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Research Paper

Towards development of new technique for cryopreservation of date palm (*Phoenix dactylifera* L.) *in vitro* grown cultures

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ABSTRACT

The method of cryopreservation was described as very simple with the potential application of a wide range of date palms. The undifferentiated tissue cultures have been successfully frozen by later freezing methods of renewed seedlings. Shoot tips of date palm were cultured on MS-medium containing 10 mg / l 2,4-D + 3mg / l 2iP. The potential of dehydration caused by air drying to cryopreservation of date palm tissue cultures through direct immersion in liquid nitrogen was subjected. The embryogenic callus of about 65% of the water content caused by 20 min of air drying period recorded the highest percentage of survival and *in vitro* conversion to plants. Among the different types of sugars (sucrose) used as pre-treatment agents in the pre-plant average, sucrose was the best for the persistence of cryogenic palm tree cultures. The highest survival rate (80%) and switch to plants (75%) were observed with 1 M sucrose. Cryopreservation of date palm embryogenic callus through encapsulation is a new method we tried to introduce to our laboratory and spread for others also. Date palm (*Phoenix dactylifera* L) tree is a member of the family Palmace (Arecaceae) and inhabit tropical and sub-tropical habitat. The Arecaceae is a family of woody perennial monocots and consists of about 200 genera and 2500 species. The entire tree of date palm is utilized to provide food, fiber, fuel and furniture. Date palm trees are tolerance to environmental stresses ranging from salinity to drought and high temperature, in addition to low maintenance and yield harvesting costs. The encapsulation is a simple process that can be done inserting the propagule singly into an alginate drop and then falling it into a complexing solution (20 to 35 min) to form calcium alginate capsules that are approximately 5 mm in diameter. The presence of nutritive elements into the capsule (artificial endosperm) has a function similar to endosperm of zygotic seeds. RAPD was extensively used to study the genetic stability of cryogenic tissue cultures from date palm. According to RAPD analysis, the seedlings derived from cryopreserved cultures were similar to those derived from untreated cultures, both of which were similar to the cultivated plants in the field. Finally, whole plants of chilled cultures were able to adapt to free living conditions after a stage of adaptation.

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Key words: Embryogenesis, Khalas, Date palm, cryopreservation, vitrification, RAPD analysis.

INTRODUCTION

Date palm (*Phoenix dactylifera* L.) is a member of the family *Palmace* (*Arecaceae*), inhabiting tropical and sub-tropical habitat. It consist of woody perennial monocots comprising about 200 genera and 2500 species (Jain et al., 2011). Date palm is native to almost all the Middle east countries concentrating in Arabic Peninsula and economically the most conspicous commercial crops with average number of trees estimated >62.0 million (Alkhateeb, 2006; Jain et al.,

2011). With the consumption of the new millennium and what will be accompanied by a new technology and with growing concern of over population growth and environmental threats, the world is beginning to be aware of the state of plant genetic resources, especially in relation to food, pastures and forests, and the need for immediate and urgent regulatory and awareness action. With the awareness of the plant kingdom (as it represents the life support system), the number of people who wonder about threats to plant sources and the consequent decline in plant sources and what to do to protect plant habitats and how individuals, association members and decision-making organizations contribute to plant conservation.

The Arab homeland possesses many plant and plant genotypes, as well as, wild herbs that have a global medical importance. Therefore, these assets must be protected and registered and preserved from extinction or looting by other organizations. The industrial countries and some international organizations have legitimately and illegally collected these resources from poor countries and saved them for future exploitation (Alkhateeb, 2006; Bekheet, 2001, 2002, 2006, 2007; Yamamoto et al., 2015).

Preservation is in the natural place *in-situ* conservation. This method is intended to conserve species and species along with their wild origins and show the genetic variations of the crosses between the modern breeds and the natural plant assets in the region. In this type of conservation methods, some breeds may be lost due to the environmental conditions in addition to the high cost of maintaining these breeds in the ground and to serve them. It is considered one of the best strategies in place for the enactment of administrative laws and regulations, including the identification of the target area in which species are to be preserved (Bekheet et al., 2007; Toshikazu 2018).

Therefore, date palm tree is an excellent candidate for cultivation in arid regions of the world. Date palms is concentrated in Arabic world and considered one of the most important commercial crops in the Arab world (FAO, 1984). In the Arab countries, the average number of date palm was estimated at 62,000,000 trees.

The technology of the synthetic seed introduces some peculiarities that can bring remarkable advantages in the propagation of the plants, but, at the moment, there are also some problems that constitute the limit for the practical application, demanding the search for effective solutions which we will try to do in this project in KSA with the date palm. Long term conservation of clonal crops remains costly as it generally involves maintaining collections either in field- and/or in *in vitro*- genebank. Plant tissues cryopreservation, that is, conservation at very low temperature can drastically reduce clonal conservation cost. The present projects will explore the possibility of cryopreserving date palm. The temperature at which the nuclei reach the required thermodynamic properties (critical size and energy) to become an ice crystal is higher than that seen in homogeneous nucleation (Reid, 1986). After the traditional cryogenic protocol, the samples are cooled slowly in a cryoprotective solution, which is usually assisted by a programmable freezer. There is a direct relationship between the temperature at which heterogeneous nuclei should cause seeding and concentration of protoportal solution used. The sowing process is usually done at 2 to 3°C below the freezing point of the freezing solution. The cooling device is kept at this temperature for a few minutes to balance the temperature gradient, or the temperature may drop continuously after the slow-freezing rate.

