Brazilian Journal of Biology®

ISSN 1519-6984 (Print)
ISSN 1678-4375 (Online)

Original Article

Callus induction, shoot regeneration, and conservation of *in vitro* grown date palm (*Phoenix dactylifera*)

Indução de calos, regeneração de brotos e conservação de tamareiras (*Phoenix dactylifera*) cultivadas *in vitro*

W. G. Shahrour[a*], M. A. Shatnawi[a], R. A. Shibli[b], M. Al-Alawi[c], S. M. Abubaker[c], M. Majdalawi[d] and A. K. Alkawaldeh[e]

[a] Al-Balqa Applied University, Faculty of Agricultural Technology, Department of Biotechnology, Al-Salt, Jordan

[b] The University of Jordan, Hamdi Mango Center for Scientific Research (HMCSR), Amman, Jordan; The University of Jordan, Faculty of Agriculture, Department of Horticulture and Crop Sciences, Amman, Jordan

[c] Al-Balqa Applied University, Faculty of Agricultural Technology, Department of Plant Production and Protection, Al-Salt, Jordan

[d] Al-Balqa Applied University, Zarqa University College, Department of Basic and Applied Science, Zarqa, Jordan

[e] Al-Balqa Applied University, Zarqa University College, Department of Allied Medical Sciences, Zarqa, Jordan

Abstract

Date palm (*Phoenix dactylifera*(cv. Medjool is a significant plant, grown in Jordan. *In vitro* propagation gives operative resources for the significant propagation of date palms. Maximum callus induction was achieved from MS media supplemented with benzyl amino purine (BA) and naphthalene acetic acid (NAA). The highest plant regeneration was recorded on MS medium supplemented with dichlorophenoxyacetic acid (2,4-D) at 3.0 mg/L, and BA at 2.0 mg/L. A significant positive impact on shoot formation was recorded on MS medium supplemented with 1.0 mg/L BA with 0.5 to 1.5 mg/L NAA in both liquid and solid MS medium. To maintain survival and regrowth capacity, sucrose could be used for medium-term conservation at lower concentrations (0.1 - 0.2 M). In addition, sorbitol might be used at 0.1 M to maintain the quality of explants. The vitrification technique for long-term preservation was experimented. Embryogenic callus was used as explants for conservation. The survival as well as regrowth percentages of non-cryopreserved and cryopreserved tissue cultures were affected by their duration of treatment with the vitrification solution plant vitrification solution 2 (PVS2) and modified plant vitrification solution 2 (MPVS2). Results showed that using PVS2 for 60 minutes for cryopreserved calli was more effective than other treatments. After storage in liquid nitrogen, the highest survival rate (65%) and regrowth rate (40%) were achieved.

Keywords: conservation, date palm, embryogenic calli, *Phoenix dactylifera*, vitrification.

Resumo

A tamareira (*Phoenix dactylifera* cv. Medjool) é uma planta significativa, cultivada na Jordânia. A propagação *in vitro* fornece recursos operacionais para a propagação significativa de tamareiras. A indução máxima de calos foi alcançada a partir de meio MS suplementado com benzil amino purina (BA) e naftaleno ácido acético (ANA). A maior regeneração das plantas foi registrada em meio MS suplementado com ácido diclorofenoxiacético (2,4-D) a 3,0 mg/L e BA a 2,0 mg/L. Um impacto positivo significativo na formação de brotos foi registrado em meio MS suplementado com 1,0 mg/L BA com 0,5 a 1,5 mg/L NAA em meio MS líquido e sólido. Para manter a sobrevivência e a capacidade de regeneração, a sacarose pode ser usada para conservação em médio prazo em concentrações mais baixas (0,1 - 0,2 M). Além disso, o sorbitol pode ser utilizado a 0,1 M para manter a qualidade dos explantes. A técnica de vitrificação para preservação em longo prazo foi experimentada. Calos embriogênicos foram utilizados como explantes para conservação. A sobrevivência, bem como as porcentagens de novo crescimento de culturas de tecidos não criopreservadas e criopreservadas, foram afetadas pela duração do tratamento com a solução de vitrificação, a solução de vitrificação de plantas 2 (PVS2) e a solução de vitrificação de plantas modificada 2 (MPVS2). Os resultados mostraram que o uso de PVS2 por 60 minutos para calos criopreservados foi mais eficaz do que outros tratamentos. Após armazenamento em nitrogênio líquido, foram alcançadas as maiores taxas de sobrevivência (65%) e de rebrota (40%).

Palavras-chave: conservação, tamareira, calos embriogênicos, *Phoenix dactylifera*, vitrificação.

