBA) and α-naphthalene acetic acid (NAA). The best proliferation of shoot per explant (9.67) and rooting were shown on medium supplemented with 0.5 mg l^{-1} BA + 0.5 mg l^{-1} NAA. The largest number of roots was obtained on medium supplemented with 0 mg l^{-1} IBA + 1 mg l^{-1} NAA (9.71). The longest (8.75 cm) and thickest (4.3 cm) roots were achieved on medium supplemented with 1 mg l^{-1} IBA + 1 mg l^{-1} NAA. Minimum microshoots were obtained in control plants. In all stages of this experiment, regenerated plants were transferred to cocopeat and perlite (1:1) after hardening and they showed 100% of survival.

Keywords: *Aloe vera* L., Micropropagation, IBA, NAA , BA

Introduction

Aloe vera L. belongs to the Liliaceae family and is an important medicinal plant. Aloe has been used for pharmaceutical, food, and cosmetic industries (Gui et al., 1990; Meyer and Staden, 1991). Although *A. vera* L. is propagated vegetatively in its natural state, but propagation rate is too low for commercial production (Meyer and Staden, 1991). One of the major applications of plant tissue culture is micropropagation or rapid multiplication. Compared to conventional propagations, microprogation has the advantage of allowing rapid propagation in limited time and space. The microprogation of elite or selected plants have shown good results, which benefits the forestry, agriculture and horticultural industries (Drew, 1979). *A. vera* L. has been cultured *in vitro* by various researchers (Natali et al., 1990; Roy and Sarkar, 1991; Abrie and Staden, 2001; Corneanu et al., 1994; Chaudhuri and Mukandan, 2001). Based on previous researches, it is beleived that the best explants for micropropagation of aloe are shoot tips and axillary buds (Meyer and Staden, 1991). Plant growth regulators and explants are very important factors for successful plant regeneration. Natali et al. (1990) reported a rapid highly effective plant micropropagation from meristems. Richwine et al. (1995) reported and Velcheva et al. (2005) developed a system for *in vitro* regeneration of aloe, using young inflorescences as explants. Little work has been done on callus culture of aloe species, because the establishment of primary cultures is difficult, owing to the secretion of the phenolic substances by explant. Roy and Sarkar (1991) reported a rapid propagation method by the formation of shoots from calli of *A. vera* L. Polyvinylpyrrolidone (PVP) was used to reduce the secretion of phenolic substances from the explants. Thus, the use of phenolic attractive substances in regeneration media is reportedly suitable (Ramsay and Gratton, 2000). Blacking or browning are the major problems in meristem culture. Blacking most likely is caused by oxidation of phenols which are released from the cut surface of the meristem. To eliminate the blacking and browning, some antioxidants such as ascorbic acid, PVP and citric acid have already been used (Minas, 2007; Ramsay and Gratton, 2000; Liao et al., 2004). The presence of the plant growth regulators in media are necessary for shoot and root initiation (Aggarwal and Barna, 2004; Debiasi et al., 2007; Liao et al., 2004). Natali et al. (1990) showed micropropagation of *A. vera* L. by culturing shoot apices on medium containing 2,4-D and kinetin. Meyer and Staden (1991) reported axillary shoot formation using only IBA, whereas Roy and Sarkar (1991) and Natali et al. (1990) got shoots on medium containing 2,4-D and kinetin. Richwine et al. (1995) reported the induction of shoots using zeatin. Liao et al. (2004) studied the effects of benzyladenine (BA), α-naphtaleneacetic acid (NAA) and sucrose on bud initiation from explants. Sucrose and BA were recognized the most important factors affecting the bud initiation and promoted efficient multiplication. Abrie and Staden (2001) cultured aloe plantlets on medium containing BA alone, or with combination of BA and NAA. The plantlets rapidly formed axillary and adv-

Table 1. Effect of different culture media on shoot proliferation

Culture medium	Mean of shoot proliferation
QL	2.250 a*
MS	2.208 a
1/2MS	1.417 a

*: Values followed by the same letter are not significantly different (P <0.01) using DNMRT.

