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Optimizing *in vitro* Cryopreservation of Date Palm (*Phoenix dactylifera* L.)

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Abstract: Cryopreservation, the storage of materials at ultra-cold temperature, is a useful method for long-term storage of a wide variety of plant germplasm including buds, seeds, seed parts, twigs and cell and tissue cultures. The objective of this study was to define the optimal concentrations of key components of the cryoprotectant solution based on sucrose combinations with either dimethyl sulfoxide (DMSO) or glycerol for date palm (*Phoenix dactylifera* L.) cell suspension. Callus induced from shoot tip explants isolated from young offshoots of cv. Khalas, the most important commercial cultivars in Saudi Arabia, was used to establish cell suspension cultures. The cells were pretreated in sucrose solution and kept in a cryoprotectant solution containing sucrose at a concentration corresponding to that used in the pre-treatments (0.1, 0.25, 0.5, 0.75 and 1 M) and supplemented with either DMSO at 0, 5 and 10% or glycerol at 0, 1 and 2 M. After freezing in liquid nitrogen for 14 day, cell samples were revived and tested for survivability and competency in terms of colony formation, callus re-growth and subsequent somatic embryogenesis. The highest colony formation (21 colonies), greatest callus growth (0.12 g) and highest embryos number (11 embryos) was recovered from cell cryoprotected in 10% DMSO supplemented with 0.75 M sucrose. This study has resulted in cryoprotectant solutions suitable for date palm cells suspension; thus provided the necessary fundamental knowledge for establishing a germplasm bank based on cryopreservation approach for date palm conservation.

Key words: Cryopreservation, cryoprotectant, date palm, *in vitro*, DMSO, glycerol, sucrose

INTRODUCTION

Date palm (*Phoenix dactylifera* L.) is a dioecious tall evergreen heterozygous fruit tree which is normally vegetatively propagated through offshoots. Seed propagation produces off-types plants because of cross pollination; therefore, it is not a valid method to clone known cultivars (Al-Khayri, 2005). Date palm germplasm is difficult to store using conventional means and vegetatively propagated material has high potential risk of spreading pest and pathogens. Cryopreservation has an advantage of long-term storage without subjecting to frequent subculturing which is known to induce somaclonal variation (Jain, 2011). Cryopreservation is currently the only safe and cost-effective method for long term conservation of the germplasm of species that are vegetatively propagated or have seeds that are recalcitrant to storage (Shibli, 2000). Therefore, cryopreservation is the most appropriate available technique for long-term conservation of date palm germplasm (Bekheet, 2011).

An important prerequisite for successful cell cryopreservation is the availability of a regeneration

system to insure the recovery of complete plants after cell revival. *In vitro* plant regeneration has been achieved in date palm through somatic embryogenesis (Mounir and Ismail, 2004; Al-Khateeb, 2006a, b; Al-Khayri, 2007; 2010; Sghaier-Hammami *et al.*, 2010; Al-Khayri, 2011; Fki *et al.*, 2011) as well as organogenesis (Abahmane, 2011). Several studies demonstrated the utilization of cell suspension culture to improve date palm somatic embryogenesis (Zouine and El-Hadrami, 2004, 2007; Othmani *et al.*, 2009) and investigate *in vitro* physiological responses (Al-Khayri and Al-Bahrany, 2004).

Successful cryopreservation requires the optimization of numerous variables including the size of specimen, the correct type and concentration of cryoprotectant, sample water content and rate of freezing and thawing (Mycock *et al.*, 1995). Key components of the protectant solutions normally used in plant materials cryopreservation include Polyethylene Glycol (PEG), sorbitol, dimethyl sulfoxide (DMSO), glycerol and sucrose (Haggman *et al.*, 1998; Mathur *et al.*, 2003; Moges *et al.*, 2004; Winkelmann *et al.*, 2004; Jain, 2011). These components have been tested in the *in vitro*