*e-mail: wesamshahrour@bau.edu.jo
Received: March 9, 2024 – Accepted: May 9, 2024

1. Introduction

Date palm (*Phoenix dactylifera* L.) is a member of the Arecaceae family; distributed in North Africa and the Middle East (Al-Khayri and Naik, 2017). Date palms tolerate harsh climatic and soil conditions. Moreover, *P. dactylifera* is well known to be salt tolerant, and needs a long intensively hot summer, without rain or an excessively humid atmosphere (Aschale, 2022; Abdelghaffar et al., 2023). Several methods have been used to proliferate date palms among them offshoot, seed, and *in vitro* methods. Seed methods do not warrant true-to-type palms. In comparison, using off-shoots till now is the main method to ensure true-type plants. Moreover, date palm plantations and diversity have decreased due to the effects of environmental stress such as drought, salinity, insects, and diseases. However, due to their nature, vegetative propagation in date palms is very limited. Therefore, the application *in vitro* methods would be the right choice for date palm propagation (Subaih et al., 2007; Al-Khayri and Naik, 2017; Abdalla et al., 2022). Moreover, it has become essential to develop new methods that permit the preservation of date palm genetic resources for a long time without a substantial decrease in vitality, genetic stability, and low survival.

Tissue culture offers a great advantage where different materials are propagated and preserved (Shatnawi, 2013; El-Bahr et al., 2016; Elaziem et al., 2022; Shahrour et al., 2024). Therefore, *in vitro* propagation is a valuable method because it offers a means of resolving the problems of generating large numbers of homogenous female date palm seedlings (Subaih et al., 2007; Abul-Soad and Mahdi, 2010; Aschale, 2022). When using *in vitro* techniques, it is necessary to study different factors affecting the production of homogenous explants. *In vitro* conservation offers another method of field conservation (Shatnawi et al., 2007; Aschale, 2022; Elaziem et al., 2022). *In vitro,* slow-growth techniques are highly suggested for preserving *in vitro* cultures in many conservation centers (Bekheet et al., 2007; Shatnawi, 2011; El-Bahr et al., 2016; Al-Khateeb et al., 2019).

Cryopreservation is the storage of plant materials in a cryogenic medium such as liquid nitrogen LN -196 °C (Shatnawi, 2006; Bekheet et al., 2007; Metwali et al., 2020). Because of its minimum space requirements, genetically stable remains, maintenance efforts free from pathogens, and large quantities of materials that can be conserved, cryopreservation has become a significant tool for preservationists. One of the base methods in cryopreservation is the vitrification method (Shatnawi et al., 2011; Metwali et al., 2020). The establishment of an active technique for cryopreservation can be a hard and laborious process that needs the employment of dehydration processes, cooling rate, freezing rate, thawing rate, and plant materials to accomplish the achievement of the cryopreservation method (Shatnawi, 2013; Alansi et al., 2019; Metwali et al., 2020). Different vitrification methods using protoplasts, cell suspensions, meristems, and somatic embryos are used effectively to cryopreserve several plant species (Shatnawi et al., 2004; Rabba'a et al., 2012; Araújo de Oliveira et al., 2021).

Using PVS2 solutions as a cryoprotectant solution in which living tissues can be chilled devoid of ice formation either intra or extra-cellular (Shatnawi et al., 2011). Such solutions increase the osmotic potential of the external medium (Shibli et al., 2012). The length of treatment of the explant and the vitrification solution is an important parameter that affects the survival of frozen (cryopreserved) tissue (Bekheet et al., 2007). Performing the dehydration step at 0 °C has been found to reduce vitrification solution toxicity, and a similar result on performing the dehydration step at 0 °C was reported on *Phoenix dactylifera* by Subaih et al., (2007) and Gonzalez-Arnao et al. (1988) on *Ananas comosus*. Therefore, in the current investigation, a simple protocol for callus induction, shoot regeneration, and conservation of *in vitro* growth date palm (*Phoenix dactylifera*) was initiated.

2. Material and Methods

2. 1. Location of the study and plant materials

Date palm plants collected from Jordan Valley in the southern part of Jordan (longitude: latitude, 35.487345: 31.0379772). Seeds of date palm (*P. dactylifera* cv, Majhool) were selected for this study.

2.2. Callus induction

"Seeds were treated with 6% sodium hypochlorite (5.25%). Then seeds were plunged in 70% alcohol for one minute, sterile seeds were rinsed with sterile deionized water three times. Embryos were then inoculated on MS (Murashige and Skoog, 1962; Al Shhab et al., 2022) media that were accompanied by different combinations of growth regulators (BA and NAA, or BA and 2,4-D). For comparison callus induction under light and dark conditions, all the prepared MS media with growth regulators were separated into two categories, one hold on under dark conditions and another hold on under light with photoperiods of 16/8 h light/dark, and grow at 24 ± 2 °C".

2.3. Embryogenic calli formation

The callus cultures MS media with different concentrations of BA, 2,4-D, or BA with 2,4-D in darkness for up to six months for embryogenic calli formation were used. For the growth of embryogenic calli, the callus was subculture to a new MS medium containing different combinations of NAA and BA, with 1.0 g activated charcoal on a solid medium (8 g/L agar).

2.4. Medium-term conservation

Embryogenic calli were grown on MS medium supplemented with sucrose, mannitol, or sorbitol at 0.0, 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 M in 250 mL culture vessels for 10 or 20 weeks. Each experiment was repeated three times and each experiment contained 20 replicates.

2.5. Long-term storage of date palm

2.5.1. Vitrification methods

The vitrification method was achieved by inserting embryogenic calli in cryotubes (20 replicates for each treatment with 10-12 embryogenic calli), with 1.0 ml

loading solutions for 10 or 20 minutes at 0 °C. Then loading solutions were taken out, and exchanged with vitrification solutions of plant vitrification solution 2 (PVS2) or modified plant vitrification solution 2 (MPVS2) for up to 180 minutes at 0 ºC.