After sowing, ice occurs in the extracellular solution. This external crystallization enhances the flow of water from cytoplasm and vesicles into outer space, which eventually freezes (Farrant, 1980; Taylor, 1987). Water is left in the cells of the protoplast to restore the osmotic equilibrium resulting from the ice transition. When the outer solution becomes more concentrated the cell is overloaded (Panis, 1995, 2002) according to the cooling rate and the slow-cooled cooling unit before the contents inside the cells freeze when the samples are immersed in liquid nitrogen (Pitt, 1992). However, collective freezing is often insufficient to remove enough water from complex biological systems such as embryos and access advice, and it is difficult to eliminate the relative amount of intracellular water from these structures. In addition, it is difficult to control the drought during the protocol and this depends on the freezing. The cooling system, which is often used to ensure an appropriate level of freezing for the successful freezing of different plant cells, involves lowering the temperature at 0.5 to 1°C and -1 to -40°C, followed by rapid immersion in liquid nitrogen (PANIS, 2005). According to Toner (1993), the cooling rate, which reduces cell survival to 50%, corresponds to the cooling rate at which 50% of cells are detected to form intracellular ice.

Due to the flow of water, the cellular solution is frozen at a temperature lower than the freezing point, or is sufficiently concentrated to form amorphous glass (Engelmann, 2000, 2003, 2004). Therefore, depending on the amount of water inside the remaining cells at the moment of immersion of the samples in liquid nitrogen, the following physical events can occur: large lethal crystals form if the water content is high and small and harmless crystals if the water content is not high enough, or amorphous glass is formed if the cellular viscosity is so high that any residual water inside the cells becomes glazed (Benson et al., 2006). Unstable glasses are obtained during rapid submersion in liquid nitrogen from slightly concentrated samples; such glasses are unstable because nitrogen removal can occur when re-heating and return to a liquid or crystalline state (Benson et al., 2006). To avoid instability in non-crystalline solids produced, melting must be performed quickly by placing the cryovials in a water bath at + 40°C for a few minutes. If warming is carried out quickly, there is not enough time for the nitrogen removal

