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Cryopreservation of date palm tissue cultures by vitrification and assessment of genetic stability using ISSR analysis

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Abstract

In this work, a successful cryopreservation method of date palm tissue cultures by vitrification was established. Callus and somatic embryos of three date palm cultivars were exposed to dimethylsulfoxide (DMSO) and Plant Vitrification Solution 2 (PVS2) for different durations before plunging into liquid nitrogen for cryoprotection. After freezing phase, the cryopreserved cultures were thawed and cultured on recovery media. Among different times of exposure to DMSO (10%), 60 min gave best results of survival and regrowth of the date palm tissue cultures. Regarding recovery, 'Zaghlool' cultivar gave the highest values of callus fresh mass (0.5 g) and growth value (1.00). Also, the highest differentiation percentages (75%) and number of proliferated shootlets (8.50) were recorded with 'Zaghlool' cultivar. Concerning PVS2, survival and regrowth gradually increased as exposure time increasing till 30 min and then decreased. Unlike of callus cultures, the survival and regrowth rates of somatic embryos were relatively higher at all PVS2 exposure time. With regard to recovery, the genotype effect took same trend in cryopreservation of callus cultures, since 'Zaghlool' was superior in growth parameters in comparison to the other two cultivars. On the other hand, genetic stability of the cryopreserved date palm tissue cultures was assessed using ISSR markers. Among four screened primers, three gave monomorphic bands; all of these banding profiles were similar to those of non-cryopreserved cultures. Generally, the results of ISSR showed high genetic similarity suggesting that cryopreservation using vitrification does not affect genetic stability of date palm tissue cultures. The results of this study demonstrated PVS2 was more effective in cryoprotection of date palm tissue cultures compared with DMSO and cryopreservation by vitrification is a suitable tool for conservation of genetic stable date palm germplasm.

Keywords: cryoprotective agents; date palm; ISSR analysis; liquid nitrogen

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Introduction

Date palm (*Phoenix dactylifera* L.) tree plays an important role in the Egyptian agriculture sector representing a significant part in the reclamation programme. Beside the nutritional values and health benefits of the fruits, the date palm by-products are used by Egyptians. Date palm plantations are spread all over Egypt, wherever water is available, and the palm is considered the major component of the Egyptian oasis ecosystem. There are many factors threat the genetic diversity and expansion of date palm plantains. At the present, the most common method used to preserve the genetic resources of date palm is as whole plants in the field. The high variability of seeds, together with difficulties in the distribution and exchange of healthy plant material from field, support tissue culture and cryopreservation as being the best alternative approaches for germplasm conservation of date palm. Preservation of plant cells, meristems and somatic embryos are used for the storage of date palm germplasm using minimum of space and maintenance. The miniaturization of explants allows reduction in space requirements and consequently labor cost for the maintenance of collections. In this respect, cryopreservation is, so far, the only viable procedure for in vitro long-term germplasm conservation of date palm and other vegetatively propagated plant species (Bekheet *et al*., 2007). Cryopreservation is achieved in liquid nitrogen at −79 and −196 °C, by preventing ice crystal formation. The major advantage of plant material at such temperature is that both metabolic process and biological deterioration are considerably slowed or even halted. In addition, cryopreserved material remains genetically stable, thus affording an advantage over conventional conservation methods. In classical cryopreservation techniques, dehydration of samples takes place both before and during freezing (freeze-induced dehydration), whereas in new, vitrification-based techniques, dehydration takes place only before freezing. The vitrification method was developed by Sakai *et al*. (1990) and has been applied to many kinds of plants, including tropical and subtropical species. In this context, many chemical solutes like DMSO, sucrose, and PVS were used as cryoprotectants (Panis *et al*., 2002; Mandumpal *et al*., 2011) to decrease the free water content in cells. Vitrification-based procedures are more appropriate for multicellular organs (shoot tips, embryos) which contain a variety of cell types, each with unique requirements under freeze-induced dehydration. In this respect, different cryopreservation methods i.e., vitrification, dehydration and encapsulation-dehydration were investigated for cryostorage of date palm explants (Bekheet *et al*., 2007; Fki *et al*., 2013; Metwali *et al*., 2020). Despite that, survival and regrowth frequencies of the cryostored date palm cultures still do not meet the commercial applicable levels.

On the other hand, during cryopreservation processes, plant materials are exposed to a variety of stresses, both physico-chemical and physiological (Harding, 2004). These may cause a loss of genetic integrity of the conserved material. Thus, maintaining the genetic stability of conserved material and assessing it from time to time is very important. Several techniques, including biochemical, molecular and cytological techniques, are employed to assess the genetic stability of conserved plant material. Practically, Inter-Simple Sequence Repeat (ISSR) technique proved to be useful in areas of genetic diversity, phylogenetic studies, gene tagging, genome mapping and evolutionary biology in a wide range of plant species (Zietkiewicz *et al*., 1994). In this respect, Karim *et al*. (2010) used ISSRs to estimate genetic diversity among accessions of high-quality date palm varieties. Similarly, ISSR technique was used for determination the molecular phylogeny of date palm cultivars (Munshi and Osman, 2010). Moreover, the genetic variation in the tissue culture clones of date palm was detected using ISSR analysis (Thummar *et al*., 2015). This work describes an effective method for the cryopreservation of callus and somatic embryo cultures of date palm by vitrification process followed by evaluation of genetic stability after cryopreservation using ISSR marker.

