# EFFECT OF PROLINE IN COMPARISON TO PVS2 IN CRYOPRESERVATION OF SOMATIC EMBRYO AND CALLUS CULTURES OF DATE PALM

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## Abstract

In this work, the amino acid proline was investigated as cryoprotectant in cryostorage of date palm, *Phoenix dactylifera* L., tissue cultures. Among various levels of proline, it was found that 15% is the most effective one on survival. This concentration was compared to Plant Vitrification Solution 2 (PVS2). Generally, the maximum viability percentages of the two types of cryoprotectants were registered at 60 min exposure period. Proline was superior to PVS2 on callus viability. Moreover, healthy and good appearance calli were observed with the proline treatments. In contrast, the highest viability percentages of somatic embryos were observed with PVS2 solution. Regarding regrowth, the recovery parameters of both callus and somatic embryos increased as exposure period increased till 60 min and then decreased. The maximum callus fresh mass and growth value were registered when callus was cryoprotected by 15% proline. With respect to somatic embryos, proline gave good differentiation percentages, but they were slightly lower than PVS2. The highest differentiation percentage (70%) was recorded with PVS2 for 60 min in cryoprotection. However, the highest numbers of proliferated shootlets were recorded with proline in cyoprotection.

***Key words**:* Date palm; Cryopreservation; Proline; Shootlets; PVS2.

## INTRODUCTION

The date palm (*Phoenix dactylifera* L.) is considered one of the most important fruit crops cultivated in arid regions. Besides utilization of the tree by-products, date palm fruits possess high nutritional values (Barreveld 1993). Moreover, date palm tree is playing an important role fighting desertification in Egypt since it has represents a significant part in the reclamation programme. As the heterozygous species, date palm is commercially propagated vegetatively by offshoots. Low number of plants is produced from mother tree by the reproduction method. Moreover, conservation of date palm germplasm in the form of offshoots or in field is difficult due to high cost of farming practices. The in vitro culture techniques offer advantages over the vegetative methods of propagation for a rapid and large scale multiplication of superior cultivars of date palm. Furthermore, biotechnology techniques are potentially used for the preservation of date palm genetic resources. At this point, date palm germplasm has been conserved under aseptic conditions using different types of explants (Bekheet *et al*. 2002, Bekheet *et al.* 2017a, de Oliveira *et al.* 2021, Metwali *et al.* 2020). These types of explants allow reduction in space requirements and simplify the quarantine procedures of germplasm samples. Cryopreservation, the storage at very low temperatures (between -79ºC and -196ºC) is applied for long duration storage of plant accessions. The in vitro cryopreserved plant materials don't require subculture and hypothetically the hereditary material can stay flawless for an uncertain period (Kaczmarczyk *et al.* 2008). In this respect, numerous cryopreservation protocols have been optimized for in vitro storage of

different plant species (Burritt 2008). The cryopreservation procedure comprises a number of steps including preculture in media with osmotically active compounds, treatment with cryoprotective agents, storage at -196°C, thawing, and recovery of growth. However, successful cryopreservation requires the optimization the type and concentration of cryoprotectant. Cryoprotectants are chemical compounds which prevent plant cells or tissues from damage due to freezing. They reduce the ice formation at any temperature by increasing the total concentration of all the solutes present in the cells. The most commonly employed cryoprotective substances are glycerol and dimethylsulfoxide (DMSO). Generally, cryoprotectant must be able to penetrate the cell and cause osmotic dehydration. Also, cryoprotectant must be non-toxic to the cell at the concentrations required for their efficacy (Sakai *et al.* 1990). The most common cryoprotectants used in plant cryopreservation are sucrose, glycerol, DMSO, methanol (and sometimes ethanol) and glycols.

Because of its ability to act as an osmoprotectant, proline has been used in cryoprotection of plant cultures. Proline enters into plant cells to avoid the osmotic injury caused by dehydration during cryopreservation (Liu *et al.* 2021). In addition, proline reduces mechanical injury to cells by breaking hydrogen bonds between ice structures (Yang *et al.* 2017). Numerous studies have recognized the importance of proline for plant cold tolerance (Duncan and Widholm 1987, Ait-Barka and Audran 1997, Hoffman *et al.* 2010). Moreover, proline which has been implicated in protection against cold stress in a wide range of plants is nontoxic at high concentrations (Javadian *et al.* 2010). Meanwhile, several studies show that cold stress induces the accumulation of amino acids, such as proline and glutamine, which play important protective roles in freezing tolerance (Taji *et al.* 2002, Cook *et al.* 2004, Kaplan and Guy 2004). Proline contributes to stabilizing sub-cellular structures (*e.g.* membranes and proteins) and buffering cellular redox potential under stress conditions (Ashraf and Foolad 2007). In early reports, we developed different protocols for the cryopreservation of date palm using traditional cryoprotectants, *i.e*. DMSO, ethylene glycol and glycerol (Bekheet *et al.* 2007, Bekheet 2017b). The main problem of such compounds is their toxicity effects especially at high level exposure. The present work aims to investigate the role of proline as cryoprotectant in the survival and regrowth of cryopreserved tissue cultures of date palm cv Samany.