entitious buds. Chaudhuri and Mukandan (2001) also reported that the formation of multiple shoots *in vitro* is a function of cytokinin and auxin concentrations. The presence of only cytokinin or auxin resulted in formation of callus or roots. Best multiplication of shoot was obtained on medium containing BA + adenine sulphate + IAA. Rooting was performed spontaneously on bud initiation medium and the rooting rate was significantly improved in the presence of other plant growth regulators (Abrie and Staden, 2001; Feng et al., 2000; Hongzhi, 2000). In some studies, rooting was performed on MS media lacking growth regulators (Velcheva et al., 2005; Aggarwal and Barna, 2004). Maximum shoot growth and rooting were obtained on MS medium supplemented with IBA (Meyer and Staden, 1991). Development of efficient systems for regeneration of aloe is currently in high demand, particularly for plant genetic transformation and cloning techniques (Campestrini et al., 2006; Velcheva et al., 2005). Acclimatization of rooted plantlets in the pots containing a mixture of sand, silt and compost under greenhouse conditions with 70-90% moisture is suited for young plants survival (Natali et al., 1990; Hirimburegama and Gamage, 1995). The objective of the present investigation is to determine the influence of some factors such as plant growth regulators, like BA, IBA, NAA and sucrose and phenolic attractive compounds on rapid *in vitro* propagation of *A. vera* L.

Materials and methods

Shoot tip explant of *Aloe vera* L. was obtained from the off shoot-derived plants. The explants were collected from the plants free of symptoms of disease and pest problem growing in the commercial greenhouse. After cutting stem into pieces with 1-2 buds, the extra leaves were removed and shoots were used for cultivation. Explants were washed under running tap water for 30 min. Stems with buds were surface sterilized with 2% (w/v) NaOCl and Tween 20 for 10 min followed by three rinses with sterile distilled water for 30 min. The surface disinfected stems were cut into 1-cm segments each with buds. Again, explants were sterilized with 1% (v/v) Savlon for 2 min followed by three rinses with sterile distilled water under laminar air flow hood. Then, explants were taken out and dipped on 70% (v/v) ethanol for 30 sec and 1% (w/v) $HgCl_2$ for 5 min and washed five times with sterile deionized water. Explants were cultured in test tubes containing basal MS (Murashige and Skoog, 1962) medium supplemented with plant growth regulators, BA (0.5, 1 and 2 mg l^{-1}) and NAA (0.5 and 1 mg l^{-1}). Sucrose (3%) was used as carbon source and media were solidified with Agar–agar (0.7%). The pH was adjusted to pH 5.7 prior to autoclaving at 121°C and 102 kpa for 20 min. Four shoots per test tube were inoculated and six replicates taken. In other experiment, QL and 1/2 MS media were used instead of MS medium, also sugar and glucose were used as carbon sources instead of sucrose. Anti-phenolic substances including active charcoal (0 and 2 g l^{-}), ascorbic acid (0 and 200 mg l^{-1}) and citric acid (0 and 200 mg l^{-1}) were added to culture media. Newly formed shoots, 3-4 cm in length, were detached individually from the parent explant and transferred to rooting media. Three types of rooting media were used on MS basal media lacking plant growth regulators and other MS basal media containing BA and NAA with the same concentrations of shoot induction as well IBA and NAA (both 0 and 1 mg l^{-1}). Four shoots per culture bottle were used and five replicates were applied per treatment. Data were recorded after 15 days of culture. Some characters such as root and leaf number, crown and root diameter, proliferation rate, and root and plantlet length were calculated following rooting. Cultures were kept in growth chamber (Fig 1) under 16 h photoperiod with light intensity of 2000-2500 lux provided by cool-white fluorescent tubes at 24°C and 70% relative humidity of the air. Data were recorded after 30 and 60 days of culture. Matured plantlets were washed with sterile distilled water and sterilized with a fungicide solution (1%) and transferred into the plastic pots (10-cm in diameter) containing a mixture of cocopeat and perlite (1:1) (Fig 2). The top of the pots were covered with transparent plastic (Fig 3) and grown in a greenhouse at 24 ± 1°C and 70% RH with periodic irrigation (3 days) for 10 days. Then plants were shifted to shadehouse with less humidity level and indirect sunlight. In shadehouse, plants were watered two times a day (morning and evening). The experimental design was factorial with R.C.B.D design, which was done with unequal repetition. All experiments were carried out in three to six replicates. Data were subjected to ANOVA (analysis of variance) and significant differences between treatments means were determined by DNMRT using SPSS and MSTATC software packages.