Materials and Methods

Establishing in vitro cultures of date palm

Shoot tips of date palm cultivars i.e., 'Zaghlool', 'Siwy' and 'Bartmoda' isolated from 100-125 cm offshoots were used as plant materials for establishing *in vitro* cultures. The trimmed shoot tips were dipped in 70% ethanol for a few minutes. The shoot tips (5-6 cm) were treated with sodium hypochlorite (2.5%), for 20 minutes and then mercuric chloride solution (0.2%) for 20 minutes and finally rinsed thoroughly with sterile distilled water for surface sterilization. After that, shoot tips were kept in a cold sterilized antioxidant solution of citric acid (150 mg/l) and ascorbic acid (100 mg/l) for 30 minutes to prevent the browning. The disinfected shoot tips were shortened by removing the leaf primordia surrounding the meristematic regions. These explants were cultured on MS medium supplemented with 10 mg/l 2,4-D + 3 mg/l 2iP +1.5 g/l activated charcoal and the cultures were incubated at complete dark condition in order to avoid browning of date palm cultures.

Proliferation of embryonic callus and somatic embryos

After several subcultures on fresh medium, cultures with signs of callogenesis were transferred on medium contained 10 mg/l 2,4-D + 3 mg/l 2iP without charcoal in order to get stock callus cultures. The obtained callus cultures were divided into two parts according their texture. The compact callus was used as non-differentiated explant in cryopreservation experiments. For embryonic cultures, the friable callus were transferred onto 3 mg/l 2,4-D + 1 mg/l 2iP containing medium. The embryonic calli (with globular structures) were cultured on MS + 1 mg/l 2.4-D + 0.5 mg/l 2ip +1 mg/l NAA and incubated at 16 h photoperiod condition for somatic embryos differentiation.

Cryopreservation by DMSO and PVS2

Callus (250 mg) and somatic embryos (clusters with 2-3 somatic embryos) of the three date palm cultivars taken from 2 weeks sub-culture were used as cryopreservation materials. The explants were pre-cultured on growth regulator-free MS medium containing 2 M sucrose for 3 days and incubated at ± 25 °C, under 16/8 h (light/dark) photoperiod. To investigate the effect of exposure time to DMSO on cryoprotection of date palm tissue cultures, the explants (callus and somatic embryos) were placed in DMSO (10%) in liquid MS medium for 20, 40, 60 or 80 min. In another experiment, PVS2 (Sakai *et al*., 19910) in a growth regulators-free MS liquid medium was used for cryprotection. In this experiment, the explants were exposed to PVS2 for 10, 20, 30 and 40 min before cryostorage. Twenty cryovials contained one explant for each were immersed directly into liquid nitrogen tank (at −196 °C) and stored for three months. The cultures plunged into liquid nitrogen (LN) without vitrification treatments were used as control treatments.

Thawing and recovery of cryopreserved cultures

By the end of storage period, cryovials were taken from liquid nitrogen container and rapidly thawed in water bath at 37 °C for 2-3 min until the ice had melted. Then the vials were filled with 1 ml MS liquid medium plus 1M sucrose (unloading solution) for 30 min at room temperature and then blotted on filter paper. The cultures were transferred onto regrowth media (3 mg/l 2,4-D + 1 mg/l 2iP for callus and MS + 1 mg/l 2.4-D + 0.5 mg/l 2ip +1 mg/l NAA for somatic embryos) and then all the cultures were incubated under standard conditions of illumination and temperature. Data of survival (% of explants that survived and remained creamy for callus and white or green color for somatic embryos) were registered. Also, regrowth (% of explants that showed signs of further development) frequencies were recorded (3 days for survival and 8 weeks for regrowth). For recovery investigation, callus and somatic embryos of the three cultivars indicated the increase in size were transferred into recovery medium and incubated on standard incubation conditions. Various growth parameters were recorded after 10 weeks of culturing including fresh mass and growth value (for callus) and differentiation (%) and number of shootlets (for somatic embryo cultures).

Culture medium and incubation conditions

Tissue culture media were solidified with 0.7% agar and supplemented with 30 g/l sucrose, plus (per liter) 170 mg NaH_2PO_4, 100 mg myo-inositol, 200 mg glutamine, 5 mg thiamine-HCl, 1 mg nicotinic acid, 1 mg pyridoxine-HCl. The pH was adjusted to 5.8 before autoclaving at 121 °C and 1.5 ib/m^2 for 25 min. In all treatments, the growth regulators were added to the culture medium prior to autoclaving. For both recovery and re-growth, the cultures were normally incubated at 25 ± 2 °C and 16 h photoperiod provided by white fluorescent tubes (3000 lux light intensity).