## MATERIAL AND METHODS

In vitro cultures (callus and somatic embryos) of date palm cv Samany were established using shoot tip explants excised from offshoots. Shoot tips were trimmed off till reached 30 cm and then transferred to the laminar flow cabinet for surface sterilization. The gradual removal of white young leaves resulted in 5-6 cm shoot tips and these were kept in a cold sterilized antioxidant solution of citric acid (150 mg/l) and ascorbic acid (100 mg/l) to prevent browning. The shoot tips were disinfested with 50% (v/v) commercial sodium hypochlorite for 20 minutes and then these were rinsed thoroughly with sterile distilled water three times. The apical meristems (about 2 cm long and 1 cm wide) were excised and cultured in glass jars contained 20 ml of tissue culture medium. The culture medium consisted of Murashige and Skoog (1962) (MS) basal medium plus ( $L^{-1}$) 170 mg sodium dihydrogen phosphate ($NaH_2PO_4$), 100 mg myo-inositol, 200 mg glutamine, 5 mg thiamine-HCl, 1 mg nicotinic acidand 1mg pyridoxine-HCl. For callus induction (Fig. 1A), 10 mg/l 2, 4-dichlorophenoxyacetic acid (2, 4-D) + 3 mg/l 6-(γ,γ-dimethylallylamino) purine (2iP) were used. However, 1mg/l 2.4-D + 0.5mg/l 2ip + 1 mg/l

naphthaleneacetic acid (NAA) were added into culture medium for proliferation of somatic embryos (Fig.1B). Cultures were maintained by sub-culturing every month on their induction media.

In order to identify the suitable level of proline to be such as a cryoprotectant solution in date palm cryopreservation, different concentrations, *i.e*. 5, 10, 15 and 20%, were investigated. Callus segments and somatic embryo clusters were treated with the proline concentrations for one hr prior to following cryopreservation procedures and then they were cryostored in a liquid nitrogen container. The percentage of explants that survived and remained viable (creamy colour for callus and white or green colour for somatic embryo clusters) was calculated from three replicates (20 explants).

For cryopreservation using proline and PVS2, callus inocula (about 250 mg) and somatic embryo clusters taken from 2 weeks sub-cultured date palm tissue cultures were used as cryopreservation plant materials. The explants were pre-cultured on growth regulator-free MS-medium containing 2M sucrose for 3 days and incubated at ±25°C under 16/8 h (light/dark) photoperiod. The pre-treated cultures were placed in sterile cryovials (2 ml) (Fig. 1C) and loaded with 1 ml of 2M glycerol plus 0.4M sucrose in a liquid MS medium for 60 min. The loading solution was then removed using a sterile syringe and then the explants were exposed to Plant Vitrification Solution2 (PVS2) which consisted of 30% glycerol, 15% ethylene glycol, 15% DMSO (all v/v), and 0.4M sucrose (Sakai *et al.* 1990) or proline (15%) for 20, 40, 60 and 80 min as cryoprotectants. After removing the cryoprotectants, the cryovials containing the explants were immersed directly into a liquid nitrogen tank.

For thawing and recovery, cryovials with frozen date palm tissue cultures, *i.e*. callus and somatic embryos were taken after two months of cryopreservation and thawed in water bath at 37°C for about 2-3 min until the ice melted. Then the vials were filled with 1ml MS liquid medium plus 1M sucrose (unloading solution) for 30 min at room temperature and then the explants were blotted on a piece of sterile filter paper. The cultures were transferred onto recovery media (MS + 10mg/l 2,4-D + 3mg/l 2iP for callus and MS + 1mg/l 2.4-D + 0.5mg/l 2ip +1mg/l NAA for somatic embryos) and the cultures were incubated on normal incubation conditions. The viability of the cryopreserved date palm cultures was performed using the 2, 3, 5-triphenyltetrazolium chloride (TTC) test. Samples were taken after one week of culturing on recovery media and washed three times in distilled water to eliminate all the traces of nutrient solution. The samples were put into 10 ml vials containing 3 ml 1% TTC solution and then incubated on a rotary shaker (100 rpm) at room temperature in the dark for 6h. TTC solution was discarded and the cultures were rinsed with sterile distilled water. Cultures were scored as alive (viability %) if the tissues stained a pink or red color and dead if they remained white.