Results and discussion

The type of explant used on *in vitro* conditions affected the plant proliferation. Shoot tip, axillary bud, meristem, underground stem and inflorescence have been used as explant (Liao et al., 2004; Aggarwal and Barna, 2004; Velcheva et al., 2005). In this study, the shoot tip was used as the main suitable explant. Using axillary buds as the explants has been proven to be the most successful and efficient micropropagation procedure for aloe yet (Meyer and Staden, 1991). The most researchers proposed the use of shoot tip and apical meristem for micropropagation of aloe (Debiasi et al., 2007; Natali et al., 1990; Meyer and Staden, 1991; Liao et al., 2004; Aggarwal and Barna, 2004; Campestrini et al., 2006). Natali et al. (1990) and Budhiana (2001) reported rapid and highly effective plant micropropagation from vegetative meristem and shoot tip, respectively. Richwine et al. (1995) reported and Velcheva et al. (2005) developed a system for *in vitro* regeneration of aloe using young inflorescence as explant. Velcheva et al. (2005) showed that the young inflorescence of *A. barbadensis* is a reliable explant for *in vitro* aloe micropropagation as previously reported for *A. barbadensis* (Richwine et al., 1995). Some of the reported aloe micropropagation studies had been performed using mainly underground stems as explant (Zhou et al., 1999; Roy and Sarkar, 1991; Kawai et al., 1993; Corneanu et al., 1994; Hirimburegama and Gamage, 1995). Such explants suffer from a relative high contamination level and phenolic substances. Seeds and meristems were

Table 2. Effect of different phenolic attractives compound on shoot proliferation and plantlet length

Phenolic attractive compound	Mean of shoot proliferation	Plantlet length (cm)
A_1 (0 g l^{-1} active charcoal)	2.354 a *	3.401 b
A_2 (2 g l^{-1} active charcoal)	1.760 b	6.469 a
B_1 (0 mg l^{-1} ascorbic acid)	1.969 a	5.198 a
B_2 (200 mg l^{-1} ascorbic acid)	2.146 a	4.672 b
$A_1 \times B_1$	2.000 b	3.770 a
$A_1 \times B_2$	2.708 a	3.031 a
$A_2 \times B_1$	1.938 b	6.625 a
$A_2 \times B_2$	1.583 b	6.313 a
C_1 (0 mg l^{-1} citric acid)	2.083 a	4.964 a
C_2 (200 mg l^{-1} citric acid)	2.031 a	4.906 a
$A_1 \times C_1$	2.250 a	3.490 a
$A_1 \times C_2$	2.458 a	3.313 a
$A_2 \times C_1$	1.917 a	6.438 a
$A_2 \times C_2$	1.604 a	6.500 a
$B_1 \times C_1$	1.979 a	5.313 a
$B_1 \times C_2$	1.958 a	5.083 a
$B_2 \times C_1$	2.188 a	4.615 a
$B_2 \times C_2$	2.104 a	4.729 a
$A_1 \times B_1 \times C_1$	1.833 a	4.063 a
$A_1 \times B_1 \times C_2$	2.167 a	3.479 a
$A_1 \times B_2 \times C_1$	2.667 a	2.917 a
$A_1 \times B_2 \times C_2$	2.750 a	3.146 a
$A_2 \times B_1 \times C_1$	2.125 a	6.563 a
$A_2 \times B_1 \times C_2$	1.750 a	6.688 a
$A_2 \times B_2 \times C_1$	1.708 a	6.313 a
$A_2 \times B_2 \times C_2$	1.458 a	6.313 a

***** Values followed by the same letter are not significantly different ($P < 0.01$) using DNMRT.

also used as explants for callus induction, and plant regeneration (Abrie and Standen, 2001; Sanchez et al., 1988). Little work has been done on callus culture of aloe species because establishment of primary cultures is difficult owing to the secretion of the phenolic substances by explant. Groenewald et al. (1975) used also seeds as explants for the initiation of callus, but failed to obtain regeneration. Roy and Sarkar (1991) showed the rapid propagation in the formation of shoots from calli of *A. vera* L. Polyvinylpyrolidine (PVP) was used to reduce the secretion of phenolic substances from the explant. Therefore, use of phenolic attractive substances in regeneration media is suitable (Ramsay and Gratton, 2000). The best explants for tissue culture of aloe are shoot tips, meristems and axillary buds. Meristems and axillary buds were used for micropropagation of other plants such as rosa, blackberry, tulip and chrysanthemum (Minas, 2007; Jafari Najaf-Abadi and Hamidoghli, 2009; Chu et al., 1993; Chen, 2006). Mamidala and Nanna (2009) showed successful plant regeneration using leaf explants. Meristem culture is a very useful approach giving high quality plantlets with high virus-free rate.