Genetic stability of cryopreserved cultures using ISSR analysis

Samples of the recovered cultures taken randomly from the three cultivars and two cryopreservation treatments were investigated for their genetic stability in comparison to non-cryopreserved cultures using ISSR analysis. The genomic DNA was isolated from the cryopreserved tissue cultures following the steps of CTAB (Porebski *et al*., 1997). ISSR scorable primers presented in Table 1 were screened for PCR amplification. PCR was performed in 25 µl reaction volume containing 1X PCR buffer, 1.75 mM $MgCl_2$, 5 mM of each dNTPs, 40 µM oligonucleotide primer from each of the ISSR primers, 25 ng genomic DNA and 1 U of Taq DNA polymerase. The optimized PCR conditions for ISSR amplifications were consisted of an initial denaturation step for 5 min at 94 °C, followed by 35 cycles each of 20 s at 94 °C for denaturation, 60 sec. at 50 °C for annealing, 90 sec at 72 °C for extension. The final extension was done for 7 min at 72 °C. The PCR products were separated by electrophoresis in a 2% agarose gel containing ethidium bromide (0.5 µg/ml) in 1X TBE buffer at 90 volts. Gel was photographed under UV light with Tracktel GDS-2 gel documentation system.

Table 1. Four selected primers used in ISSR analysis

No.	Name	Sequence
1	UBC810	GTGTGTGTGTGTGTGTCA
2	UBC834	GAGAGAGAGAGAGAGAGAGAT
3	UBC-823	TCTCTCTCTCTCTCTCC
4	TA-1	(AG)10C

Experimental design and statistical analysis

The experiments were conducted in a completely randomized design with three replicates of each treatment. Results obtained were presented as mean values ± standard deviation (SD). The significant difference between the means was determined using one-way analysis of variance (ANOVA) and Least Significant Difference (LSD) test at $p < 0.05$, using Statistix (version 8.0).

Results

Cryoprotection of callus and somatic embryos by DMSO

Survival, regrowth and recovery of callus cryoprotected by DMSO

Suitable cryoprotectant is very effective in enhancing survival of the cryopreserved plant material. This experiment was carried out to study the effect of exposure time (20, 40, 60 or 80 min) to the DMSO on survival, regrowth and recovery of date palm callus cultures. Obvious variations in survival and regrowth rates of the treated and non-treated calli were observed. Data exhibited in Table 2 reveal that survival and regrowth of cryopreserved calli tended to increase with increasing the exposure time to DMSO up to 60 min and then decreased. Generally, high survival (60-70%) and regrowth (53-61%) were observed with 60 min exposure treatment. The reduction in regrowth compared to survival percentage may be attributed to partial damage of the calli due to osmotic shock and ice crystallization of some frozen cells. Among the three cultivars, 'Zaghlool' gave the best results of survival and regrowth followed by 'Siwy' cultivar. The maximum survival (70 %) and

regrowth (61 %) percentages were recorded with 'Zaghlool' when callus was exposed to 60 min to DMSO before plunging in liquid nitrogen (Table 2). Regarding recovery, results of Figure 1 reveal that the viable callus cultures of the three cultivars grew well and the fresh mass increased on MS +10 mg/l 2,4-D + 3 mg/l 2iP using exposure to DMSO. Concerning genotypes effect, the three cultivars responded differently to cryoprotection by DMSO. In this respect, 'Zaghlool' gave the best results of callus fresh mass (0.5 g) and growth value (1.00). It was noticed that the recovered calli were compact with light brown colour in 'Zaghlool' and 'Siwy' cultivars and compact with dark brown colour in 'Bartmoda' cultivar.

Table 2. Effect of exposure to the DMSO on survival and regrowth of cryopreserved callus cultures

Exposure time (min.)	Survival frequency (%)			Regrowth frequency (%)		
	Zaghlool	Siwy	Bartmoda	Zaghlool	Siwy	Bartmoda
Control	25.0±5.0^{c}	21.7±7.6^{b}	23.3±2.9^{c}	18.3±2.9^{d}	00.0±00.0	00.0±00.0
20	45.0±8.7^{b}	30.0±5.0^{b}	30.0±8.7^{c}	45.0±5.0^{c}	28.3±2.9^{c}	20.0±5.0^{c}
40	65.0±13.2^{a}	56.7±2.9^{a}	45.0±5.0^{b}	56.7±5.8^{b}	43.3±5.8^{b}	46.7±2.9^{ab}
60	70.0±5.0^{a}	63.3±2.9^{a}	60.0±10.0^{a}	61.7±2.9^{a}	56.7±2.9^{a}	53.3±5.8^{a}
80	66.7±5.8^{a}	56.7±2.9^{a}	50.0±10.0^{ab}	60.0±10.0^{b}	48.3±10.4^{ab}	45.0±5.0^{b}

All values were presented as the average of the three replicates ± standard deviation, small letter express significant differences (LSD) which analyzed by Statistix 8.0.