1- **PVS2** containing "30% (w/v) glycerol, 15% (w/v) dimethyl sulfoxide (DMSO), 15% ethylene glycol and 14% (w/v) sucrose in MS medium (Sakai et al., 1990)".

2- **MPVS2** containing" 30% (w/v) glycerol, 20% (w/v) DMSO, 30% ethylene glycol and 14% (w/v) sucrose in MS medium" at 0 °C for 0.0 to 180 min.

2.5.2. Freezing and thawing

Rapid freezing is performed by plunging embryogenic calli directly into LN. The storage duration in LN was at least one hour (Shibli et al., 2006). Rapid thawing is performed at 45 °C in a water bath. Embryogenic calli were subcultured to MS medium hormone-free and kept in growth chamber conditions for one day. Regrowth was measured as the percentage of embryogenic calli that became green and restarted growth after eight weeks of plating out.

2.6. Experimental design

A completely randomized design was used; each treatment was repeated three times. Results were analyzed using SPSS programs version 17, at 0.05 level of probability, and Tukey"s Honestly Significant Difference (HSD) test was used (SPSS, 2017).

3. Results

Callus was initiated after eight weeks in MS media with different combinations of growth regulators (BA and NAA, or BA and 2,4-D) under light and dark conditions. Maximum callus induction was obtained on a medium supplemented with different concentrations of BA and NAA (as shown in Table 1). The highest callus formation with high somatic embryogenesis development was recorded on MS medium with 3.0 mg/L 2,4-D and 2.0 mg/L BA. In addition, shoot regeneration from calli was developed on MS medium containing both BA and 2,4-D, which was highly promoted by BA compared to other growth regulators (Table 2).

The maximum percentage of shoot formation (90%) was recorded in MS medium augmented with 1.0 mg/L BA with 0.5 to 1.5 mg/L NAA, in both liquid and solid MS medium (Table 3). Statistical analysis showed that BA had influenced shoot formation from the callus, whereas there was no shoot formation in the medium free of the hormone. The influence of BA seems to be critical to enhanced shoot formation (Table 3).

For medium-term conservation, survival and regrowth of the date palm callus decreased significantly as the concentration of sucrose increased in the medium (Table 4). The maximum survival and regrowth capacity of date palms was at concentrations of 0.1 sucrose. However, sucrose at high concentrations can decrease cell division and hinder growth and development. After 10 and 20 weeks of preservation, browning was observed on calli at 0.8 M (Table 4).

By using mannitol, no difference in survival at 0.1 or 0.2 M concentrations after ten weeks of storage periods. Increasing concentration of mannitol reduced survival and regrowth irrespective of the preservation period (Table 5). While, for sorbitol, a significant reduction in survival with increasing sorbitol concentration. Medium containing 0.1 M sorbitol resulted in 95% survival after 10 weeks, while after 20 weeks survival as 60% (Table 6).

Regards cryopreservation of embryogenic calli, significant variances were achieved in survival and regrowth of non-cryopreserved (-LN) calli when they were treated with PVS2 or MPVS2. Embryogenic calli treated with PVS2 or

Table 1. Callus induction in different treatments on *Phoenix dactylifera* after an eight-week culture period on solid media.

Growth regulator (mg/L)	Callus induction	
	Light	Dark
BA and NAA		
0.5 + 0.5	+++	+
0.5 +1.0	+++	+
0.5 + 1.5	+++	++
1.0+0.5	+++	++
1.0 + 1.0	+++	++
BA and 2,4-D		
0.5 + 0.5	+	+
0.5 + 1.0	++	+
0.5 + 1.5	+++	+
1.0 + 0.5	+	+
1.0 1.0	+	+

+ Low callus formation; ++ Medium callus formation, ++++ High callus formation.

Table 2. Effects of different growth regulator concentrations on plant regeneration medium after eight and sixteen weeks on solid media.

Growth regulator	Eight weeks	Sixteen weeks
Callus induction		
0.0 (control)	-	-
3.0 mg/L BA	-	-
3.0 mg/L 2.4-D	-	-
1.0 mg/L L 2.4-D + 2.0 mg/L BA	-	-
1.0 mg/L 2.4-D +1.0 mg/L BA	-	+
1.0 mg/L 2.4-D + 1.0 mg/L NAA	+	++
3.0 mg/L 2.4-D + 2.0 mg/L BA	++	++++
3.0 mg/L 2.4-D + 1.0 mg/L BA	=	++

- Zero callus formation; +: percentage callus formation (low); ++: percentage callus formation (medium); ++++: percentage callus formation (high).

Table 3. Influence of various growth regulators on the percentage of callus induction, percentage of shoot regeneration frequency, and maximum shoot length of *Phoenix dactylifera* after eighth-week growth periods.

