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Recovery and Histological Changes of Date Palm Somatic Embryos After Crypreservation By Dehydration

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Abstract

Cryopreservation of plant materials is the storage in liquid nitrogen (LN) in such a way, that viability is maintained and regeneration is obtained after rewarming. In this study, cryopreservation of date palm somatic embryos was examined through dehydration by air drying (20, 40, 60 and 80 minutes) or osmotic dehydration caused by sucrose (0.5, 1.0 and 1.5 M). After two months of cryostorage, survival, re-growth and recovery parameters were registered. Furthermore, the ultrastructure of somatic embryos cells was studied after cryostorage. Among three drying periods, the best results of survival were observed with 60 min exposure to air-flow cabinet. Also, the highest values of recovery parameters i.e., number of secondary embryos, embryos germination and numbers of proliferated shootlets were obtained using 60 min exposure period. Regarding osmotic dehydration, the maximum values of survival (50 %) and re-growth (45 %) were counted when somatic embryos were dehydrated by 1.0 M sucrose prior to cryopreservation. Also, the highest number of second embryos (5.90) was registered when somatic embryos were dehydrated with 1.0 M and 85 % of the embryos were able to germinate into healthy propagule. However, using 1.5 M sucrose for dehydration induced highest number of proliferated shootlets (5.50) after ten weeks of culturing on recovery medium. Histological screening revealed that cryopreservation by dehydration caused minimal cellular damage within the cells of date palm somatic embryos. Comparing to the non-cryopreserved cultures, some cell walls were broken and vacuole size and cytoplasm density were increased. The cells suffered some damage could divide and regenerate into normal tissues. Depending on our finding, we can conclude that dehydration especially osmotic dehydration (1.0 M sucrose) is an effective method can be used for cryopreservation of date palm tissue cultures.

Keywords: Date palm; cryostorage; dehydration; histological analysis; somatic embryos.

1. Introduction

Date palm (*Phoenix dactylifera* L.) tree plays an important role in the economic and social lives of the people. Beside the nutritional values and health benefits of the fruits, every part of the tree is used. As a dioecious and heterozygous plant species, date palm genetic resources are preserved conventionally as whole plants on farms **[1]**. Field genebank provides ready access to conserved material for research as well as for commercial use. While *in situ* conservation is vital to keep the evolution of the species and permit novel diversity to be created via natural selection processes, it demonstrates numerous disadvantages for conservation. Whereas, the collections are exposed to natural disasters and attacks by pests and pathogens.

The conventional preservation programs of date palm are recently equipped with up-to-date biotechnology methods like the use of in vitro preservation. Nowadays, tissue culture strategies are indispensable for the manipulation of any genetic resources including date palm **[2-6]**. Two types of *in vitro* genebanks for conservation of such species have been reported: (a) slow growth, and (b) cryopreservation. The principle long-term *in vitro* conservation method is cryostorage between -79°C and -196°C in liquid nitrogen (LN) **[7]**. Successful cryopreservation requires the optimization of numerous variables including the size of specimen, type and concentration of cryoprotectant and sample water content. Cryopreservation can be achieved by using different procedures such as, dehydration, vitrification and encapsulation-dehydration **[8]**. Dehydration is natural mechanism for cryopreservation since explants can be safely stored in liquid nitrogen when they have low moisture contents **[9]**. Dehydration increases cytoplasmic viscosity and so impedes intracellular ice-crystal growth during cooling **[10]**. Usually, dehydration is obtained by using a laminar flow air stream, silica gel or natural osmotic agents such as sucrose **[8]**. Cryopreservation procedures may result in cell distortion, cell and nuclear shrinkage, nuclear envelope rupture and plasmolysis **[11]**. During cryostorage, freezing induces damages at different cellular levels. In this respect, modifications in cells within and after cryopreservation were investigated for both shoot-tips and suspension culture cells **[12-16]**. These reports indicated a range of cellular alterations after various steps involved in cryopreservation. Minimal studies have been performed to preserve the germplasm of date palm using dehydration before cryostorage. In earlier study on date palm, **Bekheet *et al*. [17]** described a