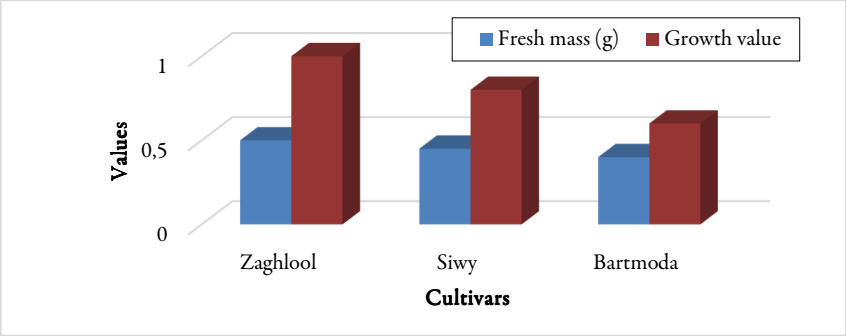

Figure 1. Recovery of callus cultures of date palm cryopreserved by DMSO.
Growth value = final fresh mass – initial fresh mass/ initial fresh mass

Survival, regrowth and recovery of somatic embryos cryoprotected by DMSO

This experiment aimed to investigate the effects of DMSO as cryoprotective agent on cryostorage of somatic embryos of date palm. In this regard, survival, regrowth and recovery of somatic embryos cryoprotected by DMSO for 20, 40, 60 and 80 min. prior to cryostorage were assessment. Our findings reveal that survival and regrowth values took similar trend of callus cultures since the best results were obtained when cultures were exposed to DMSO for 60 min. Otherwise, significant differences among exposure periods to DMSO were observed (Table 3). Meanwhile, low survival and regrowth percentages were observed with control treatment. Also, cultivars refer to the differences of both survival and regrowth values. At this point, 'Zaghlool' cultivar registered the maximum percentages of survival (80%) and regrowth (70%) (Table 3).

On the other side, relatively high differentiation and shootlets number were obtained after recovery of somatic embryos of date palm exposed to the DMSO. Interestingly, the most noteworthy recovery parameters were counted with 'Zaghlool' cultivar. At this point, the highest differentiation percentages (75%) and number of proliferated shoottlets (8.50) were recorded with this cultivar (Figure 2). The results proved that prolonging to the DMSO solution before cryostorage is relatively harmful for 'Bartmoda' explants since lowest recovery parameters were noticed. It is important here to mention that, the recovered somatic embryo cultures produced healthy plantlets as shown in Figure 3 (A, B, C).

Table 3. Effect of exposure to DMSO on survival and regrowth of cryopreserved somatic embryos

Exposure time (min.)	Survival frequency (%)			Regrowth frequency (%)		
	Zaghlool	Siwy	Bartmoda	Zaghlool	Siwy	Bartmoda
Control	30.0±5.0^{c}	26.7±2.9^{d}	25.0±5.0^{b}	23.3±2.9^{d}	20.0±5.0^{c}	18.3± 2.9^{b}
20	53.3±2.9^{b}	46.7±2.9^{c}	40.0±10.0^{b}	51.7±2.9^{c}	40.0±5.0^{b}	32.5±3.5^{b}
40	68.3±2.9^{a}	60.0±5.0^{b}	60.0±10.0^{a}	61.7±2.9^{b}	51.7±2.9^{a}	48.3±5.8^{a}
60	80.0±10.0^{a}	71.7±5.8^{a}	65.0±8.7^{a}	70.0±5.0^{a}	60.0±8.7^{a}	56.7±7.7^{a}
80	73.3±11.5^{a}	63.3±2.9^{b}	61.7±7.6^{a}	70.0±5.0^{a}	55.0±5.0^{a}	53.3±10.4^{a}

All values were presented as the average of the three replicates ± standard deviation, small letter express significant differences (LSD) which analyzed by Statistix 8.0.

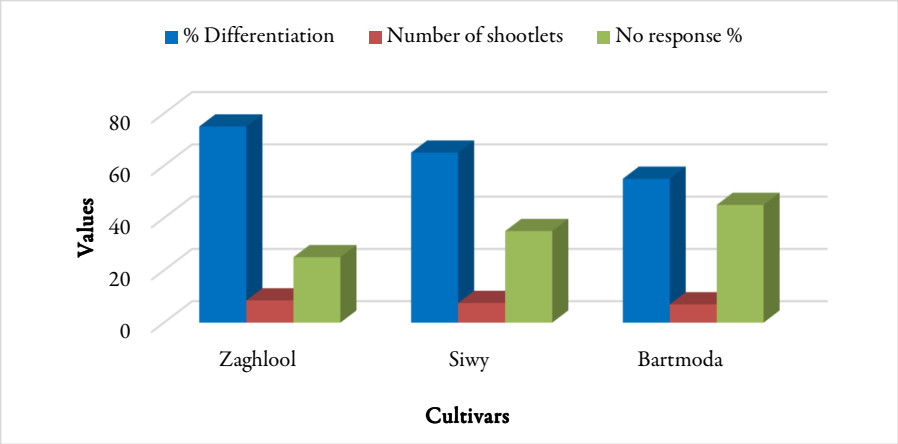

Figure 2. Recovery of somatic embryos of date palm cryopreserved by DMSO

Figure 3. A) Recovery of somatic embryos cryopreserved by DMSO after three weeks, B) six weeks and C) ten weeks of culturing on medium contained MS + 1 mg/l 2,4-D + 3 mg/l 2ip

