African Journal of Biotechnology Vol. 12(12), pp. 1419-1430, 20 March, 2013
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB12.2857
ISSN 1684–5315 ©2013 Academic Journals

Full Length Research Paper

Cryopreservation of *in vitro*-grown shoot tips of apricot (*Prunus armeniaca* L.) using encapsulation-dehydration

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Accepted 14 February, 2013

***In vitro* grown apricot (*Prunus armeniaca* L.) cv. El-Hamawey shoot tips were successfully cryopreserved using an encapsulation-dehydration procedure. Shoot tips were encapsulated in calcium-alginate beads before preculture on woody plant (WP) medium supplemented with different sucrose concentrations; 0.1, 0.3, 0.5, 0.75 and 1.0 M for 2, 4, 6 and 8 days at 25°C. Encapsulated shoot tips were then dehydrated by incubation in the sterile air flow of a laminar air-flow cabinet for 0.0 - 6 h and immediately plunged into liquid nitrogen (LN, -196°C). The highest survival (100%) and regrowth (92.3%) rates were obtained with encapsulated unfrozen apricot shoot tips that were precultured on WP medium containing 0.5 M sucrose for two days and followed by dehydration for 2 h. After recovering from liquid nitrogen and rapid thawing in a water bath at 38°C for 2 to 3 min, the greatest survival (74.5%) and regrowth (71.8%) were obtained and encapsulated shoot tips were precultured for two days in WP medium containing 0.75 M sucrose dehydrated for 2 h, desiccated to 19.55% moisture content and conserved three months in liquid nitrogen. After two years in liquid nitrogen storage, the greatest regrowth percentage (60.4%) was obtained when encapsulated cryopreserved shoot tips were precultured for two days in medium containing 0.75 M sucrose and also with 2 h of dehydration. This study has successfully developed a simple and effective protocol for the cryopreservation of apricot shoot tips. Genetic stability of plantlets derived from cryopreserved shoot tips was evaluated using random amplification of polymorphic DNA (RAPD) markers.**

Key words: Apricot (*Prunus armeniaca* L), cryopreservation, encapsulation/dehydration, RAPD.

INTRODUCTION

Apricot is a member of the genus *Prunus*, which comprises other economically important crops like peach, almond and plum. Apricot (*Prunus armeniaca* L.) is an important stone – fruit tree growing in temperate climates. The main growing areas are China, the Mediterranean European countries, Turkey and USA. Apricots are mainly used to prepare juices and for fresh consumption, with a fair amount of 2.7 million tons of fruit per year world production being destined to comfitures, drying and canning (FAO, 2006). Apricot is a good candidate for *in vitro* propagation because scion selections are currently budded onto seedlings (Hartmann and Kester, 1975). Data on *in vitro* apricot culture are quite limited in comparison to other *Prunus* species. Some reports indicate that the capacity to produce axillary shoots is low (Skirvin et al., 1979; Kataeva and Kramarenko, 1989).

Cryopreservation techniques (including cryopreservation of *in vitro* grown shoot tips) that are effective for a wide range of genotypes are important for both gene banks and breeders collections. These techniques save time and labor costs and increase the security of germplasm collections (Chang and Reed, 2001). Cryopreservation is an important tool for long-term storage of biological materials. It offers a safe and cost-effective option for long-term conservation of genetic resources in many plant species. At the temperature of liquid nitrogen (LN, -196°C), all the metabolic activities of cells are at a standstill. Thus, they can be preserved in such a state for a long period (Sakai, 1995). Many new cryopreservation techniques such as simple freezing, vitrification, encapsulation/dehydration and encapsulation/vitrification have been reported for successful use for many cells, tissues

and organs of plant species (Engelmann, 2000; Fahy et al., 1984; Grout, 1995). However, for successful cryopreservation, many factors are involved, such as starting materials, pretreatment conditions, cryprocedures and post-thaw treatment (Reinhoud et al., 2000). Therefore, in order to accomplish successful cryopreservation for each species and cultivars, a separate study must be carried out.

Encapsulation-dehydration includes encapsulation of plant material in calcium alginate beads, followed by pregrowth treatment in a medium containing high levels of sucrose ranging from 0.3 to 1.5 M for at least one day (Shatnawi et al., 1999). The alginate beads are then dehydrated before freezing using either air-drying in a laminar flow hood or by exposure to silica gel (Al-Ababneh et al., 2003). Encapsulation of plant material with calcium alginate induced a short delay only in the development of meristems (Clavero-Ramirez et al., 2005). Encapsulation-dehydration was described to have many advantages over virification (Lipavska and Vreugdenhil, 1996). Vitrification method involves very delicate steps to handle compared to encapsulation-dehydration (Charoensub et al., 2004). Also, cryoprotectant solution used in vitrification is complex and it is composed of cryoprotectant cocktails while sucrose is the most used in encapsulation-dehydration methods (Wang et al., 2005). Toxicity caused by cryoprotectants especially dimethyl sulfoxide (DMSO) is a major problem in vitrification and could be avoided using encapsulation-dehydration method (Lipavska and Vreugdenhil, 1996). The random amplification of polymorphic DNA (RAPD) technique is considered to be a fast, simple and efficient method for evaluating the genetic stability of cryopreserved material and can be used after the completion of a freezing experiment as a quick complement to other genetic stability evaluation methods (Hirai and Sakai, 2000). This technique has been successfully used to study the genetic stability of cryopreserved materials of *Arachis* species (Gagliardi et al., 2003), *Diascorea bulbifera* (Dixit et al., 2003), and *Vitis vinifera* (Zhai et al., 2003).

The objective of this study was to establish efficient techniques for cryopreservation of apricot (*P. armeniaca* L.) cv. El-Hamawey shoot tips by encapsulation/dehydration technique and to study genetic stability after cryopreservation using RAPD techniques to define a reliable method for the germplasm preservation.

MATERIALS AND METHODS

Preparation of explant for cryopreservation

Shoots formation obtained from shoot tips cultured on woody plant (WP) medium supplemented with 1.0 mg/l Zeatin, 0.1 mg/l, IAA 3% sucrose and 2.5 g/l phytagel. The pH was adjusted to 5.8 before autoclaving (Hemaid, 2012). Shoot tips approximately 5 mm long, obtained from shoot cultures after up to three subcultures on the WP medium supplemented with 4 mg/l benzyl adenine (BA) and 0.5 mg/l 2-isopentenyl adenine (2iP) (Figure 1). The shoot tips were then subjected to a series of treatments before cryopreservation. All cultures were incubated at 25 ± 2°C under a 16/8 h (light/dark) photoperiod of 45 to 50 µmol $m^{-2}s^{-1}$ irradiance provided by Phillips cool white fluorescent tubes.