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method for cryopreservation of undifferentiated tissue cultures of date palm using two methods of dehydration. Also, **Fki *et al.* [18]** mentioned that sucrose treatment improves survival of date palm meristems after cryopreservation. The effect of dehydration caused by air drying on cryoprotection of undifferentiated tissue cultures of date palm was studied by **Solliman *et al.* [19]**. In this respect, dehydration was combined with encapsulation techniques in order to develop effective protocol of date palm cryopreservation using embryogenic calli **[20]**. So, recognize cryopreservation methods of somatic embryos using physical dehydration (desiccation) or natural osmotic agents (sucrose) are essential for conservation of date palm genetic resources. This work describes simple procedures for cryopreservation of somatic embryos of date palm using dehydration caused by air drying or sucrose as osmotic agent and histological analyses of the recovered cultures.

2. Materials and Methods

2.1. Establishment of in vitro cultures and somatic embryo formation

Shoot tips of Zaghlool date palm cultivar were isolated from offshoots and used as plant materials for establishing *in vitro* cultures. For surface sterilization, shoot tips (6-8 cm) were treated with sodium hypochlorite (2.5 %), for 20 minutes and then mercuric chloride solution (0.2%) for twenty min. and washed with sterile distilled water **[17]**. The disinfected shoot tips were shortened by removing the leaf primordia surrounding the meristematic regions. These explants were cultured on Murashige and Skoog (MS) medium **[21]** fortified with 10 mg/l 2,4-Dichlorophenoxyacetic acid (2,4-D) + 3 mg/l 6-(γ,γ-Dimethylallylamino)Purine (2iP) +1.5 g/l activated charcoal and the cultures were incubated at complete dark condition. 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. For embryonic cultures, the friable callus was 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 Naphthaleneacetic Acid (NAA) and incubated at 16 h photoperiod condition for somatic embryos differentiation.

2.2. Cryopreservation by dehydration using air drying and sucrose

To examine the potential of dehydration by air drying, small clusters (2-3 somatic embryos) of date palm cultures were aseptically taken and exposed to the air flow of the laminar air-flow cabinet for 20, 40, 60 and 80 minutes. Also, sucrose was employed as osmotic agent in dehydration of date palm somatic embryo cultures in order to investigate its role in decreasing of freezing injury. In this experiment, somatic embryo clusters were grown in MS medium without growth regulators and contained 0.5, 1.0 and 1.5 M of sucrose for one week. The cultures dehydrated by both air drying and osmoticum by sucrose were transferred to 2.0 ml sterilized cryotubes. Each treatment was consisted of 20 replicates. The cryotubes (which contained small clusters of somatic embryos) were cryostored for two months.

2.3. Thawing, viability and regrowth of cryopreserved cultures

After two months of cryostorage, cryotubes included somatic embryo cultures were taken from liquid nitrogen container and rapidly thawed in water bath at 37°C for almost two to three minutes till the ice has melted according to the methods described by **Bekheet [22].** After that, the explants were moved onto MS + 1 mg/l 2,4-D + 3 mg/l 2ip which incubated under standard conditions of illumination and temperature for one week. Viability was investigated using 1% of 2,3,5-triphenyltetrazolium chloride (TTC). Samples were scored as alive (survival %) if the tissues stained a pink or red color and dead if they remained white color. Also, regrowth (% of cultures that showed signs of further development) frequencies were recorded after four weeks of incubation at standard growth conditions.

2.4. Recovery of cryopreserved cultures

For recovery of the cryopreserved cultures, somatic embryos of each dehydration treatment that showed signs of further development were transferred onto glass jars contained 20 ml of MS + 1 mg/l 2,4-D + 3 mg/l 2ip + 0.5 mg/l Gibberellic Acid (GA3) and amended with 50 g/l sucrose (according the results of our primary experiments) and then incubated under standard conditions. Repetitive somatic embryo frequency (number of secondary embryos per cluster), percentage of embryo germination and number of proliferated sheetlets per cluster were recorded after 10 weeks of incubation.

