CRYOPRESERVATION OF *IN VITRO* ESTABLISHED SHOOT TIP EXPLANTS OF DATE PALM CV. ZAGHLOOL

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(Manuscript received 28 Nov. 2007)

Abstract

Cryoprotection procedures for *in vitro* established shoot tip explants of Date palm cv. Zaghlool prior to cryoprservation process in liquid nitrogen were investigated in this study. This work aimed to develop simple cryoprotection procedure that can be handled easily. The first cryoprotection procedure was to study the effects of different sucrose concentrations (0.1, 0.3, 0.5, and 0.7Mol) in preculturing medium and the incubation under at 5°C or 27°C for four weeks. The second cryoprotection procedure was to study the effects of the same previous sucrose concentrations in preculturing medium and the incubation under at 27°C for four weeks followed by desiccation procedure for two hours in an open petri dish exposed to continual current stirel air in the laminar airflow cabint. The third cryoprotection procedure was to study the effects of abscisic acid (ABA) concentrations (2.0, 4.0, 6.0 and 8.0 mg/L) in preculturing media and the incubation under at 5°C or 27°C for four weeks. The fourth cryoprotection procedure was to study the effects of the same previous concentrations of (ABA) in preculturing media and incubation under at 27 ° C for four weeks followed by desiccation procedure for two hours in an open petri dish exposed to continual current of stirel air in laminar airflow cabinet . All shoot tip explants obtained from the four cryoprotiction procedures were plunged in liquid nitrogen Dewar at (-196 °C) for one hour after thawing process in water bath at 40 °C. All cryopreserved shoot tip explants were cultured on recovery medium for 3 weeks to examine their potential for surviving after cryopreservation process. The results of explants viability showed that all of the tested pretreatments gave quiet results for survival. The optimal procedure that we developed was the cryoprotiction of cultured shoot tip explants on preculturing medium supplemented with 0.5 M of sucrose concentration for four weeks and incubated at 27°C followed by air desiccation treatment prior to cryopreservation process. The cryopreserved explants from this cryoprotiction procedure revealed high survival and could resume their life cycle in normal manner on the recovery medium. Date palm explants cryopreservation needs more studies to achieve better results in germplasm conservation.

INTRODUCTION

Date palm *(Phoenix dactylifera L.)* is an important commercial food crop in many Arab countries. Cultivation is largely confined to a belt across the top of Africa

(including the Morocco, Egypt and Sudan) and most of the Near East commercial plantations have also been established in the new world in particular in California.

It must not be forgotten that the date palm is still (and will remain for the foreseeable future) a significant subsistence crop. The way forward a primary aim for all countries concerned in date production should be to establish a dedicated date palm tissue culture laboratory concentrating on *in vitro* culture of local superior varieties for both commercial and small holder supplies. In tandem the expansion of investigation studies and the establishment of germplasm resource centres should be occurring in part to help counteract the local crops resulting from tissue culture. (Watanab and Pehu, 1997)

Plant *in vitro* technology offers a potential solution to the long term conservation of germplasm (Grout, 1990). The advantage of this technology is that a variety of cells and tissues can be stored: for instance protoplasts, single cells and organized tissues, for such as, meristems and somatic embryos (Bajaj, 1983). In addition the technology can also be useful for the generation of novel genetic resources through, for example, the exploitation of somaclonal variations and these must be conserved (Mycock *et al.*, 1991)

Management of large-scale *in vitro* collections posses numerous practical problems. Moreover, risks of contaminations and somaclonal variation increase with time. It is therefore essential to develop long term conservation techniques for the germplasm of numerous potentially useful varieties of date palm (Engelmann *et al.*, 1995)

One of the principle long-term *in vitro* conservation methods is cryostorage. Cryopreservation is generally understood as storage between -79 and -196°C, the lower extreme being the temperature of liquid nitrogen. The major advantage of storage of biological material at such temperatures is that both metabolic processes and biological deterioration are considerably slowed or even halted (Kartha, 1981). In addition, it is believed that cryopreserved materials remains genetically stable thus affording an advantage over conventional conservation methods (Withers, 1980, 1983). It is particularly important that cryopreserved cells, tissue and organs be capable of producing plants identical to the unfrozen phenotypes (Kobayashi *et al.*, 1990). *In vitro* gene banks for long term conservation maintain the genetic stability during storage (Harding and Benson, 2000) only a limited amount of work has been carried out on cryopreservation of date palm, using *in vivo* (seeds, pollen, and apices) or *in vitro* calli. The first experiments were performed in the (1980) with embryogenic calli of different varieties (Tisserat *et al.*, 1981, Finkle *et al.*, 1982 and Urich *et al.*,

1982) using classical freezing process. Plantlets could be regenerated from the cryopreserved calli.

In any cryogenic procedure, cells and meristems must be sufficiently dehydrated to be capable of vitrifing before immersion into liquid nitrogen (LN) (Sakai and Yoshida, 1976, Fabre and Dereuddre 1990).

