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Optimization of in vitro germination and cryopreservation conditions for preserving date palm pollen in the USDA National Plant Germplasm System

Annie Carolina Araújo de Oliveira¹ · Ana da Silva Léo² · MaryLou Polek³ · Robert Krueger³ · Ashley Shepherd⁴ · Gayle M. Volk⁴

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Abstract

Date palm (*Phoenix dactylifera* L.) is a staple crop across arid and semi-arid regions around the world. Both genebanks and date palm breeders benefit by having clearly defined methods for the long-term preservation of pollen. Methods were developed to cryopreserve pollen from date palm trees in the USDA-ARS National Plant Germplasm System. First, in vitro pollen germination conditions were optimized using five cultivars. Then, date palm pollen equilibrated over saturated salt solutions of $\text{Ca}(\text{NO}_3)_2$ or MgCl_2 at 23 °C (46% and 33% RH, respectively) or in a room set at 23% RH and 5 °C. Pollen was kept at these conditions for one to 56 days and then placed into liquid nitrogen vapor (LNV). Viability was assessed for fresh, moisture-equilibrated (non-LNV and LNV-exposed), and pollen stored in LNV for 9 months. The highest level of in vitro pollen germination was observed for date palm cultivar Thoory BC3 ($60 \pm 2\%$) when it was rehydrated for 2 h and then incubated at 23 °C on Marquard medium with 15% sucrose. Viability remained stable for pollen that was stored in LNV for 9 months. In addition, pollen also remained viable if it was cooled to and warmed from LNV for up to 60 cycles. These data suggest that date palm pollen can be successfully placed in genebank cryostorage.

Key message

Using optimized in vitro germination conditions, date palm pollen can survive at least 9 months in LNV and can be cooled to and warmed from LNV at least 60 times.

Keywords *Phoenix dactylifera* L. · Liquid nitrogen · In vitro germination

Abbreviations

ARS Agricultural Research Service
 $\text{Ca}(\text{NO}_3)_2$ Calcium nitrate

$\text{Ca}(\text{NO}_3)_2 \cdot 2\text{H}_2\text{O}$ Calcium nitrate dihydrate
 $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ Copper sulfate pentahydrate
DW Dry weight
FW Fresh weight
 H_3BO_3 Boric acid
 KNO_3 Potassium nitrate
LNV Liquid nitrogen vapor
MC Moisture content
 MgCl_2 Magnesium chloride
 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ Magnesium sulfate heptahydrate
NCGRCD National clonal germplasm repository for citrus and dates
NPGS National Plant Germplasm System
RH Relative humidity
TW Tare weight
UC-VARS University of California Coachella Valley agricultural research station

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USD United States dollars
USDA United States Department of
Agriculture

Introduction

Date palm (*Phoenix dactylifera* L.), Arecaceae, has both social and economic importance. It is a crop primarily cultivated for its edible fruits. Dates are rich in carbohydrates, especially sugars and antioxidants (Ghnimi et al. 2017). Moreover, dates are a staple food in arid and semi-arid regions of the Middle East and North Africa (Mahamoud et al. 2019). The species was introduced into the Americas and is grown commercially in the United States and Mexico (Wright 2016; Ortiz-Urbe et al. 2019). In 2018, world date production reached 8.5 million tons, with an estimated market value of 9.8 billion USD (FAO Stats 2020).

There is a wide range of differences among the fruits of date palm cultivars with respect to ripening stage, shape, size, color, texture and flavor; however, many varieties are only locally grown and are not extensively available. As a result, most of the market share is comprised of only a few elite cultivars and the gene pool is becoming narrower (Chao and Krueger 2007). On the matter of conservation, several countries, including Egypt, Algeria and the United States, maintain field date palm genebank collections (Bekheet and El-Sharabasy 2015; Bouguedoura et al. 2015).

The National Clonal Germplasm Repository for Citrus and Dates (NCGRCD) is part of the USDA-ARS National Plant Germplasm System (NPGS), which has a field collection at the University of California Coachella Valley Agricultural Research Station (UC-CVARS) in Thermal, CA (Krueger 2015). The NPGS date palm collection plays a critical role in providing genetic resources for national and international efforts to broaden the genetic base of breeding and production programs. The field collection is subject to biotic and abiotic stresses, making it necessary to develop technologies to secure the NPGS date palm collection germplasm at a secondary facility, such as the National Laboratory for Genetic Resources Preservation (NLGRP) in Fort Collins, CO. The NLGRP serves as a back-up location for plant collections in the NPGS (Byrne et al. 2018).