The viable cultures taken from the best treatments of exposure to the two types of cryoprotectants (PVS2 and proline at the four exposure periods) were cultured on the standard media and incubated at normal growth conditions. To assess the effectiveness of proline in comparison to PVS2 on recovery of the cryopreserved cultures, various parameters were recorded after 10 weeks of culturing including fresh mass and growth value (for callus), and differentiation (%) and number of proliferated shootlets (for somatic embryo cultures). Tissue culture media also contained 0.7% agar and 30 g/l sucrose and pH was adjusted to 5.8 before autoclaving at 126ºC and 1.5 Ib/M2 for 20 min. Cultures were incubated in growth chamber at 25±2ºC under dark condition for cultures induction and later at sixteen hour light

(2000 Lux) and eight hour dark for cultures maintenance. Data were statistically analysed from 20 replicates using standard error (SE) (Snedecor and Cochran 1967).

$$\text{Growth value} = \frac{\text{Final fresh mass - Initial fresh mass}}{\text{Initial fresh mass}}$$

## RESULTS AND DISCUSSION

The survival and regeneration rates are key factors determining the success rate of cryopreservation protocols. In this part of study, survival of callus and somatic embryo cultures of date palm cv. Samany in response to cryopreservation using different concentrations of proline was investigated. The data in Table 1 showed that survival percentages tended to increase with increasing of proline up to 15% and then decreased. At low proline level (5%), high percentages of the cryopreserved callus and somatic embryo cultures turned brown and then died. On the other hand, obvious variations in the survival percentages of callus and somatic embryo cultures were obtained suggesting that survival is affected by the culture type (Table 1). This variation may be due to the difference between the two explants in their contents of the intercellular water. The highest percentage (70%) of survival was registered by exposing somatic embryos to 15% of proline before cryostorage in liquid nitrogen. The obtained results demonstrated that the differentiated cultures (somatic embryos) were more responding to exposure to proline as cryoprotectant comparing with the non-differentiated (callus) cultures. Also, the results proved the applicability of using the amino acid proline as cryoprotectant in cryostorage of date palm tissue cultures.

**Table 1. Survival of callus and somatic embryo cultures of date palm cv. Samany after cryoprotection using proline.**

| Proline concentration (%) | Survival (%) | |
|---|---|---|
| | Callus | Somatic embryos |
| 5 | 20±5.00 | 25±10.00 |
| 10 | 45±7.50 | 50±8.20 |
| 15 | 65±6.80 | 70±5.90 |
| 20 | 55±9.10 | 60±7.00 |

± Standard Error (SE)

Cryoprotectors are chemicals which decrease cryodestruction. The cryoprotectant commonly used is DMSO; however, this is not appropriate for all cell lines. Also, the toxicity of traditional cryoprotectants, such as glycerol and DMSO prompt the search for other compounds. In this respect, proline has also been used for cryopreservation of callus, meristems and embryos cultures (Nanjo *et al.*1999). In the present investigation, the role of proline in decreasing freezing injury in date palm tissue cultures was effective and clear. Increasing proline till 15% led to obtain highest survival rates with the two types (callus and somatic embryo) of date palm tissue cultures. This illustrates the importance of recognize appropriate proline concentration. However, taking into account the entire protocol of cryopreservation, the method used here for cryoprotection is cheap and safe comparing with other cryoprotection methods. Many reports showed the efficiency of proline in the cryostorage of plant tissue

cultures since exogenously addition of proline can enhance freeze-resistance. In this regard, proline was found to be an effective cryoprotectant for the cryostorage of cultured cells of *Zea mays* L. (Withers and King 1979). In this context, Jain *et al.* (1996) used proline in the cryopreservation of embryogenic cells of aromatic indica rice varieties. Also, addition of abscisic acid and proline to the pre-treatment medium improved the percentage of shoots surviving freezing of *Begonia* species (Burritt 2008). In this respect, Kleinhans (1998) mentioned that the degree to which cells shrink and re-swell after addition of a membrane-permeable cryoprotectant depends on the concentration of the cryoprotectant and the relative permeability of the membrane to water and to the cryoprotectant.