The most common cryoprotectants used (in cryoprotectant solutions) included agents like dimethyl sulfoxide (DMSO), ethylene glycol, 1, 2, -propandiol and glycerol which is basal component for vitirfiction solution, (Sakai *et al.*, 1990, Uragami *et al.,* 1989 and Nishizawa *et al.,* 1993) while in the preculture stage only a limited number of sugars such as sucrose, glucose, sorbitol and manitol have been used in plant tissues (Moran *et al.*, 1999, Touchell, 1995, Thierry *et al.*, 1997 and Watanabe, *et al.,* 1999)

Simple freezing avoids the use of vitrification solutions, which are often phytotoxic to the plant tissue (Takagi, 2000).

Preculture and pretreatment also influenced the growth of cells after cryopreservetion in liquid nitrogen (Winkelmann, *et al.*, 2004).

Several authors indicated that cold hardening and /or preculturing with a high concentrations of sugar in the medium are essential to successful cryopreservation of *In vitro* cultured plant materials (Kartha *et al.*, 1979, 1980, Engelmann *et al.*, 1995, Reed 1988, 1990, 1992, Dereddure *et al.*, 1990, Niino, *et al.*, 1992, Kartha and Engelmann 1994).

Cell dehydration has been achieved by new approach involving an air desiccation period (from 20 min to several hours) where the explants are held in the air current of laminar flow cabinet (Jekkel, *et al.*, 1998).

Abscisic acid (ABA) can be used as cryoprotectant factor (Mycock, *et al.*, 1995) while ABA had no effect on the reduction in water content its effect may be due to tolerance to desiccation induced by ABA treatment.(Jekkel, *et al.,* 1998).

The aim of this study was to investigate some pretreaments for cryoprotection of date palm cv. Zaghlool established shoot tip segments explants before cryopreservation in liquid nitrogen and the effect of these pretreatment on the regrowth of shoot tip explants after cryopreservation in liquid nitrogen in order to avoid the used of vitrification solutions which have some toxic effects because of containing agent like dimethyle sulfoxid (DMSO). In addition, in the vitrification method its difficult to treat carefully a large number of merisitim tips at the same time.Thus, we tried to develop new cryoprotection procedures which are simple and can be handled easily.

MATERIALS AND METHODS

This research was carried on Central Laboratory of Date Palm Research and Development, Agriculture Research Center, Cairo, Egypt.

During the period of (2004-2007).

Plant material and explant preparation:

Selected of healthy young offshoots 5-7 kg in weight which were carefully separated from adult date palm *Phoenix dactylifera* L. (cv. Zaghlool) grown in the field of ministry of Agriculture at Rashid, Albehaera, Egypt.

The selected young offshoots were carefully brought to the laboratory immediately. At first, the adventitious roots, older leaves, outer most sheath of leaves and fibers were carefully removed with hock saw or serrated knife. All leaves are peeled away from the shoot terminal portion. Shoot tip containing the meristem surrounded by its leaf primordial washed in running tap water for one hour. The explants were soaked in cold antioxidant solution (0.15 g/l ascorbic acid + 0.10 g/l citric acid) for 30 min to avoid culture browning. The sterilization steps for shoot tip surface were carried out under aseptic conditions the explants were treated with 70% ethanol for 30 min, followed by immersion in (0.5 g/l) mercuric chloride ($HgCl_2$) for 5 min and then washed with sterilized distilled water for one time. After that, additional leaf primordial were removed from sterilized explant. The explants were then exposed to double surface sterilization by commercial (Clorox, 5.25% w/v, Sodium hypoclorite NaOCl). The first one by 30% Clorox for 15 min followed by thoroughly washing with sterilized water for one time. The second one by 50% Clorox for 20 min and then washing the explants with sterilized distilled water for three times. All surface sterilized solutions contained one drop of Tween 20 per 100 ml solution. Final, some surrounding leaf primordial were carefully removed.

Establishment of shoot tip explants:-

Sterilized shoot tip explants containing apical meristem, surrounded with about 4-6 leaf primordia were excised and were used as an initial explant material. Each shoot tip sliced longitudinally into 3 equal parts. Sterilized shoot tip segments were established on starting medium which consists of basal nutrient medium formula of (Murashing and Skoog 1962) supplemented with 100 mg/L myo-inositol + 0.4 mg/L thiamine HCl + 3 g/l activated charcool + 30 g/L sucrose, medium were solidified with 5 g/L agar. Culture medium was distributed in culture tubes 25 × 150 mm (15 ml of the medium per tube). The culture tubes were immediately capped with polypropylene closure autoclaved at 121C° at 15/bs/inch^2 for 20 min.

One shoot tip segment was cultured in each tube. The cultured were incubated on this hormone free medium for 10 days under complete dark at 27±1C° to be sure that explants were free of any fungial or bacterial contamination. Each

treatment = 3 replicate and each replicate = 3 culture tubes and each tube contained one explant.

All uncontaminated explants which derived from starting medium were divided into four groups and each group was exposed to one of the four different pretreatment which were used as a cryoprotection procedures prior to immersion in liquid nitrogen container. The work aimed to examine the potential of these pretreatments to increase the tolerance of shoot tip explants for cryopreservation process and to enhance the cryopreseved shoot tip explants to resume their development on recovery medium.

The following four pretreatments were investigated prior to the immersion of shoot tip explants in liquid nitrogen.

