Jordan Journal of Agricultural Sciences, Volume **3**, No.**2**, 2007

Cryopreservation of Date Palm (*Phoenix dactylifera*) Embryogenic Callus by Encapsulation-Dehydration, Vitrification and Encapsulation-Vitrification

***Walid S. Subaih*[*], *Mohamad A. Shatnawi*[**] *and Rida A. Shibli*[*]✉**

ABSTRACT

Cryopreservation of date palm (*Phoenix dactylifera)* embryogenic callus via encapsulation-dehydration, vitrification and encapsulation-vitrification was investigated. High survival (80%) out of the encapsulation-dehydration method was obtained when cryoprserved calli were pretreated with 0.3 sucrose for 2 days followed by 2 h of dehydration. The highest (33.3 or 40%) regrowth was obtained with 0.1 M sucrose after 2 h or 4 h of dehydration; with 0.3 M sucrose after 2 h of dehydration; or with 0.5 M sucrose after 4 h of dehydration. Viability of calli decreased with increasing sucrose concentration and dehydration period. In vitrification, direct exposure of calli to 100% Plant Vitrification Solution 2 (PVS2) decreased the viability of calli. Survival of 80-93.3% and 40-53.3% regrowth rates were achieved with two- or four- step dehydration, using PVS2 at 25 ˚C for 20 min intervals prior to freezing. Cryoprotection by using 1.0 M sucrose plus 15% Dimethyle Sulfooxide (DMSO) and dehydration using 2.0 M glycerol plus 0.4 M sucrose or 0.5 M sucrose plus 10% DMSO all produced 60 or 66.7% survival rates after freezing. In encapsulation-vitrification, cryopreservation of encapsulated calli after treatment with 100% PVS2 (at 25 ˚C for 5 to 10 min) resulted in 60% survival and there were no differences in regrowth rates (ranging between 20 to 33.3%) in response to PVS2 dehydration period (5 to 90 min.).

KEYWORDS: Date palm, cryopreservation, encapsulation, dehydration, vitrification.

1. INTRODUCTION

Cryopreservation is the only viable long-term storage method for germplasm of plant species with recalcitrant seeds, and vegetative propagated species (Al-Ababneh, 2001; Shibli et al., 1999; Towill, 1996 b). In cryopreservation, exposure to ultra-low temperature (-196 ^{o}C) with liquid nitrogen causes the cell division, metabolic and biochemical processes to stop (Niino et al., 1992; 1995) and thus plant material can be stored without deterioration or modification for an unlimited period of time (Lambardi et al., 2000; Mannonen et al., 1990). Also, the genetic stability and regeneration potential of the cryopreserved plant materials are maintained (Al-Ababneh, 2001; Ford et al., 2000; Mannonen et al., 1990). Theoretically, the plant materials can be stored without any changes for an indefinite period of time (Engelmann, 1997).

Cryopreservation techniques, include two-step slow freezing (Chen et al., 1984), vitrification (Moukadiri et

*✉ Department of Plant Production, Faculty of Agriculture, Jordan University of Science and Technology, Irbid, Jordan. Email: shibli@just.edu.jo.

** Department of Biotechnology, Faculty of Agriculture Technology, Al-Balqa Applied University, Salt, Jordan. Received on 23/3/2006 and Accepted for Publication on 20/12/2006.

al., 1999), encapsulation-vitrification (Hirai and Sakai, 1999), and encapsulation-dehydration (Gonzalez-Arnao et al., 1996; 1998; 1999; Shibli, 2000; Shibli et al., 1999). Of these methods, encapsulation-dehydration is widely used because it is applicable to many species (Shibli, 2000). Encapsulation- dehydration cryopreservation methods are based on a successive osmotic and evaporative dehydration of plant cells (Swan et al., 1999). Dehydration techniques allow more flexibility when handling large sample numbers because the processing is less time-critical than with vitrification (Sakai et al., 2000). Encapsulation-dehydration also avoids the use of harmful cryoprotectants as compared to other methods (Moges et al., 2004; Shibli et al., 1999).

Two-step freezing involves a slow cooling step induced by crystallization of the external medium to –30 or –40 °C for tissue dehydration followed by dipping into liquid nitrogen (Al-Ababneh, 2001; George and Sherrington, 1984; Scottez et al., 1992; Towill, 1996 a). This method is complex and requires an expensive cooling apparatus to provide the required cooling temperature (Scottez et al., 1992; Tahtamouni, 1999; Moges et al., 2004). The method also has the risk of ice formation during the slow cooling step and this may kill cells (Niino et al., 1992; Towill, 1996 a; Tahtamouni, 1999).

For vitrification, the plant material is dehydrated with a high concentration of cryoprotectants then directly dipped into liquid nitrogen (Hirai and Sakai, 1999; Sakai et al., 1990). The two methods, encapsulation-dehydration and vitrification, are simple, inexpensive and preserve high genetic stability (Hirai et al., 1998; Kartha and Engelmann, 1994; Niino et al., 1992). Encapsulation-vitrification is a hybrid of these techniques which minimizes any potential injury from vitrification(Sakai et al., 1990; Engelmann, 1997; Moges et al., 2004).

Successful cryopreservation must avoid the formation of ice crystals inside cells during immersion into the Liquid Nitrogen (LN) by lowering cell water content prior to rapid cooling. Several pre-treatments procedures, including cold acclimation, exposure to ABA, immersion in concentrated sugar solutions, and extensive dehydration, can enhance the cryopreservability of plant cells (Martinez and Revilla 1998; Moukadiri et al., 1999; Shibli et al., 1999; Shibli, 2000; Tahtamouni and Shibli, 1999).