Cryoprotection of callus and somatic embryos by PVS2

Survival, regrowth and recovery of callus cryoprotected by PVS2

Due to possible toxic effects of the PVS2 solution which can compromise plant cell viability, the exposure time must be optimized. To determine the optimal time of exposure to PVS2, survival and regrowth of cryopreserved callus of date palm was examined in response to exposure durations. Data of survival and regrowth of vitrified callus affected by exposure times to PVS2 are illustrated in Table 4. It is apparent from this table that various durations resulted in a variable rate of growth parameters presented as survival and regrowth percentages. The lowest survival and regrowth percentages were observed with control treatment (without exposure to PVS2). At this treatment, most of the cryopreserved callus cultures turned brown and

then died. Generally, survival and regrowth gradually increased as exposure time to PVS2 increased till 30 min and then decreased. The highest survival (75%) and regrowth (73.3%) rates were observed when callus of 'Zaghlool' cultivar was treated with PVS2 for 30 min. before plunging in LN (Table 4). The obtained results proved that exposure to PVS2 is less harmful for callus compared with DMSO. On the other hand, recovery of callus cultures in response to cryopreservation by PVS2 was also evaluated. Data showed that, callus fresh mass was increased two to three times (depending on the cultivar) after ten weeks of culturing on recovery medium. This illustrate that using suitable period of vitrification agent is effective for recovery of date palm callus after cryopreservation. The highest fresh mass (0.7 g) and growth value (1.80) were registered with 'Zaghlool' followed by 'Siwy' and then 'Bartmoda' cultivar (Figure 4). It was noticed that, callus growth parameters presented as fresh mass and growth values were higher using PVS2 than those determined with cryoprotection by DMSO alone. Regarding callus appearance, most of calli obtained after recovery were compact and their color varied from light to dark depending on the cultivar.

Table 4. Effect of exposure to PVS2 on survival and regrowth of cryopreserved callus of date palm cultivars

Exposure time (min.)	Survival frequency (%)			Regrowth frequency (%)		
	Zaghlool	Siwy	Bartmoda	Zaghlool	Siwy	Bartmoda
Control	25.0±8.7^{b}	20.0±5.0^{d}	20.0±5.0^{d}	21.7±2.9^{d}	18.3±5.8^{c}	16.7±2.9^{d}
10	65.0±5.0^{a}	50.0±5.0^{c}	46.7±2.9^{c}	56.7±5.8^{c}	45.0±5.0^{b}	41.7±2.9^{c}
20	68.3±5.8^{a}	58.3±2.9^{b}	55.0±5.0^{b}	65.0±5.0^{b}	56.7±2.9^{a}	53.3±2.9^{b}
30	75.0±5.0a	71.7±2.9^{a}	68.3±2.9^{a}	73.3±2.9^{a}	65.0±5.0^{a}	63.3±2.9^{a}
40	70.0±5.0a	66.7±5.8^{a}	60.0±5.0^{b}	70.0±5.0^{ab}	60.0±10.0^{a}	55.0±5.0^{b}

All values were presented as the average of the three replicates ± standard deviation , small letter express significant differences (LSD) which analyzed by Statistix 8.0.

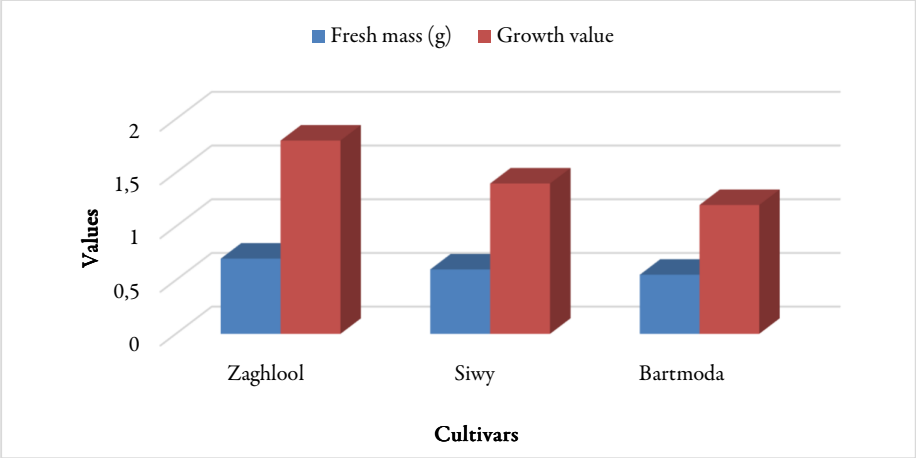

Figure 4. Recovery of callus cultures of date palm cryopreserved by PVS2
Growth value = final fresh mass – initial fresh mass / initial fresh mass