Pretreament (1): The effect of different sucrose concentrations in preculturing media and incubation temperature at 5°C or 27°C.

In this pretreatment, the first uncontaminated group of shoot tip explants were trancplanted from starting medium to preculturing medium which consisted of the same composition of starting medium with the addition of 10 mg/L 2, 4-D (dichlorophenoxy acetic acid), 3 mg/L 2ip (2-isopentenyl adenine) combined with different sucrose concentrations (0.1, 0.3, 0.5 and 0.7 M). Alcohol sugar (mannitol) was added in constant concentration at (0.3 M) to induce extra osmotic cryopotection. The culture tubes were divided into two groups, the first group was incubated under 5°C for cold hardening and the second group was incubated under 27°C. All culture tubes were incubated at complete dark for four weeks.

Pretreatment (2): The effect of different sucrose concentrations in preculturing media and incubation temperature at 27°C followed by desiccation procedure.

In this pretreatment the second uncontaminated group of shoot tip explants were removed from starting medium and recultured on preculturing medium which consists of the same composition of starting medium with the addition of 10 mg/L 2, 4-D, 3 mg/L 2ip combined with different sucrose concentrations (0.1, 0.3, 0.5 and 0.7M). Alcohol sugar (mannitol) was added in constant concentration at (0.3M) to induce extra osmotic cryoprotection.

The culture tubes were incubated at 27°C under complete dark. for four weeks. At the end of incubation period, each explant was removed from the preculture medium and placed in an open sterilize petridish and subjected to continual of stirel air in the laminar airflow cabinet for 2 hours to desiccate the explants before placing them in cryotubes for cryopreservation process in liquid nitrogen.

Pretreatment (3): The effect of different abscisic acid (ABA) concentrations in preculturing media and incubation temperature at 5°Cor 27°C.

In this pretreatment, the third uncontaminated prepared group of shoot tip explants were removed from starting medium and recultured on preculturing medium which consists of the same composition of starting medium with the addition of 10 mg/L2, 4-D, 3 mg/L 2ip combined with different ABA concentrations (0.0, 2.0, 4.0, 6.0 and 8 mg/L).

The culture tubes were divided into two groups. The first group was incubated under 5°C for cold hardening and the second group was incubated under 27°C. All culture tubes were incubated at complete dark for four weeks.

Pretreatment (4): the effect of different abscisic acid (ABA) concentrations in preculturing media and incubation temperature at 27°C followed by desiccation procedure.

In this pretreatment the fourth uncontaminated group of shoot tip explants were removed from starting medium and reculture on preculturing medium which consists of the same composition of starting medium with the addition of 10 mg/L2, 4-D, 3 mg/L 2ip combined with different ABA concentration (2.0, 4.0, 6.0 and 8 mg/L).

The culture tubes were incubated at 27°C under complete dark, for four weeks. At the end of incubation period, each explant was removed from the preculture medium and placed in an open sterilized petridish and subjected to continual current of stirel air in the laminar airflow cabinet for 2 hours to desiccate the explants before placing them it in cryotubes for cryopreservation process in liquid nitrogen.

Cryopreservation process:

At the end of incubation period of all the previous cryoprotection pretreatments the shoot tip explants were ready to place in cryotubes for cropreservation process. Individual explant was placed in 1.8 ml cryotube, then plunged into liquid nitrogen container for one hour. After frozen storage, cryotubes with explants were exposed to water bath at 40°C for rapidly thawing.

After crypreservation process cryopreserved shoot tip explants from each pretreatment were recultured on recovery medium which consists of the same composition of starting medium with the addition of 10 mg/L2, 4-D, 3 mg/L 2ip. The culture tubes were incubated for three weeks at 27°C ± 2 under complete dark to evaluate the survival percentage.

All survived cryopreserved explants cultured on recovery medium for three weeks were continuously reculturing on the same recovery medium for four months and incubated at 27°C under complete dark to determine the effects of each croyprotection

pretreatment prior cryopreservation on keeping the survival of explants and the ability of the explants to resume their normal development cycle.