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cryopreservation of different tissues such as callus, cell cultures and somatic embryos in a variety of plant species (Bajaj, 1983). They include banana *Musa* spp. (Panis and Thinh, 2001), coffee *Coffea arabica* (Dussert *et al.*, 1997), oil palm *Elaeis guineensis* (Dumet *et al.*, 1993), tea *Camellia sinensis* (Kim *et al.*, 2002), grapevine *Vitis vinifera* L. (Wang *et al.*, 2002; Gonzalez-Benito *et al.*, 2009), ash *Fraxinus excelsior* L. (Ozudogru *et al.*, 2010), Persian cyclamen *Cyclamen persicum* (Winkelmann *et al.*, 2004) Ginkgo *Ginkgo biloba* (Popova *et al.*, 2009) and cassava *Manihot esculenta* Crantz (Danso and Ford-Lloyd, 2011), Thai orchid *Cleisostoma arietinum* (Maneerattanarungroj *et al.*, 2007), Golden nugget orchid *Mokara* (Safrinah *et al.*, 2009), lily *Lilium ledebourii* (Baker) Boss. (Kaviani *et al.*, 2008) and rice *Oryza sativa* (Fatima *et al.*, 2002).

In date palm, research progress towards defining the requirements for cryopreservation has been limited and only a small number of genotypes were tested. The date palm genotype tested thus far are cv. Zaghlool (Bekheet *et al.*, 2007), cv. Medjool (Subaih *et al.*, 2007) and an unspecified cultivar (Mycock *et al.*, 1995). These studies utilized different type of tissues including nodular cultures (Bekheet *et al.*, 2007), embryogenic callus (Subaih *et al.*, 2007), somatic embryo (Mycock *et al.*, 1995), immature somatic embryo (Mycock *et al.*, 1997), pollen (Tisserat *et al.*, 1985).

Previous studies related to date palm cryopreservation examined the effect of different components of cryoprotectant including DMSO, glycerol, sucrose and polyethylene glycol (PEG) (Subaih *et al.*, 2007; Mycock *et al.*, 1995; Mycock *et al.*, 1997; Tisserat *et al.*, 1985). Due to the specificity of genotype requirements, it is necessary to empirically determine the optimum cryoprotectant components of different genotypes (Ellis *et al.*, 2006). The current study was conducted to define the requirements suitable for the most commercially important Saudi date palm cultivar, Khalas. Furthermore, this study investigated the behavior of date palm cell suspension to cryopreservation; whereas none of the previous studies has addressed this type of culture. This study was designed to investigate the response of date palm *in vitro* cells to concentrations of key components of cryoprotectant solution based on sucrose combinations with either DMSO or glycerol. The study evaluated the competency of thawed cryopreserved cells in terms of colony formation, callus re-growth and subsequent somatic embryogenesis.

MATERIALS AND METHODS

Explant preparation and culture establishment: Tissue culture procedures to establish date palm *in vitro* cultures and callus proliferation were according to previously

described methods (Al-Khayri, 2001; Al-Khayri and Al-Bahrany, 2001). Young offshoots of cv. Khlass were separated from mother trees, outer leaves were removed exposing the shoot tip regions that were excised and immediately placed in a chilled antioxidant solution consisting of ascorbic acid and citric acid, 150 mg L⁻¹ each, to prevent browning. The shoot tip tissue, about 8 cm long, was surface sterilized in 70% ethanol for 1 min followed by 15 min in 1.6% w/v sodium hypochlorite (30% v/v Clorox, commercial bleach) containing 3 drops of Tween 20 (Sigma Chem Co, St. Louis, MO) per 100 mL Clorox solution. The tissue was then rinsed with sterile distilled water four times and placed again in sterile antioxidant solution in preparation for explant excision. The tissue surrounding the shoot tips was removed until the leaf primordia was exposed and detached at the base. The shoot tip terminal, about 1 cm long, was sectioned longitudinally into four to eight sections. Leaf primordial and terminal tip explants were used for culture initiation.