Pollen storage methods are needed for use in date palm breeding programs due to the lack of flowering synchrony. Because of their dioecious nature, male flowers usually emerge before female flowers, making it necessary to store pollen to perform crosses in breeding programs (Zaid and de Wet 2002; Mesnoua et al. 2018a). Conventional methods, involving room temperature (25–30 °C) and low temperature (4 °C, – 20 °C, – 80 °C) storage have been successfully reported by Shaheen et al. (1986), Boughediri et al. (1995), Maryam et al. (2015) and Anushma et al. (2018).

Cryopreservation, usually in liquid nitrogen (– 196 °C) or its vapor phase (– 165 to – 190 °C) is a preferred method for long-term storage (Reed 2008).

Many factors influence the viability and longevity of cryopreserved pollen samples, including the moisture content of the tissues that are exposed to these conditions (Connor and Towill 1993). Moisture content adjustment can be achieved by desiccating the material over saturated salts such as $\text{Ca}(\text{NO}_3)_2$ and MgCl_2 or by placement in low-humidity environmental chambers (Volk 2011; Walters 2015). Pollen of different species, such as pineapple and other bromeliads (Souza et al. 2018), coconut (Machado et al. 2014), grapes (Ganeshan 1985), and orchids (Vendrame et al. 2008), has been stored at ultralow temperatures. Cryostorage of date palm pollen was achieved by Tisserat et al. (1983), Mortazavi et al. (2010), and Anushma et al. (2018), although the pollen moisture contents were not described.

The aim of this study was to optimize in vitro germination for date palm pollen and to identify a cryopreservation protocol that could be implemented as a standard procedure in the NPGS.

Materials and methods

Pollen collection

Mature unopened spathes of date palm cultivars Dayri BC2 (PI 555413), Fard #4 (PI 555411), Jarvis #1 (PI 555443), Thoory BC3 (PI 555445) and Thoory X (Halawy BC3) F1 (PI 555430) were harvested from male plants in February 2019 from the USDA-ARS UC-CVARS in Thermal, California. Spathes were sent to the NLGRP in Fort Collins, Colorado for processing. Upon arrival, spathes were kept at room temperature (23 °C) until they cracked. Then, inflorescences were shaken to release the pollen, either immediately (Harvest 1), 2 days (Harvest 2), or 4 days (Harvest 3) after the spathe opened.

Pollen moisture content

Fresh pollen (Harvest 1) was collected from each of the five date palm cultivars and moisture-adjusted for 18 h or 72 h over saturated salts of $\text{Ca}(\text{NO}_3)_2$ at 23 °C (46% RH), MgCl_2 at 23 °C (33% RH), or in a room set at 23% RH and 5 °C to determine the duration needed for moisture equilibration. Moisture content (MC) was calculated by weighing 1.0–5.0 mg of pollen on a precision balance before and after 4 days of drying at 90 °C, according to the equation: $\text{MC} = [(\text{FW} - \text{DW})/(\text{DW} - \text{TW})]$, where MC is the moisture content ($\text{g g}^{-1} \text{ dw}$), FW is the fresh weight (g), DW is the dry weight (g) and TW is the tare weight (g).

Pollen viability assessment

Pollen viability was determined by using in vitro germination methods. Pollen was first placed in a high moisture environment for rehydration and then it was plated onto germination medium. After overnight incubation, germinated pollen grains were counted. The rehydration condition, germination medium, and incubation temperature were optimized using the following experiments.

Germination medium

First, the culture medium was optimized for pollen germination. Freshly harvested pollen from the day the spathe opened (Harvest 1) from each of the five date palm cultivars was placed in a saturated H₂O environment (100% RH) for 2 h at 23 °C and then dusted (by tapping a clean paintbrush that was dipped in the hydrated pollen) onto a Petri dish containing one of five media formulations: Medium A (15% sucrose and 0.8% agar); Medium B (Ca(NO₃)₂·2H₂O (0.300 g L⁻¹), H₃BO₃ (0.100 g L⁻¹), KNO₃ (0.100 g L⁻¹), MgSO₄·7H₂O (0.200 g L⁻¹), 15% sucrose and 2% agarose (Marquard 1992)); Medium C (Brewbaker and Kwack (1964) modified with Ca(NO₃)₂·2H₂O (0.200 g L⁻¹), H₃BO₃ (0.050 g L⁻¹), 15% sucrose and 1% agar (Mortazavi et al. 2010)); Medium D (Ca(NO₃)₂·2H₂O (0.417 g L⁻¹), H₃BO₃ (0.200 g L⁻¹), KNO₃ (0.101 g L⁻¹), MgSO₄·7H₂O (0.217 g L⁻¹), 20% sucrose and 1% agar (Maryam et al. 2015)); Medium E (Brewbaker and Kwack (1964) modified with 15% sucrose and ethylenediamine tetraacetic acid (EDTA; 0.100 g L⁻¹) and 2% agarose (Tisserat et al. 1983)). Plated pollen was incubated at 23 °C overnight in the dark. One hundred pollen grains were counted in each of three Petri dishes. The percentage of viable pollen for each treatment was determined based on the number of pollen grains that had pollen tubes that were at least the length of the pollen grain, as observed using a 10 × objective in a Leica DM LM microscope (Buffalo Grove, IL, USA).