RESULT AND DISCUSSION

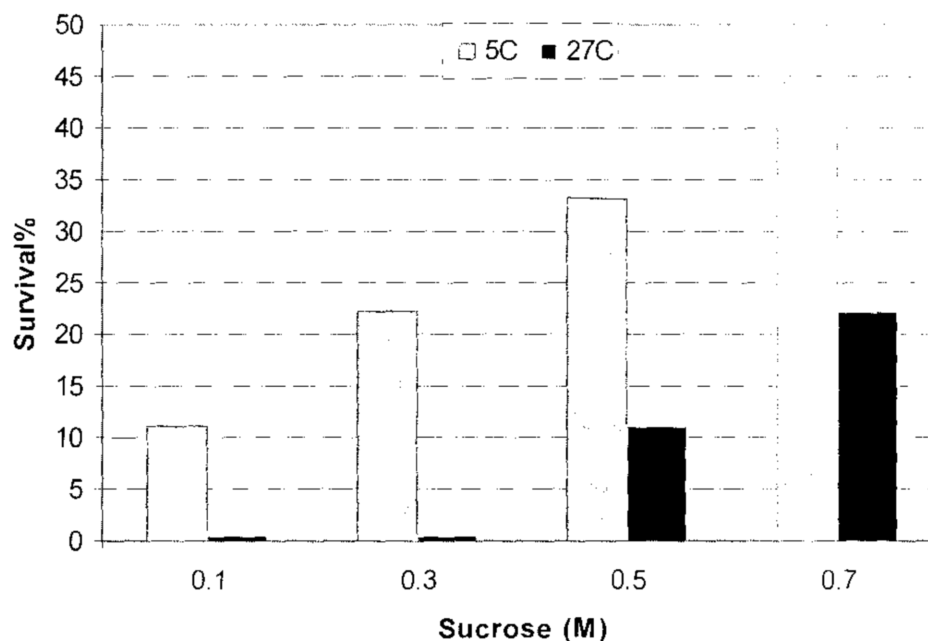
Pretreatment (1): The effect of different sucrose concentrations in preculturing media and incubation temperature at 5°C or 27°C on the survival percentage of cryopreserved shoot tip explants of date palm (cv. Zaghlool) cultured on recovery medium for three weeks.

Data in Table and Fig (1) showed the survival percentages of cryopreserved shoot tip explants cultured on recovery medium for three weeks after cryoprervation. The survival percentage of cryopreserved shoot tip explants was affected significantly by different sucrose concentrations added to the preculturing media and incubated under 5°C or 27°C for four weeks as pretreatment before cryeopreservation treatment. Shoot tip explants cultured on preculturing medium supplemented with 0.7 M of sucrose for 4 weeks before cryopreservation gave the highest significant mean value of survival percentage (33.33%) of cryopreserved shoot tip explants cultured on recovery medium for three weeks after cryopreservation. Data also showed that the reduction in sucrose concentrations in preculturing medium before cryopreservation was paralled with the reduction in the survival percentage for cryopreserved explants on recovery medium after cryopreservation.

Panis *et al.,* (1996) mentioned that sucrose has an important role as a freeze – hardening factor or cryoprotective substance for cryopreservation of banana meristems and the indirect effect of sucrose could be the accumulation of endogenous compounds induced by a mild osmotic stress, that then offer protection against further water stress and cryopreservation.

Table and Fig (1):The effect of different sucrose concentrations in preculturing media and incubation temperature at 5°C or 27°C on the survival percentage of cryopreserved shoot tip explants of date palm (cv. Zaghlool) cultured on recovery medium for three weeks

Sucrose M (A)	Incubation temperature (B)		Mean (A)
	5°C	27°C	
0.1	11.11	00.00	05.55 d
0.3	22.22	00.00	11.11 c
0.5	33.33	11.11	22.22 b
0.7	44.44	22.22	33.33 a
Mean (B)	22.22 a	08.33 b	
Mean separation by L.S.D at 0.05			
	A = 0.13	B = 0.09	AB = 0.18



Sucrose reduces moisture content slowly due to osmotic action (Uragami, 1991). As a result of its uptake, it decreases the freezing point and the amount of freezable water present in tissues. A considerable amount of sucrose uptake and its subsequent dissociation into glucose and fructose during preculture in the presence of high sucrose level was demonstrated using axillary buds of asparagus (Uragami *et al.*, 1990).

Sucrose cryopretection reduces the amount of freezable water in tissues by osmotic effect (Panis and, Thinh 2001) while the OH groups of sucrose replace water interact with membrane phospholipids (Turner *et al.,* 2001). Consequently, sucrose stabilizes cellular membranes and proteins during dehydration and freezing, thus maintaining the liquid crystalline state of the membrane bilayers (Pains *et al.,* 1996 and Thomashow, 1999). Addition effects of sucrose cryoprotection are an increase in endogenous sugar level in cytosol and the accumulation of specific hydrophilic proteins such as dehydrins, which are also known to stabilize the cell membrane (Jitsuyama *et al.,* 2002).

Our results is disagree with (Panis *et al., 1996)* who found that increasing sucrose level in preculturing medium for banana meristem at 0.6 M or 0.7 M for 2-4 weeks at 25°C before direct plunging in liquid nitrogen lowered survival rate and was concomitant white blackening of tissue on regrowth medium. The best results were obtained when banana meristem precultured on medium with sucrose at 0.3 – 0.5 for 2-4 weeks at 25° before direct plunging in liquid nitrogen which exhibited high recovery occurred on regrowth media after cryopreservation.

Regarding to the effect of incubation temperature 5°C or 27° C on shoot tip explants cultured on preculturing medium for 4 weeks before cryopreservation, data in Table (1) showed that survival percentages of crypreserved explants cultured on recovery medium was significantly higher in the cryopreserved shoot tip explants derived from preculturing medium that incubated at 5°C for 4 weeks before cryopreservation compared with the survival percentage of those incubated at 27°C (22.22% and 8.33% respectively)

Cold acclimation is described as prior exposure of plants to cold, non freezing temperature that increases their tolerance to subsequent freezing (Kalengamaliro, *et al.,* 2000). Also when plants are subjected to low temperature compounds can accumulate and changes in membrane structures occur, that enable constituent cells to with stand subsequent freeze injury. Plants vary considerably in their ability to frost injury, so that it is not surprising that the tolerance of cells or tissues to low temperature storage is also variable. In many species conditioning plants by keeping them in the cold (1-4° C) for several days is found to increase the probability of success cryopreservation of shoot tip apical meristem (George, 1993).