Callus initiation, proliferation and maintenance: The medium used consisted of MS salts (Murashige and Skoog, 1962) supplemented with (L⁻¹) 170 mg NaH₂PO₄, 125 mg myo-inositol, 200 mg glutamine, 5 mg thiamine-HCl, 1 mg nicotinic acid, 1 mg pyridoxine-HCl, 30 g sucrose and 7 g agar (purified Agar-agar/Gum agar) (Sigma). This basal medium was used throughout the system with modifications made according to each stage. The callus initiation medium contained (L⁻¹) 100 mg 2,4-dichlorophenoxyacetic acid (2,4-D) (452.5 µM) and 3 mg 2-isopentenyladenine (2iP) (14.7 µM) and 1.5 g activated charcoal (acid-washed, neutralized) (Sigma). These cultures were maintained in darkness at 24°C±3°C for 12 week during which they were transferred at a 3-week interval. In the end of this period, resultant callus was separated and transferred to callus proliferation medium that contained (L⁻¹) 10 mg naphthaleneacetic acid (NAA) (53.7 µM), 30 mg 2iP (147 µM) and 1.5 g activated charcoal. These cultures were maintained for additional 3 week at 24°C±3°C and a 16-h photoperiod (50 µmol m⁻² sec⁻¹) provided from cool-white fluorescent lamps. To proliferate embryogenic callus, the callus was transferred to a medium containing (L⁻¹) 10 mg NAA (53.7 µM), 6 mg 2iP (29.6 µM) and 1.5 g activated charcoal. These cultures were maintained for 9 week during which they were transferred at a 3-week interval. Embryogenic callus was maintained in the dark on a medium containing (L⁻¹) 10 mg NAA (53.7 µM) and 1.5 mg 2iP (7.4 µM) as callus source for subsequent experiments designed to develop a cryopreservation method for date palm.

Cryopreservation procedures: The cryopreservation protocol used in this study was according to Withers and King (1980). Date palm callus from 12-week-

old maintenance cultures was chopped into 1-3 mm pieces and cultured (2 g callus per flask) in a pre-treatment liquid medium (25 mL per flask), which consisted of MS medium supplemented with sucrose at 0.1, 0.25, 0.5, 0.75 and 1 M. These pre-treatment cultures were incubated at 24°C±3°C in complete darkness on a gyratory shaker set at 100 rpm for 2 days. Following the pre-treatment period, the cell clusters were allowed to settle to the bottom of the culture flask, the liquid medium was decanted and cooled cryoprotectant solutions were added.

The cryoprotectant solution treatments consisted of MS medium supplemented with the following: Sucrose at a concentration corresponding to that used in the pre-treatments (0.1, 0.25, 0.5, 0.75 and 1 M) supplemented with either DMSO at 0, 5, or 10% or glycerol at 0, 1, or 2 M. The mixtures were left for 1 h shaking to mix the viscous cryoprotectant solution with the cell suspension. Aliquots of 1 mL cell suspension were dispensed into 2 mL capacity cryopreservation ampoules (cryovials), sealed and labeled. The cryovials were gradually cooled by refrigerating at 4°C for 2 h, then at -20°C for another 2 h and finally submerged in Liquid Nitrogen (LN) for storage until testing.

To thaw the cryopreserved date palm cell suspension, ampoules stored for 14 days were transferred to a water bath set at 40°C until samples were completely thawed. The ampoules were wiped with 70% ethanol to assure sterility. Thawed cell suspension samples were withdrawn with a sterile Pasteur pipette and transferred to the surface of a semisolid medium containing 7 g L⁻¹ agar in Petri dishes (25 mL culture medium per plate). The cell suspension was evenly distributed on the agar surface and excess liquid medium was removed. The dishes were sealed with a double layer of Parafilm and incubated in a culture room at 24°C±3°C in complete darkness.

To assess the effectiveness of cryopreservation treatments, various parameters were observed including cell colonies re-growth and amount of resultant microcalli in terms of fresh weight. Callus masses recovered from the cryopreserved cells were transferred to a hormone-free regeneration medium to examine their capacity for develop somatic embryos.

Experiment design and statistical analysis: To assess the effectiveness of cryopreservation treatments, two different experiments were conducted involving 2-factor factorial design. The first experiment involved two main factors, sucrose concentration at five levels (0.1, 0.25, 0.5, 0.75 and 1 M) and DMSO at three levels (0, 5, or 10%). The second experiment involved two main factors, sucrose concentration at five levels (0.1, 0.25, 0.5, 0.75 and 1 M) and glycerol at three levels (0, 1, or 2 M).

Assessments of effectiveness of cryoprotectant treatments in terms of recovering callus growth and preserving regeneration capacity were based on five thawed cryopreserved samples per treatment. Data included cell colony number, callus fresh weight and number of resultant somatic embryos. The data from each experiment were subjected to Analysis of Variance (ANOVA) and the means were separated, where appropriate, with a Least Significant Difference (LSD) at 5% significance level.