2.5. Media composition and environment conditions Media were augmented with 0.7% agar and fortified with 30 g/l sucrose (except recovery medium), (L^{-1}) 170 mg NaH_2PO_4, 100 mg myo-inositol, 200 mg glutamine, 5 mg thiamine-HCl, 1 mg nicotinic acid, 1 mg pyridoxine-HCl **[22]**. The pH was adjusted to 5.8 before autoclaving at 121 °C and 1.5 Ib/M^2 for twenty-five minutes. In all experiments, the hormones were supplemented to media before autoclaving. For establishment in vitro cultures and somatic embryo formation, the cultures were incubated at 25 ± 2 °C under dark conditions. While, incubation at sixteen hours photoperiod obtained by white fluorescent (3000 lux light intensity) was used for viability, re-growth and recovery.

2.6 Histological analysis

To determine cellular change resulted from cryopreservation, specimens of the date palm somatic embryos cryopreserved by the two dehydration methods in addition to the non-cryopreserved embryos were fixed in FAA (ten ml formalin, five ml glacial acetic acid, and thirty-five ml distilled water and fifty ml ethyl alcohol 95 %) for, at minimum, two days. The samples were treated with 50% ethyl alcohol and subsequently dehydrated in normal butyl alcohol series. Thereafter, they were embedded in paraffin wax of melting point 56 C. LeicaRM2125 microtome was used to section them at a thickness of twenty micron, double stained with safranin and fast green, cleared in xylene and finally mounted in Canada balsam. Samples were analyzed microscopically and photomicrography.

2.7. Experimental design and statistical analysis

Statistical analysis was performed using Statistix (version 8.0). One-way analysis of variance (ANOVA) was used for statistical analysis. Three replicates were used for each treatment. Data were presented as means ± standard deviation (SD) and were compared with Least Significant Difference (LSD) test at a 5% probability level.

3. Results

3.1. Cryopreservation of somatic embryos by dehydration using air drying

3.1.1. Effect of dehydration using air drying on survival and re-growth

The experiment was conducted to investigate the impact of dehydration by air-flow on survival and re-growth of cryopreserved somatic embryo of date palm. For this purpose, the cultures were dehydrated by air-flow of the laminar air-flow cabinet for 20, 40, 60 and 80 minutes before immersion in liquid nitrogen. The obtained data demonstrate survival as well as re-growth of cryopreserved cultures was strongly affected by air drying duration. The survived embryos remain in white color or turned to green, while dead ones became brown. Data of **Figure (1)** reveal that lowest survival and re-growth rates were obtained with non-dehydrated cultures. However, the viability parameters progressively raised as growing duration of exposure to air air-flow until sixty minutes and then decreased. The maximum rates of survival (50 %) and re-growth (40 %) were observed with sixty minutes exposure to air-flow cabinet **(Figure 1)**. The viable somatic embryos were able to grow and obviously increased in size.

3.1.2 Effect of dehydration using air drying on recovery

In this part of study, the effect of exposure duration to air-flow on recovery parameters of cryopreserved somatic embryos was investigated. Different treatments of dehydration time i.e., 20, 40, 60 and 80 min. were compared to control (0.0 exposure duration). Results presented in **Table (1)** show that recovery parameters i.e., number of secondary embryos per cluster, embryos germination percentages and numbers of proliferated shootlets per cluster are significantly increased as increasing exposure period to air-flow. The highest number of second embryos (5.70), embryos germination (80%) and number of proliferated shootlets (5.00) were obtained with 60 min exposure period. Data exhibited in **Figure (2)** obviously refer that the recovered somatic embryos were able to differentiate and develop into healthy plantlets. On contrast, the most lessened growth parameters of recovery were registered with control (0.0 exposure time) treatment (**Table 1**). It is worth to mention that, our protocol for dehydration is less time and labor-consuming than other methods.