Data revealed clearly that the survival percentages on recovery medium for cryoprserved explants precultured on preculturing medium supplemented with different sucrose concentrations (0.1, 0.3, 0.5 and 0.7 M) and incubated under 5°C for 4 weeks before plunging into liquid nitrogen were significantly higher compared with the survival percentages of those incubated under 27°C.

Niino *et al.,* (1997) succeeded in cryopreserved *in vitro* grown shoot tip of cherry by subculturing at 5 °C cold hardener for 45 days then preculture on Murashige and Skoog medium supplemented with 0.7 M sucrose for one day at 5°C using one step vitrification before plunging in liquid nitrogen.

In this pretreatment, all survived cryopreserved explants for three weeks on recovery medium exhibited vitrification appearance and couldn't resume their normal development by giving callus initiation after recultured on recovery medium for four months later.

Pretreatment (2): The effect of different sucrose concentrations in preculturing media and incubation temperature at 27°C for four weeks followed by desiccation procedure on the survival percentage of cryopreserved shoot tip explants of date palm (cv. Zaghlool) cultured on recovery medium for three weeks.

Data in Table and Fig (2) showed that the highest significant difference of survival percentages (66.66% and 55.55%) of cryopreserved shoot tip explants cultured on recovery medium after cryopreservation in liquid nitrogen was achieved when shoot tip explants were cultured on preculturing medium supplemented with sucrose at 0.5 M and 0.7 M respectively and incubated at 27°C for four weeks followed by desiccation treatment in laminar air flow for 2 hours.

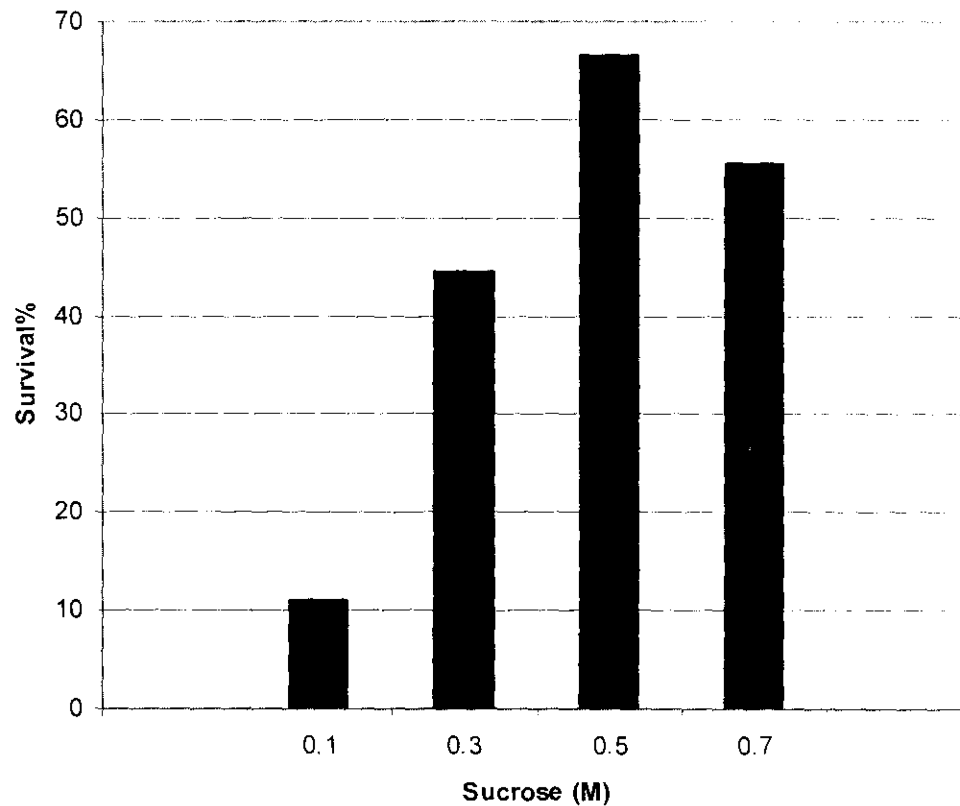
In this pretreatment the cryopreserved shoot tip explants were revealed good potential for resuming their growth when recultured for four months on recovery medium and they could produce callus initiation in the later period similar to non cryopreserved shoot tip explants.

Sufficient dehydration for explants prior to the cryopreservation process gave the most satisfied results that for most plant cells and organ cultures, injury during freezing is associated with intracellular ice formation in highly vacuolated cells, there for survival of cryopreserved shoot tips could be increased by reducing water content of the cells (Wang, *et al.,* 2000).

Sucrose cryoprotection as well as air desiccation are both simplified vitrification – based method used to avoid the crystallization of tissue water during freezing (Wolfe and Bryant 2001, Zhang, *et al.,* 2001) as a high sucrose concentration and dehydration cause cytosol water to vitrify during freezing (Wolfe and Bryant 2001).