Encapsulation of preconditioned shoot tips

Shoot tips (5 mm long) of *in vitro* grown plantlets were precultured for one day on a solid WP medium containing 0.3 M sucrose in the dark at 25 ± 2°C. Shoot tips were suspended in a calcium-free liquid medium with 3% (w/v) Na-alginate. Shoot tips were pipetted with some alginate solution, which was dispensed as drops into a liquid WP medium 0.1 M calcium chloride, where alginate formed solid beads through polymerization with calcium.

Preculture of encapsulated shoot tips

Encapsulated shoot tips were precultured on hormone-free liquid WP medium supplemented with different concentrations [0.0 (control), 0.1, 0.3, 0.5, 0.75 or 1.0 M] of sucrose and incubated for 2, 4, 6 and 8 days at 25°C. After each incubation period, beads were transferred to fresh medium of the same composition in a new 100 ml flask on rotary shaker (100 rpm) and incubated in darkness at 4°C for one day. Each bead was containing one shoot tip, and data collected were percentages of survival and regrowth.

Dehydration of precultured encapsulated shoot tips

To determine the optimum drying time, the encapsulated shoot tips were dehydrated on sterilized filter paper in uncovered 9-cm Petri dishes for various periods (0.0 to 6 h) in the air current of a laminar flow cabinet at room temperature. Liquid nitrogen tolerance was tested for each drying time at 2 h intervals.

Determination of moisture content (MC)

Beads moisture content was measured during dehydration (prior to freezing). Beads were weighed every 2 h, to produce desiccation curves for each osmoticum. Beads moisture content percentage (MC %) was determined on a fresh weight basis using the following equation: Percentage of bead moisture content = 100*[(Fresh weight - Dry weight) / Fresh weight].

Growth recovery after cryopreservation

The beads were placed in 2 ml sterile cryovials and directly immersed into liquid nitrogen while the beads (non-cryopreserved) were then transferred to a recovery medium for growth. The beads were kept in liquid nitrogen for at least two years. After cryopreservation, regrowth of shoot tips were tested by taking samples every three months, cryogenic vials containing beads were thawed in a water bath at 38°C for 2 - 3 min. Encapsulated non-cryopreserved (-LN) and encapsulated cryopreserved (+LN) beads were inoculated onto a solid WP recovery media containing 1.0 mg/L Zeatin, 0.1 mg/L IAA and 0.1 M sucrose, then kept in the dark for three days. Survival rates were tested for non–frozen and frozen encapsulated shoot tips by transferred to the normal growth conditions as described previously and kept for further recovery. After four weeks, shoot tips were checked for any recovery signs.

Shoot multiplication

Microshoots (2 cm) were subculture on WP medium supplemented with 4 mg/l BA and 0.5 mg/l 2iP (Hemaid, 2012). For each replicate,

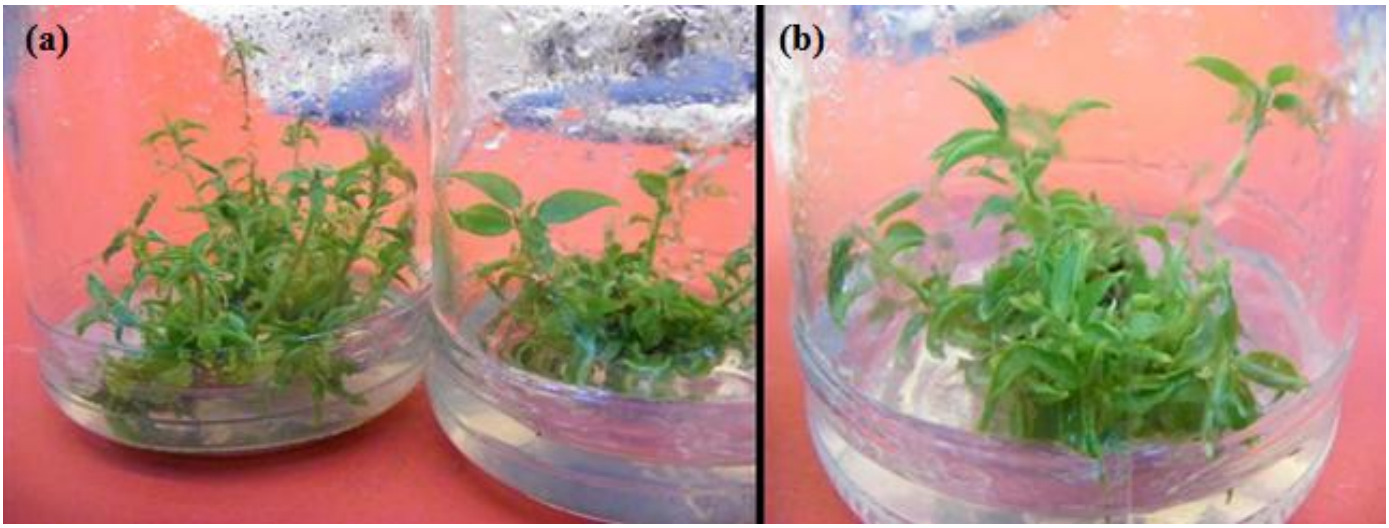

Figure 1: Shoot formation on WP medium supplemented with: (a) 4 mg/l BA and 0.5 mg/l 2iP and (b) 3 mg/l BA and 0.5 mg/l 2iP.

50 ml medium was dispensed into 250 ml culture vessels.

Rooting and acclimatization of regenerated plants

Shoots derived from multiplication stage (about 3 cm in length) were transferred to liquid Murashige and Skoog (MS) medium containing with 2 mg/L IBA, 0.5 mg/L NAA and 40 mg/L phloroglucinol. Cultures were incubated in the dark for 7 days and then transfer a 16 h photoperiod for 3 weeks (Hemaid, 2012). The plantlets were transferred to pots containing a mixture media of peatmoss and sand (1:1), plastic pots enveloped in polyethylene bags were incubated under 3000 Lux light intensity derived from cool white fluorescent lamps for 16 h photo period at 28 ± 2°C in growth cabinets. After four weeks polyethylene bags were completely opened and after three weeks more polyethylene bags were removed and plantlets were maintained under greenhouse conditions.

Random amplified polymorphic DNA (RAPD) analysis

RAPD analysis was carried out to test genetic stability of plantlets regenerated from cryopreserved and non-cryopreserved shoot tips, the genomic DNA was isolated from rooted plantlets growing *in vitro* conditions. For RAPD analysis, 10 oligonucleotide primers were chosen but however only five primers produced bands (Table 4).