In cryopreservation of plant materials, it is essential to recognize suitable method to identify the viable plant cells. In this experiment, viability of the cryopreserved date palm tissue cultures, *i.e*. callus and somatic embryos were performed using TTC test. Our observation indicated that different frequencies of viability (cultures with red colour) were obtained after cryostorage of callus and somatic embryos of date palm cv. Samany. The lowest frequencies of viability were registered at 20 min exposure period to the cryoprotectants, *i.e*. proline and PVPS2 solutions. The viability percentages of both two types of date palm tissue cultures increased as increasing of exposure time to the cryoprotectants till 60 min and then decreased (Table 2) the highest value of callus viability (60%) was registered when callus cultures treated with proline for 60 min. However, the best viability value of somatic embryos (70%) was observed with cryoprotection with PVS2 for 60 min before cryostorage. It is important to mention that, viability of callus treated with proline as cryoprotectant was higher (mean 45.25%) than PVS2 (mean 40.00%). In contrast, the highest viability percentages of somatic embryos were observed with PVS2 (Table 2 and Fig. 1D).

**Table 2**. **Viability of callus and somatic embryo cultures of date palm cv. Samany after cryopreservation using proline and PVS2 as cryoprotectants.**

| Exposure time (min) | Viability (%) | | | |
|---|---|---|---|---|
| | Callus | | Somatic embryos | |
| | Proline | PVS2 | Proline | PVS2 |
| 20 | 30±3.30 | 25±4.00 | 30±2.50 | 35±1.70 |
| 40 | 45±5.00 | 40±5.40 | 45±4.70 | 55±5.00 |
| 60 | 60±4.50 | 55±5.30 | 65±3.80 | 70±5.60 |
| 80 | 50±5.10 | 40±4.10 | 55±4.90 | 50±4.00 |
| **Mean** | **45.25±4.47** | **40.00±4.72** | **48.75±3.97** | **52.50±4.07** |

± Standard Error (SE)

The viability of the cryopreserved plant tissue is highly dependent on the effeteness of cryoprotectant. The general properties of cryoprotective compounds are that they have low molecular weight and can permeate plant cells. In this investigation and to compare the efficiency of proline and PVS2 as cryoprotectants, the viability of the cryopreserved date palm tissue cultures, *i.e*. somatic embryos and callus were assessed using the TTC test. The viability of the cultures after cryopreservation was assessed by the visual observation of growth. The viability of the cultures largely increased with exposure duration and reached a maximum when treated for 60 min and then decreased at 80 min. The observation proved that, proline is effective in the cryopreservation of date palm tissue cultures especially the non-differentiated (callus) cultures. At this point, the TTC test results indicated that the

highest viability of callus was observed with cryoprtotection with 15% proline for 60 min before cryopreservation. Also, acceptable results of viabilities was obtained when somatic embryo explants was cryoprotected with proline. These findings are in accordance with those reported by Withers and King (1979). In their study on cryopreservation of *Zea mays*, they reported that proline has a potential in the recovery of cryopreserved cell in comparison to dimethylsulfoxide or glycerol. Similarly, Burritt (2008) mentioned that freeze tolerance can be achieved using medium contained proline prior to cryopreservation. In their study on cryopreservation of in vitro grown *Prunus* rootstock, Brison *et al.* (1995) added dimethylsulfoxide and proline to a pre-culture medium prior to the cryostorage. In sweet potato and black spruce, the optimal exposure time to cryoprotection solution was reported to be 60 min at 25°C (Hirai and Sakai 2003, Touchell *et al.* 2002).


Fig. 1. Pictorial presentation: **A**. Callus induction on MS medium plus 10mg/l 2,4-D + 3mg/l 2iP; **B**. Somatic embryo proliferation on MS medium 1mg/l 2.4-D + 0.5mg/l 2ip + 1mg/l NAA; **C**. The cryoprotected cultures in cryovials; and **D**. Viable somatic embryos cryoprotected by PVS2.

After cryopreservation using proline or PVS2 as cryoprotectants, the regrowth of callus and somatic embryos of Samany date palm cultivar was investigated. Data of Table 3 reflected the effect of proline and PVS2 on fresh mass, growth value of the recovered callus cultures. The obtained results revealed that viable callus cultures grew well, and both fresh mass and growth value increased with the increasing of exposure time to both cryoprotectants till 60 min and then decreased. It was noticed that the growth parameters of callus cultures were higher with proline at all exposure periods in comparison to PVS2 with 547.50 mg average fresh mass and 1.19 average growth values. Moreover, healthy and good appearance calli were observed with the proline treatments. This may be due to the non-toxicity of proline on the cultured cells. The highest fresh mass (700 mg) and growth value (1.80) were obtained

when callus was cryoprotected by proline (15%) for 60 min (Table 3). In contrast, the most lessened growth parameters of callus were observed with cryoprtection with PVS2 for 20 min. The revelations in Table 3 demonstrate that proline can be used for cryopreservation date palm callus.