Table and Fig (2) The effect of different sucrose concentrations in preculturing media and incubation temperature at 27°C for four weeks followed by desiccation procedure on the survival percentage of cryopreserved shoot tip explants of date palm (cv. Zaghlool) cultured on recovery medium for three weeks.

Sucrose M	Survival%
0.1	11.11 **d**
0.3	44.44 **c**
0.5	66.66 **a**
0.7	55.55 **b**
Mean separation by L.S.D at 0.05 = 1.04	



Enhancing the acquisition of freezing tolerance by sucrose treatment in embryonic tissues which coupled with air desiccation results in a high post-thawing embryogenic competence this conclusion is in agreement with our results (Danso and Ford-Lioyd, 2004).

Air – dry desiccation method is easier to apply than the more complicated cryoprotectant method (JekkeL *et a.,* /1998).

Pretreatment (3): The effect of different abscisic acid (ABA) concentrations in preculturing media and incubation temperature at 5°C or 27°C. on The survival percentage of cryopreserved shoot tip explants of date palm (cv. Zaghlool) cultured on recovery medium for three weeks.

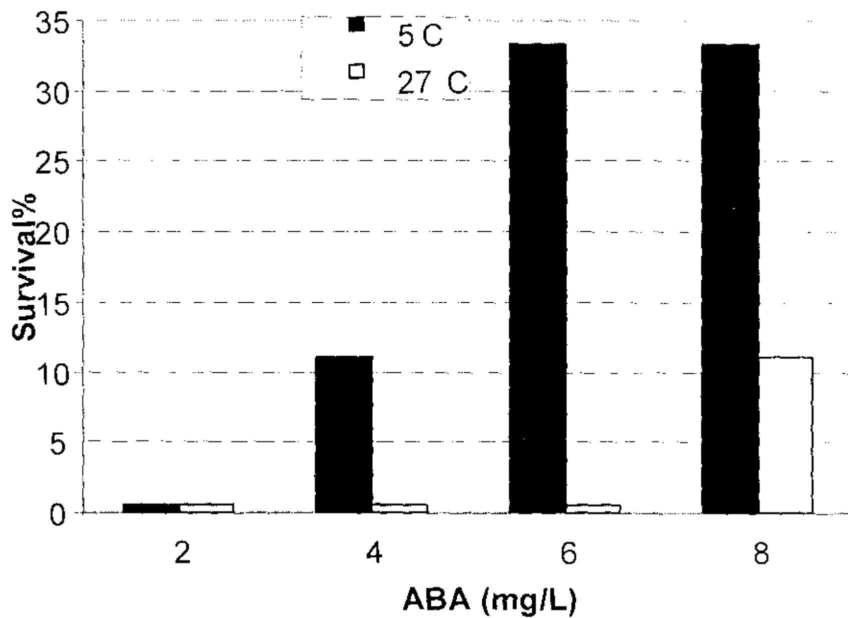
Data in Table and Fig (3) showed that survival percentages of cryopreseved shoot tip explants cultured for three weeks on recovery medium were affected significantly by different ABA concentration added to preculturing medium and incubated under 5°C or 27°C for four weeks as pretreatment before cryopreservation. Shoot tip explants precultured on preculturing medium supplemented with 8 mg/L of ABA before cryopreservation recorded the highest significant mean value of survival percentage (22.22%) of cryopreserved. Shoot tip explants precultured on preculturing medium supplemented with 2 mg/L of ABA for four weeks before cryopreservation process failed completely to survive when cultured on recovery medium for three weeks.

The effect of incubation temperature at 5°C or 27°C of shoot tip explants cultured on preculturing media for 4 weeks before cryopreservation exhibited a significant differences.

Shoot tip explants cultured on preculturing medium and incubated at 5°C for 4 weeks before cryopreservation showed the highest survival percentages (19.44%) on recovery medium compared with those incubated at 27°C which gave the lowest significant value of survival percentage on recovery medium (2.77%).

Table and Fig (3)The effect of different abscisic acid (ABA) concentrations in preculturing media and incubation temperature at 5°C or 27°C on the survival percentage of cryopreserved shoot tip explants of date palm (cv. Zaghlool) cultured on recovery medium for three weeks.

ABA mg/l (A)	Incubation temperature (B)		Mean (A)
	5°C	27°C	
2.0	00.00	00.00	00.00 **d**
4.0	11.11	00.00	05.55 **c**
6.0	33.33	00.00	16.66 **b**
8.0	33.33	11.11	22.22 **a**
Mean (B)	19.44 **a**	02.77 **b**	
Mean separation by L.S.D at 0.05			
	A = 0.13	B = 0.09	AB = 0.18



Shoot tip explants precultured on preculturing medium supplemented with 6 mg/L or 8 mg/L ABA and incubated under 5 °C for 4 weeks before immersion in liquid nitrogen had the same value of survival percentages (33.33%).

Shoot tip explants precultured on preculturing medium supplemented with 2 mg/L ABA and incubated under 5 °C or 27 °C and also Shoot tip explants precultured on preculturing medium supplemented with 4 mg/L or 6 mg/L ABA and incubated under 27°C failed completely to show any survival when cultured on recovery medium for three weeks after cryopreservation.