Date palm (*Phoenix dactylifera*) is a monocotyledonous plant, that is expected to be salt-tolerant and can withstand long hot summers (Dowson 1982). Setting a priorities list for the conservation of date palm genetic resources is much needed. Conservation of date palm via cryopreservation has been reported for shoot meristem (Bagnoil and Engelmann, 1991, 1992; Bagnoil et al., 1992) and somatic embryos (Mycok et al., 1995). To our knowledge, no studies have reported the cryopreservation method of date palm embryogenic callus. This study investigates alternative cryopreservation methods for date palm embryogenic callus and the recovery of the cryopreserved calli.

2. MATERIALS AND METHODS

Offshoots from 2-3 year-old date palm trees cv. Medjool were used as a source of apical buds. Expanded leaves and tissues were removed and buds were excised. Buds were immediately plunged in a cold antioxidant solution (150 mg/l citric acid and 100 mg/l ascorbic acid) and kept overnight in a refrigerator. Approximately, 0.5-1.5 cm pieces of buds were surface sterilized for 35-40 minutes in a 3% sodium hypochlorite solution followed by 3 washings in an autoclaved distilled water. The explants were cultured on a modified MS (Murashige and Skoog, 1962) medium containing 2.26 µM 2,4-Dichlorophenoxy acetic acid (2,4-D). The pH of the medium was adjusted to 5.7 before adding agar (8 g/l). After a period of 3-4 months, formed calli were transferred to a fresh growth regulator-free modified MS medium for embryogenic callus

establishment. Subculturing was performed every month using a fresh growth regulator-free modified MS medium to establish sufficient callus stock for experimentation.

The cultures were incubated in the growth room at 28±2 ˚C, with a 16-h light/8-h dark photoperiod provided by cool-white fluorescent lamps at a Photosynthetic Photon Flux Density (PPFD) of 50-60 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

2.1. Encapsulation-dehydration

Calli were precultured on a growth regulator-free solid MS medium containing 0.3 M sucrose for 2 days. Precultured calli were suspended in 3% (w/v) sodium alginate in a growth regulator- and calcium-free liquid MS medium supplemented with 0.1 M sucrose. The callus clump was picked up individually using a 10 ml sterile pipette with some alginate solution and then dispensed into a liquid MS medium containing 100 mM calcium chloride and 0.1 M sucrose to form beads of about 4 mm in diameter (Al-Ababneh, 2001; Moges et al., 2003). The beads were kept in the calcium chloride solution for 30 min for polymerization.

Beads, each containing one piece of callus, were transferred to a growth regulator-free liquid dehydration MS medium supplemented with various concentrations (0.1, 0.3, 0.5, 0.75 or 1.0 M) of sucrose and incubated on rotary shaker providing gentle shaking for 2 days. The dehydration solution was then removed using a sterile pipette and beads were dehydrated in the air on sterilized filter paper in uncovered 9-cm Petri dishes in a laminar airflow cabinet for 0, 2, 4, 6 or 8 h at room temperature. Half of the beads, from each sucrose-dehydration treatment, was then transferred to a recovery medium for rehydration and growth, while the other half of the beads was placed in 2-ml sterile cryogenic vials (each containing 5 beads) and rapidly cooled by direct immersion into Liquid Nitrogen (LN). Beads were stored in LN at least for 1 h.

After cryopreservation, cryogenic vials containing beads were thawed in a water bath at 38 °C for 2-3 min. Encapsulated-dehydrated non-cryopreserved and encapsulated-dehydrated cryopreserved calli were inoculated onto a solid MS recovery medium containing 0.1 M sucrose and then kept in dark for 3 days. Survival percentage was tested for half of the non-frozen and frozen encapsulated callus using 2,3,5-Triphenyl Tetrazolim Chloride (TTC) test (Steponkus and Lamphearm, 1967) following the procedures outlined by Al-Ababneh (2001). The other half of the beads was transferred to normal growth conditions. After two weeks, calli were checked under a binocular microscope for any signs of recovery.

For Moisture Content (MC) determination during dehydration, beads devoid of callus were prepared and treated as described before. After each dehydration time, beads were weighed, dried in an oven at 90 °C for 16 h and then reweighed to determine its content of moisture (Al-Ababneh, 2001; Shibli et al., 2001).

2.2. Vitrification

Effect of PVS2 Concentration

Calli were precultured on a growth regulator-free solid MS medium containing 0.3 M sucrose for 2 days. The precultured calli were placed in sterile cryogenic vials and loaded with 1 ml of loading solution (MS medium plus 2.0 M glycerol and 0.4 M sucrose) at 25 °C for 20 min. The loading solution was removed using a sterile syringe and replaced by 1 ml of vitrification solution (PVS2) containing (w/v) 30% glycerol, 15% DMSO and 15% EG in a hormone-free liquid medium with 0.4 M sucrose. One-third of the cryoprotected calli was treated with a highly concentrated vitrification solution (100% PVS2) for 20 min while the other one-third was treated first with 60% PVS2 for 10 min and then replaced by a 100% PVS2 for another 10 min. The rest of the calli was treated with a step-wise increased concentration (20%, 40%, 60% and then 100%) of PVS2 at 25 °C for 20 min (5 min for each concentration).

Cryogenic vials with the calli and the vitrification solution were directly plunged into LN and stored for at least 1 h.

Using a water bath at 38 °C, the cryogenic vials were thawed for 2-3 min. The vitrification solution for the vitrified non-frozen and frozen calli was then removed and replaced by a liquid MS medium containing 1.2 M sucrose (unloading solution), which was changed three times (10 min each). Treated calli were then cultured on a solid MS recovery medium containing 0.1 M sucrose and incubated in dark for three days.