Isolation of genomic DNA for RAPD technique

Young leaves from the cryopreserved and regenerated plantlets as well as control plantlets were used for DNA extraction. A cetyltrimethyl ammonium bromide (CTAB) protocol (Murray and Thompson, 1980) was adopted with slight modification for DNA extraction. Leaf material (0.5 g) was first washed in running tap water and then in distilled water two to three times after chopping the leaves to a size of 1 cm^2 using a sterilized scalpel with disposable blades. They were then dried by spreading on tissue paper. Dried leaves were then pulverized in liquid nitrogen in a precooled mortar and pestle by rapid grinding to a fine powder. The dry powder was transferred to a 2 ml centrifuge tube, mixed thoroughly with 1 ml extraction buffer (0.7 N NaCl, 1% CTAB, 50 mM Tris–HCl (pH8.0), and 10 mM ethylenediaminetetraacetic acid (EDTA)), 100 µl (% or conc.) polyvinylpyrrolidone (PVP), 50 µl (% or conc.) sodium dodecyl sulphate (SDS), and 20 µl β- mercaptoethanol and incubated in a water bath at 65°C for 1 h. The mixture was then centrifuged at 15,000 rpm for 10 min at 4°C. The upper phase was collected and subjected to repeated chloroform/ isoamyl alcohol (24:1) extraction, until the interphase disappeared. The final supernatant was collected and added to one-tenth volume of 3.0 M sodium acetate and double volume of chilled absolute isopropyl alcohol. Extracts were placed in a refrigerator at 4°C for 30 min and then centrifuged at 10,000 rpm for 10 min at 4°C to pellet the DNA. The supernatant was discarded and the pellet washed in 70% alcohol and centrifuged at 10,000 rpm for 5 min at 4°C. The supernatant was again discarded and the pellet air dried for 20 min. The pellet was then dissolved in 0.5 ml of 1× Tris–EDTA buffer (10 mM Tris HCl, 1 mM EDTA- pH 8.0) and stored at −20°C.

Agarose gel electrophoresis

Agarose gel electrophoresis was carried out in a horizontal gel electrophoresis unit. The Tris-acetate-EDTA (TAE) buffered gels (0.04 Tris acetate, 0.001 M EDTA at pH8.0) were either 0.7% w/v agarose for visualizing genomic DNA and 1% w/v agarose for amplified products. After cooling to about 50°C, ethidium bromide was added to a final concentration of 0.5 µg/ ml. The DNA sample was mixed with the required volume of gel loading buffer (6.0× loading dye 1.17 M (40%) sucrose, 0.25% bromophenol blue). Each well was loaded with 20 µl of sample. One of the wells was loaded with 5 µl of molecular weight marker along with required volume of gel loading buffer. Electrophoresis was performed at 75 V until the loading dye reached the length of the gel. The gel was visualized using an ultraviolet–visible (UV–Vis) transilluminator.

Polymerase chain reactions (PCR) reaction

DNA amplification was done using arbitrarily designed primers (Operon Inc., California, USA) adopting the procedure of William et al. (1990) with required modifications. Polymerase chain reactions (PCR) of genomic DNA were performed in 12.5 µl reaction mixtures

Table 4. Distribution of monomorphic and polymorphic bands in plantlets from cryopreserved shoot tips and non-cryopreserved shoots of apricot.

Primer	Number of scorable bands per primer	Total number of monomorphic bands	Total number of polymorphic bands	Length of amplified DNA fragment
OPE A-01 5`- CAGGCCCTTC-3`	9	9	0	300-2500 bp
OPE A-11 5`- CAATCGCCGT-3`	7	6	1	450-1600 bp
OPE B-17 5`-AGGGAACGAG-3`	8	5	3	200-2000 bp
OPE N-13 5`-AGCGTCACTC -3`	6	5	1	300-2000 bp
OPE P-09 5`- GTGGTCCGCA-3`	7	5	2	250-2500 bp
Total number of bands	37	30	7	

containing 1.25 µl 10× PCR buffer, 5 pM primer, 200 µM each of dNTPs, 0.75 units of Taq, and 20 ng genomic DNA. The PCR programme included an initial denaturation step at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 45°C for 1 min, and extension at 72°C for 2 min, then 35°C for 1 min. The synthesis step of the final cycle was extended further by 5 min. Finally, the products of amplification were cooled to 4°C. The DNA fragments produced and the PCR molecular weight markers were visualized by agarose gel electrophoresis, stained with ethidium bromide, and photographed with the help of gel doc system.

Experimental design and statistical analysis

Treatments were arranged in a complete randomized design (CRD). Each treatment was replicated five times with five shoot tips per replicate. The collected data were statistically analyzed using Statistical Analysis System (SAS). Means were separated according to the least significant difference (LSD) at 0.05 probability level.

RESULTS AND DISCUSSION

Cryopreservation by encapsulation/dehydration was studied for shoot tips of apricot. This technique combined two cryoprotective treatments; precultured with sucrose and air dehydration. Both treatments have been used to investigate the optimal duration of dehydration, the most favorable sucrose concentrations and sucrose preculture for inducing cytoplasmic vitrification in order to avoid the formation of intracellular ice crystals during rapid cooling in LN (Wilkinson et al., 1998).

Effect preculture and different sucrose concentrations on shoot tips of apricot

Significant variations in survival and regrowth of non-cryopreserved shoot tips were obtained along with the different concentrations of sucrose in the preculture medium and the duration of preculture for apricot (*P. armeniaca* L.) cv. El-Hamawey (Table 1). High survival (98.4 to 100%) rates were obtained after preculture of the shoot tips of apricot with 0.0, 0.1, 0.3 or 0.5 M sucrose at two or four days. Complete survival and regrowth of shoot tips were obtained along with 0.0, 0.1, 0.3 and 0.5 M at two days; while, the percentages of survival and regrowth (98.4 and 86.8%, respectively) after preculture in 0.5 M sucrose at four days. The minimum survival and regrowth of shoot tips were obtained at 1.0 M sucrose for all preculture duration. From the results, preculture of shoot tips in encapsulated-dehydration step was decided and performed for two days at 25°C on the WP medium with 0.5 M sucrose.

The results are similar to the findings of Al-Ababneh et al. (2003) in which high survival and regrowth were obtained for shoot tips of bitter almond (*Amygdalus communis* L.) when shoot tips were precultured on 0.1 or 0.3 M sucrose. Moges et al. (2004) reported that 95 - 100% of African violet shoot tips were survived after two days of preculture with 0.3 and 0.5 M sucrose. Moreover, Baghdadi et al. (2010) reported that maximum survival (100%) rates were obtained after preculture of crocus calli on 0.1 and 0.5 M sucrose irrespective of preculture duration.

