Agronomía Costarricense 32(2): 41-52. ISSN:0377-9424 / 2008
www.mag.go.cr/rev_agr/inicio.htm www.cia.ucr.ac.cr

SOMATIC EMBRYOGENESIS, PLANT REGENERATION AND ACEMANNAN DETECTION IN ALOE (*Aloe barbadensis* MILL.)[1]

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Palabras clave: *Aloe barbadensis*, embriogénesis somática, cultivo in vitro, semillas.
Keywords: *Aloe barbadensis*, somatic embryogenesis, in vitro culture, seeds.

Received: 02/05/08 Accepted: 07/07/08

RESUMEN

Embriogénesis somática, regeneración de plantas y detección de acemanan en aloe (*Aloe barbadensis* Mill). La presente investigación tuvo como objetivo la regeneración de plantas de aloe (*Aloe barbadensis* Mill.) vía embriogénesis somática. Para la desinfección de los explantes se evaluó 2, 3, 4, 5, 10 y 15 min de sonicación en combinación con 4% v/v de NaOCl. El mayor porcentaje de sobrevivencia (85%) y de menor contaminación (15%) se logró con 5 min de sonicación. Se obtuvo callos embriogénicos friables a partir de meristemos apicales, bases de hojas jóvenes y embriones cigóticos. El mejor explante para la inducción de callos embriogénicos fue la base de hojas jóvenes con 89%, cultivadas en el medio complementado con 2,5 mg.l^{-1} de 2,4-D, 2 mg.l^{-1} de BAP y 40 mg.l^{-1} de sulfato de adenina. El mayor número de brotes se obtuvo a partir de callos embriogénicos producidos de embriones cigóticos, en el medio con 0,05 mg.l^{-1} de 2,4-D y 2 mg.l^{-1} de BAP. El análisis de cromatografía líquida de alta resolución (HPLC) reveló que la concentración de acemanan en callos embriogénicos (0,8-2,1 mg.ml^{-1}) fue menor en comparación con la de hojas frescas (85 mg.ml^{-1}). El protocolo de cultivo establecido en la presente investigación puede ser utilizado tanto para la propagación como para la transformación genética.

ABSTRACT

A method for plant regeneration via somatic embryogenesis was established in aloe (*Aloe barbadensis* Mill.). For explant disinfection, treatments involved 2, 3, 4, 5, 10 and 15 min sonication, in combination with 4% v/v NaOCl. Explant source and growth regulators were investigated. The highest survival rate (85%) and the lowest contamination (15%) were obtained with 5 min sonication. Friable embryogenic calluses were produced from apical meristems, leaf bases, and zygotic embryos of aloe. The best explants for callus induction (89%) were the leaf bases when cultured on callus induction medium with 2.5 mg.l^{-1} 2,4-D, 2 mg.l^{-1} BAP, and 40 mg.l^{-1} adenine sulphate. The highest number of shoots was obtained from embryogenic calluses derived from zygotic embryos on a medium supplemented with 0.05 mg.l^{-1} 2,4-D and 2 mg.l^{-1} BAP. High Performance Liquid Chromatography (HPLC) analysis revealed that acemanan concentration in embryogenic calluses (0.8-2.1 mg.ml^{-1}) was much lower than fresh leaves (85 mg.ml^{-1}). The protocol obtained in this investigation could be a useful tool not only for the propagation of this important medicinal plant but also for genetic transformation.

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INTRODUCTION

Aloe (*Aloe barbadensis* Mill) is one of the economically important crops of the Liliaceae family, and has been used worldwide in pharmaceutical, food and cosmetic industries, or in traditional medicine, due to the plethora of biological activities of some of its primary and secondary metabolites (Campestrini et al. 2006, Tanabe and Horiuchi 2006).

Bioactive compounds isolated from the gel of *Aloe* species have been known to have anti-inflammatory, anti-tumor, anti-ulcer, anti-cancer, anti-bacterial, and anti-viral properties (Reynolds and Dweck 1999, Campestrini et al. 2006, Tanabe and Horiuchi 2006). Examples of these compounds include enzymes, mono and polysaccharides, amino acids, vitamins, anthraquinones, saponins, salicylic acid, lignin, and steroids (Campestrini et al. 2006). Acemannan, a major carbohydrate fraction of aloe gel, has been known to have anti-viral and antitumoral activities in vivo through activation of immune responses (McDaniel and McAnalley 1987, Hart et al. 1989, Reynolds and Dweck 1999, Lee et al. 2001). Moreover, Lee et al. (2001) proposed that acemannan has the capacity to promote differentiation of immature dendritic cells.