Data on survival and recovery rates were collected for calli before and after dipping it into LN. After dark incubation, survival percentage was tested using TTC test. The rest of the incubated calli was transferred to the normal growth conditions described before and then examined for any recovery signs after two weeks of transfer.

Effect of Cryoprotectant Combinations

Calli were precultured on a growth regulator-free solid MS medium containing 0.3 M sucrose for 2 days. The precultured calli were placed in sterile cryogenic vials and loaded with 1 ml of a loading solution consisting of a growth regulator -free liquid MS medium supplemented with 1 or 2 M sucrose, 2 M glycerol plus 0.4 M sucrose, or a combination of DMSO (5 or 10%) and sucrose (0.3, 0.5, 0.75, 1 or 2 M) at 25 °C for 20 min. Half of the cryogenic vials with the calli and the cryoprotective solution were plunged directly into LN and stored for at least 1 h. The other half of the cryogenic vials was treated without using LN. Data on survival percentages were collected for calli before and after dipping it into LN.

Effect of the Type of Loading and Vitrification Solution

Calli were precultured on a solid MS medium containing 0.3 M sucrose for 2 days. The precultured calli were placed in sterile cryogenic vials and loaded with 1 ml of a loading solution consisting of liquid MS medium supplemented with 2 M glycerol plus 0.4 M sucrose, or a combination of DMSO (5 or 10%) and sucrose (0.5 or 0.75) at 25 °C for 20 min. The loading solution was then removed using a sterile syringe and then replaced by 1 ml of 100% PVS2 (30% DMSO plus 1 M sucrose, or 15% DMSO plus 1.0 M sucrose in a hormone-free liquid MS medium) for 20 min at 25 °C. The cryogenic vials with the calli and the vitrification solution were plunged directly into LN and kept for 1 h at least. After thawing and unloading, treated calli were cultured on a solid MS recovery medium containing 0.1 M sucrose and incubated in the dark for three days. Survival rate was determined for half of the treated calli. The other half was transferred to growth room conditions and then examined for any recovery signs after two weeks of transfer.

Effect of Duration of Exposure to the Loading Solution

Calli were precultured on a solid MS medium containing 0.3 M sucrose for 2 days. The precultured calli were placed in sterile cryogenic vials and loaded with 1 ml of a cryoprotectant mixture of 2 M glycerol plus 0.4 M sucrose in a liquid MS medium at 25 °C for 10, 20, 30, 60 or 90 min. The loading solution was then removed using a sterile syringe and replaced by 1 ml of highly concentrated vitrification solution (30% (w/v) glycerol, 15% (w/v) DMSO and 15% (w/v) EG) in a growth regulator-free liquid MS medium supplemented with 0.4 M sucrose at 25 °C for 20 min. The cryogenic vials with the calli and the vitrification solution were immersed directly into LN and stored for at least 1 h. Data on survival and regrowth were collected as described above.

2.3 Encapsulation-vitrification

Calli of date palm were precultured on a solid MS medium containing 0.3 M sucrose for 2 days. Precultured calli were suspended in calcium-free MS medium supplemented with 3% sodium alginate and 2 M glycerol plus 0.4 M sucrose. The calli were picked up

using 10 ml sterile pipette with some alginate solution and then dispensed into a hormone-free liquid MS medium containing 100 mM calcium chloride and 2 M glycerol plus 0.4 M sucrose so as to form beads. The beads were kept in the calcium chloride solution for 30 min for polymerization.

Encapsulated beads were dehydrated with a highly concentrated PVS2 solution [30% glycerol, 15% DMSO and 15% EG (w/v) in a growth regulator-free liquid MS medium supplemented with 0.4 M sucrose] at 25 °C for 5, 10, 30, 60 or 90 min. The cryogenic vials with the beads and the vitrification solution were immersed directly into LN and held for at least 1 h. Cryogenic vials were then thawed in a water bath at 38 °C for 2-3 minutes. The PVS2 solution was removed and replaced by a liquid MS medium containing 1.2 M sucrose as an unloading solution for 10 min. Half of the treated calli was transferred to a solid MS recovery medium containing 0.1 M sucrose and kept in the dark for three days. Survival rates of the calli before and after freezing were assessed. The other half of the calli was transferred to the growth room conditions, and calli with and without LN exposure were examined for any recovery signs after two weeks of transfer.

Experimental Design and Statistical Analysis

Treatments in each experiment were arranged in a completely randomized design. Each treatment was replicated three times with 5 callus clumps per replicate. Data were statistically analyzed using the MSTATC software (Michigan State University, 1988). Means were separated according to the Least Significant Difference (LSD) test at 0.01 probability level.

3. RESULTS AND DISCUSSION

3.1 Encapsulation-dehydration

Survival and regrowth of the non-cryopreserved encapsulated calli was decreased with increasing the dehydration period especially at 0.1 to 0.5 M concentrations of sucrose (Table 1). The reduction in survival and regrowth may be due to the osmotic shock at higher sucrose concentrations. Wang et al. (2002) found that a maximum growth of encapsulated shoot tips of ´Troyer' citrange was obtained when the sucrose concentrations ranged from 0.15 M to 0.29 M. Similarly, Al-Ababneh (2001) reported that survival and regrowth of encapsulated non-cryopreserved sour orange shoot tips were decreased with increasing sucrose concentration and dehydration duration.