In the present study, MS, $^1/_2$MS and QL soild culture media were applied. The effect of culture medium on shoot proliferation was significant. However, MS and QL media with 2.08 and 2.25 shoots per explant, were the better than $^1/_2$ MS medium with 1.417 shoots (Table 1).

Debiasi et al. (2007) and Michel et al. (2008) have shown that use of MS medium culture has the best result on *in vitro* multiplication. Some studies revealed that liquild medium is better than soild medium. Use of liquid medium considerably reduces the cost of plant production for the commercial purposes. Aggarwal and Barna (2004) tested both solid and liquid media for shoot proliferation in *A. vera* L. and observed liquid medium has merits. The average number of shoot in liquid medium was 4.80 ± 2.5, while in solid medium was 4.08 ± 2.0. Growth of cultures was faster in liquid medium than soild medium. In contrary, the microshoots inoculated on soild medium showed better rooting response than liquid medium. The 100% of shoots showed rooting and the mean number of roots per shoots were 2.7 ± 1.2, but in liquid medium, only 18% of shoots showed rooting and the mean number of roots per shoots was 0.2 ± 0.5. The shoots inoculated on liquid medium were failed to give any further rooting response even after 3-4 weeks. Budhiana (2001) proposed soild MS medium for micropropagation of *A. vera* L. Liao et al. (2004) demonstrated that the best media for bud initiation and root induction were semi-soild MS and $^1/_2$ MS, respectively. Also, the studies of Chen (2006) on *Aglaonema* revealed more growth of explants on $^1/_2$ MS

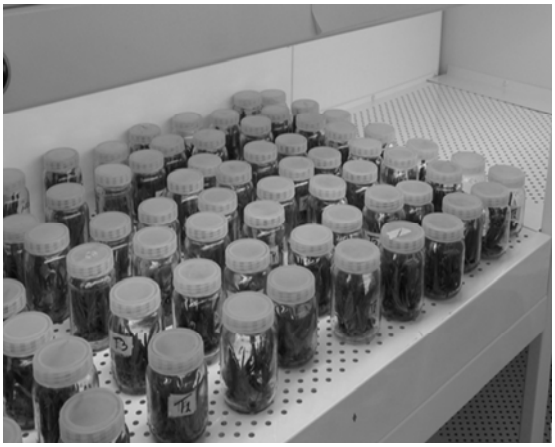

Fig 1. Tissue culture propagation of *Aloe vera* L., shoot proliferation and rooting after 8 weeks of culture

Table 3. Effect of different carbohydrate sources on shoot proliferation

Carbohydrate source	Mean of shoot proliferation
Sucrose	4.375 a *
Glucose	3.333 a
Sugar	3.167 a

*: Values followed by the same letter are not significantly different (P <0.01) using DNMRT.

Fig 2. Hardening process of *Aloe vera* L. plantlets. Plantlets were transferred to plastic pots containing a mixture of cocopeat and perlite (1:1)

medium in comparison with MS medium for semi-solid system. Most researchers proposed soild MS medium for shoot and root induction (Velcheva et al., 2005; Campestrini et al., 2006; Abrie and Staden, 2001). Aggarwal and Barna (2004) showed that the use of MS liquid medium considerably reduces the cost of producing plants for the commercial purposes. Active charcoal (A) (0 and 2 g l^{-1}), ascorbic acid (B) (0 and 200 mg l^{-1}), and citric acid (C) (0 and 200 mg l^{-1}) were added to the media.