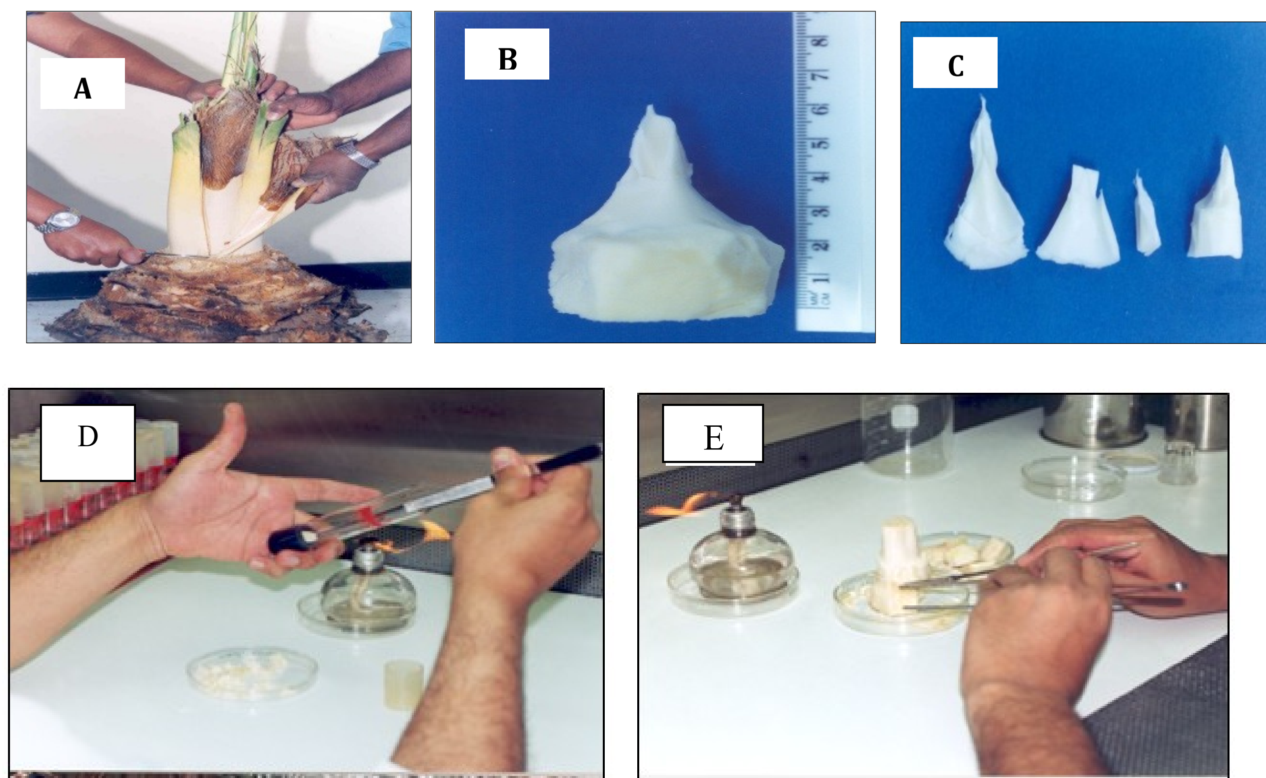

Figure 1: Preparation of Date palm tissue culture material (somatic embryo, shoot tips and other explant tissues) from offshoot, Khalass, Berhi and Sukary cultivar.

process to occur, and recovery is likely to occur after the end of the disease (Mazur, 2004). Based on the DSC and microscopic studies (Rall et al., 1984; Rall and Polge, 1984), temperature rise was reported to be much more damaging than rapid warming, especially in the case of a slow freezing system, where degrees of the final heat of the freeze is about 30 to -40°C before immersion in liquid nitrogen (Mazur, 2004; Yamamoto et al., 2015).

MATERIALS AND METHODS

Preparation of plant material

The study was conducted on plant date palm cultivars Khalass, Berhi and Sukary. The terminal shoot tips and surrounding leaf primordia excised from offshoots of date palm cultivar 'Khalas' were carefully and immediately put into antioxidant solution containing 150 g/L ascorbic acid and 100 g/L citric acid (Figure 1). The shoot tip were sterilized twice using Benlate fungicide for 20 min followed by multiple washes with sterile water. Thereafter, the shoot tips were placed in 20% chlorox solution with few drops of Tween-20 for at least 20 min. Shoot tips were then washed thrice with sterile water and kept in the antioxidant solution until explants were excised.

Preparation of plant material

Leaves of the mentioned date palm cultivar offshoots was carefully removed to expose the terminal shoot tip. The shoot tip was immediately put into antioxidant solution containing 150 g/L ascorbic acid, 100 g/L and citric acid. The shoot tip was sterilized twice by placing it in Benlate fungicide for 20 min and cleaned several times with sterile water and thereafter, transfered to a solution containing 20% chlorox with few drops of Tween for 20 min. Shoot tip tissues was then washed thrice with sterile water and kept in a sterile solution containing 150 g/L ascorbic acid and 100 g/L citric acid until explants are excised under sterilize conditions. Terminal and lateral buds were removed from the shoot tip tissue section and placed on culture medium.

Culture media and multiplication stages

The explants were subsequently transferred to MS-media for culture initiation (100 mg/L 2,4-D and 2iP 3 mg/L), culture swelling (10 mg/L NAA and 6 mg/L 2iP) and formation of embryogenic calli (10 mg/L NAA and 30 mg/L 2iP) for 9, 16 and 3 weeks, respectively. All the incubations were carried out at 25±2°C in a 16 h photoperiod provided from cool white florescent lamps.

Experimental treatments and design

The resultant cultures were placed onto agar-solidfied phytohormone-free MS medium containing 0.7 M each of DMSO and sucrose. The calli were washed with liquid MS medium and then transferred to hormone free medium supplemented with 30 g/L sucrose for the production of embryos and plantlets. Each treatment was replicated 10 times. Cultures were incubated at 25±2°C in 16 h of day light supplied by 65/80 Warm White Weisse 3500 fluorescent tubes. Data was recorded for total number of somatic embryos, total number of germinating embryos and fresh and dry weights of the cultures after 16 weeks of culturing.

Freezing procedures and recovery

The crytubes (which contained date palm cultures) was kept at 0 <C for 2 h and then plunged directly into liquid nitrogen (<-196°C) for 48 h.

Freezing

1) Transfer the date palm calli aseptically and place in a 10 × 35 mm disposable Petri dish containing 2 ml of cryo-date medium;
2) Transfer the calli of date palm into a cryotube (Nunc 1.8 cc) containing 200 ml of cryoprotectant solution (CPA) [1.5 M dimethylsulphoxide (DMSO, Sigma) in M2 medium supplemented with 10% fetal bovine serum and 25% glycerol)];
3) Keep the sample in the CPA at 20°C (room temperature) for 10 min and then place the cryotubes on ice for approximately 45 min;
4) After the 45 min equilibration, place the cryotubes in an ice bath at - 5°C for 10 min;
5) Add the medium inside the cryotube with a Pasteur pipette containing a small amount of frozen CPA in the tip that has been pre-cooled to -20°C. After date palm calli place the cryotubes in a controlled rate freezer pre-equilibrated at -5°C;
6) Start the controller to cool at a rate of - 1.0°C per minute to -80°C. At that point the date palm cryotubes can be transferred to liquid nitrogen storage for long or short periods. The methods employed vary according to the storage duration request (Bekheet et al., 2002, 2006, 2007; Abdulaziz and Jameel, 2012).