Survival, regrowth and recovery of somatic embryos cryoprotected by PVS2

In order to investigate the cryoprotection of somatic embryos by PVS2, various exposure times (10, 20, 30 and 40 min.) were evaluated. Unlike of callus cultures observations, the survival and regrowth rates of somatic embryos were relatively higher at all PVS 2 exposure durations (Table 5). The best results of survival and regrowth of the three cultivars of date palm were obtained when the somatic embryos were exposed to PVS2 for 30 min. before plunging in LN. Augmenting exposure duration above 30 min led to relatively decreasing the growth parameters. The decline in regrowth with increased duration may be due to the osmotic shock. Our findings indicate that, the maximum survival (76.7 %) and regrowth (70.3 %) rates of somatic embryos were registered with 'Zaghlool' cultivar on PVS2 for 30 min. Concerning the recovery of somatic

embryo cryopreserved by PVS2, the proliferation parameters were higher than those obtained in cryoprotection by DMSO. It is clear from the data displayed in Figure 5 that vitrification by PVS2 was perfect for recovery of date palm somatic embryos after cryopreservation. It was found that the explants grew slowly during the first two weeks and then increased. Healthy and green shootlets were obtained after ten weeks of culturing on MS + 1 mg/l 2.4-D + 3 mg/l 2ip (Figure 6: A, B, C). The highest differentiation (78 %) and number of shootlets (8.00) were registered with 'Zaghlool' cultivar treated by PVS2 for 30 min. However, the lowest growth parameters presented as differentiation percentages and number of shootlets was registered with 'Bartmoda' cultivar Figure (5).

Table 5. Effect of exposure to PVS2 on survival and regrowth of cryopreserved somatic embryos of date palm cultivars

Exposure time (min.)	Survival frequency (%)			Regrowth frequency (%)		
	Zaghlool	Siwy	Bartmoda	Zaghlool	Siwy	Bartmoda
Control	36.7±2.9^{c}	31.7±2.9^{c}	30.0±5.0^{d}	30.0±8.7^{b}	28.3±2.9^{c}	21.7±2.9^{c}
10	65.0±5.0^{b}	56.7±5.8^{b}	50.0±5.0^{c}	61.7±2.9^{a}	48.3±2.9^{b}	43.3±2.9^{b}
20	73.3±2.9^{a}	65.0±5.0^{ab}	61.7±2.9^{b}	68.0±10.0^{a}	60.0±5.0^{a}	56.7±5.8^{a}
30	76.7±2.9^{a}	73.3±2.9^{a}	71.7±2.9^{a}	70.3±2.9^{a}	66.7±2.9^{a}	61.7±2.9^{a}
40	73.3±2.9^{a}	68.3±7.7^{a}	61.7±2.9^{b}	65.0±5.0^{a}	60.0±10.0^{a}	56.7±2.9^{a}

All values were presented as the average of the three replicates ± standard deviation, small letter express significant differences (LSD) which analyzed by Statistix 8.0.

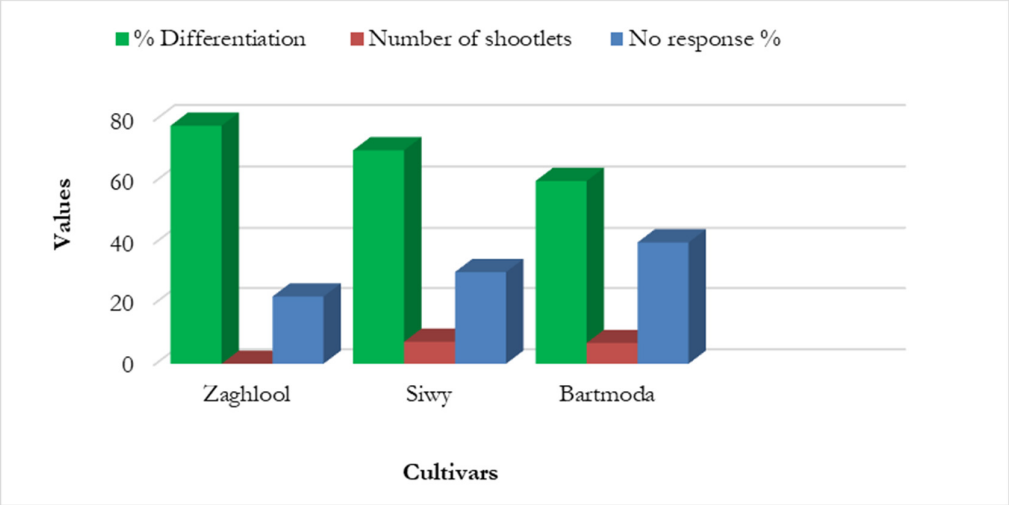

Figure **5**. Recovery of somatic embryos of date palm cryopreserved by PVS2

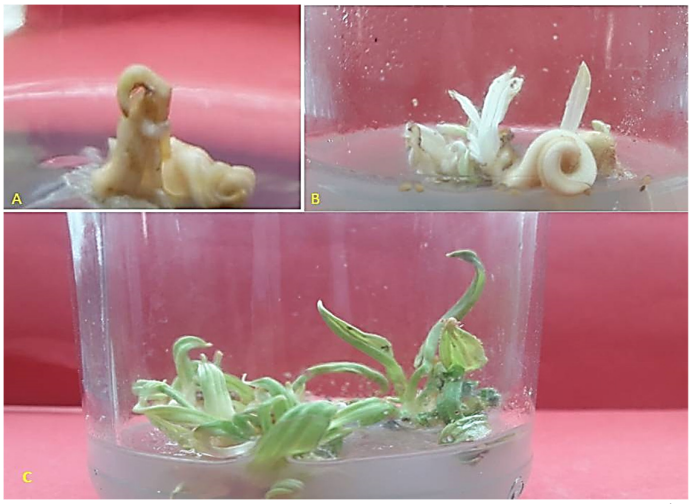

Figure 6. A) Recovery of somatic embryos cryopreserved by PVS2 after three weeks, B) six weeks and C) ten weeks of culturing on medium contained MS + 1 mg/l 2.4-D + 3 mg/l 2ip