Encapsulation-dehydration: In sterile airflow

There was a significant interaction effect of dehydration solution (sucrose concentration) and dehydration duration on survival and regrowth of non cryopreserved and cryopreserved shoot tips of apricot (*P. armeniaca* L.) cv. El-Hamawey (Table 2). In our current study, minimum survival (12.5%) was observed when non-cryopreserved shoot tips were suspended in 1.0 M sucrose for two days with 6 h dehydration in sterile airflow (Table 2). The greatest survival (100%) was obtained when non-cryopreserved (-LN) shoot tips of apricot were pretreated with 0.5 and 0.75 M sucrose for two days followed by 2 h dehydration period, where the beads attained 58.42 and 19.55% moisture content, respectively (Figure 2). The moisture content of the beads decreased with increasing dehydration. The regrowth rate of encapsulation non-cryopreserved shoot tips decreased with increasing sucrose concentrations and dehydration period.

From the results, the survival ratio and regrowth of all encapsulated non-cryopreserved shoot tips decreased with decreasing bead water content and increasing dehydration time (Table 2). The dehydration step was neces-

Table 1. Effect of preculture duration and sucrose concentration on survival and regrowth percentages of non-cryopreserved (-LN) shoot tips of apricot (*Prunus armeniaca* L.) cv. El-Hamawey.

Preculture duration (day)	Sucrose conc. (M)	Survival (%)	Regrowth (%)
2	0.0	100[a]	100[a]
	0.1	100[a]	100[a]
	0.3	100[a]	100[a]
	0.5	100[a]	100[a]
	1.0	78.3[c]	48.5[e]
4	0.0	100[a]	100[a]
	0.1	100[a]	100[a]
	0.3	100[a]	96.2[b]
	0.5	98.4[a]	86.8[c]
	1.0	23.2[f]	12.4[f]
6	0.0	100[a]	100[a]
	0.1	100[a]	100[a]
	0.3	95.3[b]	75.5[d]
	0.5	65.4[d]	48.5[e]
	1.0	14.5[f]	10.2[f]
8	0.0	100[a]	100[a]
	0.1	100[a]	100[a]
	0.3	60.5[d]	38.5[e]
	0.5	30.8[e]	14.8[f]
	1.0	0.0[g]	0.0[g]

Means within column, for each preculture period, having different letters are significantly different according to LSD at ≤ 0.05.

Table 2. Effect of sucrose concentration and dehydration duration on survival and regrowth percentages of encapsulated non-cryopreserved (-LN) and cryopreserved (+LN) shoot tips of apricot (*Prunus armeniaca* L.) cv. El-Hamawey.

Sucrose (M)	Dehydration duration (h)	Survival %		Regrowth %		MC%
		+LN	-LN	+LN	-LN	
0.5	0	0.0[h]	100[a]	0.0[g]	98.2[a]	76.55
	2	0.0[h]	100[a]	0.0[g]	92.3[ab]	58.42
	4	9.3[g]	76.5[c]	8.3[f]	72.5[c]	46.12
	6	15.4[f]	62.3[e]	11.2[e]	56.3[e]	38.65
0.75	0	68.2[c]	100[a]	52.2[bc]	97.7[a]	54.13
	2	74.5[a]	100[a]	71.8[a]	87.2[b]	19.55
	4	54.3[d]	78.2[c]	50.6[c]	42.4[f]	17.35
	6	38.5[e]	67.8[e]	29.3[d]	31.5[g]	14.75
1	0	71.2[b]	82.3[b]	58.5[b]	80.5[b]	48.24
	2	68.3[c]	72.7[d]	54.5[bc]	60.8[d]	17.32
	4	18.4[f]	46.5[f]	12.5[e]	40.2[f]	16.38
	6	10.6[g]	12.5[g]	7.8[f]	8.5[h]	12.48

MC%, Moisture content percentage for bead. Means within column having different letters are significantly different according to LSD at P ≤ 0.05.

Figure 2. Encapsulated shoot tips of apricot (*Prunus armeniaca* L.) cv. El-Hamawey; Calcium alginate beads formed by encapsulation of explants using 3% sodium alginate.

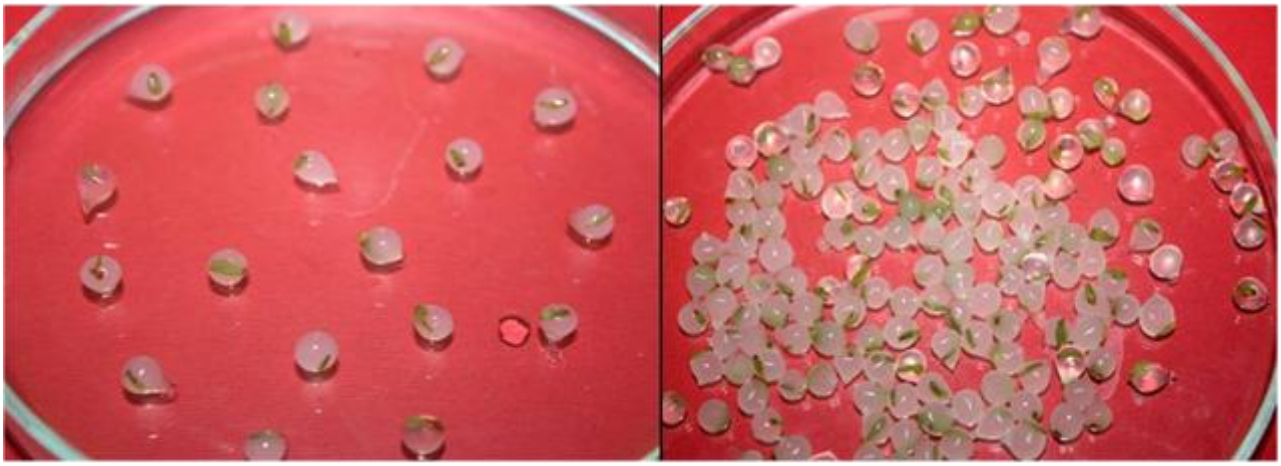

Figure 3. Preculture encapsulated shoot tips of apricot in WP medium supplemented with 0.75 M sucrose for 2 days followed by 2 h dehydration period.

sary to avoid the formation of intracellular ice crystals even though increasing dehydration time led to damage of encapsulated shoot tips. Table 2 shows the effect of LN on the survival ratio and regrowth of apricot at various times of dehydration. Most of cryopreserved shoot tips (with LN treatment) gave significantly lower values than treated control (without LN treatment). It confirms that the damage of ice crystal occurs in the cells. However, increasing sucrose concentration in preculture treatment and dehydration times could improve the survival ratio and regrowth. Therefore, survival percentage of precultured beads after plunging into LN were increased until 2 h of dehydration from 0.75 M and 1.0 M sucrose preculture condition and then decreased again. The greatest survival (74.5%) and regrowth (71.8%) for cryopreserved (+LN) shoot tips of apricot was obtained when shoot tips were pretreated with 0.75 M sucrose for two days followed by 2 h dehydration, where the beads attained 19.55% moisture content (Figure 3). Also, the highest regrowth (58.5%) was obtained with 1.0 M sucrose for two days without air dehydration (Table 2).