Thawing

The methods employed vary according to the storage duration request (Bekheet et al., 2007; Abdulaziz and Jameel, 2012).

Recovery

For recovery, the cultures were rapidly thaw in warm water bath (<37°C) and then inoculated on regrowth medium. Four weeks after inoculation, survival was evaluated by examining the colors of cultures: the green ones survived, while the brown colored died. Also, the conversion to differentiated cultures was observed. The methods employed vary according to the storage duration request described by Bekheet et al. (2007) and Abdulaziz and Jameel (2012).

In vitro conservation of date palm germplasm

The methods employed vary according to the storage duration request described by Bekheet et al. (2007) and Abdulaziz and Jameel (2012).

Synthetic seed (synseeds) culture media and carry substrates

The methods employ vary according to the storage duration request according to Bekheet et al. (2007) and Bushra et al. (2010). Quarter-strength MS medium without $CaCl_2.H_2O$, was supplemented with 30 g/L^{-1} sodiumalginate, adjusted to pH 6.0 and used as coating material (date palm synseeds medium). Afterwards, synseeds were cultured in Erlenmeyer flasks containing 1/4 MS medium supplemented with 30 g/L^{-1} Sucrcose, 8 g/L^{-1} agar and 1 g/L^{-1} activated charcoal and adjusted to pH 6.2 (S medium).

Embryogenic callus segments was mixed with sterilized syseeds media, sucked up into a modified 10 ml pipette, and then dropped into sterilized 14.7 g/L^{-1} $CaCl_2.H_2O$ solution. Synseeds was let there from 3 to 5 min for hardening, then picked out, washed with sterilized distilled water, and ready to be cultured on various media [This process followed by the method described by Nhut et al. (2005).

RAPD-PCR analysis

The methods employed vary according to the storage duration request (Bekheet et al., 2007). The amplified DNA fragments were separated on 2% agarose gel and stained with ethidium bromide. The methods employed vary according to the storage duration request (Bekheet et al., 2007; Abdulaziz and Jameel, 2012; Toshikazu, 2018).

Primer sequence (5′-3′)

Table 1 shows the primers used and their annealing temperatures.

Table 1: Primers used and their annealing temperatures.

Primer	Sequence 5'- 3'	Annealing Temperature (°C/s)
Moh1	5'TGGCGACCTG3'	
Moh 2	5'GAGGCGTCGC3'	
Moh 3	5'CCCTACCGAC3'	36
Moh 4	5'TCGTTCCGC3'	
Moh 5	5'CACCTTTCCC3'	
Moh 6	5'GAGGGAGAG3'	
Moh 7	5'GTTCCGCTCC3'	
Moh 8	5' GTGAGGCGTC 3'	
Moh 9	5' GGACCCAACC 3'	
Moh 10	5' TTGGCACGGG 3'	
Moh 11	5' CTCAGTCGCA 3'	
Moh 12	5' GGTGCGGGAA 3'	
Moh 13	5' GGTGACTGTG 3'	
Moh 14	5'GCTAAATCATAC3'	
Moh 15	5' GTTTCGCTCC 3'	
Moh 16	5' GTAGACCCGT 3'	
Moh 17	5' AAGAGCCCGT 3'	

Table 2: Effect of different concentrations of plant growth regulators added to MS-medium on *in vitro* morphogenesis of date palm.

S/No	MS-medium supplemented with:	Nodular culture Frequency (%)	Culture fresh weight (g)	Differentiated cultures (%)
1	1 mg/L NAA	20	0.68	_
2	2 mg/L NAA	30	0.89	14
3	5 mg/L 2,4-D	50	1.10	19
4	10 mg/L 2,4-D	60	1.30	23
5	1 mg/L NAA+3 mg/L 2iP	25	0.99	_
6	2 mg/L NAA+3 mg/L 2iP	35	1.08	17
7	5 mg/L 2,4-D+3 mg/L 2iP	70	1.30	52
8	10 mg/L 2,4-D+3 mg/L 2iP	80	1.37	51

Electrophoresis

The methods employed vary according to the storage duration request (Bekheet et al., 2007).

RESULTS AND DISCUSSION

In this study, we studied the effect of the main components of the cryopreservation solution on the survival and re-growth of date palm suspension. Recycled cells reappeared in culture plates within 4 weeks after plating regardless of preservation components. The methods employed vary according to the storage duration request (Bekheet et al., 2007; Abdulaziz and Jameel, 2012). When combinations of DMSO and sucrose were used as protective agents, the growth rate of the affected colonies was significantly affected by both sucrose and DMSO concentrations, as demonstrated by a significant two-way interaction detected by the variance analysis (Table 1).