The effect of ascorbic acid and citric acid was not significant on shoot proliferation ($P \leq 0.05$). The average number of shoot per explant was 2.35 and 1.76 in the media, without and with 2 g l^{-1} active charcoal, respectively (Table 2). No significant difference was shown between AC, BC and ABC. Only, AB had significant effect on shoot proliferation ($P \leq 0.05$). The best media were the combination of $A_1B_2C_2$ and A_1B_2 with 2.75 and 2.71 shoots per explant, respectively. The least shoot proliferation was observed on $A_2B_2C_2$ medium (Table 2). Thus, the combination of active charcoal, ascorbic acid and citric acid in media is not suitable. Our results do not support the results obtained by Aggarwal and Barna (2004). These researchers observed that application of citric acid in culture medium increased the shoot proliferation of *A. vera* L. Highest number of shoot per explant was formed on medium containing 10 mg l^{-1} citric acid. The average number of shoot per explant was 5.0 ± 1.9. On the medium with lack of citric acid, the average number of shoot was 3.3 ± 0.9. Higher concentration of citric acid (100 mg l^{-1}) was found to be less promotive (4.2 ± 2.3).

Browning and blacking are two major limiting factors for establishing of tissue culture in aloe species (Abrie and Staden, 2001). Blacking most likely is caused by oxidation of phenols which are released from the cut surface of the meristem. To eliminate the blacking, based on previous experience (Casselle and Minas, 1983b), several measure were taken consist of, the use of antioxidant ascorbic acid in high concentration in the culture medium, and the use of phytagel rather than agar for solidifying the media but agar contains Mn and Mg ions are necessary for oxidase activation. The studies of Minas (2007) on 3 types of gardenia, tulip and charysanthemum showed that by using of 55.7 mg l^{-1} ascorbic acid, none of the cultures of meristem tip showed blacking. Meristem tip used in this study is the most likely suitable explant for cloning of pathogen-free and genetically unique micro-plants. Previous studies indicated that browning could be alleviated by adding anti-oxidants such as PVP to the culture medium (Roy and Sarkar, 1991; Sudripta et al., 1999). Liao et al. (2004) showed that *A. vera* L. var. chinesis (Haw.) Berger (chinese aloe) explants, become seldom brown, when 0.6 g l^{-1} PVP was included in the medium. Our studies on the effect of phenolic attractive substances on plantlets length revealed that active charcoal increased and ascorbic acid decreased length of plantlets (Table 2). Statistical analysis of results showed that active charcoal had significant effect on plantlet length ($P \leq 0.01$). The highest length of plantlets were observed on media containing 2 g l^{-1} active charcoal, i.e. $A_2B_1C_2$ (6.688 cm), A_2B_1 (6.625 cm) and $A_2B_1C_1$ (6.563 cm) (Table 2). The culture medium with lack of ascorbic acid was significantly ($P \leq 0.05$) better than medium with 200 mg l^{-1}. On the other hand, the least length of plantlet was seen on media containing 200 mg l^{-1} ascorbic acid, i.e. $A_1B_2C_1$ (2.917 cm), A_1B_2 (3.031), and $A_1B_2C_2$ (3.146 cm). The presence of citric acid in media with or without active charcoal and ascorbic acid had positive effect on plantlets length. Sucrose, glucose and sugar were added to the culture media. None of 3 carbon sources have significant effect on shoot proliferation. The average number of shoots per plant in medium containing sucrose (4.375) was more than glucose (3.333) and sugar (3.167) contained media, but these differences were not significant (Table 3). Liao et al. (2004) reported that among the three factors, namely sucrose, BA and NAA, sucrose was the most important for bud initiation followed by BA and NAA.