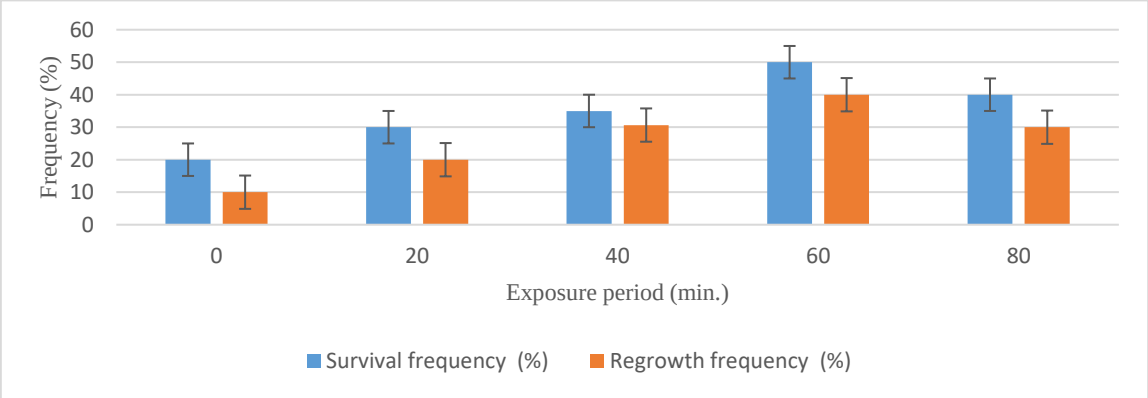

Figure 1. Effect of dehydration caused by air-flow cabinet on survival and regrowth of cryopreserved somatic embryos of date palm.

Table 1. Recovery of somatic embryos of date palm cryopreserved by air-flow cabinet.

Exposure period (min.)	No. second embryos/ cluster	Embryos germination (%)	No. proliferated sheetlets/ cluster
0.0	2.00 ±0.6^{b}	10 ±1.9^{c}	1.80 ±0.5^{c}
20	3.30 ±0.5^{b}	40 ±8.7^{c}	2.50 ±0.7^{b}
40	4.60 ±0.8^{a}	60 ±5.0^{b}	3.00 ±0.8^{a}
60	5.70 ±0.11^{c}	80 ±10.0^{a}	5.00 ±0.9^{a}
80	4.10 ±0.9^{a}	65 ±10.0^{ab}	4.00 ±0.8^{a}

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

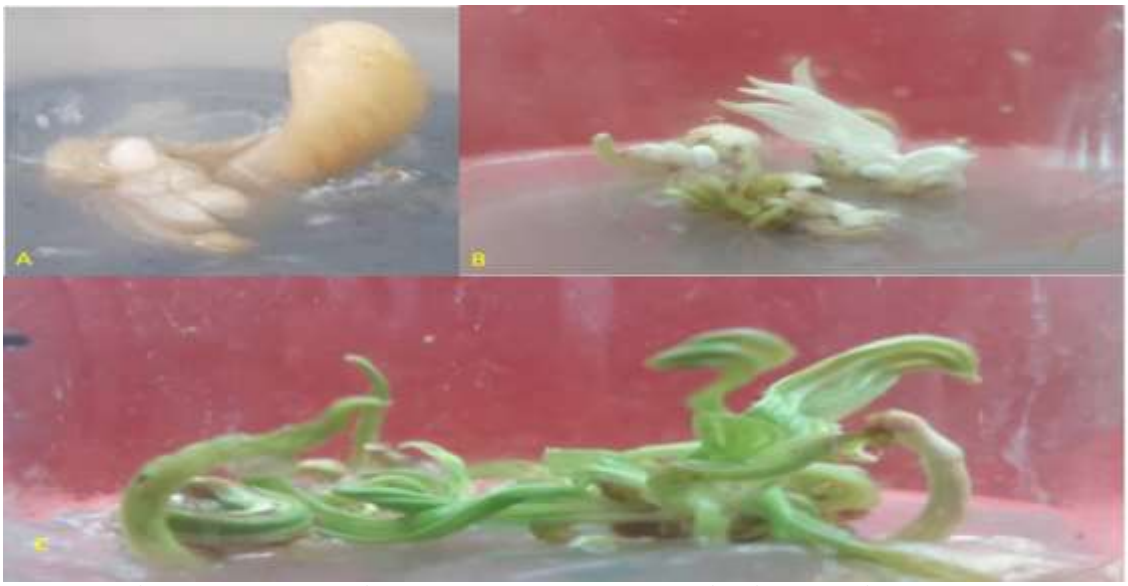

Figure 2. Proliferation and germination of somatic embryos cryopreserved by air-flow for 60 min. after three A), six (B) and ten weeks (C) of culturing on recovery medium contained MS + 1 mg/l 2,4-D + 3 mg/l 2ip + 0.5 mg/l GA3.