Shoot tips from the *in vitro* cultures were subjected to a series of conditioning stages and could survive encapsulation, dehydration and cryopreservation. Encapsulating the explants allows exposure to extreme treatments, including preculture with high sucrose concentrations and desiccation to low moisture content that would be highly damaging or lethal to non-encapsulated samples (Engelmann et al., 2008). In this study, the best result was encapsulated shoot tips to be cryopreserved were precultured in a medium supplemented with 0.75 M sucrose for two days followed by 2 h dehydration compared to the other treatments. Cryopreserved shoot tips of apricot (*P. armeniaca* L.) displayed increasing survival

Table 3. Effect of storage duration on survival and regrowth percentage of cryopreserved (+LN) shoot tips apricot (*Prunus armeniaca* L.) cv. El-Hamawey after liquid nitrogen storage.

Storage duration (month)	Survival (%)		Regrowth (%)	
	0.75 M sucrose	1.0 M sucrose	0.75 M sucrose	1.0 M sucrose
3	74.5[a]	71.3[a]	71.8[a]	58.5[a]
6	72.3[b]	71.2[a]	70.8[a]	58.4[a]
9	71.4[b]	69.4[c]	68.2[b]	50.4[c]
12	73.8[a]	65.5[e]	65.3[c]	51.3[b]
15	73.5[a]	69.2[c]	66.5[c]	48.5[d]
18	71.4[b]	70.5[b]	65.8[c]	45.5[e]
24	70.2[c]	68.3[d]	60.4[c]	42.3[f]

Encapsulated shoot tips were precultured on WP medium supplemented with 1.0 M sucrose for two days without further air dehydration and 0.75 M sucrose for two days, then dehydrated for 2 h in the air current of a laminar flow cabinet before immersion into liquid nitrogen.

rates with increasing sucrose concentrations until 0.75 M and then decreased again. This could be attributed to the fact that high sucrose concentrations retard shoot tips growth, with tissue blackening seen as one of the symptoms of cellular death (Panis et al., 1996). In this experiment, the low survival rate was obtained when shoot tips were precultured in medium containing 1.0 M sucrose for 2 days with 6 h of dehydration prior to cryopreservation. It had been reported that sucrose concentrations ranging from 0.5 to 0.75 M are most frequently used for preculture in cryopreservation experiments (Yin and Hong, 2009). Sucrose preculture induces sugar accumulation, reduces water content and prevents ice crystallization in cells. It was reported that the accumulation of sucrose within plant tissues contributes to their viability when freezable water is removed to the point of reaching a glassy state during vitrification in the presence of liquid nitrogen (Verma et al., 2010).

Growth recovery after cryopreservation

Shoot tips of apricot (*P. armeniaca* L.) cv. El-Hamawey were stored for different duration (Table 3). The survival percentage of the apricot shoot tips did not differ with storage duration. After recovering from LN and rapid thawing in water bath at 38°C for 2 - 3 min, the greatest survival percentage of encapsulated cryopreserved shoot tips (71.3%) was obtained following the combination of preculture in WP medium containing 1.0 M sucrose for two days without further air dehydration after three months and (68.3%) after 24 months in liquid nitrogen storage. The survival percentage of encapsulated cryopreserved shoot tips (74.5%) was obtained with WP medium containing 0.75 M sucrose for two days followed by dehydration 2 h under the sterile air of a laminar flow hood after three months and (70.2%) after 24 months in liquid nitrogen storage (Table 3). Furthermore, the regrowth percentage of encapsulated cryopreserved shoot tips (60.4%) was precultured WP medium containing 0.75 M sucrose for two days with 2 h of dehydration and (42.3%) was precultured WP medium supplemented with 1.0 M sucrose for two days without further air dehydration after 24 months in liquid nitrogen storage (Figure 4).

Encapsulation-dehydration is generally widely used because it is simple, inexpensive and provides a high level of genetic stability (Sakai et al., 1990; Khoddamzadeh et al., 2011). This method depends on a successive osmotic and evaporation dehydration steps performed on plants material (Shibli et al., 2001). The encapsulation-dehydration technique for cryopreservation was initially reported for shoot tips of *Solanum* sp. (Fabre and Dereuddre, 1990) and additional plant species, including pear, apple, sugarcane and potato (Sakai and Engelmann, 2007). Recently, many researches illustrate the protocol of encapsulation-dehydration which is consisted of the encapsulation of plant material in alginate beads followed by a dehydration step in a medium containing a high concentration of sucrose ranging from 0.1 to 1.0 M for at least one day (Shibli et al., 2009; Subaih et al., 2006). The decline in survival and regrowth of encapsulated noncryopreserved shoot tips with increased dehydration may be due to the osmotic shock at higher concentrations (Maruyama et al., 1998). As the moisture content inside the beads containing apricot shoot tips decreased the survival rates after cryopreservation increased. Similar to our results, Subaih et al. (2006) reported that high (80%) survival was obtained when date palm calli were pretreated with 0.3 M sucrose for two days followed by 2 h dehydration where the beads attained 55.4% moisture content and the highest regrowth (33.3 to 40%) of date palm was obtained with 0.1 M sucrose after 2 or 4 h of dehydration.