In this pretreatment all survived cryopreserved explants on recovery medium for 3 weeks after cryopreservation process failed to resume their growth when recultured for four months later and they turned to blacken appearance.

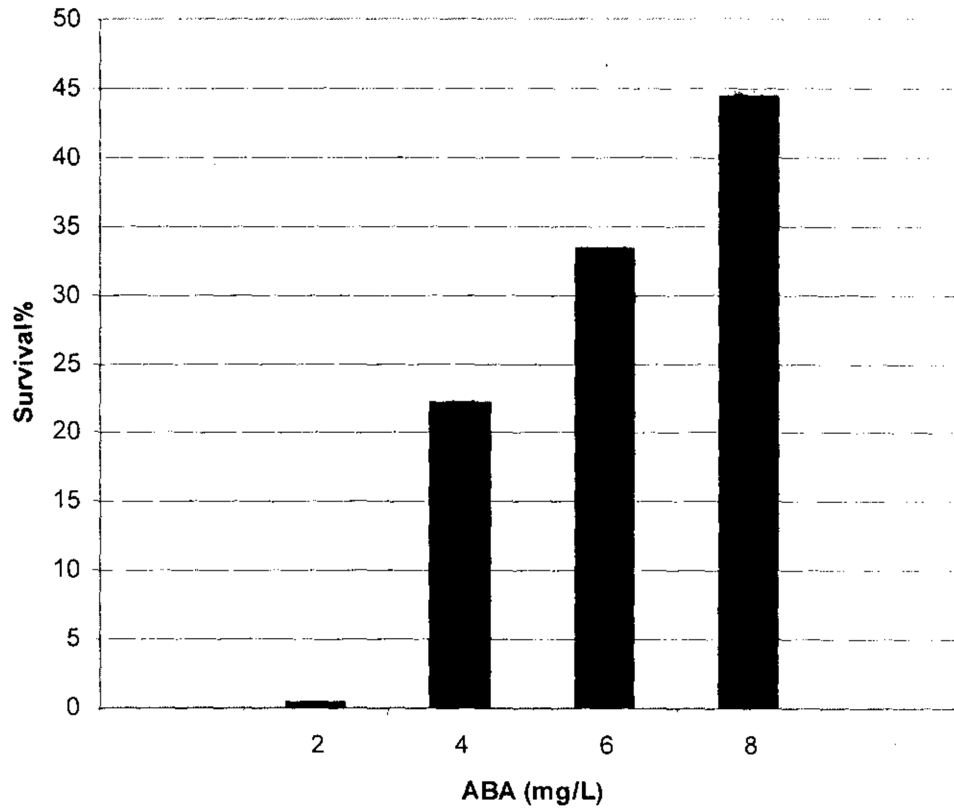
In this pretreatment, ABA didn't gave a sufficient dehydration for explants to be capable of vitrifing before immersion into liquid nitrogen this suggestion agreed with (Jekkel, *et al.*, 1998) who found that ABA had no effect on the reduction in water content but it increased the tolerance for desiccation.

Pretreatment(4): The effect of different abscisic acid (ABA) concentrations in preculturing media and incubation temperature at 27°C followed by desiccation procedure on the survival percentage of cryopreserved shoot tip explants of date palm (cv. Zaghlool) cultured on recovery medium for three weeks.

Data in Table and Fig (4) showed that the pretreatment for cultured shoot tip explants on preculturing media with different ABA concentrations and incubated at 27°C for four weeks followed by a desiccation procedure for these explants in laminar air flow for 2 hours before plunging them in liquid nitrogen had a significant effect on the survival percentage of cryopreserved shoot tip explants cultured on the recovery medium.

Table and Fig (4) the effect of different abscisic acid (ABA) concentrations in preculturing media and incubation temperature at 27°C followed by desiccation procedure on the survival percentage of cryopreserved shoot tip explants of date palm (cv. Zaghlool) cultured on recovery medium for three weeks.

ABA mg/L	Survival%
2.0	0.000 **d**
4.0	22.22 **c**
6.0	33.33 **b**
8.0	44.44 **a**
Mean separation by L.S.D at 0.05 = 0.297	



The highest significant mean value of survival percentage (44.44%) was observed when shoot tip explants precultured on preculturing medium supplemented with 8 mg/L of ABA while shoot tip explants precultured on preculturing medium supplemented with 2 mg/L of ABA lost their survival on recovery medium after cryopreservation.

In this pretreatment, all cryopreserved explants survived on recovery medium could´t resume their normal growth and turned black after recultured for four months later on recovery medium.

Blackness is normal reaction of living tissues to stress as mentioned by (Panis, *et al.,* 1996) and may be that due to the retarding effect of ABA as growth retardants. On other hand, our result are in disagreement with those found by (Jekkel, *et al.*, 1998) who successeded in cryopreservation of somatic embryos of *Aseculus hippocastumen* cultured on nutritive media containing ABA at 0.75 µM then subjected to 4 hours period of air desiccation.