RESULTS AND DISCUSSION

Colony number: Colony numbers were used as an indicator of the cell-maintaining capability to divide and form colonies that subsequently develop into microcalli. This depends upon the effectiveness of the cryoprotectant components to provide conditions supportive of cell survivability. These components exert influence on water outflow from the cells as well as the penetration of the cryoprotectants and consequently affect survivability of cryopreserved cells (Bagniol *et al.*, 1992). In this study, we investigated the effect of the key components of cryopreservation solution on the survival and re-growth of date palm cell suspension. Cryopreserved cells re-growth was visible in the culture plates within 4 weeks after plating regardless of the preservation components. When combinations of DMSO and sucrose were used as protectant agents, the number of resultant colony growth was significantly influenced by both sucrose and DMSO concentrations as indicated by a two-way significant interaction revealed by analysis of variance (Table 1). Generally, at a given concentration of sucrose, the colony number increased in response to higher concentrations of DMSO (Fig. 1). This trend was less pronounced at both the lowest, 0.1 M and highest,

Table 1: Analysis of variance of the effect of sucrose and DMSO concentration in the cryoprotectant solution on cell colony count, amount of callus re-growth and resultant embryo number recovered from date palm embryogenic cell suspension

Factor	df	SS	MS	F-value	p-value
Cell colony count					
Sucrose conc.	4	379.653	94.913	30.816	0.0001
DMSO conc.	2	127.760	63.880	20.740	0.0001
Suc.+DMSO conc.	8	143.307	17.913	5.816	0.0001
Error	60	184.800	3.080		
Callus re-growth					
Sucrose conc.	4	0.034	0.008	8.867	0.0001
DMSO conc.	2	0.025	0.013	13.142	0.0001
Suc.+DMSO conc.	8	0.020	0.002	2.564	0.0180
Error	60	0.057	0.001		
Embryo number					
Sucrose conc.	4	46.747	11.687	10.624	0.0001
DMSO conc.	2	35.707	17.853	16.230	0.0001
Suc.+DMSO conc.	8	23.093	2.887	2.624	0.0160
Error	60	66.000	1.100		

p-values less than 0.05 are significant

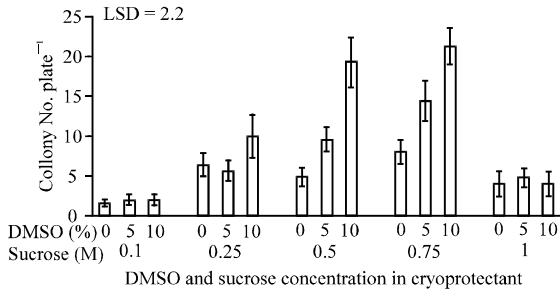


Fig. 1: Effect of sucrose and DMSO concentrations in the cryoprotectant solution on cell colony count recovered from date palm embryogenic cell suspension

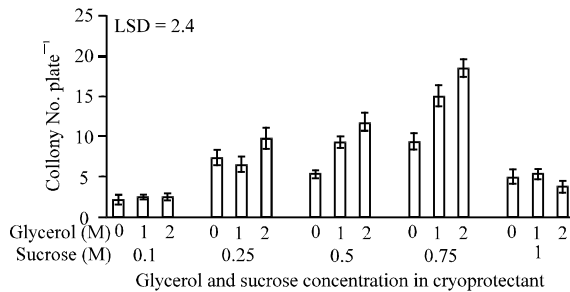


Fig. 2: Effect of sucrose and glycerol concentrations in the cryoprotectant solution on cell colony count recovered from date palm embryogenic cell suspension

Table 2: Analysis of variance of the effect of sucrose and glycerol concentration in the cryoprotectant solution on cell colony count, amount of callus re-growth and resultant embryo number recovered from date palm embryogenic cell suspension

Factor	df	SS	MS	F-value	p-value
Cell colony count					
Sucrose conc.	4	363.813	90.953	25.937	0.0001
Glycerol conc.	2	86.427	43.213	12.323	0.0001
Suc.+glycerol conc.	8	123.307	15.413	4.395	0.0003
Error	60	210.400	3.507		
Callus re-growth					
Sucrose conc.	4	0.0089	0.0022	11.2896	0.0001
Glycerol conc.	2	0.0106	0.0053	26.6936	0.0001
Suc.+glycerol conc.	8	0.0041	0.0005	2.5774	0.0173
Error	60	0.0119	0.0002		
Embryo number					
Sucrose conc.	4	42.6133	10.6533	11.2535	0.0001
Glycerol conc.	2	36.9867	18.4933	19.5352	0.0001
Suc.+glycerol conc.	8	15.9467	1.9933	2.1056	0.0491
Error	60	56.8000	0.9467		