Due to the importance of this plant for the pharmacological, medical, and cosmetic industry, the vegetative propagation of aloe has been insufficient to meet the increasing demand (Hosseini and Parsa 2007). Therefore, there is a need for propagation methods; which can increase commercial production (Campestrini et al. 2006, Silva et al. 2007).

In this sense, the mass production of aloe plants through in vitro propagation is an alternative. Micropropagation of aloe offers several advantages over conventional vegetative propagation (Campestrini et al. 2006, Hosseini and Parsa 2007, Silva et al. 2007). Moreover, genetic transformation of aloe varieties requires development of efficient in vitro culture systems to obtain competent explants for plant transformation and regeneration (Velcheva et al. 2005).

Plant regeneration from in vitro cultured cells can be accomplished through somatic embryogenesis or organogenesis. In several aloe species, somatic organogenesis is the most common regeneration pathway and has been obtained from apical meristems (Natali et al. 1990, Meyer and van Staden 1991, Richwine et al. 1995, Campestrini et al. 2006, Hosseini and Parsa 2007), root meristems (Roy and Sarkar 1991), anthers (Keijzer and Cresti 1987), leaf bases of young seedlings (Cavallini et al. 1993, Hosseini and Parsa 2007), inflorescences (Velcheva et al. 2005), underground stems (Liao et al. 2004) and young shoot explants of in vitro grown plants (Chukwujekwu et al. 2002). Nevertheless, there is no report of successful plant regeneration through somatic embryogenesis of *A. barbadensis*.

The main objective of this work was to set up a protocol for callus induction and plant regeneration through indirect somatic embryogenesis (ISE) by studying the influence of plant growth regulators and explant source; and, to compare the acemannan content in the inner gel of fresh leaves with the one from embryogenic calluses.

MATERIALS AND METHODS

Plant material and explant disinfection

Aloe plants (*Aloe barbadensis* Mill.), provided by Sábila Industrial (Carrington Laboratories Inc) (Liberia, Costa Rica), were used as source of explants (Figure 1A). Plants were pretreated with 0.5% (v/v) NaOCl prior to explant disinfection and maintained in the greenhouse. Immature inflorescences and flowers, mature ovaries and seeds, rhizome discs, apical meristems, and leaf bases from 4-months-old plants were used as explants for embryogenic callus induction. These explants were washed with tap water, followed by sonication (Bransonic 2200, Ultrasonic Cleaner) for 2, 3, 4, 5, 10 or 15 min, in 4% (v/v) NaOCl. Then, explants were washed 3 times with sterile distilled water, immersed in 70% ethanol for 1 min, and washed again 3 times with sterile distilled water.