Growth regulators (mg/L)	Callus induction (%)	Shoot regeneration (%)		Shoot number/Explant		Shoot length (cm)	
BA and NAA							
mg/L		**Agar**	**Liquid**	**Agar**	**Liquid**	**Agar**	**Liquid**
control	-	-	-	-	-	-	-
0.5 + 0.5	70%	70% b	70% b	2.3 c	3.7 c	1.27 ab	1.31 ab
0.5 + 1.0	70%	70% b	70% b	5.5 b	6.2 b	1.53 a	1.64 a
0.5 + 1.5	80%	65% b	70% b	5.3 b	6.1 b	1.33 ab	1.58 a
1.0 + 0.5	100%	90% a	90% a	7.9 a	8.2 a	1.58 a	1.65 a
1.0 + 1.0	90%	85% a	85% a	5.3 b	5.8 b	1.54 a	1.63 a
1.0 + 1.5	100%	90% a	90% a	5.2 b	3.7 c	1.28 ab	1.62 a
BA							
0.5	-	-	-	-	-	-	-
1.0	-	-	-	-	-	-	-
1.5	20%	20% c	20% c	0.4 d	0.9 d	0.80 c	0.80 c
2.0	20%	20% c	30% c	0.5 d	1.1 d	0.90 c	1.11 bc
BA and 2,4-D							
0.5 + 0.5	30%	-	-	-	-	-	-
0.5 + 1.0	40%	-	-	-	-	-	-
0.5 + 1.5	30%	-	-	-	-	-	-
1.0 + 0.5	30%	-	-	-	-	-	-
1.0 + 1.0	20%	-	-	-	-	-	-
1.0 + 1.5	20%	-	-	-	-	-	-

Means within the column having different letters are significantly different according to Tukey HSD at $p \leq 0.05$.

Table 4. Survival, regrowth, and browning of *in vitro* preserved date palm calli as influenced by sucrose concentration 10 and 20 weeks after initiation of culture.

Sucrose Conc. (M)	Survival (%)		Regrowth (%)		Browning (%)	
	10 weeks	**20 weeks**	**10 weeks**	**20 weeks**	**10 weeks**	**20 weeks**
0.0	83.0 b	65.0 b	75.0 b	55.0 b	9.0 d	10.0 d
0.1	100 a	90.0 a	95.0 a	85.0 a	9.0 d	25.0 c
0.2	75.0 b	45.0 c	75.0 b	35.5 c	15.0 d	65.0 b
0.4	55.0 c	35.0 c	25.0 c	9.0 d	35.0 c	95.0 a
0.6	35.0 d	15.0 d	0.0 d	0.0 d	70 b	95.0 a
0.8	0.0 e	0.0 d	0.0 d	0.0 d	100 a	100 a
1.0	0.0 e	0.0 d	0.0 d	0.0 d	100a	100 a

Means within the column having different letters are significantly different according to Tukey HSD at $p \leq 0.05$.

MPVS2 for 20 and 40 minutes, resulted in 90 to 100% survival of non-cryopreserved embryogenic calli (Table 7). There was a reduction in survival due to partial damage of the embryogenic calli after rehydration with vitrification solutions. The results showed that survival and regrowth of cryopreserved tissue cultures were influenced by the duration of treatment with the vitrification solution (PVS2 and MPVS2, Table 7). Using PVS2 calli, formation after cryopreservation ranged from 15 to 65%. The regrowth percentage of calli using MPVS2 ranged from 10 to 35% after cryopreservation (Table 7). Frozen calli treated with PVS2 for 60 min exhibited regrowth rates of 65%, while longer

Table 5. Survival, regrowth, and browning of *in vitro* preserved date palm calli as influenced by mannitol concentration after 10 and 20 weeks of culture.

Mannitol Conc. (M)	Survival (%)		Regrowth (%)		Browning (%)	
	10 weeks	20 weeks	10 weeks	20 weeks	10 weeks	20 weeks
0.0	65.0 a	50.o ab	60.0 a	35.0 b	30.0 d	55.0 c
0.1	70.0 a	65.0 a	60.0 a	40.0 b	30.0 d	55.0 c
0.2	65.0 a	45.0 b	35.0 b	30.0 b	55.0 c	75.0 b
0.4	45.0 b	0.0 c	35.0 b	0.0 c	80.0 b	80 b
0.6	25.0 c	0.0 c	0.0 c	0.0 c	90.0 a	90.0 ab
0.8	0.0 d	0.0 c	0.0 c	0.0 c	100 a	100 a
1.0	0.0 d	0.0 c	0.0 c	0.0 c	100 a	100 a

Means within the column having different letters are significantly different according to Tukey HSD at $p \leq 0.05$.

Table 6. Survival, regrowth, and browning of *in vitro* preserved Date palm calli as influenced by sorbitol concentration after 10 and 20 weeks of culture.