The greatest survival (93.3-100%) of encapsulated non-cryopreserved calli was obtained when calli were cultured with 0.1 or 0.3 M sucrose for 2 days with or without further 2 h of air dehydration (Table 1). Whereas, the greatest regrowth (73.3-80%) of calli with white color was obtained when calli were cultured with 0.1 or 0.3 M sucrose with or without 2 h of dehydration. Paul et al. (2000) reported that high sucrose concentration improved dehydration resistance. Furthermore, Sarkar and Naik (1998) also reported that sucrose is an important pregrowth additive for the acquisition of dehydration tolerance.

Survival capacity was diminished if calli were dehydrated 8 h at any level of sucrose concentration (Table 1), while a complete loss of regrowth capacity has only occurred when using 1.0 M sucrose for 2 days, irrespective of dehydration period. Matsumoto et al. (1998) found that preculture of *Wasabia japonica* on solidified MS medium containing 0.3 M sucrose for one day produced higher rate of shoot formation. On the contrary, Vandenbussche and Proft (1996) demonstrated that no survival was achieved after the preculture of encapsulated shoot tips of *Beta vulgaris* with 0.3 M or 0.5 M sucrose solution. Al-Ababneh (2001) found that a very low rate of survival and regrowth or complete loss of them were observed for encapsulated non-cryopreserved shoot tips of sour orange after 8 h of dehydration regardless of the concentration of sucrose.

A complete loss of survival and regrowth for

encapsulated cryopreserved (+LN) calli occurred when calli were cultured with 0.1 or 0.3 M sucrose for 2 days without air dehydration (Table 2). This might be attributed to the formation of extra- and intra-cellular ice crystals as a result of high moisture content (Moges et al., 2004; Plessis et al., 1993). Without dehydration, the moisture contents of beads treated with 0.1 or 0.3 M sucrose were 92.5% or 87.2%, respectively (data not shown) . Furthermore, Al-Ababneh (2001) found that no survival of encapsulated cryopreserved sour orange shoot tips was achieved without dehydration with bead moisture contents of 75-85%.

The greatest survival (80%) of cryopreserved (+ LN) calli (Table 2) was obtained when calli were pretreated with 0.3 M sucrose for 2 days followed by 2 h of dehydration where the beads attained 55.4% moisture content. While the highest regrowth (33.3-40%) was obtained with 0.1 M sucrose after 2 or 4 h of dehydration, with 0.3 M sucrose after 2 h of dehydration, or with 0.5 M sucrose after 4 h of dehydration. Moges et al. (2004) reported complete survival (100%) of encapsulated cryopreserved African violet (*Saintpaulia ionantha* Wendl.) shoot tips that were precultured with 0.3 M sucrose for 2 days with 2 h of dehydration. Whereas, Martinez and Revilla (1998) reported a very high recovery percentage of encapsulated cryopreserved hop (*Humulus lupulus* L.) shoot tips after 2-days-sucrose preculture and 4 h of dehydration.

Table(1): Survival and regrowth of encapsulated non-cryopreserved (-LN) embryogenic calli of Date palm as influenced by sucrose concentration during preculture and dehydration period.

Sucrose Conc. (M)	Dehydration Period (h)	Survival (%)	Regrowth (%)	CallusColor
0.1	0	100 a[z]	80 a	W
	2	93.3 ab	80 a	W
	4	66.7 c	33.3 de	YW
	6	33.3 e	20 fg	B
	8	0 i	0 i	-
0.3	0	86.7 b	60 b	W
	2	93.3 ab	73.3 a	W
	4	66.7 c	26.7 ef	YW
	6	26.7 ef	6.7 hi	B
	8	26.7 ef	0 i	-
0.5	0	66.7 c	46.7 c	YW
	2	66.7 c	40 cd	YW
	4	53.3 d	20 fg	YB
	6	26.7 ef	6.7 hi	B
	8	6.7 hi	0 i	-
0.75	0	33.3 e	20 fg	B
	2	53.3 d	26.7 ef	B
	4	26.7 ef	13.3 gh	B
	6	20 fg	0 i	-
	8	0 i	0 i	-
1.0	0	13.3 gh	6.7 hi	B
	2	20 fg	6.7 hi	B
	4	13.3 gh	0 i	-
	6	0 i	0 i	-
	8	0 i	0 i	-

W = White, YB = Yellowish Brown, YW = Yellowish White, B = Brown. [z] Means within column having different letters are significantly different according to LSD at $P \leq 0.01$.

Table (2): Survival and regrowth of encapsulated cryopreserved (+LN) embryogenic calli of Date palm as influenced by sucrose concentration during preculture and dehydration period.

Sucrose Conc.(M)	Dehydration Period(h)	Survival(%)	Regrowth(%)	Callus Color
0.1	0	0 jz	0 f	-
	2	73.3 a	33.3 ab	YB
	4	60 b	33.3 ab	YB
	6	33.3 ef	20 cd	B
	8	40 de	13.3 de	B
0.3	0	0 j	0 f	-
	2	80 a	40 a	W
	4	60 b	26.7 bc	YB
	6	40 de	13.3 de	B
	8	40 de	13.3 de	B
0.5	0	33.3 ef	13.3 de	B
	2	53.3 bc	33.3 ab	YB
	4	46.7 cd	33.3 ab	YB
	6	20 gh	13.3 de	B
	8	26.7 fg	13.3 de	B
0.75	0	26.7 fg	0 f	-
	2	46.7 cd	20 cd	B
	4	20 gh	6.7 ef	B
	6	20 gh	0 f	-
	8	13.3 hi	6.7 ef	B
1.0	0	0 j	0 f	-
	2	33.3 ef	0 f	-
	4	6.7 ij	0 f	-
	6	0 j	0 f	-
	8	0 j	0 f	-

W = White, YB = Yellowish Brown, B = Brown.