**Table 3. Regrowth of callus cultures of date palm cv. Samany cryoprotected by proline and PVS2 after ten weeks of culturing on recovery medium.**

| Exposure time (min) | Regrowth | | | |
|---|---|---|---|---|
| | Fresh mass (mg) | | Growth value | |
| | Proline | PVS2 | Proline | PVS2 |
| 20 | 420±17.20 | 400±18.00 | 0.68 | 0.60 |
| 40 | 550±19.30 | 510±20.20 | 1.20 | 1.04 |
| 60 | 700±21.10 | 650±19.50 | 1.80 | 1.60 |
| 80 | 520±15.00 | 500±16.90 | 1.08 | 1.00 |
| **Mean** | **547.50±18.15** | **515.00±18.65** | **1.19** | **1.06** |

± Standard Error (SE)

With respect to somatic embryo explants, they differentiated slowly during the first three weeks of culturing on regrowth media and then resumed growth since healthy and green shootlets were obtained after eight weeks. The results presented in Table 4 indicate that differentiation percentages in PVS2 treatments were higher compared to proline. In contrast, the numbers of proliferated shoottlets were slightly higher when the cultures were cryoprotected by proline solution. As it was found in cryoprotection of callus, 60 min exposure time gave the best results of the growth parameters in both types of cryoprotectants, *i.e.* proline and PVS2. The highest differentiation percentage (70%) was recorded with cryoprotection with PVS2 for 60 min. However, the maximum number of proliferated shoottlets (7.10) was obtained with cryoprotection by proline for 60 min. A remarkable reduction of the two growth parameters (differentiation percentage and number of proliferated shoottlets) were obtained with 80 min exposure time. The results proved that proline is effective as cryoprotectant in cryostorage of somatic embryos cultures of date palm.

**Table 4. Regrowth of somatic embryos of date palm cv. Samany cryoprotected by proline and PVS2 after ten weeks of culturing on recovery medium.**

| Exposure time (min) | Regrowth | | | |
|---|---|---|---|---|
| | Differentiation (%) | | Proliferated shootlets | |
| | Proline | PVS2 | Proline | PVS2 |
| 20 | 30 ±5.10 | 40±7.20 | 4.00 ± 0.20 | 3.50 ± 0.24 |
| 40 | 50±3.50 | 55±2.80 | 5.20 ± 0.27 | 5.00 ± 0.32 |
| 60 | 65±1.90 | 70±3.00 | 7.10 ± 0.52 | 6.50 ± 0.19 |
| 80 | 55±3.20 | 60±2.20 | 5.90 ± 0.30 | 6.00 ± 0.22 |
| **Mean** | **50.00 ±3.42** | **56.25 ±3.80** | **5.55 ± 0.32** | **5.25 ± 0.24** |

±Standard Error (SE)

The recovery of cryopreserved plant tissue is a critical part of the cryopreservation process and it depends on, among other factors, the subsequent culture conditions. The results of the present investigation clearly reveal the positive effect of proline on the recovery and regrowth of both callus and somatic embryos cultures of Samany date palm cultivar after cryostorage in liquid nitrogen. Application of cryoprotection with 15% proline for 60 min was the most effective concentration for combating the

harmful cryostorage injury since high growth parameters of the two types of date palm tissue cultures were observed at this treatment. In comparison to PVS2, proline had higher positive effects on regrowth of callus culture of Samany date palm cultivar. The results are in line with those obtained by Marković *et al.* (2013) on the grapevine that used 50 or 50µM proline in pre-treatment for cryoprotection of in vitro grown shoot cultures. Also, the addition of proline to cryoprotectant solution up to 86.9 mM was effective on the cell viability of *Arabidopsis thaliana* after cryopreservation (Ogawa *et al.* 2012). Likewise, pretreatment for 3-4 days using with 10-5M ABA and 100 mM proline increased survival of cryopreserved moss (Christianson 1998). Pretreatment with solution contained proline and ABA was also used for the cryopreservation of several plant species (Burritt 2012). Cryopreserved cultures remained viable for at least one year at -80°C. In several studies it was found that proline not only protects plant cells, but it also enhances their growth thereby helping the plant to tolerate cold stress (Hoque *et al.* 2007, Ashraf and Foolad 2007).

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