In general, at a given concentration of sucrose, the number of colonies increased in response to higher concentrations of DMSO. In comparison, when sucrose were used as protective agents, the number of re-growth of the resulting colonies was also significantly affected by sucrose concentrations as shown by a significant bi-directional interaction detected by variance analysis (Tables 1 and 2). Thawed samples formed colonies at low rates when the protoplaut solution contained 0.1 µg sucrose regardless of the sucrose level. Using another severe sucrose concentration, 1.5 m, the same growth limit was observed. In both cases, no statistically significant differences were detected between sucrose levels. In contrast, a significant difference in the number of colonies was observed in response to changes in DMSO level when sucrose was used at 0.5 and 1. 5 m and to some extent at

Table 3: DMSO agents and the number of re-growth of the resulting colonies of Khalass, Berhi and Sukary date palm. Analysis of variance in the effect of sucrose concentration and DMSO in carotoprotein solution on cell count, amount of callus re-growth and fetal yield number retrieved from date palm embryo.

S/No	MS-medium supplemented	Nodular culture frequency (%)	Culture fresh weight (g)	Differentiated cultures (%)
1	none	10	0.88	_
2	2	40	0.99	13
3	5	44	1.05	18
4	10	50	1.40	21
5	15	47	1.19	24
6	20	45	1.28	19

Table 4: Effects of different sucrose at diiferent concentrations of (0.5, 0.75, 1.0 and 1.5 M) added to pre-culture medium on survival and conversion of cryopreserved date palm cultures at -196°C.

Sugar	Concentration (M)	Water content (%)	Survival (%)	Conversion (%)
None	-	92.23	12	-
Sucrose	0.5	70.40	60	56
Sucrose	0.75	69.30	66	55
Sucrose	1.0	66.40	72	60
Sucrose	1.25	67.20	69	62
Sucrose	1.5	60.10	70	61

0.25 m, respectively. The highest colony formation, approximately 12 to 18 colonies, was recovered from a previous cell of 0.5, 0.75, 1.0, 1.25 or 1. 5 m sucrose treatment, respectively, and then cryopreserved in a cryoprotectant solution supplemented either with 0.5 or 0.75 M sucrose (Table 4).

Accordingly, DMSO appears to be more suitable than sucrose for cryopreservation to suspend the date palm cell in relation to colony formation.

Callus growth

When DMSO was used as an adjuvant, the amount of callus growth in terms of fresh weight of calcium was significantly affected by both sucrose and DMSO concentrations, as demonstrated by a significant two-way interaction revealed by variance analysis (Table 2). The amount of callus spread from regenerating the resulting colonies is an excellent indicator of the state of frozen cells, because it reveals the ability of cells to divide and elongate. Crystalline-induced palm-cell sample cells that formed colonies after thawing, developed microcoli differed in weight depending on protoplactant treatments (Table 3).

In all sucrose concentrations tested, calcium weight was generally increased with increased levels of DMSO. This suggests the inclusion of DMSO into the protective solution required for effective cryopreservation of the date palm cell suspension. The highest weight of 0.12 g was obtained from the pre-treated cell with 0.75 m sucrose and then frozen in a cold-resistant protein solution containing 10% DMSO with 0.75 m sucrose. Therefore, this is the ideal solution for freezing of date palm in laboratory-treated cells. The role of these components in the cryopreservation of palm pollen was clarified. For example, Tisserat et al. (1985) found that palm pollen survived 3 months at -69°C in a greenhouse protein solution containing 10% DMSO. The viability of cold pollen was also affected by storage temperature, as reported by Mortazavi et al. (2010) who obtained the highest survival rate using a very low temperature (-196°C). In a study by Bekheet et al. (2007), the role of sucrose was studied in cryopreservation of the ideological cultures in the field of the laboratory. They observed that the addition of sucrose separately to a vegetarian medium in 1 m gave the highest survival rate (80%) of date palm tree cultivation (Table 5). Each of these stages has special requirements that vary from one stage to the other in order to obtain a high response to the implanted tissue.

Method of production of embryogenesis

The embryonic embryos are obtained from the formation of embryonic callus, which is a continuous source of embryos, and can be replicated by replicating it for the purpose of forming embryonic nodes, which later develop into vegetative embryos.

This technique relies on the use of a food environment with high concentrations of oxinate to help form callus tissue, which can be multiplied by transport and agriculture in the

Table 5: Effect of exposure to vitrification solution at 28 and 0°C on survival and conversion of cryopreserved date palm.

Exposure time (min)	Survival (%)		Conversion (%)	
	Exposure at 28°C	Exposure at 0°C	Exposure at 28°C	Exposure at 0°C
none	10	25	15	10
30	55	60	45	40
60	60	74	56	55
90	75	80	70	57
120	65	78	66	50
150	50	75	65	48

Starting from the bottom of young leaves, soft tissues, shoot tips or axillary buds of date palm offshoots.