3.2 Cryopreservation by dehydration using sucrose as osmotic agent

3.2.1 Effect of sucrose concentration on survival and re-growth

In this investigation, sucrose in 0.5, 1.0 and 1.5 M levels was used as osmotic agent in cryopreservation of date palm somatic embryos. Data presented in Figure (3) indicate that 80 % of non-treated cultures were killed by freezing. Noticeable variations in survival re-growth of cryopreserved somatic embryos were obtained among the various concentrations of sucrose used for osmotic dehydration. Obviously, the results indicate that increasing sucrose levels led to obtain high survival rates after cryostorage. Not only produced high survival after cryopreservation, dehydration treatments with sucrose also increased re-growth rates which observed as healthy and developed cultures. It is apparent from Figure (3) that the greatest values of survival (50 %) and re-growth (45 %) were registered when somatic embryos were dehydrated by 1.0 M sucrose prior to cryopreservation.

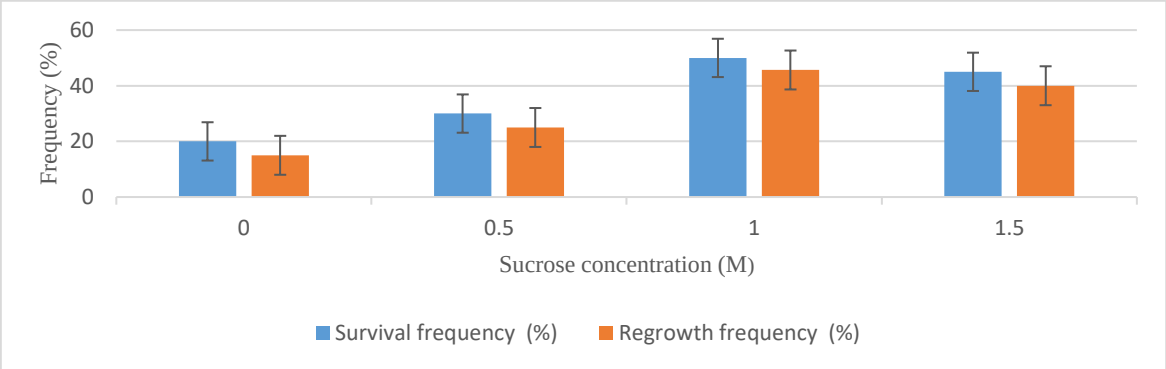

Figure 3. Effect of dehydration using sucrose on survival and regrowth of cryopreserved somatic embryos of date palm.

3.2.2 Effect of sucrose concentration on recovery

In this part of study, assessment of recovery parameters of the somatic embryos of date palm cryopreserved by dehydration resulted from sucrose treatments were carried out. The findings of the current study indicated the positive impact of sucrose on the recovery parameters i.e., number of secondary embryos per cluster, percentages of embryos germination and numbers of proliferated shootlets. In comparison to control (0.0 sucrose), all the recovery parameters were significantly affected by sucrose concentrations. Maximum number of second embryos per cluster (5.90) was registered when somatic embryos were dehydrated with 1.0 M sucrose (Table 2 and Figure 4). The results also reveal that 85 % of the embryos were able to germinate into propagule without necrosis at 1.0 M sucrose dehydration. However, treatment with 1.5 M sucrose for dehydration induced the highest number of multiplied shootlets (5.50) after ten weeks on recovery medium (Table 2). Regarding the effect of the dehydration caused by sucrose on recovery of somatic embryos of date palm, the registered growth parameters were relatively higher comparing to those observed with dehydration caused by air-flow cabinet. Depending on our finding, we can conclude that dehydration using sucrose is a simple and effective method for cryopreservation of date palm tissue cultures by osmotic dehydration.