Rooting and acclimatization of regenerated plants

Shoots from multiplication stage were separated and transferred to liquid MS medium obtained with 2 mg/L IBA, 0.5 mg/L NAA and 40 mg/L phloroglucinol when

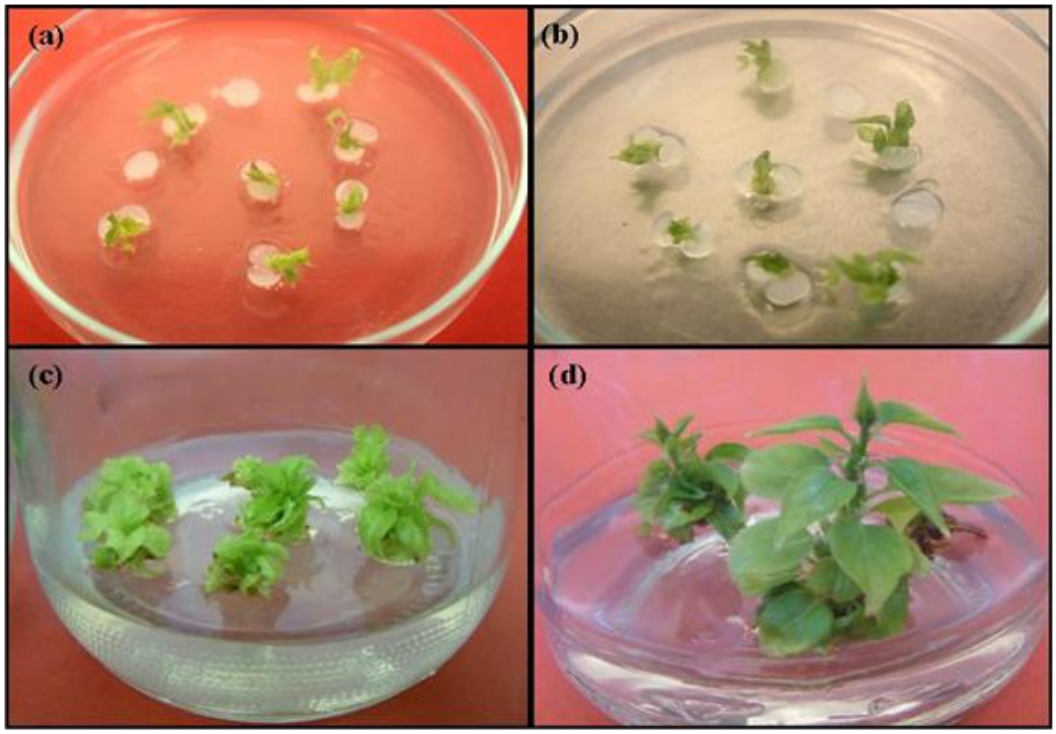

Figure 4. Regeneration of encapsulated cryopreserved shoot tips of *Prunus armeniaca* L in recovery WP medium supplemented with 1.0 mg/l zeatin and 0.1 mg/l IAA after two weeks (**a and b**). Multiple shoot formation from encapsulated microshoots on WP medium supplemented with 4 mg/l BA and 0.5 mg/l 2iP after three weeks (**c**) and after five weeks (**d**).

Table 4. Distribution of monomorphic and polymorphic bands in plantlets from cryopreserved shoot tips and non-cryopreserved shoots of apricot.

Primer	Number of scorable bands per primer	Total number of Monomorphic bands	Total number of Polymorphic bands	Length of amplified DNA fragment
OPE A-01 5`- CAGGCCCTTC-3`	9	9	0	300-2500 bp
OPE A-11 5`- CAATCGCCGT-3`	7	6	1	450-1600 bp
OPE B-17 5`-AGGGAACGAG-3`	8	5	3	200-2000 bp
OPE N-13 5`-AGCGTCACTC -3`	6	5	1	300-2000 bp
OPE P-09 5`- GTGGTCCGCA-3`	7	5	2	250-2500 bp
Total number of bands	37	30	7	

shoots were maintained in the dark for seven days before transfer to a 16 h photoperiod for root induction (Figures 5a and b). Transfer to phytagel based solid medium was not suitable for root induction. The rooted plantlets were transferred to a mixture of peatmoss and sand (1:1) in the pots for further development and hardening (Figures 5c and d). The sterilized mixture of peatmoss and sand was used with good results. Use of autoclaved mixture of peatmoss and sand (1:1) was found mandatory for the acclimation of apricot (Hemaid, 2012).

Genetic stability of cryopreserved materials

To verify the genetic fidelity of plantlets regenerated from cryopreserved shoot tips, the RAPD patterns were compared with those of control plantlets. A total of 10 primers were used for initial screening with plantlets derived from cryopreserved and non-cryopreserved shoot tips, and five primers produced amplification products in DNA samples. All five primer produced clear and reproducible bands. Primers and their nucleotide sequence are presented in Table 4. The number of scorable bands of each primer varied from six to nine. The five primers produced 37 bands for plantlets derived from cryopreserved and non-cryopreserved shoot tips.

The size of these amplification products ranges from between 200 to 2500 bp (Table 4).