p-values less than 0.05 are significant

1 M, sucrose concentrations. In both of these cases, no significant differences were detected between DMSO levels. In contrast, significant differences in colony numbers were observed in response to changing DMSO level when sucrose was used at 0.5 and 0.75 M, also to some extent at 0.25 M. The highest colony formation, 19

to 21 colonies, were recovered from cell pretreated in 0.5 or 0.75 M sucrose, respectively, then cryopreserved in a cryoprotectant solution containing 10% DMSO supplemented to either 0.5 or 0.75 M sucrose. This illustrates the importance of incorporating the appropriate concentration of sucrose in combination with DMSO in the cryoprotectant solution. In a related study, using embryogenic suspension cultures of Persian cyclamen, *Cyclamen persicum*, Winkelmann *et al.* (2004) obtained an average of 14 colonies per plate from cryopreserved cells subjected to a cryoprotectant solution containing 0.6 M sucrose and 10% DMSO. Without DMSO, the average number of cell colonies re-growth per plate was reduced to only 2 colonies. Similarly, we found that when DMSO was not included low rates of colony re-growth was obtained ranging from 2 to 8 depending on the sucrose concentration (Fig. 1).

Comparably, when sucrose and glycerol were used as a protectant agents, the number of resultant colony re-growth was also significantly influenced by both sucrose and glycerol concentrations as indicated by a two-way significant interaction revealed by analysis of variance (Table 2). Thawed cell samples formed colonies at low rates when the cryoprotectant solution contained 0.1 M sucrose regardless of the glycerol level (Fig. 2). Using the other extreme sucrose concentration, 1 M, the same growth reduction was observed. In both cases, no significant differences were detected between glycerol levels. In contrast, significant difference in colony number was observed in response to changing glycerol level when sucrose was used at 0.5 and 0.75 M and to some extent at 0.25 M. The highest colony formation, approximately 12 to 18 colonies, were recovered from cell pretreated in 0.5 or 0.75 M sucrose, respectively, then cryopreserved in a cryoprotectant solution containing 2 M glycerol supplemented with either 0.5 or 0.75 M sucrose. Accordingly, DMSO appears to be more suitable than glycerol for the cryopreservation of date palm cell suspension in relation to colony number formation.

Callus growth: When DMSO was used as a protectant agent, the amount of callus growth in term of callus fresh weight was significantly influenced by both sucrose and DMSO concentrations as indicated by a two-way significant interaction revealed by analysis of variance (Table 1). The amount of callus proliferation from the re-growth of resultant colonies is an excellent indicator of the status of the cryopreserved cells, since it reveals the capacity of the cells to divide and elongate. Cryopreserved date palm cell samples that formed colonies after thawing, developed microcalli that differed in weight depending on the cryoprotectant treatments. In

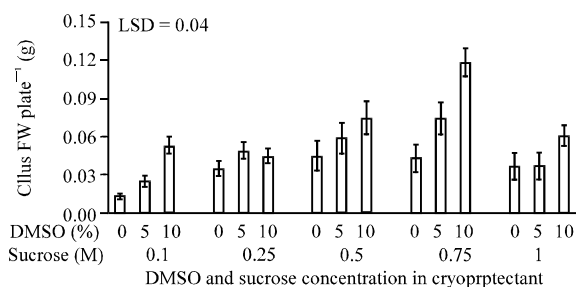


Fig. 3: Effect of sucrose and DMSO concentrations in the cryoprotectant solution on cell amount of callus re-growth recovered from date palm embryogenic cell suspension