[z] Means within column having different letters are significantly different according to LSD at $P \leq 0.01$.

In the current study, survival and regrowth of encapsulated cryopreserved calli tended to increase with increasing the sucrose concentration up to 0.3 M and the duration of dehydration up to 4 h (Table 2). This might indicate that increased sucrose concentration in the pregrowth medium would lead to accumulation of solutes inside the cells resulting in maintaining the integrity of plasma and inner membranes during dehydration and freezing (Moges et al., 2004; Plessis et al., 1993). Tahtamouni and Shibli (1999) reported that moisture content in beads pretreated with 0.75 M sucrose decreased more than in those pretreated with 0.3 M sucrose when exposed to the same dehydration period.

The reduction in regrowth compared to survival percentage (Table 2) may be attributed to partial damage of the calli due to osmotic shock after rehydration and ice crystallization of some cells in the calli (Al-Ababneh et al., 2003); or maybe due to unfavorable growth condition (Al-Ababneh, 2001; Moges et al., 2004). A

high concentration of sucrose inside plant cells was beneficial in establishing a vitrification state during freezing; so preventing the damage caused by dehydration and freezing (Wang et al., 2002).

3.2 Vitrification

Effect of PVS2 Concentration Solution

Significant variations in survival and regrowth rates of non-cryopreserved calli were obtained among the different concentrations of PVS2 (Table 3). High survival (86.7-100%) was obtained for non-cryopreserved calli treated with a four or two step-wise increased concentration of PVS2 at 25 ˚C for 20 min (5 min for each concentration). Survival of vitrified cell reported to be red, while cryopreserved cell reported to be light red. Moges et al. (2004) reported no significant differences in terms of survival and regrowth of non-cryopreserved African violet shoot tips among the different concentrations of PVS2, moreover, a complete survival and regrowth of shoots with green color were obtained for non-cryopreserved shoot tips, irrespective of the concentration of PVS2. Similarly, Al-Ababneh (2001) reported a complete survival and 97.5% regrowth with green appearance for non-cryopreserved sour orange shoot tips after dehydration with 100% PVS2 at 25 ˚C for 10 min and then at 0 ˚C for 10 min.

After cryopreservation, a high survival (93.3%) was observed when calli were treated with a four or two step-wise increased concentration of PVS2 (Table 3), whereas, the maximum regrowth (40-53.3%) of calli was obtained when calli were dehydrated with two or four step-wise increased concentration of PVS2. Sakai et al. (1991) reported high survival (90%) of cells of naval orange after dehydration with 100% PVS2 solution at 25 ˚C for 3 min prior to freezing. Direct exposure of shoot tips to the vitrification solution reduced survival and regrowth due to osmotic shock induced by the vitrification solution (Al-Ababneh et al., 2003; Moges et al., 2004).

In the current study, a higher recovery rate of cryopreserved calli was obtained when calli were dehydrated with step-wise increased concentration of PVS2 than in the case of direct dehydration with a highly concentrated PVS2 (Table 3). Moges et al. (2004) found that a complete survival of African violet shoot tips was observed after cryopreservation, irrespective of the concentrations of PVS2, whereas, the maximum regrowth (90%) of shoots with yellowish appearance was obtained when shoot tips were dehydrated with four-step increased concentration of PVS2.

Table (3): Survival and recovery of non-cryopreserved (-LN) and cryopreserved (+LN) embryogenic calli of Date palm as influenced by concentration of vitrification solution.

PVS2 Conc.	Survival (%)	Red Color Intensity	Regrowth (%)	Callus Color
Vitrified non-cryopreserved (-LN)				
A	66.7 b[Z]	Red	33.3 b	YB
B	86.7 a	Red	53.3 ab	W
C	100 a	Red	66.7 a	W
Vitrified cryopreserved (+LN)				
A	33.3 b	Light red	13.3 b	B
B	80 a	Light red	40 a	YB
C	93.3 a	Light red	53.3 a	YW

(A) Direct exposure to 100% PVS2, (B) Two step (treating with 60 % PVS2 followed by 100% PVS2), and (C) Four-step (loading with 20%PVS2 followed by treating with 40%, 60% and then 100% PVS2).

[z] Means within column having different letters are significantly different according to LSD at $P \leq 0.01$.

Effect of Cryoprotectant Combinations

Significant variations in survival rates of non-cryopreserved and cryopreserved calli were obtained among the various cryoprotectants (Table 4). The variations obtained in survival for the various cryoprotectant combination tested might be due to their differences in respect to permeability inside plant

tissues, ability to induce osmotic stress and toxicity effect (Al-Ababneh et al., 2003; Moges et al., 2004). Increasing the concentration of cryoprotectants in the medium leads to reduced survival percentages due to their toxic effect at higher concentrations (Al-Ababneh et al., 2003). Gazeau et al. (1998) found that using DMSO as a cryoprotectant was effective in increasing intra-cellular viscosity and thus avoiding the formation of ice crystals.

Table (4): Survival of non-cryopreserved (-LN) and cryopreserved (+LN) embryogenic calli of Date palm as influenced by loading solution.