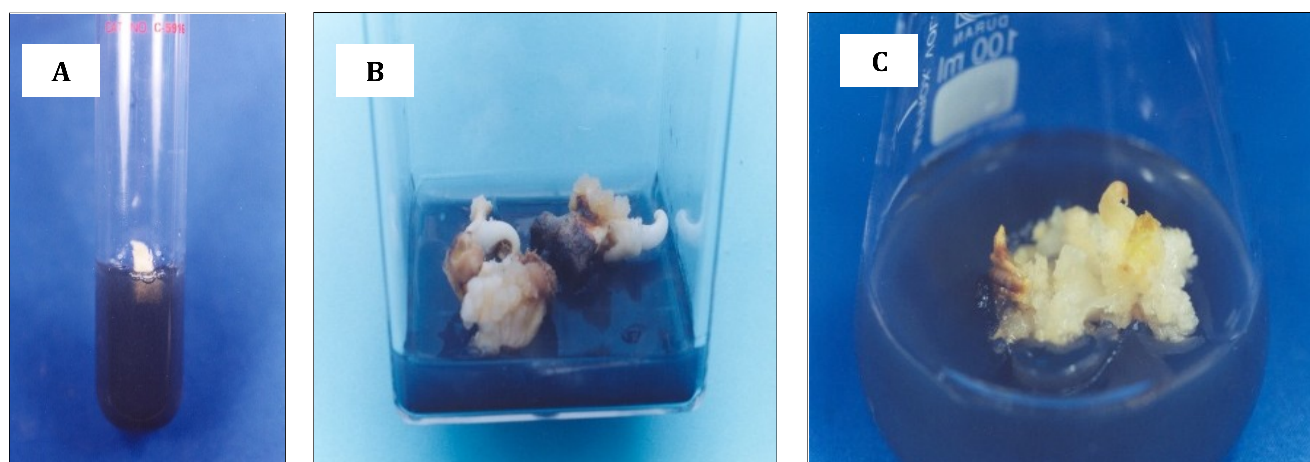

Figure 2: Production of embryogenesis from calli of Khalass, Berhi and Sukary cultivar.

media to help the rapid reproduction of its constituent cells. After propagation, the nutrient environment is altered to obtain fetal callus, which in turn produces vegetative embryos that develop to give complete plants after transfer to an environment suitable for rooting.

This method is characterized by its abundant production of plants in a short period, but it is possible that it may produce plants that are not identical or incompatible with the mother plant due to the passage of the callus, which usually if used for a long time is sometimes presented to the occurrence of genetic mutations, as observed in several plants, including palm oil, banana and strawberry etc. Figure 2 shows that the formation of embryonic embryos passes through several different stages including each of its requirement stage of the environment, hormones and lighting.

Somatic embryogenesis

The ultimate test of the effectiveness of cryopreservation protocol is the ability of cells preserved to maintain physical embryonic development. Jain (2011) reported that the growth and germination of the fetal body in date palm remains normal when treated with a protein mixture of sucrose. The rate of growth and rate of embryo germination remained normal after cryopreservation. Mater (1987) observed that freezing cultured cultures at -250°C for 4 months did not affect the possibility of physical fetal development.

Our study identified the optimal concentrations of the main components of the appropriate cryopotectin solution for cryopreservation to suspend the date palm cell. The effects of non-glowing catalysts, sucrose and breakthrough cryoprotectants and DMSO were determined on cell growth and redifferentiation ability. The highest colony formation, callus weight and number of embryos were associated with a pre-prepared cell at 0.75 m sucrose and then kept in cryoprotectant solutions containing 10% DMSO supplemented to 0.75 M sucrose. These cryoprotectants, which are most suitable for heat storage, are therefore considered as suspension of date palm cells. The benefit of knowledge from this study would benefit future research aimed at establishing germplasm banks in vitro to preserve the genetic diversity of date palm.