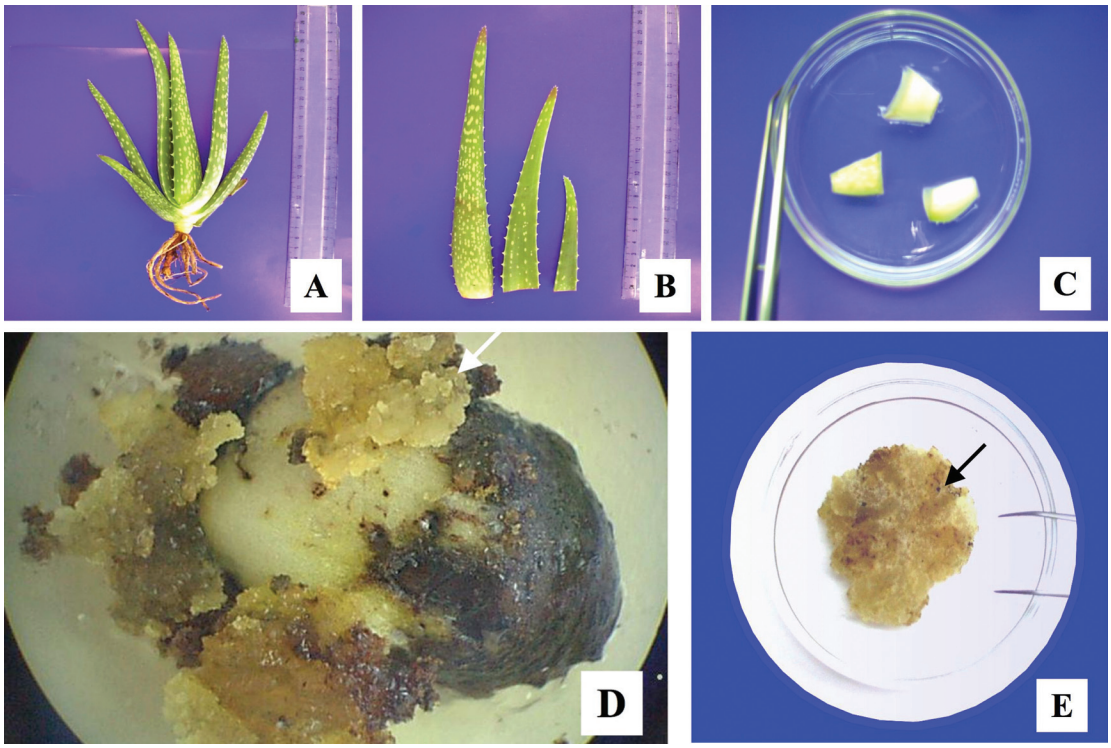

Fig. 1. Somatic embryogenesis in aloe (*A. barbadensis* Mill.). A) Four-month-old plants grown in the greenhouse and used as source of explants, B) Young leaves, C) Leaf bases used as explants for embryogenic callus induction, D) Embryogenic callus fragment (arrow) obtained after 4 weeks of culture, E) Embryogenic callus (arrow) obtained after 12 weeks of culture.

Mature seeds were surface-sterilized following the procedure described by Valdez et al. (1996). Briefly, seeds were immersed in an aqueous 30% (v/v) Domestos™ (Lever Ltd. Warrington, G.B.) solution for 35 min and then rinsed 5 times in sterile distilled water. Disinfected seeds were soaked overnight in sterile distilled water to facilitate embryo isolation. Zygotic embryos were excised under a binocular microscope.

Induction of somatic embryogenesis

In a first experiment, in order to determine the optimal explant source for embryogenic callus induction, explants were cultured in Petri dishes (100 mmx20 mm) containing 20 ml of MSC 00 (Murashige and Skoog 1962) medium with 30 $g.l^{-1}$ sucrose and 3 $g.l^{-1}$ Phytagel), MSC 01 (MSC 00 supplemented with 0.25 2,4-D $mg.l^{-1}$ and 1 $mg.l^{-1}$ kinetin) or MSC O2 (MSC 00 supplemented with 1 $mg.l^{-1}$ 2,4-D and 0,25 mgl^{-1} kinetin). Once the best explant source was determined, in a second experiment, the leaf bases were cultured in Petri dishes containing 20 ml of MS mineral salts and vitamins, BAP (1, 1.5 and 2 $mg.l^{-1}$), 2,4-D (1.5, 2 and 2.5 $mg.l^{-1}$) and adenine sulphate (40 and 80 $mg.l^{-1}$) (Table 4), 30 $g.l^{-1}$ sucrose and 3 $g.l^{-1}$ Phytagel. In a third experiment, disinfected zygotic embryos were cultured in Petri dishes containing 20 ml of callus induction medium MSC 15 (MSC 00 supplemented with 2.5 $mg.l^{-1}$ 2,4-D and 2 $mg.l^{-1}$ BAP) or MSC 17 (MSC 00 supplemented with 2 $mg.l^{-1}$ 2,4-D and 2 $mg.l^{-1}$ BAP). The pH was adjusted to 5.6 with NaOH before autoclaving for

21 min at 121 °C and 1.05 kg.cm^{-2}. Cultures were maintained in the dark at 26±2°C. Embryogenic calluses were transferred to maintenance medium (MS medium supplemented with 1.5 mg.l^{-1} 2,4-D, 1 mg.l^{-1} BAP and 80 mg.l^{-1} adenine sulphate) after 8 weeks of culture initiation. Callus formation frequency [(explants with embryogenic callus/ total of explants)*100] was evaluated after 14 weeks of culture.