Cryoprotectants Combination	-LN		+LN	
	Survival	Red Color Intensity	Survival	Red Color Intensity
5%DMSO+0.3M sucrose	46.7 de[z]	Red	20 d	Red
10%DMSO+ 0.3 M Sucrose	46.7 de	Red	13.3 de	Red
5% DMSO+ 0.5 M Sucrose	73.3 ab	Red	33.3 c	Light red
10% DMSO + 0.5M sucrose	80 ab	Red	46.7 ab	Red
5% DMSO+ 0.75 M sucrose	73.3 ab	Red	40 bc	Red
10%DMSO+0.75 M sucrose	66.7 bc	Red	40 bc	Red
5%DMSO+1.0 M sucrose	33.3 ef	Light red	0 e	-
10%DMSO+1.0 M sucrose	33.3 ef	Light red	0 e	-
5%DMSO+2.0 M sucrose	20 fg	Light red	0 e	-
10%DMSO+ 2.0 M sucrose	6.7 g	Light red	0 e	-
1.0 M sucrose	40 de	Light red	13.3 de	Light red
2.0 M sucrose	6.7 g	Light red	0 e	-
1.2 M sucrose	53.3 cd	Light red	0 e	-
2.0 M glycerol + 0.4M sucrose	86.7 a	Red	53.3 a	Red

[z] Means within column having different letters are significantly different according to LSD at $P \leq 0.01$.

In the current study, maximum survival (86.7%) of non-cryopreserved calli was achieved when calli were cryoprotected with 0.4 M sucrose plus 2 M glycerol at 25 ˚C for 20 min (Table 4). High survival rates (73.3-80%) for non-cryopreserved calli were also achieved with 0.5 M sucrose plus 5 or 10% DMSO; or with 0.75 M sucrose plus 5% DMSO. Survival of vitrified cell reported to be red, while cryopreserved cell reported to be light red (Table 4). The high survival obtained for non-cryopreserved calli might be because the cryoprotectants mixture tested was probably not toxic or the period of exposure was not long enough to cause a damaging effect on cell wall activity (Al-Ababneh, 2001; Moges et al., 2004). After cryopreservation, the highest survival (53.3%) of calli was obtained when calli were cryoprotected with 0.4 M glycerol plus 2.0 M sucrose. Moges et al. (2004) found that the maximum survival (95%) of non-cryopreserved African violet shoot tips was achieved when shoot tips were cryoprotected with 0.4 M sucrose plus 2 M glycerol at 25 ˚C for 20 min. Furthermore, Al-Ababneh (2001) reported that higher survival (96.7%) of non-cryopreserved sour orange shoot tips was obtained after using 5% DMSO with 1.0 M sucrose for 20 min at 25 ˚C.

Very low survival (6.7%) of non-cryopreserved calli was obtained when calli were pretreated with 2.0 M sucrose alone or combined with 10% DMSO (Table 4). Moreover, a complete loss of survival after freezing was exhibited when calli were cryoprotected with a mixture of 1.0 or 2.0 M sucrose plus 5 or 10% DMSO as well as with 1.2 or 2.0 M sucrose alone. Moges et al. (2004) reported a complete loss of survival of non-cryopreserved African violet shoot tips which occurred when shoot tips were pretreated with 2.0 M sucrose alone. This might be attributed to osmotic stress resulting from the increased sucrose concentration (Al-Ababneh, 2001; Moges et al., 2004).

After cryopreservation, the survival rates of calli decreased (Table 4); this might indicate that the cryoprotectants mixture was probably not able to

produce high freezing tolerance. Mycock et al. (1995) found that the reduction in survival and regrowth after cryopreservation was shown to be associated with intra - cellular ice formation due to insufficient dehydration. Variation in color intensity was observed for both non-cryopreserved and cryopreserved calli (Table 4). This might indicate that not all cells or tissues survived due to cryo-injury (Benson et al., 1994; Moges et al., 2004).

Effect of the Type of Loading and Vitrification Solution

Significant differences in terms of survival of cryopreserved calli were obtained for the different loading solutions and vitrification solutions combination tested (Table 5). Sarkar and Naik (1998) demonstrated that loading phase was necessary to reduce osmotic shock caused by direct exposure of precultured explants to a highly concentrated PVS2. In this study, no differences in terms of survival rate were obtained by using different loading solutions for each cryoprotectant mixture for the vitrified non-cryopreserved (-LN) calli (Table 5). Sakai et al. (1991) reported that complete vitrification of cryopreserved plant tissues would eliminate the concerns for the potentially damaging effects of intra- and extra-cellular crystallization and could lead to high survival percentages.

After cryopreservation, the highest survival (60-66.7%) was obtained after cryoprotection with 1.0 M sucrose plus 15% DMSO and dehydration with 2.0 M glycerol plus 0.4 M sucrose or 0.5 M sucrose plus 10% DMSO prior to freezing (Table 5). However, survival cell was reported to be red (Table 5). There were no significant differences in terms of regrowth among non-cryopreserved or cryopreserved calli (Table 6). Gonzalez-Arnao et al. (1993) demonstrated that not all of the survived shoot tips were able to regrow due to the fact that only one group of cells that are often localized in primordia of leaf tissues and meristematic dome area remained alive after stress of freezing and thawing. Tahtamouni and Shibli (1999) reported that explants of wild pear that lost their colors died. Color changes of the explants may be due to osmotic stress caused by vitrification solution (Sakai et al., 1991) and water depletion during dehydration (Shibli et al., 1992). It might also be due to the synthesis of toxic material resulting from stresses during cryopreservation (Engelmann, 1997).

Table (5): Survival of non-cryopreserved (-LN) and cryopreserved (+LN) embryogenic calli of Date palm as influenced by loading solution type and vitrification solution combinations.