Primer OPE A-01 produced nine bands, zero polymor-

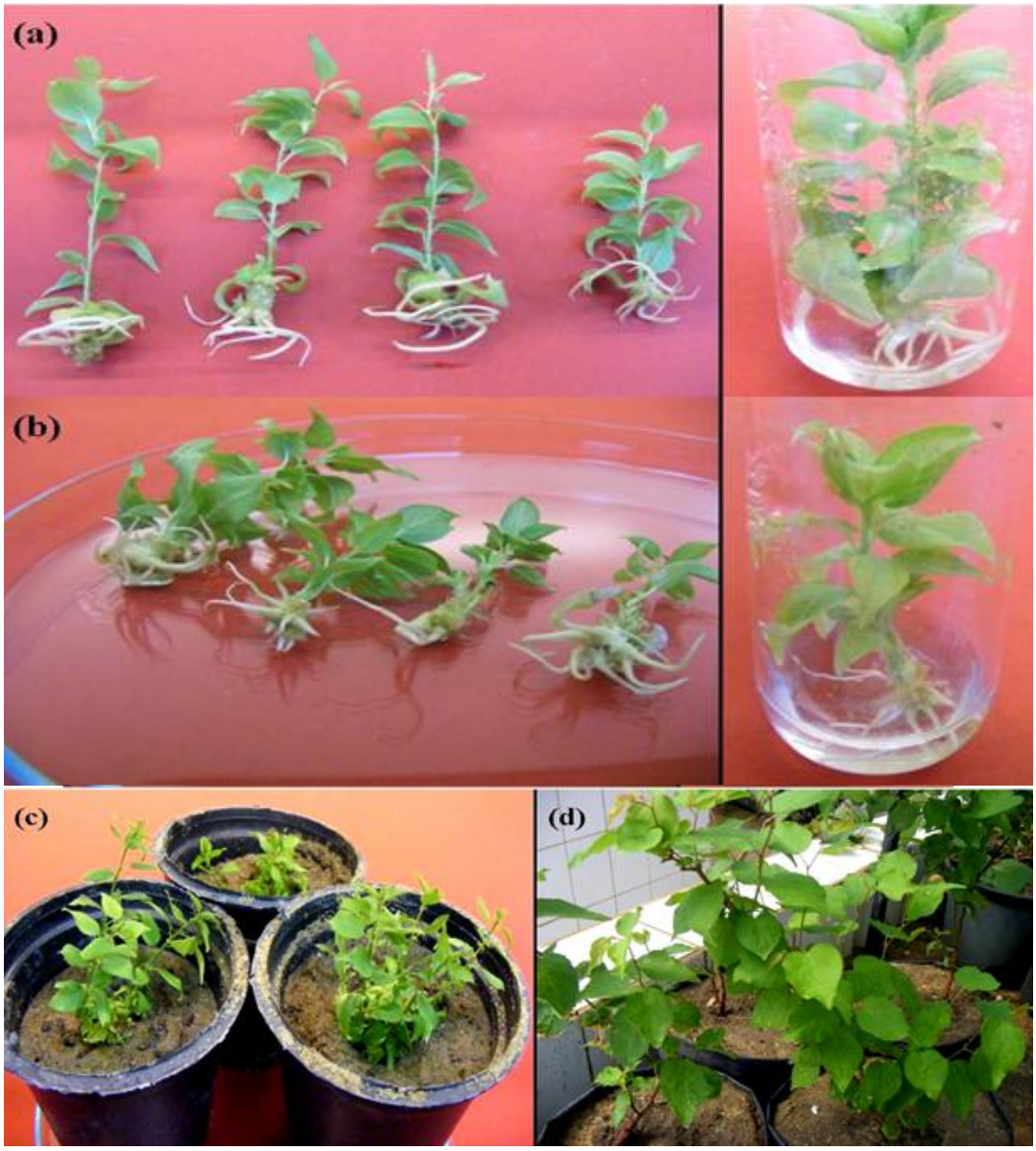

Figure 5. (a) Rooted plantlets regenerated from non-cryopreservation shoot tips of apricot on MS medium supplemented with 2 mg/l IBA and 0.5 mg/l NAA. **(b)** Rooted plantlets regenerated from cryopreservation shoot tips of apricot on MS medium supplemented with 2 mg/l IBA and 0.5 mg/l NAA. **(c)** Acclimatized plantlet in pots after 4 weeks of culture. **(d)** One year-old growing in greenhouse.

phic band and nine bands were monomorphic with molecular mass between 300 to 2500 bp, with primer OPE A-11 produced seven bands, one polymorphic band with molecular mass 650 bp were absent in plantlets derived from cryopreserved shoot tips after six months and six bands were monomorphic (Figure 7). Primer OPE P-09 produced seven bands, two polymorphic bands with molecular mass 250 and 2500 bp were absent in plantlets derived from cryopreserved shoot tips after 18 months and five bands were monomorphic. Also, using primer OPE N-13 produced six bands, one polymorphic band with molecular mass 300 bp was absent in plantlets derived from cryopreserved shoot tips after six, 12 and 18 months and five bands were monomorphic. While, primer OPE B-17 produced eight bands, three polymorphic bands with molecular mass 400, 700 and 2000 bp were absent in plantlets derived from cryopreserved shoot tips after 18 months in liquid nitrogen and five bands were monomorphic (Figure 6). A total of 30 bands (81.1%) were monomorphic and seven bands (18.9%) were polymorphic, indicating that the genetic fidelity was maintained between the plantlets derived from cryopreserved shoot tips after two years in DNA and methylation variations following cryopreservation. However, the design of

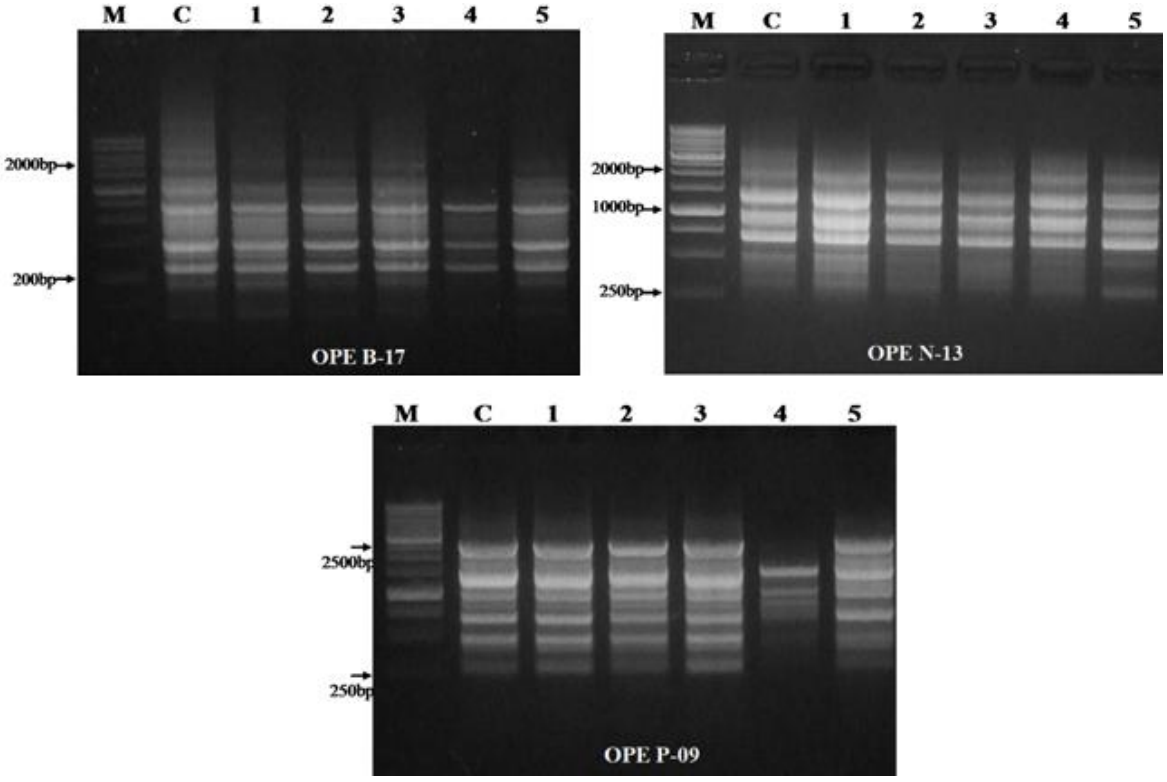

Figure 6. RAPD banding profiles of DNA samples extracted from the *in vitro* plantlets of apricot. Amplification products were generated by primers OPE B-17, OPE P-09 and OPE N-13 from control (Lane C) and plantlets regenerated from cryopreserved shoot tips after three months in LN (Lane 1), after six months (Lane 2), after 12 months (Lane 3), after 18 months (Lane 4), after 24 months (Lane 5). Lane M, 1 Kb base pair ladder.