Table 2. Recovery of somatic embryos of date palm cryopreserved by sucrose.

Sucrose concentration (M)	No. second embryos/ cluster	Embryos germination (%)	No. proliferated shootlets/ cluster
0.0_	1.90 ±0.7[b]	10 ±1.5[b]	1.50 ±0.6[a]
0.5	3.50 ±0.9[c]	60 ±7.7[c]	2.60 ±0.5[b]
1.0	5.90 ±0.5[a]	85 ±9.0[a]	5.00 ±0.3[c]
1.5	4.10 ±0.10[ac]	70 ±10.0[ac]	5.50 ±0.9[c]

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

Figure 4. Proliferation and germination of somatic embryos cryopreserved by 1 M sucrose as after three A), six (B) and ten weeks (C) of culturing on recovery medium contained MS + 1 mg/l 2,4-D + 3 mg/l 2ip + 0.5 mg/l GA3.

3.3 Histological analysis of cryopreserved somatic embryos

In this part of study, histological investigation was performed on somatic embryos of date palm submitted to a cryopreservation process using dehydration. The observations indicated that some cells of date palm plant were injured during cryopreservation by dehydration methods. Cross sections of non-cryopreserved cells proved the existence of a complete outer layer of the cell wall (**Figure 5**), while the injured cryopreserved cells demonstrated the breakage of this layer (**Figure 6**). Also, one manifestation of injures, the cytoplasm ruptured and had collapsed, composing of deformed cells. In contrast, the healthy cells had small vacuoles and normal density cytoplasm (**Figure 5**). However, the damaged cells still have the possibility to regenerate as long as there is a cell that can survive and develop new cells. The results obtained showed that date palm somatic embryos withstand freezing in liquid nitrogen as intact structures. Moreover, the cells suffered some damage could divide and restore the culture. Histological observations revealed that the optimized dehydration protocol caused somewhat cellular damage within the cells because cryoprotection help to reduce ice crystal formation during exposure to liquid nitrogen.

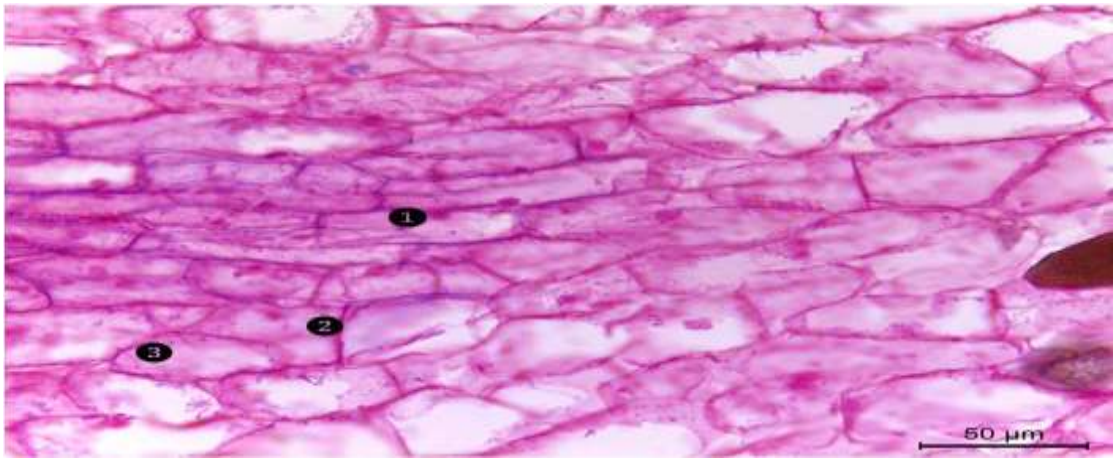

Figure 5. Cross section of non-cryopreserved somatic embryos showing normal cells (1: small vacuoles, 2: normal cell wall and c: normal cytoplasm density).