Loading Solution	Cryoprotectant Mixture	-LN		+LN	
		Survival (%)	Red Color Intensity	Survival (%)	Red Color Intensity
1	A	73.3 a[z]	Red	46.7 cd	Light red
	B	80 a	Red	60 ab	Red
	C	80 a	Red	60 ab	Red
2	A	66.7 a	Red	40 d	Light red
	B	80 a	Red	40 d	Light red
	C	80 a	Red	66.7 a	Red
3	A	66.7 a	Red	53.3 bc	Red
	B	66.7 a	Red	53.3 bc	Red
	C	80 a	Red	66.7 a	Red

(**1**) 0.5 M sucrose + 10% DMSO, (**2**) 0.75 M sucrose + 5% DMSO, (**3**) 2.0 M glycerol + 0.4 M sucrose, (**A**) 100% PVS2, (**B**) 30% DMSO + 1.0 M sucrose, and (**C**) 15% DMSO + 1.0 M sucrose.

[z] Means having different letters are significantly different according to LSD at $P \leq 0.5$.

Table (6): Regrowth of non-cryopreserved (-LN) and cryopreserved (+LN) embryogenic calli of Date palm as influenced by loading solution type and vitrification solution combinations.

Loading Solution	Cryoprotectant Mixture	-LN		+LN	
		Regrowth (%)	Callus Color	Regrowth (%)	Callus Color
1	A	60 a[z]	Y	33.3 a	YB
	B	66.7 a	Y	40 a	YB
	C	66.7 a	Y	40 a	YB
2	A	60 a	Y	40 a	YB
	B	60 a	Y	33.3 a	YB
	C	66.7 a	Y	33.3 a	YB
3	A	60 a	Y	33.3 a	YB
	B	60 a	Y	40 a	YB
	C	60 a	Y	40 a	YB

Y= Yellow, YB = Yellow Brown

(**1**) 0.5 M sucrose + 10% DMSO, (**2**) 0.75 M sucrose + 5% DMSO, (**3**) 2.0 M glycerol + 0.4 M sucrose, (**A**) 100% PVS2, (**B**) 30% DMSO + 1.0 M sucrose, and (**C**) 15% DMSO + 1.0 M sucrose.

[z] Means having different letters are significantly different according to LSD at $P \leq 0.01$.

Effect of Duration of Exposure to the Loading Solution

High survival (80-86.7%) for non-cryopreserved calli was obtained irrespective of the duration of exposure to loading solution (Table 7); while higher regrowth (73.3%) for non-cryopreserved calli was obtained after 10 or 20 min of exposure to loading solution. After cryopreservation, no significant variations were obtained irrespective of the duration of exposure to loading solution (Table 7). The highest regrowth (46.7%) of cryopreserved calli was obtained after 20 min of exposure to loading solution. Sakai et al. (1990) reported that 20 min of exposure of nucellar cells of naval orange to loading solutions was sufficient to reduce the toxic effect of concentrated PVS2. However, Moges et al. (2004) found a complete survival rate for non-cryopreserved African violet shoot tips irrespective of the duration of exposure to loading solution, while higher regrowth (90 or 100%) rates for non-cryopreserved African violet shoot tips were obtained after 10 or 20 min of exposure to loading solution. Increased duration of exposure to loading solution did not improve the recovery of non-cryopreserved and cryopreserved calli (Table 7). This might be due to excess dehydration of cells which would lead to the accumulation of solutes that could, in its turn, cause osmotic stresses to the cells or have chemical toxicity effect on them (Moges et al., 2004; Sakai et al., 1990). Sarkar and Naik (1998) reported that direct exposure of sucrose- precultured shoot tips to concentrated PVS2 was detrimental to the viability of vitrified potato shoot tips.

3.3 Encapsulation-vitrification

Although dehydration duration did not affect survival; up to 60-73.3% regrowth of the encapsulated-vitrified non-cryopreserved calli were obtained when calli were dehydrated with concentrated PVS2 at 25 °C for 30 to 60 min (Table 8). Induction of dehydration and freezing tolerance was achieved through encapsulation and then osmoprotection by a mixture of 2 M glycerol plus 0.4 M sucrose followed by dehydration with highly concentrated vitrification solutions (Hirai and Sakai, 1999). Reduced dehydration duration increased the survival of the non-cryopreserved shoot due to reduced

osmotic shock (Dumet et al., 1993).

After cryopreservation, the greatest survival (60%) of calli were obtained when encapsulated calli were dehydrated with concentrated PVS2 at 25 ˚C for 5 to 10 min (Table 8). No variation in regrwoth percentage occurred in response to dehydration period. Similarly, Moges et al. (2004) found that greatest survival (80-85%) and regrowth (70-80%) of cryopreserved African violet shoots with pale green or yellow color were obtained when encapsulated shoot tips were dehydrated with concentrated PVS2 at 25 ˚C for 5 to 30 min. Furthermore, Al-Ababneh et al. (2002) also found the maximum recovery of sour orange shoot tips to happen after dehydration with concentrated PVS2 at 0 ˚C for 2-3 h. Matsumoto et al. (1995) reported that maximum shoot formation of encapsulated apical meristems of wasabi was achieved when meristems were dehydrated with PVS2 at 0 ˚C for 70-100 min.

4. CONCLUSIONS

The dehydration of encapsulated calli with sterile airflow resulted in higher recovery rates. Dehydration of calli with step-wise increased concentrations of PVS2 provided better recovery rates than in the case of exposure to a highly concentrated PVS2 solution. The use of a mixture of 2 M glycerol plus 0.4 M sucrose as a cryoprotectant for 10 or 20 min provided better viability of calli before and after cryopreservation. Increasing the duration of exposure to the cryoprotectant solution decreased the viability of calli. Dehydration of encapsulated and cryoprotected (Encapsulation - Vitrification) calli with a highly concentrated PVS2 solution for 5 -90 min showed no variations in regrowth rates after cryopreservation, giving a wider range of success when using this procedure. However, further studies should be initiated to study genetic stability after cryopreservation.