Plant regeneration

After 16 weeks of culture on callus induction medium, embryogenic callus segments, induced from leaf bases, were cultured on 4 different regeneration media (RM) complemented with different concentrations of plant growth regulators and organic compounds (Table 1). On the other hand, embryogenic callus segments, induced from mature seeds, were transferred to RM composed of MS mineral salts supplemented with different concentrations and combinations of BAP (0.5, 1, 2 and 4 mg.l^{-1}), ANA (0.2 and 0.5 mg.l^{-1}) and 2,4-D (0.03, 0.05, 0.1 and 0.2 mg.l^{-1}) (Table 5). After 6 weeks of culture on RM, the regenerated plantlets were transferred to a half strength MS medium.

Explants were cultured under 12 h light photoperiod (30 μmol.m^{-2}.s^{-1}) at 26±2°C. Number of shoots regenerated from embryogenic callus segments was recorded after 12 weeks of culture on RM.

Macroscopic features of embryogenic calluses segments, globular and torpedo shape somatic embryos were observed using a ZEISS light microscope and photographed with a digital camera.

Detection of acemannan

Acemannan content was determined by High Performance Liquid Chromatography

Table 1. Composition of culture media used for the regeneration of somatic embryos in aloe (*A. barbadensis* Mill.) shoots.

Component	RM-01	RM-02	RM-03	RM-04
Macroelements	MS	MS	MS	MS
Microelements	MS	MS	MS	MS
Fe-EDTA	MS	MS	MS	MS
Thiamine (mg.l^{-1})	20	10	10	5
Glycine (mg.l^{-1})	20	2	-	-
Cysteine (mg.l^{-1})	40	10	-	-
Casein hydrolyzate (mg.l^{-1})	200	200	-	400
Malt extract (mg.l^{-1})	800	-	-	200
Nicotinic acid (mg.l^{-1})	-	1	-	-
Piridoxine (mg.l^{-1})	-	1	-	-
2,4-D (mg.l^{-1})	1	-	1	-
BAP (mg.l^{-1})	4	4	4	4
Myo-inositol (mg.l^{-1})	200	200	200	-
Adenine hemisulphate (mg.l^{-1})	60	60	-	-
Sucrose (g.l^{-1})	30	40	40	40
Phytagel (g.l^{-1})	3	3	3	3
pH	5.6	5.6	5.6	5.6

(HPLC) according to Okamura et al. (1996). Briefly, the inner gel from fresh leaves and embryogenic calluses (6 g fresh weight) was powdered and lyophilized using a Hull liophilizator. Then, samples were dissolved in 0.5% acidic solution of sodium and shaken for 8 h. The acidic aqueous solution was filtered through a 0.22 µm filter. The acemannan content was detected using HPLC (Shimatzu, SIL10-AS) composed of a TosoHaas column (TKSgel G5000PWxl) (TOSOH Bioscience, LLC), equipped with SIL-10A injector and UV lamp (Perkin Elmer 235C). As reference, standard acemannan (0.2%) was used.

RESULTS

Explant disinfection

Establishment of in vitro cultures of aloe was limited by phenolic oxidation (Figure 2A) and contamination (Table 2). Then, in the present study several disinfection treatments involving sonication were evaluated. Contamination of explants ranged from 0-32% and explant survival rate from 27-85% (Table 2). Although contamination was completely eliminated with 10 and 15 min sonication, the survival rate decreased. The highest survival rate and the lowest contamination percentage were obtained with 5 min sonication (Table 2).

Induction of somatic embryogenesis

In order to induce embryogenic callus formation, in a first experiment, immature inflorescences, immature flowers, mature ovaries, rhizome dics, apical meristems, and leaf bases were cultured on MS-00, MS-01 and MS-02 media (Table 3). Among these explants, embryogenic callus was only obtained using apical meristems and leaf bases (Figure 1D). In contrast, when immature inflorescences, immature flowers, mature ovaries, and rhizome discs were cultured on MSC 01 and MSC 02, induction of embryogenic calluses was not observed. The highest embryogenic callus frequency was obtained when leaf bases were cultured on MSC 01 (0.25 2,4-D $mg.l^{-1}$ and 1 $mg.l^{-1}$ kinetin) (Figure 1B-C). When high concentrations of 2,4-D and low kinetin concentrations were used (MSC

Table 3. Effect of explant source on embryogenic callus induction in aloe (*A. barbadensis* Mill.) after four weeks of culture on MSC 00, MSC 01 and MSC 02.