Sorbitol Conc. (M)	Survival (%)		Regrowth (%)		Browning (%)	
	10 weeks	20 weeks	10 weeks	20 weeks	10 weeks	20 weeks
0.0	65.0 b	40.0 b	60.0 a	65.0 a	0.0 f	30.0 d
0.1	95.0 a	60.0 a	70.0 a	60.0 a	10.0 e	30.0 d
0.2	70.0 b	40.0 b	40.0 c	40.0 b	40.0 d	50.0 c
0.4	50.0 c	20.0 c	25.0 d	5.0 e	50.0 c	70.0 b
0.6	30.0 d	10.0 cd	0.0 e	0.0 e	65.0 b	100 a
0.8	0.0 e	0.0 d	0.0 e	0.0 e	90.0 a	100 a
1.0	0.0 e	40.0 b	0.0 e	65.0 a	90.0 a	100 a

Means within the column having different letters are significantly different according to Tukey HSD at $p \leq 0.05$.

exposure (120 min) regrowth was significantly decreased (Table 7). Thu, the keys to successful cryopreservation depend on the concentrated non-toxic solution of cryoprotectants and the contact period with the vitrification solution. Thus, the vitrification technique used in this study is a very simple and predictable method for cryopreservation of date palm tissue resulting in a high survival and regrowth rate.

4. Discussion

In the current study, simple methods for callus induction, shoot regeneration, and conservation of *in vitro* growth date palm were initiated. Maximum callus induction was obtained on a medium supplemented with different concentrations of BA and NAA (Table 1). The healthy shoot cultures were subcultured into another culture for more growth culture (Table 1). Maximum callus formation with high somatic embryogenesis development was recorded on MS medium with 3.0 mg/L 2,4-D and 2.0 mg/L BA. Similarly, on *Catharanthus roseus* (Rahman et al., 2019), maximum callus formation was recorded on MS medium added with NAA and BA (Table 2).

Calli from the different mediums subcultured in the MS medium before moving to the regeneration medium as recorded by Wang et al. (2021) and Xiong et al. (2021). The influence of BA seems to be critical to enhanced shoot formation (Table 3). In contrast to previous investigations, endogenous levels of growth regulators such as BA may affect the growth of embryogenic calli, and this may depend on plant species (Thomas and Puthur, 2004; Guo et al., 2011).

For medium-term conservation, calli preserved at different concentrations of sucrose, mannitol, or sorbitol were free of any contamination by the end of the preservation period. On the other hand, browning exceeded 95% when calli preserved on 0.8 M sucrose after 10 and 20 weeks of preservation, similar to the previous study by Deng et al. (2012). No embryogenic calli growth was observed at 0.8 to 1.0 M sucrose after 10 and 20 weeks, this resulted in a reduction in growth percentage (Table 4). Finally, sucrose is recommended for the preservation of date palm calli at lower concentrations of 0.1 M resulting in at least 85% regrowth percentage.

No survival was recorded when mannitol exceeded 0.6 M after 20 weeks of conservation this is similar to the previous find by Ghaheri et al. (2017) on *Stevia rebaudiana*; and on *Bambusa vulgaris* (García-Ramírez et al., 2019). Mannitol at high concentrations (0.8 or 1.0 M), causes 100% browning

Table 7. Effect of contact period with PVS2 and MPVS2 on survival of non-cryopreserved, cryopreserved, and regrowth percentages of date palm calli.

Exposure duration (min)	Survival percentage non-cryopreserved calli	Survival percentage cryopreserved calli	Regrowth percentage cryopreserved calli
	PVS2		
0.0	100±0.0 a	0.0±0.0 v	0.0±0.0 e
20.0	100±0.0 a	20±0.9 e	15±2.1 cd
40.0	90±0.0 ab	35±1.4 cd	20±1.8 c
60.0	80±2.7 bc	65±2.0 a	40±2.1 a
80.0	70±35 c	50±1.6 b	30±1.6 b
100	50±19 d	45±2.5 c	20±1.7 c
120	30±2.1e	25±2.1 e	10±2.5 d
160	20±0.9 ef	15±1.4 ef	0.0±0.0 e
180	10±0.8 f	0.0±0.0v	0.0±0.0 e
	MPVS2		
0.0	100±0.0 a	0.0±0.0 v	0.0±0.0 e
20.0	100±0.0 a	10±0.9 f	0.0±0.0 e
40.0	100±0.0 a	40±2.3 c	10±0.0 d
60.0	80±4.2 bc	45±3.1bc	35±3.2 b
80.0	80±3.4 bc	40±1.5 c	30±1.5 b
100	65±2.5 c	35±1.8 cd	20±1.6 c
120	25±1.9 ef	20±2.1 e	20±0.9 c
160	20±1.6 ef	10±1.9 f	0.0±0.0 e
180	0.0±0.0 v	0.0±1.8 v	0.0±0.0 e

Means within the column having different letters are significantly different according to Tukey HSD at $p \leq 0.05$.

(Table 5). Whereas, a complete loss of regrowth capacity was also noticed when the concentrations of mannitol exceeded 0.6 M after 10 or 20 weeks of conservation (as shown in Table 5). Thus, mannitol is recommended at 0.2 M to maintain the quality of embryoenic calli survival and regrowth, similar to previous reports (Shibli et al., 2006; Chutipaijit, 2016). No survival occurred when embryogenic calli were preserved on an MS medium containing 0.6 M sorbitol (as shown in Table 6). The lost of calli might be due to the toxicity of high sorbitol concentration that resulted from a continuous accumulation of carbohydrates as a response to osmostressing. Browning of calli increased with increased concentrations of sorbitol and an extended conservation period (Table 6). Thus, sorbitol at 0.1 M could be used for the conservation of date palm calli to maintain the quality of embryogenic calli and survival and regrowth capacities.