Genetic stability of cryopreserved date palm tissue cultures using ISSR analysis

In this study, ISSR analysis was performed to investigate the genetic stability of date palm cultures cryopreserved by vitrification. DNA isolated randomly from recovered cultures was subjected to ISSR analysis. For this purpose, four random primers were screened for their ability to produce sufficient amplication products. The obtained data reveal that the four primers produced reproducible and scorable patterns with different intensity (Figure 7). The total number of bands was varied based on the primer since the highest number (6) was observed with primers 3 i.e., UBC-835 (Figure 7). Consequently, the amplification profiles were screened for the presence of polymorphisms among the analyzed plant materials. The banding patterns generated by ISSR primers were compared to determine the genetic similarity of the cryopreserved samples. The obtained results showed no polymorphism with primers 1: UBC810, 2: UBC834 and 4: TA-1. With these primers, all banding profiles of different cryopreserved samples were monomorphic and similar to those of non-cryopreserved (Figure 7). This finding reveals that no genetic variation was detected with these three primers. In spite of primer 3: UBC-835 showed polymorphism, the mean percentage of polymorphism of the four primers is 8.32%. The observed genetic instability may be due to repeated subculturing or the cryopreservation procedure.

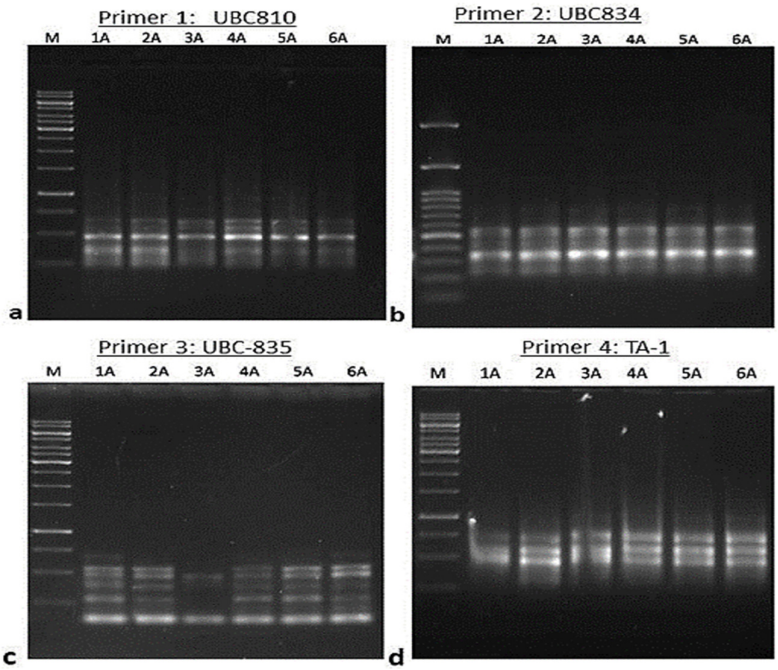

Figure 7. ISSR-PCR band profiles of date palm cultures generated by four (a, b, c and d) primers with the Lane 1A: non-cryopreserved, lane 2A- 6A: cryopreserved cultures and M: weight marker

Discussion

Treatment with the protective agent is a critical step in the cryopreservation of plant tissue cultures by vitrification. Successful vitrification requires the use of a highly concentrated non-toxic solution of cryoprotectants and the optimum exposure time to it. Otherwise, the survival and regeneration rates are key factors determining the cryopreservation protocols success and using suitable cryoprotectants are very effective in enhancing survival of the cryopreserved plant material. Due to the specificity of genotype requirements, it is necessary to empirically determine the optimum cryoprotectant components of different genotypes (Bekheet *et al*., 2020). In the present study, callus and somatic embryos cultures of three date palm cultivars were cryoprotected by DMSO and PVS2 prior to storage in liquid nitrogen. The obtained results reveal that survival and regrowth of cryopreserved calli tended to increase with increasing the exposure to DMSO up to 60 min and then decreased. However, the highest survival and regrowth rates were observed when calli were treated with PVS2 solution for 30 min. Also, somatic embryo explant took the same trend of callus since the best results of survival and regrowth were obtained with 60- and 30-min. exposure time to DMSO and PVS2 respectively. Our findings reveal that growth parameters were higher in the differentiated cultures (somatic embryos) in responding to exposure to DMSO and PVS2 comparing with non-differentiated culture (callus). These differences might be due to permeability the two solutions inside date palm cells and tissues. Among the three cultivars, 'Zaghlool' registered the maximum percentages of survival and regrowth with the two types of explants followed by 'Siwy' and 'Bartmoda'.