Explant	Culture medium		
	MS-00	MS-01	MS-02
	Embryogenic callus induction (%)		
Immature inflorescences	0	0	0
Immature flowers	0	0	0
Mature ovaries	0	0	0
Rhizome discs	0	0	0
Apical meristems	0	21	17
Leaf bases	0	75	13

Table 2. Effect of disinfection treatments on contamination, after 8 days of culture, of aloe (*A. barbadensis* Mill.) explants.

Sonication (min)	Total number of disinfected explants	Contamination (%)	Survival (%)
2	34	32	67
3	34	32	67
4	34	29	73
5	34	15	85
10	14	0	43
15	14	0	27

02) embryogenic callus induction from apical meristems and leaf bases decreased. On the other hand, no embryogenic callus was obtained when explants were cultured on induction medium devoid of plant growth regulators (MSC 00) (Table 3).

Culture of *A. barbadensis* leaf bases on MSC supplemented with a range of concentrations of BAP and 2,4-D resulted in a differential response (Table 4). The best results were obtained using 2.5 $mg.l^{-1}$ 2,4-D, 2 $mg.l^{-1}$ BAP and 40 $mg.l^{-1}$ adenine sulphate (Table 4). The embryogenic calluses obtained were characterized by their yellowish color and friability (Figure 1E).

On the other hand, when *A. barbadensis* Mill. zygotic embryos were used as an explant (Figure 2A), somatic embryogenesis began with the development of a translucid primary callus after 6 weeks of culture on calluses induction medium (Figure 2B). Figure 2C shows the differences between embryogenic calluses obtained using leaf bases and zygotic embryos of *A barbadensis*. Between the 2 media evaluated, for calluses induction, embryogenic calluses were only obtained using the MSC-15 (2.5 $mg.l^{-1}$ 2,4-D and 2 $mg.l^{-1}$ BAP) medium. No embryogenic callus was induced using 2 $mg.l^{-1}$ 2,4-D and 2 $mg.l^{-1}$ BAP after 30 days of culture. By sub-culturing these embryogenic calluses every 4 weeks on maintenance medium, it was possible to obtain 72 isogenic callus lines. Light microscopy observations showed formation of globular and torpedo

Table 4. Effect of BAP and 2, 4-D concentration on embryogenic callus formation from leaf bases of aloe (*A. barbadensis* Mill.), after 14 weeks of culture on MSC supplemented with different plant growth regulators.

Culture medium ($mg.l^{-1}$)			Explants N.°	Callus induction (%)			
BAP	2,4-D	Adenine		4 weeks	6 weeks	12 weeks	14 weeks
1.0	1.5	40	45	0	0	40	87
1.0	2.0	40	45	0	13	55	74
1.0	2.5	40	45	0	0	22	55
1.0	1.5	80	45	3	3	10	25
1.0	2.0	80	45	0	0	27	58
1.0	2.5	80	45	0	0	10	46
1.5	1.5	40	45	0	0	13	13
1.5	2.0	40	45	3	10	42	61
1.5	2.5	40	45	5	7	20	33
1.5	1.5	80	45	4.6	7	30	42
1.5	2.0	80	45	3	3	29	43
1.5	2.5	80	45	0	0	17	48
2.0	1.5	40	45	0	7	25	71
2.0	2.0	40	45	5	12	33	65
2.0	2.5	40	45	28	44	47	89
2.0	1.5	80	45	0	0	13	20
2.0	2.0	80	45	10	20	45	50
2.0	2.5	80	45	0	0	13	33