This study demonstrated that date palm embryogenic calli could be cryopreserved using vitrification. The results showed that survival and regrowth of cryopreserved embryogenic calli are affected by the duration of treatment with the vitrification solution (PVS2 and MPVS2). Thus, desiccation with the vitrification solution before storing in LN seems to be a valuable technique for cryopreservation of *in vitro* plants. Cryopreserved cultures treated with PVS2 for 160-180 minutes, showed a high percentage of damage. Our results are similar to those of Rabba'a et al. (2012) on *Teucrium polium*. They reported that cryopreserved tissue tolerated dehydration and continued life after being stored in liquid nitrogen. A study on *Vitis vinifera* concluded that vitrification of solutions reduces the injurious effect of intra and extracellular ice crystallization (Shatnawi et al., 2011). Similar observations were obtained when conserving shoot tips of *Teucrium polium* (Rabba'a et al., 2012), and *Artemisia herba-alba* (Shatnawi, 2011). Moreover, Cryopreserved vitrified embryogenic calli after thawing remained green and started to grow after five weeks. This report reported similar to previous results on *Prunus avium* (Shatnawi et al., 2007). Moreover, increased acquaintance with the vitrification solutions decreases the regrowth of non-frozen calli. For unfrozen calli, 100% survival was recorded after 20 min exposure to PVS2. Increasing vitrification solution contact up to 80 minutes decreased the survival rate of cryopreserved date palm culture. The results are similar to those obtained by Shatnawi (2013) on *Achillea millefolium*.

Lower contact with vitrification solutions reduces the injurious belonging to the high osmolarity of vitrification solution. The current results are similar to those reported by Shatnawi et al. (2011) on *Vitis vinifera* and Rabba'a et al. (2012) on *Teucrium polium*. Thus, calli preservation of date palm calli is a valuable method for the safe, long-term preservation of resources that accompany traditional fields and *in vitro* germplasm preservation activity.

5. Conclusions

A simple procedure for callus induction, shoot regeneration, and conservation of *in vitro* growth date palm was developed. Using 3.0 mg/L 2,4-D and 2.0 mg/L BA resulted in a maximum embryogenic calli formation. While in both liquid and solid medium highest shoot formation was noted on MS medium added with 1.0 mg/L BA with (0.5 - 1.0 mg/L) NAA. For medium-term conservation. MS supplemented with osmoticum agents influenced the preserved date palm embryogenic callus. Sucrose could be suggested for medium-term conservation of date palm calli at lower concentrations (0.1 - 0.2 M) to maintain survival and regrowth.

A simple and efficient cryopreservation technique was implemented. The preserved embryogenic callus was capable of regenerating embryos after reculturing on a recovery medium. Further cryopreservation methods are recommended to be assessed to enhance regrowth percentages of date palm calli. Finally, this study presented a potent and easy procedure with high viability after cryopreservation of date palm embryogenic calli.

References

ABDALLA, N., EL-RAMADY, H., SELIEM, M.K., EL-MAHROUK, M.E., TAHA, N., BAYOUMI, Y., SHALABY, T.A. and DOBRÁNSZKI, J., 2022. An academic and technical overview on plant micropropagation challenges. *Horticulturae*, vol. 82, no. 8, pp. 677-686. http://doi.org/10.3390/horticulturae8080677.

ABDELGHAFFAR, A.M., SOLIMAN, S.S., ISMAIL, T.A., ALZOHAIRY, A.M., LATEF, A.A.H.A., ALHARBI, K., AL-KHAYRI, J.M., ALJUWAYZI, N.I.M., EL-MONEIM, D.A. and HASSANIN, A.A., 2023. *In vitro* propagation of three Date Palm (*Phoenix dactylifera* L.) varieties using immature female inflorescences. *Plants*, vol. 12, no. 3, pp. 644-660. http://doi.org/10.3390/plants12030644. PMid:36771727.

ABUL-SOAD, A. A. and MAHDI, S, M., 2010. Commercial production of tissue culture date palm (*Phoenix dactylifera* L.) by inflorescence technique. *Journal of Genetic Engineering and Biotechnology*, vol. 8, no. 2, pp. 39-44.

AL SHHAB, M., SHATNAWI, M., ABU-ROMMANM, S., MAJDALAWI, M., ABUBAKER, S. and SHAHROUR, W., 2022. Antimicrobial activity and micropropagation of *Ruta graveolens* medicinal plant. *International Journal of Agriculture and Biology*, vol. 28, pp. 352-358. http://doi.org/10.17957/IJAB/15.1988.

ALANSI, S., AL-QURAINY, F., NADEEM, M., KHAN, S., TARROUM, M., ALSHAMERI, A. and GAAFAR, A.Z., 2019. Cryopreservation: a tool to conserve date palm in Saudi Arabia. *Saudi Journal of Biological Sciences*, vol. 26, no. 7, pp. 1896-1902. http://doi.org/10.1016/j.sjbs.2019.02.004. PMid:31762672.

AL-KHATEEB, A.A., MOHASSEB, H.A., AL-KHATEEB, S.A., ALDAEJ, M.I. and SHEHATA, W.F., 2019. Towards the development of a new technique for cryopreservation of date palm (Phoenix dactylifera L.) in vitro grown cultures. *Journal Biological Series*, vol. 7, no. 1, pp. 22-27. http://doi.org/10.15413/jbs2019.0120.