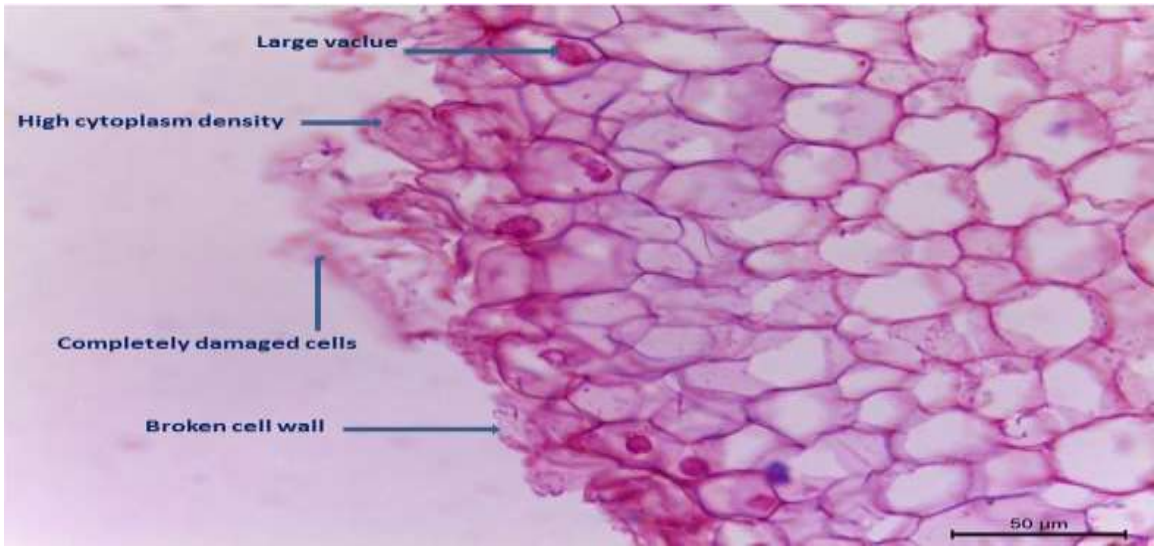

Figure 6. Cross section of cryopreserved somatic embryos of date palm showing some cellular damages caused by cryostorage.

4. Discussion

Dehydration is important for the plant materials to reach suitable water content for the subsequent cryopreservation procedure. In this respect, air drying is known as a very fast and simple method can be used for dehydration of explants that initially contained a relatively large amount of water. In the current study, the effect of dehydration by air-flow on survival and recovery of cryopreserved somatic embryo of date palm was examined. The highest rates of survival and re-growth were observed with 60 min exposure to air-flow cabinet. Also, the maximum recovery parameters presented as number of secondary embryos, embryos germination percentages and numbers of proliferated shootlets were obtained with 60 min exposure duration. This protocol was in line with a previous study **[17]** where air flow (10-50 minutes) was used for dehydration of nodular cultures of date palm before cryopreservation. Cultures of nearly 65% water content obtained from twenty minutes air drying period recorded the maximum frequency of survival and *in vitro* conversion to plantlets. In other study on cryopreservation of somatic embryoids of date palm, **Mycock *et al.* [23]** reported that drying samples within the range of 0.7-1.2 g.g-¹ permitted a 32% survival rate. In this respect, **Normah *et al*. [24]** mentioned that drying the embryonic axes of rubber seeds in laminar flow air decreased the axis moisture content and viability reached 87% at 3 hours exposure time. Recently, a simple cryopreservation method of oil palm was established for zygotic embryos using air-dried for three days without any complicated chemical pre-treatments before cryopreservation **[25]**. In this respect, physical dehydration using silica gel has also been demonstrated on cryopreservation of date palm pollen **[26]**.