ACKNOWLEDGMENT

The authors are grateful to the Deanship of Academic Research at Jordan University of Science and Technology for funding this study (Project # 89/2001).

Table (7): Survival and regrowth of non-cryopreserved (-LN) and cryopreserved (+LN) embryogenic calli of Date palm as influenced by duration of exposure to loading solution (2 M glycerol plus 0.4 M sucrose).

Duration (min)	Survival (%)	Red Color Intensity	Regrowth (%)	Callus Color
Non-cryopreserved				
10	86.7 a[z]	Red	73.3 a	W
20	86.7 a	Deep Red	73.3 a	W
30	86.7 a	Red	46.7 b	YB
60	86.7 a	Red	40 bc	B
90	80 a	red	26.7 c	B
Cryopreserved (+LN)				
10	60 a	Red	40 ab	YW
20	66.7 a	Red	46.7 a	YW
30	60 a	Red	26.7 bc	YB
60	60 a	Red	20 c	B
90	60 a	Light Red	13.3 c	B

W = White, YB = Yellowish Brown, B = Brown.

[z] Means within column having different letters are significantly different according to LSD at $P \leq 0.01$.

Table (8): Survival and regrowth of Encapsulated-Vitrified (EV) non-cryopreserved (-LN) and cryopreserved (+LN) embryogenic calli of Date palm as influenced by dehydration duration with concentrated PVS2.

PVS2 Dehyd. Duration (min)	Survival (%)	Red Color Intensity	Regrowth (%)	Callus Color
Non-cryopreserved (-LN)				
5	80 a[z]	Red	46.7 bc	YW
10	80 a	Red	46.7 bc	YW
30	80 a	Deep Red	73.3 a	W
60	73.3 a	Deep Red	60 ab	W
90	73.3 a	red	33.3 c	YB
Cryopreserved (+LN)				
5	60 a[z]	Light Red	26.7 a	YB
10	60 a	Light Red	26.7 a	YB
30	46.7 b	Light Red	33.3 a	YB
60	40 b	Light Red	26.7 a	YB
90	26.7 c	Light Red	20 a	YB

W = White, YW =Yellowish White, YB = Yellowish Brown .

[z] Means within column having different letters are significantly different according to LSD at $P \leq 0.01$.

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حفظ الخلايا الجنينية للنخيل (*Pheniox ductylifera*) في النيتروجين السائل بواسطة طريقة الكابسول وطريقة التزجيج والكابسول والتزجيج معاً

وليد صبيح*، محمد عواد شطناوي** ورضا عبد الله شبلي*✉

ملخـص

تم حفظ الخلايا الجنينية لنبات النخيل بواسطة طريقة الكابسول، وطريقة التزجيج والكابسول والتزجيج معاً. كانت أعلى نسبة حيوية (80%) بواسطة الكابسول عند معاملة الخلايا بواسطة (0.3) مول سكروز متبوعة بالتجفيف لمدة ساعتين. بينما كانت نسبة النمو (20-33%) عند معاملة الخلايا الجنينية بـ (0.1) مول سكروز بعد تجفيف الخلايا لمدة أربع ساعات، وكانت حيوية الخلايا الجنينية تقل مع زيادة التركيز وزيادة مدة التجفيف. إن معاملة الخلايا الجنينية بواسطة طريقة التزجيج بمحلول (PVS2) أدت إلى تراجع في حيوية الخلايا (80-93.2%)، بينما كانت نسبة النمو (40-53.3%) وينتج ذلك عند استخدام خطوتين أو أربع خطوات لتجفيف الخلايا بواسطة (PVS2) على درجة حرارة 25°م لمدة (20 دقيقة) قبل حفظها في النيتروجين السائل. تم حفظ النباتات في النيتروجين السائل عند معاملة الخلايا الجنينية بـ (1) مول سكروز + (15%) DMSO مضافاً إليه (2) مول جليسرول +(0.4) مول سكروز أو باستخدام (15%) DMSO وينتج عن ذلك خلايا حية (60-66.7%). إن المزج بين طريقة التزجيج والكابسول لحفظ الخلايا المعلقة بعد معاملتها بـ 100% (PVS2) لمدة (5 أو 10 دقائق على درجة حرارة 25°م) نتج عنه 60% نسبة نجاح. ولا يوجد فرق معنوي في النمو (20-33.3%) عند معاملة الخلايا الجنينية بواسطة (PVS2) والتجفيف لمدة (5-90 دقيقة)، أثبتت النتائج أن إمكانية خزن الخلايا الجنينية لنبات النخيل ممكنة في النيتروجين السائل.

الكلمات الدالة: النخيل، حفظ الخلايا الجنينية لنبات النخيل في النيتروجين السائل، الحفظ بطريقة الكابسول، الحفظ بطريقة التزجيج، الحفظ بطريقة الكابسول والتزجيج معاً.

*✉ قسم الانتاج النباتي، كلية الزراعة، جامعة العلوم والتكنولوجيا الأردنية، اربد، الأردن. البريد الإلكتروني: shibli@ju.edu.jo

** قسم التكنولوجيا الحيوية، كلية التقنيات الزراعية، جامعة البلقاء التطبيقية، السلط، الأردن. تاريخ استلام البحث 2006/3/23، وتاريخ قبوله 2006/12/20.