AL-KHAYRI, J.M. and NAIK, P.M., 2017. Date palm micropropagation: advances and applications. *Ciência e Agrotecnologia*, vol. 41, no. 4, pp. 347-358. http://doi.org/10.1590/1413-70542017414000217.

ARAÚJO DE OLIVEIRA, A.C., LÉDO, A.S., POLEK, M.L., KRUEGER, R., SHEPHERDA, A. and VOLK, G. M., 2021. Optimization of in vitro germination and cryopreservation conditions for preserving date palm pollen in the USDA National Plant Germplasm System. *Plant Cell Tissue and Organ Culture*, vol. 144, pp. 223-232. http://doi.org/10.1007/s11240-020-01907-1.

ASCHALE, N., 2022. *In vitro* protocol optimization for mass propagation of elite date palm varieties in Ethiopia: short communication. *International Journal of Novel Research in Life Sciences*, vol. 9, no. 2, pp. 104-106.

BEKHEET, S.A., TAHA, H.S., SAKER, M. M. and SOLLIMAN, M.E., 2007. Application of cryopreservation technique for in vitro grown date palm (*Phoenix dactylifera* L.) cultures. *Journal of Applied Sciences Research*, vol. 3, no. 9, pp. 859-866.

CHUTIPAIJIT, S., 2016. Changes in physiological and antioxidant activity of Indica rice seedlings in response to mannitol-induced osmotic stress. *Chilean Journal of Agricultural Research*, vol. 76, no. 4, pp. 455-462. http://doi.org/10.4067/S0718-58392016000400009.

DENG, Z., CHU, J., WANG, Q. and WANG, L., 2012. Effect of different carbon sources on the accumulation of carbohydrates, nutrient absorption, and the survival rate of Chinese Ash (*Fraxinus mandshurica*) explants *in vitro*. *African Journal of Agricultural Research*, vol. 7, no. 20, pp. 3111-3119. http://doi.org/10.5897/AJAR11.1870.

ELAZIEM, T.M., ELSALATO, M., AHMED, A.E., RAYAN, G. and ELDIS, A., 2022. In vitro propagation for conservation of the rare date palm (Phoenix dactylifera L.) 'Amri' using immature inflorescence. *In Vitro Cellular and Developmental Biology – Plant*, vol. 58, pp. 1048-1056. http://doi.org/10.1007/s11627-022-10296-3.

EL-BAHR, M.K., ABD EL-HAMID, A., MATTER, M.A., SHALTOUT, A., BEKHEET, S.A. and EL-ASHRY, A.A., 2016. *In vitro* conservation of embryogenic cultures of date palm using osmotic mediated growth agents. *Journal of Genetic Engineering and Biotechnology*, vol. 14, no. 2, pp. 363-370. http://doi.org/10.1016/j.jgeb.2016.08.004. PMid:30647634.

GARCÍA-RAMÍREZ, Y., BARRERA, G.P., FREIRE-SEIJO, M., BARBÓN, R., CONCEPCIÓN-HERNÁNDEZ, M., MENDOZA-RODRÍGUEZ, M.F. and TORRES-GARCÍA, S., 2019. Effect of sucrose on physiological and biochemical changes of proliferated shoots of *Bambusa vulgaris* Schrad Ex Wendl in temporary immersion. *Plant Cell, Tissue and Organ Culture*, vol. 137, no. 2, pp. 239-247. http://doi.org/10.1007/s11240-019-01564-z.

GHAHERI, M., KAHRIZI, D. and BAHRAMI, G., 2017. Effect of mannitol on some morphological characteristics of *in vitro Stevia rebaudiana* Bertoni. *Biharean Biology Oradea Romania*, vol. 11, no. 2, pp. 94-97.

GONZALEZ-ARNAO, M.T., ENGELMANN, F., VILLAVICENCIO, C.U., MORENZA, M. and RIO, A., 1988. Cryopreservation of citrus apices using the encapsulation-dehydration technique. *Cryo Letters*, vol. 19, pp. 177-182.

GUO, B., ABBASI, B.H., ZEB, A., XU, L.L. and WEI, Y H., 2011. Thidiazuron: a multi-dimensional plant growth regulator. *African Journal of Biotechnology*, vol. 10, no. 45, pp. 8984-9000. http://doi.org/10.5897/AJB11.636.

METWALI, E. M., KADASA, N.M., HEMAID, I.A., SOLIMAN, H.I., ALMAGHRABI, O.A. and FULLER, M.P., 2020. Cryopreservation of embryogenic callus of date palm (*Phoenix dactylifera*) cv. Magdoul through encapsulation-dehydration technology. *International Journal of Agriculture and Biology*, vol. 24, pp. 1754-1762. http://doi.org/10.17957/IJAB/15.1619.

MURASHIGE, T. and SKOOG, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum*, vol. 15, no. 3, pp. 473-497. http://doi.org/10.1111/j.1399-3054.1962.tb08052.x.