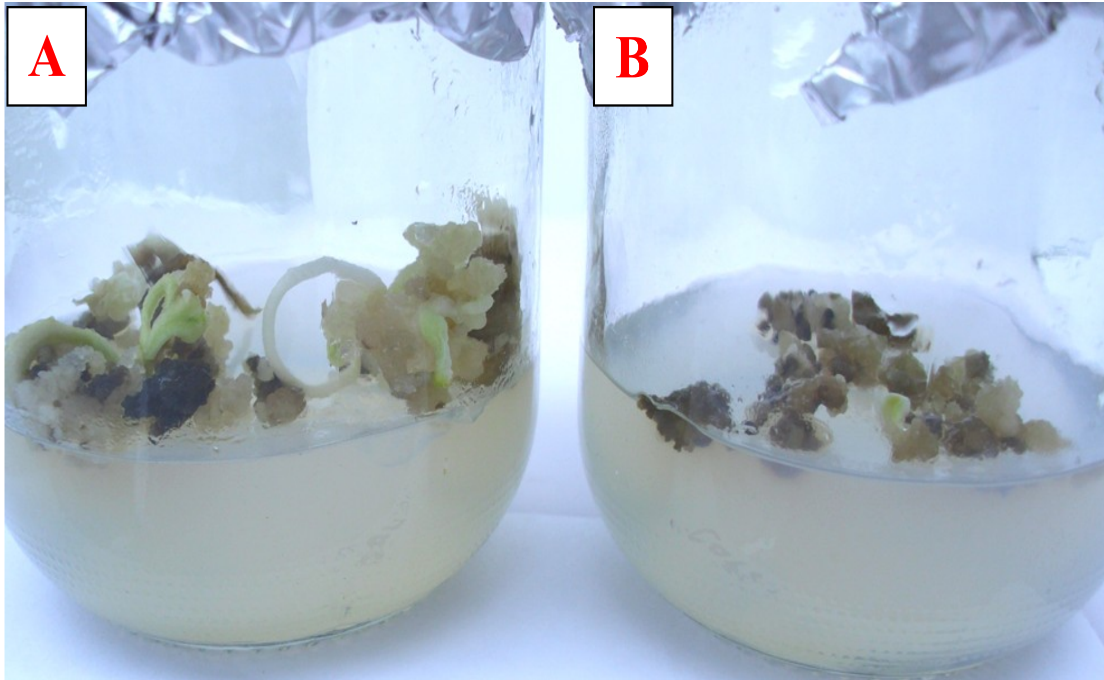

Figure 3: Callus formation after incubated under preservation in liquid nitrogen, -196°C and Revco ultra low–temperature freezers.

Technology, application and validation of plant cryopreservation, impact and applications of cryopreservation in plants

Figure 3 shows genebanks, establishment of cryo-bank and dissemination of results, preparation of Date palm somatic embryo, shoot tips and other explant tissues, Khalass, Berhi and Sukary cultivars in liquid N2 carried out.

RAPD markers

This study was carried out at the Plant Biotechnology Department, King Faisal University, Al-Hofuf, Al-Ahsa, KSA from 2014 to 2018 to evaluate an in vitro selection technique for cryopreservation of date palm cultivars, Barhy and Sukkary cultured on MS medium after 16 weeks for four sub-cultures.

Plant tissue culture is now widely accepted as an easier and safer method of producing real-type plants in palm trees. The differential behavior of varieties to feeding methods, hormones, and cultural conditions has made an interesting article for research.

Two known palm tree species in Saudi Arabia were exposed for accurate propagation in media studies using Murashige and Skoog (1962) media modified with 2,4 D, NAA, 2iP and Kinetin. The induction of callus in Sukary was 40%, whereas in Barhy it was 50%. The regeneration and production of plants rooted more than 70% of the embryos produced in Sukkary were 80%, while in Barhy it was 60%.

RAPD markers for date palm genotypes
Rapd analysis

RAPD amplification is performed in conditions similar to those of sequential polymerase interaction using genomic DNA forming species of interest, and a single short oligonucleotide primule (usually 10-mers). The majority of the genome (from bacteria on humans) contains sufficient or insufficient binding sites to shorten the arbitrary sequence, with a subset that lies in the opposite direction of each other within a distance of up to a few thousand base pairs (bp). As a result, the number of ranges with each arbitrary sequence is inflated by the action of DNA polymerase.

Differentiation of the DNA sequence between individuals in the primer binding site may result in primer failure in adhesion and in the absence of a specific bar between amplification products. The reaction products are conveniently analyzed on agarose gels and no radioactivity is needed. Each RAPD band results from the match of a primer to two sites in the genome (18 to 20 nt). A RAPD reaction which produces 5 amplified bands may therefore be screened for polymorphisms in 90 to 100 bp of DNA sequence. Data of similarity as shown in Figure 4 revealed that there is complete relationship between Date palm Barhy samples from regenerated cell line plant and tissue culture clones.

Also, Sukkary clones and regenerated cell line plants are closely related to each other and they have large number of