Date palm meristems didn't withstand freezing in LN as intact structures. It seems that the more meristematic the cells were, the more they resisted. During the steps which precedes freezing, gradient may be established both for out flow of water and penetration of cryoprotectants. In the case of date palm as for as of other plants, the central cells as well as the ones located at the base of the explant are more vacuolated and their dehydration may be more difficult or they may be less tolerant to the necessary level of dehydration. This may explain their incapability with- stand freezing. Superficial cells which are more meristematic are more likely to reversibly dehydrate since they have no or only a few vacuoles. (Engelmann *et al.*, 1995).

Water contents in dehydrated explant is an important factor in successful cryopreservation. Above a certain level (which presumably varies among species and even within a species depending on physiological status) there will be too much intracellular water and consequently lethal ice crystals damage would occur during freezing or thawing. Conversely there is a lower limit below which dehydration damage would occur (Mycock *et al.*, 1995).

So improvement of cryopreservation protocols can be achieved when the causes of freeze damage in plant cell are understood in relation to the cultural condition and growth factors of the cultures. Also the pretreatment of cells with cryoprotectants was necessary for obtaining good regrowth (Winkelmann *et al.*, 2004).

By this work we suggest that further optimization may improve the results of more successful date palm cryopreservation.

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الحفظ بالتجميد للمنفصلات النباتية المجهزة للقمم النامية لنخيل البلح (صنف الزغلول)

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باستخدام المنفصلات النباتية للقمم النامية بزراعة الأنسجة قد تم دراسة بعض معاملات الحماية من التجميد للمنفصل النباتي لنبات نخيل البلح صنف الزغلول قبل إجراء عملية الحفظ بالتجميد في جهاز النيتروجين السائل وذلك بهدف الحصول علي أبسط معاملة للحماية من التجميد يمكن إجرائها بسهولة و يسر.

ولقد تناولت أول معاملة للحماية من التجميد دراسة تأثير تركيزات مختلفة من السكروز (٠,١، ٠,٣، ٠,٥، ٠,٧ مول / لتر في بيئة الزراعة الاولية وذلك تحت درجتين حرارة ٥ أو ٢٧ مئوى للتحضين لمدة أربع أسابيع.

أما ثاني معاملة للحماية من التجميد فلقد تم دراسة تأثير نفس تركيزات السكروز السابقة في بيئة الزراعة الأولية تحت درجة حرارة تحضين ٢٧مئوى لمدة أربع أسابيع مع استخدام معاملة التجفيف الهوائي لمدة ساعتين بداخل جهاز الزراعة

وفي المعاملة الثالثة للحماية من التجميد فقد تناولت دراسة تأثير تركيزات مختلفة من حمض الأبسيسيك أسيد (٢,٠، ٤,٠، ٦,٠، ٨,٠ ملجم/لتر) والمزودة في بيئة الزراعة الاولية تحت درجتين حرارة ٥ أو ٢٧ مئوى للتحضين لمدة أربع أسابيع.

أما المعاملة الرابعة للحماية من التجميد فلقد تم دراسة تأثير نفس تركيزات حمض الأبسيسك أسيد السابقة والمزودة في بيئة الزراعة الاولية تحت درجة حرارة تحضين ٢٧ مئوية لمدة أربع أسابيع مع إستخدام معاملة التجفيف الهوائي لمدة ساعتين بداخل جهاز الزراعة.

يتم إجراء عملية الحفظ بالتجميد لجميع المنفصلات النباتية الناتجة من المعاملات الأولية للحماية وذلك بغمسها لمدة ساعة في جهاز النيتروجين السائل عند درجة -١٩٦ مئوية وبعد أن تتم عملية الإذابة في حمام مائي عند درجة ٤٠ مئوية لجميع المنفصلات النباتية التي تم تجميدها تنقل جميع المنفصلات النباتية لزراعتها على بيئة (استعادة النمو) لاختبار قدرتها على البقاء حية وذلك لمدة ٣ أسابيع بعد إجراء عملية الحفظ بالتجميد.

ولقد وجد أن جميع المنفصلات النباتية للقمم النامية والتي سبق معاملتها أولياً للحماية ثم حفظت بالتجميد قد أظهرت نتائج ضعيفة للقدرة على البقاء حية فيما عدا تلك المنفصلات النباتية للقمم النامية التي سبق معاملتها أولياً للحماية وذلك بزراعتها لمدة ٤ أسابيع على بيئة الزراعة الأولية التي تحتوي علي ٠,٥ مول/ لتر سكروز وحضنت على درجة ٢٧ مئوية ثم مع تعريض تلك المنفصلات لمعاملة التجفيف الهوائي بداخل جهاز الزراعة لمدة ساعتين وذلك قبل الغمس في جهاز النيتروجين السائل للتجميد فلقد وجد أن هذه المنفصلات النباتية للقمم النامية استطاعت أن تظهر قدرة عالية على البقاء حية بعد إجراء عملية التجميد لها بل وأن تستكمل دورة حياتها بشكل طبيعي.

وفي النهاية يمكننا القول أن الحفظ بالتجميد للأصول الوراثية لنخيل البلح تحتاج لمزيد من الدراسة للحصول على نتائج إيجابية مرجوة.