Propagation of buds from aloe species was found to be influenced by plant growth regulators and sucrose (Roy and Sarkar, 1991; Rout et al., 2001). Michel et al. (2008) demonstrated which glucose was the best sugar to promote the production of cotton callus. Shoot tip explants on medium with 0.5 mg l^{-1} BA + 0.5 mg l^{-1} NAA showed signs of proliferation after two weeks. New buds appeared from the axil of leaves and developed into shoots by the 4th week of culturing. The highest number of shoots per explant (3.15) was obtained on the medium containing 0.5 mg l^{-1} BA + 0.5 mg l^{-1} NAA. On the 8th week the highest number of shoots were formed on the medium supplemented with 0.5 mg l^{-1} BA + 0.5 mg l^{-1} NAA, produced 9.67. Also on the medium containing l mg l^{-1} BA + 0.5 mg l^{-1} NAA the average number of shoot per explant was 8.69. Shoot elongation with a maximum height was obtained on MS medium supplemented with 0.5 mg l^{-1} BA + 0.5 mg l^{-1} NAA. The medium containing 2 mg l^{-1} BA and 1 mg l^{-1} NAA did not show any significant effect on shoot proliferation. The least number of shoot per explant (nil) was achieved in hormone-free medium. The presence of the plant growth regulators, particularly cytokinin in culture medium is the most important factors for shoot proliferation (Abrie and Staden, 2001; Chaudhuri and Mukandan, 2001; Aggarwal and Barna, 2004; Liao et al., 2004; Mamidala and Nanna, 2009; Hoque, 2010; Jafari Najaf-Abadi and Hamidoghli, 2009). A range of cytokinins (BA, BAP, 2ip, Kn and zeatin) has been

Table 4. Effect of different concentrations of IBA and NAA on the number of root, root length and root diameter in *Aloe vera* L.

Phytohormanes (mg l^{-1})	The number of root	Root length (cm)	Root diameter (cm)
0 NAA (A_1)	6.25 b	7.81 a*	0.74 b
1 NAA (A_2)	9.38 a	8.17 a	3.34 a
0 IBA (B_1)	7.84 a	7.51 a	1.54 b
1 IBA (B_2)	7.79 a	8.46 a	2.54 a
$A_1 \times B_1$	5.97 a	7.44 a	0.7 c
$A_1 \times B_2$	6.53 a	8.18 a	0.78 c
$A_2 \times B_1$	9.71 a	7.59 a	2.38 b
$A_2 \times B_2$	9.05 a	8.75 a	4.30 a

*: Values followed by the same letter are not significantly different ($P < 0.01$) using DNMRT.

used for aloe micropropagation (Velcheva et al., 2005; Araujo et al., 2002; Debiasi et al., 2007; Liao et al., 2004; Namli et al., 2010). Some researchers have shown that presence of both of auxin and cytokinin is necessary for shoot proliferation (Roy and Sarkar, 1991; Rout et al. 2001; Velcheva et al. 2005). The study of Namli et al. (2010) on shoot proliferation of *Hypericum retusum* revealed that the best result was obtained on the medium supplemented with 0.5 mg l^{-1} BAP + 0.25 mg l^{-1} IBA.

Our results showed the best ratio of BA to NAA for buds initiation, shoot proliferation and rooting was 1:1 or 2:1 (Hashemabadi and Kaviani, 2008). The best rooting rate happened on MS medium supplemented with 0.5 mg 1^{-1} BA + 0.5 mg 1^{-1} NAA and 1 mg 1^{-1} BA + 0.5 mg 1^{-1} NAA (data not presented). The process of rooting on microshoots in *A. vera* L. is strongly affected by the type and concentration of the growth regulators utilized on medium, salts composition, plant genotype, and culture conditions. In most species, efficient rooting was observed on medium containing auxins. NAA and IBA are most commonly used hormones for root induction (Bhojwani and Razdan, 1992). Jafari Najaf-Abadi and Hamidoghli (2009) explained that concentration of 2 mg 1^{-1} IBA has given a bigger number of roots and the maximum root length. Some researchers reported rooting in hormone-free medium (Natali et al., 1990; Meyer and Staden, 1991; Aggarwal and Barna, 2004). Richwine et al. (1995) reported induction of roots in hormone-free medium for some other plants such as *Gasteria* and *Haworthia*. Aggarwal and Barna (2004) demonstrated that rooting of aloe microshoots was better in hormone-free medium than in medium containing IBA. After 13 days of inoculation, rooting was 100% in hormone-free medium and 90% in medium containing 1 mg 1^{-1} IBA. The number of roots per shoot was 2.8 ± 0.5 on hormone-free medium and 1.7 ± 1.1 in medium with 1 mg 1^{-1} IBA. Our findings were almost similar to this result (Table 4). But the difference was not significant, because the number of roots per shoots were 7.84 on hormone-free medium and 7.79 in medium with 1 mg 1^{-1} IBA. On medium containing IBA, roots were thin and short but on medium without IBA, roots were thick and long. The roots became thicker and longer on medium containing IBA, than the medium without IBA (Table 4). However, the thickest and longest roots were obtained on medium containing 1 mg 1^{-1} IBA + 1 mg 1^{-1} NAA (Table 4). Some other workers showed that the presence of plant growth regulators on media is necessary for rooting of shoot (Abrie and Staden, 2001; Meyer and Staden, 1991; Velcheva et al., 2005). Velcheva et al. (2005) believed that phytohormones present in the culture medium of *A. arborescens* during shoot induction and elongation play a major role during the rooting stage. These researchers reported that after transferring onto MS medium lacking growth regulators, elongated shoot started