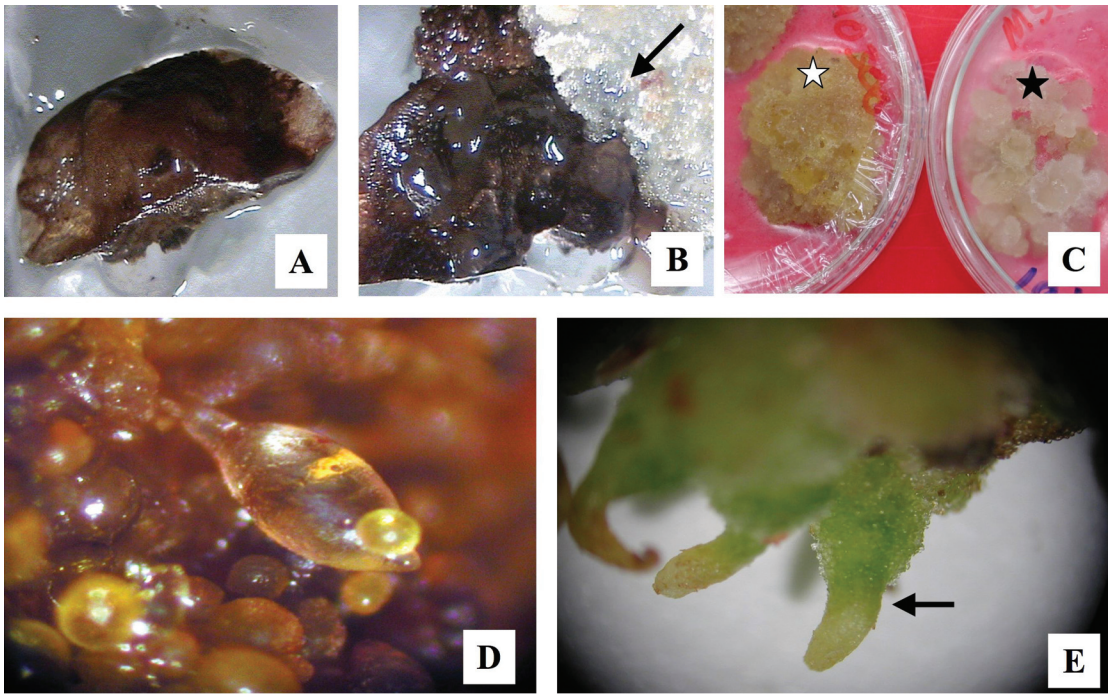

Fig. 2. Somatic embryogenesis in aloe (*A. barbadensis* Mill.). A) Seeds of aloe used as explants for embryogenic calluses induction, B) Embryogenic calluses (arrow) obtained after 12 weeks of culture on MSC-15 (2.5 $mg.l^{-1}$ 2,4-D and 2 $mg.l^{-1}$ BAP), C) Comparison of embryogenic calluses obtained from leaves (☆) and seeds (★) after 16 weeks of cultured on MSC-15, D) Globular and torpedo shaped somatic embryos, E) Shoots (arrow) obtained from embryogenic calluses after 12 weeks of cultured on regeneration medium.

shape somatic embryos on the callus surface (Figure 2D).

Plant regeneration

In relation to the regeneration, shoots were observed after 4 weeks of culture of embryogenic calluses induced from zygotic embryos on RM medium supplemented with different concentrations of BAP, ANA and 2,4-D (Figure 2E). The best results were obtained when embryogenic calluses were cultured on RM with 0.2 $mg.l^{-1}$ 2,4-D and 2 $mg.l^{-1}$ BAP, 0.05 $mg.l^{-1}$ 2,4-D and 2 $mg.l^{-1}$ BAP, 0.1 $mg.l^{-1}$ 2,4-D and 0.5 $mg.l^{-1}$ BAP or 0.5 $mg.l^{-1}$ ANA and 0.5 $mg.l^{-1}$ BAP (Table 5).

On the other hand, when embryogenic calluses induced from leaf bases, were cultured on RM1, RM2, RM3, and RM4, the formation of numerous trichomes and green areas; which did not develop into plantlets were observed (data not shown). These results suggest that, under the evaluated conditions, these embryogenic calluses had no morphogenic response.

Detection of acemannan

The HPLC analysis revealed that the polysaccharide detected from *A. barbadensis* inner gel was acemanan (Figure 3A). The polysaccharide had a retention time of 5.382, whereas, the extracts of leaf base derived embryogenic calluses showed a retention time of 5.385 (Figure 3B). The analysis showed that the acemannan concentration in embryogenic calluses was 80 times lower than the concentration in the inner gel obtained from fresh leaves (Table 6).

Table 5. Effect of ANA, 2,4-D and BAP on the regeneration of shoots from *Aloe barbadensis* Mill. embryogenic callus, after 12 weeks of culture on RM with different plant growth regulators.