Dehydration using osmotic agent such as sucrose are used to remove water from the intracellular space of plant cells and transform the cytoplasmatic solution of the cells to amorphous phase during cryopreservation. In the present study, three levels (0.5, 1.0 and 1.5 M) of sucrose were used as osmotic agent in cryopreservation of date palm somatic embryos. The results reveal that, the greatest values of survival (50 %) and re-growth (45 %) were obtained with dehydrated using 1.0 M sucrose. On the other hand, the survival rate declined at a higher concentration of sucrose (1.5 M), proving that over-dehydration might cause damage. Regarding, recovery parameters, significant differences were detected between sucrose levels. Maximum number of second embryos and the highest percentage of embryo germination were registered at 1.0 M sucrose dehydration. However, treatment by 1.5 M sucrose gave maximum number of proliferated shootlets per cluster. The exhibited data proved that, dehydration of somatic embryos of date palm caused by sucrose were relatively better comparing to dehydration caused by air-flow cabinet. The results of this study are closed with those obtained by **Solliman *et al.* [19]**. In their study on cryopreservation of undifferentiated tissue cultures of date palm using dehydration by sucrose, they reported that the maximum survival rate (80%) and switch to plants (75%) were recorded with 1 M sucrose. A study on cryopreservation of date palm meristems shows that survival rates following cryopreservation might be raised by pre-culturing the tissues on media that included elevated levels of sugar **[27]**. Likewise, desiccation of oil palm somatic embryos was combined with pre-growth in sucrose solutions to improve survival after cryopreservation **[28]**. In this respect, sucrose the non-penetrating cryoprotectant was found to withdraw water from cells by osmosis **[29]**. This dehydrating action increase intracellular viscosity and consequently reduces the amount of 'free' water available for lethal intracellular ice-crystal formation. Recently, **Ma *et al.* [30]** in their study on cryopreservation of embryogenic tissues of larch plant reported that the optimized protocol of cryoprotection involves pre-culturing on medium with 0.4 M sorbitol for 48 hours, followed by treatment with 10% DMSO, 0.4 M sucrose, and 15% PEG6000 for 1hour**.**

Cryopreservation methods (dehydrating, freezing, and thawing) could cause physical and physiochemical changes, that may lead to cell damage. Many of these changes can be avoided by cryoprotection optimization or cell repairing during recovery. Moreover, determine the cellular response to cryopreservation steps might participate in promoting the success of the protocol. In our investigation, histological analyses were performed to evaluate the tissue damages that happened throughout dehydration

and freezing of date palm somatic embryos. The obtained results indicate that some cells were injured during cryopreservation by dehydration methods. These injures involved broken of cell walls and increasing vacuole size and cytoplasm density and the cells suffered some damage could divide and regenerate into normal tissues. The reduction of cellular damages within the cells proved that cryoprotection using dehydration help to avoid ice crystal formation during storage in liquid nitrogen. Our data are in accordance with those of **Zeng *et al*. [15]** in their study on cryopreservation of papaya. They mentioned that shoot tips showed plasmolysis throughout pretreatment and dehydration and some cells were damaged. The cryopreserved cells appeared normal after culturing on recovery medium. Likewise, recovery from freezing has been investigated using traditional histological methods to assess structural changes in the crown and leaves tissues in numerous plants **[31, 32]**. The ultrastructure of apple meristem cells was studied before and after cryopreservation **[33]**. Small vacuoles were noted in cryoprotected meristem cell. Furthermore, overall treatments caused the cell size to increase when compared with the in treated cells and cells that were regrown after cryopreservation. In cryopreservation of banana meristem tips, **Helliot *et al*. [14]** mentioned that the highly vacuolized cells had lethal damage compared to those with small vacuoles. In this respect, determining viability via a histological tool may assist in observing the correlation between the remaining meristematic regions and cellular vacuolation during a later stage of recovery **[18, 34]**.

5. Conclusion

This study recognizes a simple protocol for cryopreservation of date palm somatic embryos by dehydration. It has been shown that dehydration by both air drying or sucrose osmoticum increased viability, regrowth and recovery of cryopreserved date palm somatic embryos. The survival somatic embryos after cryopreservation were able to germinate into healthy propagule. Histological analysis reveals that cryoprotection by the two methods of dehydration caused minimal cellular damage within the cells of date palm somatic embryos. The obtained results proved that this protocol of cryopreservation can be used for long-term storage as well as clonal production of date palm germplasm.

Conflict of interest

There are no conflicts of interest.

Acknowledgement

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