Fig 3. Plantlets were transferred to a box covered with transparent plastic for hardening and growth

to develop into mature, vigorous and morphologically normal plants. Optimal rooting was obtained if the shoots, which initiated on MS medium containing BA and ancimydol, were further sub-cultured into elongation medium containing BA, and finally rooted on MS media without any growth regulators. Less efficient rooting was observed when shoots initiated on MS medium containing both 19.6 µM ancymidol and 22.2 µM BA, were further elongated on MS medium containing only 4.4 µM BA or lacking growth regulators. This enabled us to produce 3-4 plants per explant to root successfully on MS medium without any plant growth regulators. Tripathi and Bitallion (1995) and Vij et al. (1980) reported the use and effect of IBA in rooting of *Hedychium roxburgii* and carnation, respectively. Abrie and Staden (2001) showed that the shoots obtained from MS medium containing 1 mg 1^{-1} BA, rooted spontaneously on the same medium, but the rooting rate was improved in the presence of 0.5 mg l^{-1} IBA. Liao et al. (2004) reported that shoots of *A. vera* L., obtained from semi-solid MS medium supplemented with 2 mg l^{-1} ± 0.3 mg l^{-1} NAA, rooted spontaneously on $^1/_2$ MS medium with the same BA and NAA level. In this case, the rooting rate was improved in the presence of 0.2 mg l^{-1} NAA. The study of Minas (2007) on tulip and chrysanthemum showed the suitable rooting of micro-plants in the same medium. In the present study, rooting percentage was improved in the presence of low concentrations of BA and NAA. The presence of NAA (1 mg l^{-1}) in the media caused an increasing of roots diameter. Optimal increasing was obtained when shoot initiation took place on MS medium supplemented with NAA + IBA (Table 4). Our findings support the data of Liao et al. (2004) and Budhiana (2001). Budhiana (2001) reported that rooting of the shoot in *A. vera*

L. was induced on medium with 0.002 or 0.02 mg l^{-1} IBA. In the previous studies, Aggarwal and Barna (2004) and Barna and Wakhlu (1994) reported 100% rooting of *A. vera* L. and rose plantlets in hormone-free medium. Meyer and Staden (1991) showed that the maximal bud growth and rooting of shoots were obtained on a modified medium of MS supplemented with IBA. Our results do not support these observations.

The present study revealed that, there is a negative correlation between rooting and BA concentration in the medium. This result is also supported by Velcheva et al. (2005) and Dubois and De Vries (1996).

The results of acclimatization showed that the 95% of plantlets were survived to grow under greenhouse conditions and were morphologically similar to mother plants. After keeping plantlets for 10 days in greenhouse, the plantlets were transferred to shadehouse under less humidity. In these conditions, the 90% of the plantlets showed survival. A mixture of light soil with good drainage is suitable for acclimatization of these plants. Researchers proposed a mixture of soil and sand (1:1) or soil and perlite or vermicolite (1:1:1) for hardening of *A. vera* L. (Hirimbur- egama and Gamage, 1995; Natali et al., 1990). Hardening of plants is the most crucial step in micropropagation. The produced plants are very soft to face ambient environmental conditions (Bhojwani and Razdan, 1992). Aggarwal and Barna (2004) used soil and farmyard manure (1:1) for hardening, and plantlets transferred to the polyhouse, then 85% of the plantlets survived. After keeping them for initial 10 days in polyhouse, plants were transferred to shadehouse under less humidity. In shadehouse, 82% plantlets were survived. Berger (chinese aloe) were poted in a mixture of potting soil, vermiculite and sand (1:1:1), acclimatized in a misthouse and young aloes were planted in the field very successfully (93%).

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