Plant growth regulator ($mg.l^{-1}$)			Total number of shoots
ANA	2,4-D	BAP	
-	0.03	0.5	62
-	0.1	0.5	111
-	0.2	0.5	98
-	0.03	1.0	23
-	0.1	1.0	90
-	0.2	1.0	95
-	0.05	2.0	135
-	0.2	2.0	125
-	0.4	2.0	34
-	0.05	4.0	95
-	0.2	4.0	98
-	0.4	4.0	111
0.5	-	0.5	118
0.5	-	1.0	99
0.2	-	0.5	72
0.2	-	1.0	62

Table 6. Concentration and retention time of acemannan isolated from the inner gel and embryogenic callus of *A. barbadensis* Mill.

Sample	Retention time (min)	Acemannan ($mg.100\ ml^{-1}$)
Gel from fresh leaves	5.382	86.5
Embryogenic callus 1	5.317	2.1
Embryogenic callus 2	5.332	0.8
Embryogenic callus 3	5.265	1.8

DISCUSSION

A protocol for the induction of *A. barbadensis* Mill. embryogenic callus and for the development of somatic embryos into shoots was successfully obtained. Moreover, acemannan was detected in aloe inner gel and embryogenic calluses based on HPLC.

It has been reported that contamination and browning of the explants and surrounding culture medium limit the establishment of aloe primary cultures under in vitro conditions (Abrie and van Staden 2001). In this study, bacterial contamination was reduced to 15% with 5 min in an ultrasonic sonicator (Table 1).

Recently, ultrasound has found multiple applications in plant tissue culture. These applications include stimulation of shoot regeneration from recalcitrant accessions, seeds germination, genetic transformation, secondary products and

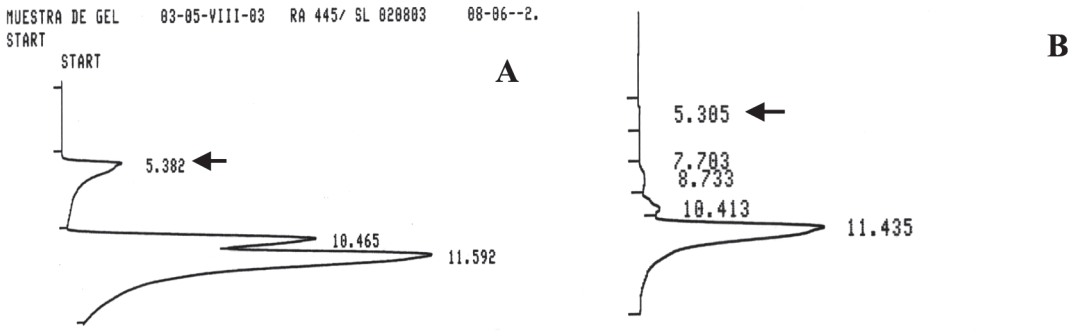

Fig. 3. HPLC analysis of acemannan (A).The retention time in the inner gel obtained from fresh leaves was 5.382, B) The retention time for the leaf base-derived embryogenic callus was 5.305. The arrows represent the peak of acemannan.

protein synthesis, and control of microorganisms (Gaba et al. 2006, Ananthakrishnan et al. 2007).

Ultrasound is an effective method for separation of bacterial cells from the explant surface, because of its strong mechanical shear stresses. Moreover, low doses of ultrasound produce a de-agglomeration while high intensities and long exposure times produce damage in the cell wall causing the death (Blume and Neis 2005). In this sense, ultrasound has been used for killing bacteria and for improving the effectiveness of disinfecting treatments (Duckhouse et al. 2004). Blume and Neis (2005), demonstrated that efficiency of NaOCl can be improved by a combined application of ultrasound treatment, due to a better chlorine dispersion in the aqueous media, which improves the fast chemical and bactericidal reaction. Aller et al. (1978), reported that sonication of a root meristem of *Allium cepa,* in the presence of detergent, eliminates external bacteria when the use of antibiotics failed. Rediske et al. (1999) reported that ultrasound, in combination with erythromycin, reduced the viability of *Pseudomonas aeruginosa*. These authors concluded that sonication destabilized the outer bacterial cell membrane enhancing the permeability of antibiotics.

Ananthakrishnan et al. (2007) indicated that ultrasound provoked the ablation of the cuticle of squash seeds allowing an enhanced uptake of water, nutrients, and plant growth regulators by the explant. In a similar manner, ultrasound of aloe explants could damage the surface and remove the cuticle wax permitting an enhanced entry of NaOCl.