RABBA'A, M.M., SHIBLI, R.A. and SHATNAWI, M.A., 2012. Cryopreservation of *Teucrium polium* L. shoot-tips by vitrification and encapsulation-dehydration. *Plant Cell, Tissue and Organ Culture*, vol. 110, no. 3, pp. 371-382. http://doi.org/10.1007/s11240-012-0158-1.

RAHMAN, N.N., ROSLI, R., KADZIMIN, S. and HAKIMAN, M., 2019. Effects of auxin and cytokinin on callus induction in *Catharanthus roseus* (L.) G. Don. *Fundamental and Applied Agriculture*, vol. 4, no. 3, pp. 928-932. http://doi.org/10.5455/faa.54779.

SAKAI, A., KOBAYASHI, S. and OIYAMA, I., 1990. Cryopreservation of nucellar cells of navel orange (*Citrussinensis Osb*. var. brasiliensis Tanaka) by vitrification. *Plant Cell Reports*, vol. 9, pp. 30-33.

SHAHROUR, W.G., SHATNAWI, M.A. and MOHAMMAD, A.A., 2024. In vitro multiplication, antimicrobial, and insecticidal activity of Capparis spinosa L. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca*, vol. 52, no. 1, pp. 13609-13609. http://doi.org/10.15835/nbha52113609.

SHATNAWI, M. A., SHIBLI, R.A., QRUNFLEH, I., BATAEINEH, K. and OBEIDAT, M., 2007. *In vitro* propagation and cryopreservation of *Prunus avium* using vitrification and encapsulation dehydration methods. *Journal of Food Agriculture and Environment*, vol. 5, no. 2, pp. 204-208.

SHATNAWI, M., ANFOKA, G., SHIBLI, R., AL-MAZRA'AWI, M., SHAHROUR, W. and AREBIAT, A., 2011. Clonal propagation and cryogenic storage of virus free grapevine (*Vitis vinifera* L.) via meristem culture. *Turkish Journal of Agriculture and Forestry*, vol. 35, pp. 173-184. http://doi.org/10.3906/tar-0912-519.

SHATNAWI, M.A., 2006. Micropropagation and germplasm storage of *Prunus amygdalus* by the vitrification method. *Jordan Journal of Agricultural Science*, vol. 2, no. 3, pp. 222-233.

SHATNAWI, M.A., 2011. Multiplication and cryogenic storage of *Artemisia herba-alba*: a medicinal plant. *Journal of Food Agriculture and Environment*, vol. 9, pp. 340-344.

SHATNAWI, M.A., 2013 [viewed 9 March 2024]. Multiplication and cryopreservation of Yarrow (*Achillea millefolium* L., Astraceae). *Journal of Agriculture Science and Technology* [online], vol. 15, no. 1, pp. 163-173. Available from: http://jast.modares.ac.ir/article-23-769-en.html

SHATNAWI, M.A., JOHNSON, K. and TORPY, F., 2004. In vitro propagation and cryostorage of *Syzygium francisii* (Myrtaceae) by encapsulation-dehydration method. *In Vitro Cellular and Developmental Biology Plant*, vol. 40, no. 4, pp. 403-407.

SHIBLI, R.A., DUWAYRI, M.A., SAWWAN, J.S., SHATNAWI, M.A. and AL-QUDAH, T.S., 2012. Regeneration via somatic embryogenesis of the endangered wild arum (*Arum palaestinum*). *In Vitro Cellular and Developmental Biology Plant*, vol. 48, pp. 335-340. https://doi.org/10.1007/s11627-012-9438-z.

SHIBLI, R.A., SHATNAWI, M.A., SUBAIH, W. and AJLOUNI, M.M., 2006. *In vitro* conservation and cryopreservation of plant genetic resources. *World Journal of Agricultural Sciences*, vol. 2, no. 4, pp. 372-382.

SPSS, 2017. *Complex samples*. Chicago: SPSS Inc.

SUBAIH, W.S., SHATNAWI, M.A. and SHIBLI, R.A., 2007. Cryopreservation of Date palm (*Phoenix dactylifera*) embryogenic callus by encapsulation dehydration, vitrification and encapsulation vitrification. *Jordan Journal of Agricultural Science*, vol. 3, no. 2, pp. 156-171.

THOMAS, T.D. and PUTHUR, J.T., 2004. Thidiazuron induced high-frequency shoot organogenesis in callus from *Kigelia pinnata* L. *Botanical Bulletin Academia*, vol. 45, pp. 307-313.

WANG, C., MA, H., ZHU, W., ZHANG, J., ZHAO, X. and LI, X., 2021. Seedling-derived leaf and root tip as alternative 257 explants for callus induction and plant regeneration in maize. *Physiologia Plantarum*, vol. 173, no. 3, pp. 1570-1581. http://doi.org/10.1111/ppl.13347. PMid:33502786.

XIONG, Y., WEI, Z., YU, X., PANG, J., ZHANG, T., WU, K., REN, H., JIAN, S., SILVA, J.A.T. and MA, G., 2021. Shoot proliferation, embryogenic callus induction, and plant regeneration in *Lepturus repens* (G. Forst.). *In Vitro Cellular & Developmental Biology. Plant*, vol. 57, no. 6, pp. 1031-1039. http://doi.org/10.1007/s11627-021-10183-3.