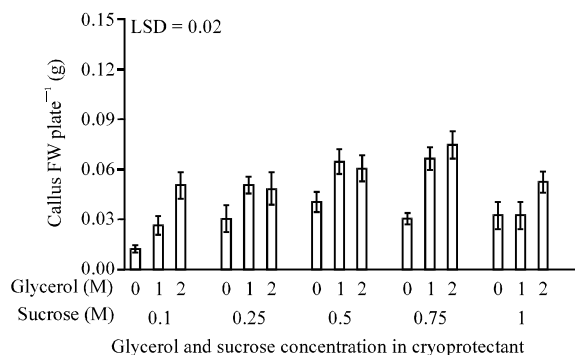


Fig. 4: Effect of sucrose and glycerol concentrations in the cryoprotectant solution on amount of callus re-growth recovered from date palm embryogenic cell suspension

all sucrose concentrations tested, callus weight generally increased as the DMSO levels increased (Fig. 3). This suggests that including DMSO to the protectant solution is required for effective cryopreservation of date palm cell suspension. The highest callus weight, 0.12 g, was recovered from cell pretreated in 0.75 M sucrose then frozen in a cryoprotectant solution containing 10% DMSO supplemented with 0.75 M sucrose; hence, this is considered an optimum cryoprotectant solution for date palm *in vitro* cells cryopreservation. The role of these components was illustrated in the cryopreservation of date palm pollen. For example, Tisserat *et al.* (1985) found that date palm pollen survived cryopreservation for 3 months at -69°C in a cryoprotectant solution containing 10% PEG, 8% glycerol and 10% DMSO. Survivability of cryostored date palm pollen was also affected by the storage temperature as reported by Mortazavi *et al.* (2010) who obtained the highest survival rate using an ultra-low temperature (-196°C). In a study conducted by Bekheet *et al.* (2007) the role of fructose, glucose, sorbitol

and sucrose on the cryopreservation of *in vitro* date palm nodular cultures was investigated. They noticed that adding sucrose separately to the preculture medium at 1 M gave the highest survival percentage (80%) of cryopreserved date palm tissue cultures.

When glycerol was used as a protectant agent, the amount of callus fresh weight produced from the revived cells was significantly influenced by sucrose and glycerol concentrations as indicated by a two-way significant interaction revealed by analysis of variance (Table 2). In all sucrose concentrations tested, the callus weight generally increased as the glycerol levels increased (Fig. 4). This suggests that including glycerol to the protectant solution is required for effective cryopreservation of date palm cells. The highest callus weight, 0.06 to 0.07 g, was recovered from cells pretreated in 0.5 and 0.75 M sucrose, respectively, then frozen in a cryoprotectant solution containing 1 and 2 M glycerol supplemented to either 0.5 or 0.75 M sucrose; hence, these treatments are considered most suitable for date palm *in vitro* cells cryopreservation.

In comparison Subaih *et al.* (2007) studying cryopreservation of date palm embryogenic callus of cv. Medjool found that cryoprotection using 1 M sucrose plus 15% DMSO and dehydration using 2 M glycerol plus 0.4 M sucrose or 0.5 M sucrose plus 10% DMSO produced best survival, 66.7%. Difference in the optimum cryoprotectant composition may be due to specificity of cultivar requirements. However, our results indicated that DMSO is more suitable than glycerol for the cryopreservation of date palm cv. Khalas as assessed by the amount of callus re-growth. In another species, Mathur *et al.* (2003) reported that survival of cryopreserved embryogenic cultures of *Pinus roxburghii* was best achieved using 0.3 M sorbitol combined with 5% DMSO.

Somatic embryogenesis: The ultimate test of the effectiveness of the cryopreservation protocol is the ability of preserved cells to maintain somatic embryogenesis. Jain (2011) reported that the growth and germination of somatic embryo in date palm remains normal when treated with cryoprotectant mixture of glycerol and sucrose and the growth rate and germination rate of somatic embryos stayed normal after the cryopreservation. Mater (1987) found that freezing callus cultures at -250°C for 4 months did not affect somatic embryogenesis potential. Similarly, in studies with the late globular to early torpedo stage of date palm somatic embryos, pretreated with a cryoprotectant solution

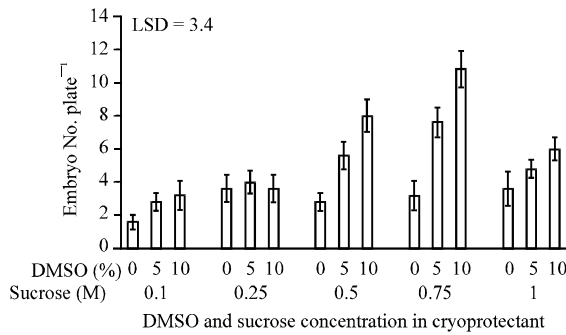


Fig. 5: Effect of sucrose and DMSO concentrations in the cryoprotectant solution on resultant embryo number recovered from date palm embryogenic cell suspension