Several investigations, in diverse species, have demonstrated that success in regeneration through somatic embryogenesis depends on the genotype, origin, physiological stage and age of the explant, and the type and concentration of plant growth regulators (Fuentes et al. 2000, Gatica et al. 2007).

Regarding explant type, the results obtained in this research, demonstrated that it is possible to induce somatic embryogenesis using leaf bases and apical meristems in *A. barbadensis* (Table 2). Generally, immature organs and meristematic tissues; which contain undifferentiated cells, are more suitable for plant morphogenesis (Hoque and Mansfield 2004). Moreover, in the present study, a system for plant regeneration of *A. barbadensis* was developed using zygotic embryos as explants. Induction of somatic embryogenesis from seeds of *A. pretoriensis* (Groenewald et al. 1975), *A. ferox* (Racchi 1988) and *A. polyphylla* (Abrie and van Staden 2001) has been reported. Nevertheless, this is the first report in which zygotic embryos of *A. barbadensis* Mill. are used for embryogenic callus induction and plant regeneration.

About the influence of plant growth regulators, the best results were obtained using 2,5 $mg.l^{-1}$ 2,4-D, 2 $mg.l^{-1}$ BAP and 40 $mg.l^{-1}$ adenine sulphate (Table 3). In somatic embryogenesis induction, investigations have been focused on the type, concentration and time of application of plant growth regulators. The plant growth regulators used in the induction phase can play an important role in somatic embryogenesis processes. Somatic embryogenesis is generally promoted, in the majority of studied species, by auxins, either alone or in combination with cytokinins. Among auxins, 2,4-D is the most frequently used, while BAP is the most employed cytokinin, followed by kinetin (Jiménez 2005). Haensch (2007), indicated that parenchyma cells of *Pelargonium x domesticum* cv. Madame Layal were stimulated to somatic embryogenesis by the application of 2,4-D in combination with BAP.

Concerning the regeneration of somatic embryos from embryogenic calluses and their subsequent development into shoots, it was shown that a combination of 0.05 $mg.l^{-1}$ 2,4-D with 2 $mg.l^{-1}$ BAP gave rise to the highest number of shoots developed from embryogenic calluses derived from zygotic embryos (Table 5). On the other hand, in the present study it was not possible to regenerate somatic embryos from embryogenic calluses derived from leaf bases. One explanation could be that continuous subculture of these calluses, on maintenance medium supplemented with 2,4-D, results in the inhibition of differentiation of

somatic embryos, as seen in other plants (Haensch 2007). Another possibility is that these embryogenic calluses had no morphogenetic capacity to respond, as reported by Meneses et al. (2005), who observed non-regenerable green areas, in rice (*Oryza sativa*) calluses. Moreover, the subculture of embryogenic callus fragments on RM medium, supplemented with BAP (4 $mg.l^{-1}$) and with or without 2,4-D (1 $mg.l^{-1}$), was not suitable for somatic embryo regeneration. This contrasts to the results of Haensch (2007), in *Pelargonium x domesticum* cv. Madame Layal, who obtained somatic embryos in a broad spectrum of 2,4-D/BAP combinations.

In the last years, a great effort has been made to produce secondary metabolites of commercial interest in plant cell cultures. Nevertheless, for many of the pharmaceutical compounds of interest, the production is low or even zero in the cultured cells, as seen in the present study. This is due to the fact that production is controlled in a tissue-specific manner and dedifferentiation results in the loss of production capacity (Verpoorte et al. 2002). Therefore, other approaches such as culture of aloe differentiated cells (leaves and shoots), induction of acemannan pathway with various elicitors, optimization of media and culture conditions, or cell line selection for high producing strains could be investigated.

The in vitro protocol reported in this study could be used for mass production of somatic embryos in bioreactors for further use as artificial seeds and to obtain competent target tissue for genetic modification.

ACKNOWLEDGEMENTS

To Paul Hanson (School of Biology, UCR) for the heplful disscusion, critical review and English correction of the manuscript. We also thank Rosaura Romero (School of Chemistry, UCR) for her collaboration in the acemannan detection. This research was supported by the University of Costa Rica and the National Commission of Scientific and Technological Investigation of Costa Rica (Consejo Nacional de Investigaciones Científicas y Tecnológicas, CONICIT).

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