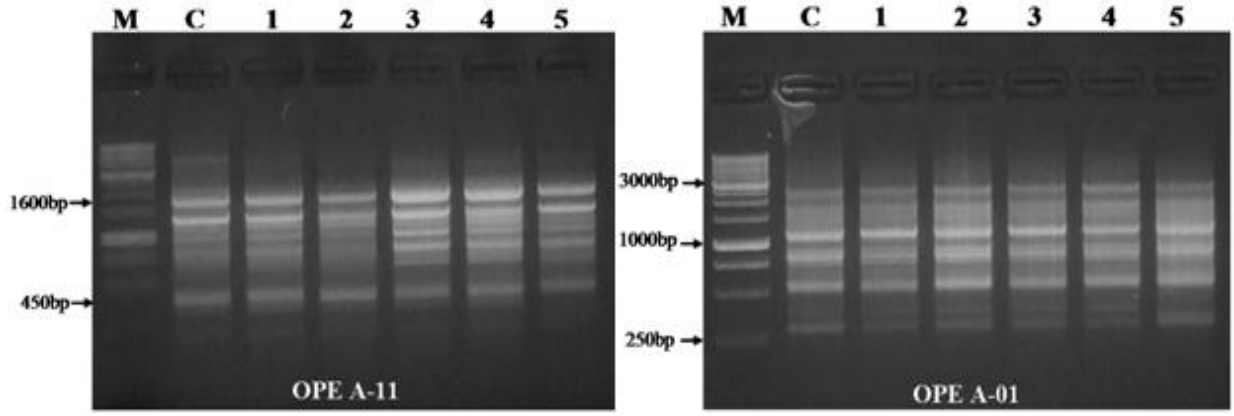

Figure 7. RAPD banding profiles of DNA samples extracted from the *in vitro* plantlets of apricot. Amplification products were generated by primers OPE A-1 and OPE A-11 from control (Lane C) and plantlets regenerated from cryopreserved shoot tips after three months in LN (Lane 1), after six months (Lane 2), after 12 months (Lane 3), after 18 months (Lane 4), after 24 months (Lane 5). Lane M, 1 Kb Base pair ladder.

the cryopreservation protocol in the present study reduces the possibility of occurrence of somaclonal variation. Eliminating callus formation at the micropropagation, explant excision, treatment, recovery and regenerating stages of the cryopreservation experiment reduced the potential for somaclonal variation to occur. Apart from that, age of the explants could increase the possibility of somaclonal variation (Peschke and Phillips, 1992).

RAPD analysis is necessary to verify the genetic stability of cryopreserved plants. No genomic variations were

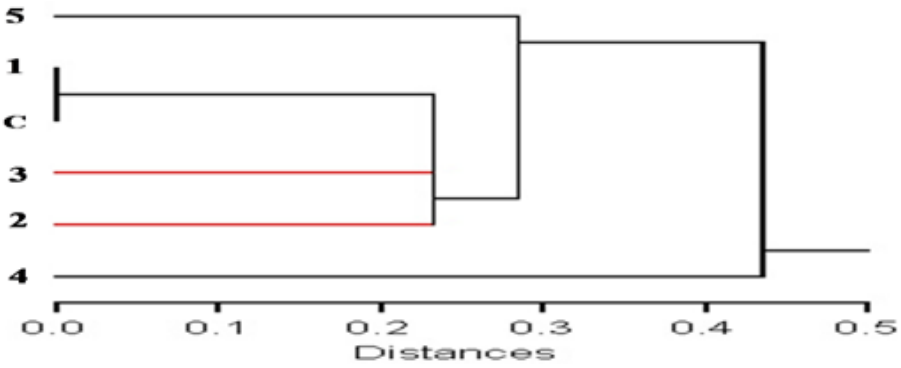

Figure 8. Dendrogram demonstrating the relationships among the plantlets regenerated from cryopreserved shoot tips and mother plant based on data recorded from polymorphism of RAPD markers. C, Mother plants; 1, plantlets regenerated from cryopreserved shoot tips after three months in LN; 2, after six months; 3, after 12 months; 4, after 18 months; 5, after 24 months.

detected in the regenerated material from most cryopreservation experiments such as embryogenic cultures of Scots pine (*Pinus sylvestris* L.) precultured using three different mixtures as cryoprotectants (Haggman et al., 1998), potato plants (*Solanum tuberosum* L.) derived from meristems cryopreservation by encapsulation vitrification (Hirai and Sakai, 2000), *Prunus* 'Ferlenain' plants (Helliot et al., 2002), *Arachis* species (Gagliardi et al., 2003), *Diascorea bulbifera* (Dixit et al., 2003), *Vitis vinefera* (Zhai et al., 2003). Success of cryopreservation may be assessed not only at the level of cell viability and the capability to recover and differentiate into whole plants but also at the DNA level, indicating genetic fidelity of the recovered plants. Therefore, the importance of the genetic stability of the plant materials becomes an important aspect during the establishment of the cryopreservation protocol (Liu et al., 2004; Dalamu et al., 2012).

In conclusion, dehydration before immersing in liquid nitrogen is the major deciding factor for obtaining a higher rate of survival and plant regeneration in cryopreservation. Fully hydrated shoot tips did not survive freezing in liquid nitrogen (LN, −196°C), and the best recovery rate was attained with encapsulated shoot tips with 19.55% moisture content. Properly dehydrated shoot tips, when subjected to freezing in liquid nitrogen and on transfer to recovery medium, developed shoots without intermediary callus formation. Our results demonstrate that encapsulation–dehydration is a compatible technique for cryopreservation of shoot tips of *P. armeniaca* L. The procedure is simple, rapid, and reproducible. It could allow the establishment of *P. armeniaca* L germplasm banks in the future. Further studies are still necessary to optimize the protocol established and to test the optimal protocol with other accessions of *P. armeniaca* L.

Abbreviations

RAPD, Random amplification of polymorphic DNA; **PCR,** polymerase chain reaction; **WP,** woody plant; **IAA,** indole-3-acetic acid; **IBA,** indole-3-butyric acid; **NAA,** naphthalene acetic acid; **BA,** Benzyl adenine; **2iP,** N6-Δ²-isopentenyl adenine; **LN,** Liquid nitrogen; **WP,** woody plant.

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