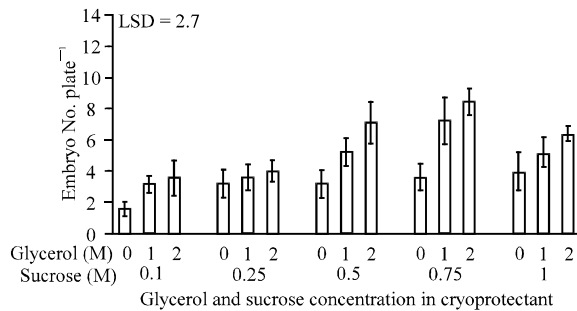


Fig. 6: Effect of sucrose and glycerol concentrations in the cryoprotectant solution on resultant embryo number recovered from date palm embryogenic cell suspension

containing 10% glycerol and 10% sucrose supported normal embryo growth and development after cryopreservation (Mycock *et al.*, 1995, 1997). We also observed that the capacity of cryopreserved cells to proliferate embryogenic callus was maintained after thawing. The number of regenerated embryos, however, was affected by a two-way strongly significant interaction between sucrose and DMSO concentration revealed by analysis of variance (Table 1). The number of embryos formed was significantly influenced by the cryoprotectant treatments. The numbers of resultant embryos was relatively low when the cryoprotectant solution contained sucrose at low levels, 0.1 or 0.25 M, as well at the highest level, 1 M. At these concentrations DMSO addition slightly improved the regeneration capacity (Fig. 5). In contrast, when 0.5 or 0.75 M sucrose was used, significant increases in numbers of embryos were observed in response to increasing the concentration of DMSO in the protectant solution. The highest embryo number, approximately 8 to 11 embryos per microcalli, was associated with cells pretreated in 0.5 or 0.75 M sucrose

then frozen in a cryoprotectant solution containing 10% DMSO supplemented to 0.5 or 0.75 M sucrose. Hence, these treatments are considered optimal for date palm *in vitro* cells cryopreservation. In addition to maintaining regeneration capacity, these treatments gave highest number of colonies and greatest callus weight. In another study, Danso and Ford-Lloyd (2011) found that post-thaw viability of cassava callus pretreated with 0.3 M sucrose was comparatively higher than 0.5 M sucrose; whereas, in date palm cell suspension 0.75 M sucrose gave the highest growth and development.

When glycerol was used as a protectant agent, the number of regenerated embryos produced from the revived cell suspension was significantly influenced by sucrose and glycerol concentrations as indicated by a two-way significant interaction revealed by analysis of variance (Table 2). The numbers of resultant embryos was relatively low when the cryoprotectant solution contained sucrose at low levels, 0.1 or 0.25 M, as well at the highest level, 1 M. At these concentrations glycerol addition slightly improved the regeneration capacity (Fig. 6). In contrast, when 0.5 or 0.75 M sucrose was used, significant increases in numbers of embryos were observed in response to increasing the concentration of glycerol in the protectant solution. The highest embryo number, approximately 7 to 8 embryos per microcalli, was associated with cells pretreated in 0.5 or 0.75 M sucrose then frozen in a cryoprotectant solution containing 2 M glycerol supplemented with 0.5 or 0.75 M sucrose. Hence, these treatments are considered optimal for date palm *in vitro* cells cryopreservation. In addition to maintaining regeneration capacity, these treatments gave highest number of colonies and greatest callus weight. According to our results, both DMSO and glycerol appears to be suitable for the cryopreservation of date palm in relation to the resultant embryo number; however, DMSO appears to be superior to glycerol.

Our study has determined the optimum concentrations of key components of the cryoprotectant solution suitable for the cryopreservation of date palm cell suspension. The effects of non-penetrating cryoprotectant, sucrose and penetrating cryoprotectants, DMSO and glycerol on cell growth and redifferentiation capacity were determined. The highest colony formation, callus weight and embryo number were associated with cell pretreated in 0.75 M sucrose then cryopreserved in a cryoprotectant solution containing 10% DMSO supplemented to 0.75 M sucrose. Thus, these cryoprotectant mixtures are considered the most favorable for date palm cells suspension cryopreservation. The knowledge gain from this study would benefit future

research aimed at establishing *in vitro* germplasm banks for the purpose of preservation date palm genetic diversity.

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