While survival rates consider post-cryostorage recovered tissues to contain viable cells, recovery demands that those tissues be able to grow back into a fully functional organ or a complete plantlet. For using vitrification technique, Kohmura *et al*. (1992) mentioned that the keys to success on cryopreservation by vitrification are to carefully control the procedures of dehydration to prevent injury by chemical toxicity or excess osmotic stresses during treatment. The results of the present investigation revealed that callus cultures of the three cultivars obtained from cryopreservation were compact and their colour varied from light to dark depending

on the cultivar. Moreover, the results proved that exposure to PVS2 are less harmful for callus compared with DMSO. On the other hand, proliferation parameters i.e., differentiation percentages and shootlets number derived from somatic embryo cryopreserved by PVS2, were higher than those obtained in cryopreservation by DMSO. Our disclosures are close with those of Fki *et al*. (2013). In their study on cryopreservation of date palm meristems, they found that perfect PVS2 treatment was 30 min. Similarly, Alansi *et al*. (2019) found that cryopreservation of date palm utilizing the vitrification convention brought about the most extreme recuperation of 53.3%, when vitrified-cryopreserved calli were subjected to PVS2 for 30 min at 25 °C. For cryopreservation of embryogenic callus of date palm, Subaih *et al*. (2007) stated that direct exposure of calli to 100% PVS2 decreased the viability of calli. Survival of 80-93.3% and 40-53.3% regrowth rates were achieved with two- or four- step dehydration, using PVS2 at 25 °C for 20 min intervals prior to freezing. Khawnium and Techato (2011) reported that, friable embryogenic cultures of oil palm were successfully cryopreserved by vitrification. The cultures were dehydrated for 60 min at 0 °C with a highly concentrated vitrification solution (PVS2) and plunged directly into liquid nitrogen. In this respect, Kartha *et al*. (1979) mentioned that, the most widely vitrification procedure used in plant cryoprservation is the treatment with PVS2 solution for periods ranging from 30 to 90 min.

Cryopreservation at −196 °C is viewed as the favoured strategy for long term protection of plant hereditary sources without changing its qualities. However, exposing to a variety of stresses during cryopreservation may cause a loss of genetic integrity of the conserved material. Thus, maintaining the genetic stability of cryopreserved plant material and assessing it from time to time is very important. In this regard, several techniques, including biochemical, molecular, cytological and phenotypic techniques, are employed to assess the genetic stability of cryopreserved plant materials. ISSR analysis is effectively used for this purpose as molecular marker. In the present study, the genetic stability of the cryopreserved date palm tissue cultures was estimated according to the ISSR data. Our findings reveal that, three primers gave monomorphic bands all of these banding profiles were similar to those of non-cryopreserved cultures. Generally, the similarity percentages of the four primers were high indicating genetic stability of the cryopreserved date palm tissue cultures. The observed variation may be occurring throughout the cryopreservation process due to osmotic shock resulted from of the used cryoprotectants. This finding may also result from the type of the cryopreserved explants. The obtained results reveal that cryopreservation by vitrification is a suitable tool for the successful preservation of a genetic stable date palm germplasm. The present results are consistent with our previous work on cryopreservation of date palm tissue cultures (Bekheet *et al*., 2007). Also, Alansi *et al*. (2017) investigated the genetic fidelity of date palm plantlets derived from somatic embryogenesis before and after cryopreservation using ISSR technique. The results indicated that plantlets derived from embryogenic calli after cryopreservation and non-cryopreservation showed high similarity (98.0 %) to their mother plants at the genetic level. In this respect, genetic fidelity was assessed using SCoT markers in regenerated date palm plantlets after cryopreservation (Al-Qurainy *et al*., 2017). Out of a total of 118 amplicons produced by 18 SCoT primers, 114 were monomorphic and the remaining was polymorphic. This low genetic variation indicates the clonal genetic stability of the regrown plantlets after cryopreservation. In their study on cryopreservation of oil palm embryogenic culture using vitrification Khawnium and Techato (2011) reported that SSR analysis did not reveal any polymorphism between cryopreserved explants and control.

Conclusions

Cryopreservation is considered an efficient strategy for long-term storage of date palm germplasm. In the present work, a successful cryopreservation method of date palm tissue cultures by vitrification was recognized. Our findings reveal that exposure callus or somatic embryo to PVS2 for 30 min are the best treatments for cryoprotection of the two types of cultures. Among the three examined cultivars of date palm, 'Zaghlool' exhibit the highest values of survival and recovery after cryostorage. According to ISSR data, high

level of monomorphic was observed indicating high level of genetic similarity of date palm tissue cultures cryoprotected by vitrification. The results demonstrate that the standardized duration of cryoprotection of date palm tissue cultures by vitrification solution could be used for wide range cultivars. Also, we can conclude that cryopreservation by vitrification is a suitable tool for preservation of genetic stable date palm germplasm.

Authors' Contributions

SB: supervised the research work, designed the experiments and wrote the manuscript. **AG**: contributed to design the experiments and preparing data. **MD**: performed tissue culture and cryopreservation experiments and contributed to data collection. **MI**: contributed to experimental design and analyzed the data. **MR**: contributed to writing and correcting the manuscript.

All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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