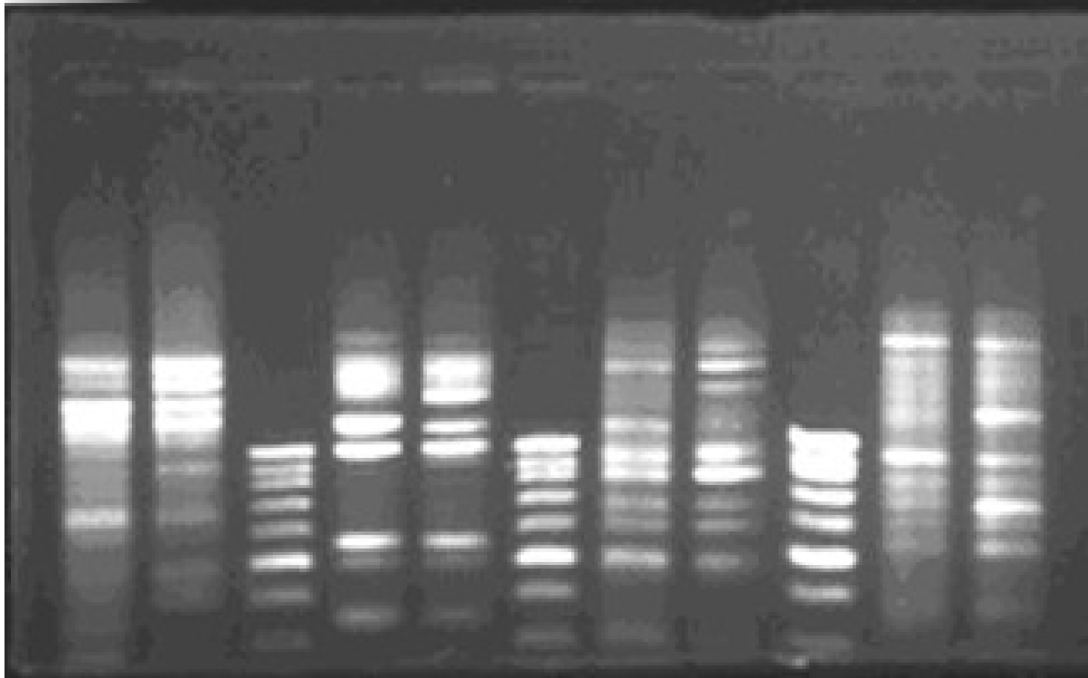

Figure 4: RAPD patterns in samples (1, 4, 6 and 8 for sukary and 2, 5, 7 and 9 for khalas) of date palm obtained with the primers (moh1 t0 moh20 primer). M is DNA marker, RAPD profile of *in vivo* grown plant, non-treated tissue cultures, cryopreserved tissue cultures of date palm.

homologous bands. High similarity was observed in vitro differentiated cultures. DNA isolated from embryo and randomally selected clones of zygotic lines differentiated in vitro were subjected to RAPD analysis. Six random primers (moh5 to moh16) were screened in RAPD analysis for their ability to produce sufficient amplication products. Figure 4 shows the results of DNA fingerprints generated by PCR amplification using the six random primers. The number of fragments generated per primer varied between 1 to 5. The total number of bands was 22 and the average percentage of polymorphism was 51.6. The primers k3 and k4 gave the highest numbers of bands (5) and percentage of polymorphism (80). As shown in Figure 4, some clones are closely related to each other and have similar patterns of bands but possess one or more different bands that can diffrentiate between them.

RAPD profiles of PCR products using primer moh5, moh7, moh12 and moh16 indicated that unique DNA fragment is present in sukary patern and this band is completely absent in the cry-embryo. However, other polymorphic bands were detected among either embryo from sukary cultivar befor or after cryopreservation with other primers tested. Data of similarity index revealed that there is high relationship in both tissues. Also, embryo cryo clones are rlatively closely related to each other and they have large number of homologous bands. As for the different cultures in the laboratory, there was a great similarity between each other. All primers (Table 1) examined produced different RAPD patterns (Figure 4). The number of fragments generated in each primer ranged from 4 to 12. The estimated genetic similarity between different date palm plants for each cultivar is shown as a lipid calligrapher (Figure 4). The highest values of genetic similarity were observed between primer moh5, ranging from 88.9 to 95.3%, respectively. This result reflects the similarity between date cultivars, but these data are not sufficient to identify unknown cryo from tissue culture. We also noted that small modifications in PCR parameters or target DNA quality could alter RAPD patterns. Thus, there may be reason to carefully consider methodological conclusions based on RAPD analysis alone.

On the other hand, the possibility of conducting compatibility analysis with an unlimited number of primers, each detection difference in several regions of the genome, provides an advantage over other techniques. Even if some raw material magnifies the corresponding regions of the genome or if the technique itself is noisy, it should be possible to build a rapid consensus of patterns of variation among the population.

RAPD profiles of PCR products using primers indicated that unique DNA fragment with bands is present in DNAs of Date palm resistance cultivars to RPW and this band is completely absent in the Date palm sensitive cultivars to RPW. However, other polymorphic bands were detected among either Date palm sensitive cultivars or Date palm resistance cultivars with other primers tested.

The random amplified polymorphic DNA (RAPD) molecular marker technique was used to determine Date

palm sensitive cultivars and Date palm resistance cultivars. In the present study, DNA samples were extracted individually from Date palm resistance and sensitive cultivar plants. After a total of all decamer primers tested, an approximate 1 to 3 primers can be used to distinguish Date palm resistance and sensitive cultivars in the future. It is feasible to identify Date palm resistance and sensitive cultivars at the early stages of plant life, which is beneficial for improving breeding programs of this dioecious species. Twenty RAPD primers revealed no polymorphism and genetic variation among date palm genotypes studied. The sukary varieties in the cluster of date palm behaved similarly in their response to salinity tolerance.

Our study shows that RAPD technique is a sensitive, precise, and efficient tool for genomic analysis in sukary varieties in Date palm. The methods employed vary according to the storage duration request (Bekheet et al., 2001, 2002, 2006, 2007; Abdulaziz and Jameel, 2012).

Conclusion

The following conclusions aer drawn from this study:

1) Conservation of genetic resources within and outside their original sites and in gene banks, in nature reserves and on farms;
2) Use of genetic resources with the required genetic diversity and regeneration as well as, genetic characterization, assessment and enhancement;
3) Documentation of all relevant data and information according to methods and systems that ensure the preservation of this information and the possibility of processing and circulation;
4) Use of plant genetic resources for food and agriculture such that they can be used to meet key challenges in food security, public health and environmental conservation.

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