Rehydration condition

Fresh pollen (Harvest 1) for all five date palm cultivars was either hydrated in a saturated H₂O (100% RH) environment for 2 h at 23 °C or over a salt solution of CuSO₄·5H₂O (96% RH) for 3 h at 23 °C. Pollen was then plated onto Medium B, incubated at 23 °C overnight, and then viability was assessed as described above.

Incubation temperature

Fresh pollen (Harvest 1) from date palm cultivars Fard #4 and Jarvis #1 was hydrated over H₂O (100% RH) for 2 h at 23 °C, plated onto germination Medium B, and then

incubated in the dark overnight at either 23 or 30 °C. Pollen viability assessments were performed as described above.

Pollen cryopreservation

Optimized pollen germination conditions were used for subsequent experiments. Date palm pollen was rehydrated over H₂O (100% RH) for 2 h at 23 °C, plated onto Medium B, incubated at 23 °C overnight, and then viability was assessed by in vitro germination as described above.

For the first cryopreservation experiment, pollen from Harvest 1, 2, and 3 for each of the five date palm cultivars was placed over saturated salts of Ca(NO₃)₂ at 23 °C, MgCl₂ at 23 °C, or in a room set at 23% RH and 5 °C for moisture equilibration. Pollen was kept in these conditions for up to 56 days (– LNV) prior to LNV exposure. Moisture-equilibrated pollen at 1, 2, 3, 4, 6, 8, 10, 14, 17, 21, 24, 30, 37, 44 and 56 days (dependent upon the quantity of pollen available) was transferred to polypropylene cryovials and then placed into the vapor phase (– 165 to – 190 °C) of liquid nitrogen (+ LNV), and held for at least 1 month. Viability assessments for – LNV and + LNV were performed using the optimized pollen germination conditions.

Freshly harvested (Harvest 1) pollen from the five date palm cultivars was used to compare germination levels for pollen that had no moisture adjustment, – LNV, + LNV, and + LNV after 9 months of storage. For the – LNV and + LNV treatments, moisture adjustment was for 1 day over saturated salt solutions of Ca(NO₃)₂, MgCl₂, or in a room at 23% RH/5°C. After moisture adjustment, pollen was rehydrated and assessed for viability (– LNV), or placed into LNV, warmed, rehydrated, and assessed for viability (+ LNV), or held for 9 months in LNV and then warmed, rehydrated, and assessed for viability.

The ability to withstand multiple freeze–thaw LNV-exposure cycles was determined. Harvest 1 pollen for each of the five date palm cultivars was equilibrated over saturated salts of Ca(NO₃)₂, MgCl₂, or in a room at 23% RH/5 °C for 4 days and then aliquoted into cryovials and then placed into LNV. Cryovials of pollen from each cultivar were warmed at 23 °C for 15 min and then returned to LNV for 1, 10, 20, 30, 40, 50, and 60 cooling and warming cycles. Viability assessments were then performed using the optimized pollen germination conditions.

Statistical analyses

Experiments were performed using a completely randomized design with three replicates, each represented by a Petri dish. Data for germination optimization, and 9-month LN storage experiments were analyzed by analysis of variance (ANOVA) and Tukey separation was used to compare means ($p \leq 0.05$; JMP 12, Cary, NC, USA). Factorial models

identified significant variables for the experiments that had multiple factors. Independent variables included cultivar, pollen harvest, moisture equilibration condition, duration of equilibration condition storage, LN exposure, duration of LN exposure, and/or LNV cooling and warming cycles, depending on the experiment. The dependent variable was percent viable pollen, as measured by in vitro germination. Data were combined for the non-significant factors and only significant factors were graphed. Regressions were calculated for the graphed data.

Results

Moisture content

MC levels varied among pollen from date palm cultivars and desiccation treatments. The MC of freshly harvested pollen ranged from $5.75 \text{ g g}^{-1} \text{ dw}$ for Fard #4 to $21.73 \text{ g g}^{-1} \text{ dw}$ for Dayri BC2. A decrease in the water content was observed after 18 h of equilibration, with an average MC of $6.22 \pm 0.38 \text{ g g}^{-1} \text{ dw}$, $4.65 \pm 0.27 \text{ g g}^{-1} \text{ dw}$, and $4.43 \pm 0.29 \text{ g g}^{-1} \text{ dw}$ recorded for $\text{Ca}(\text{NO}_3)_2$, MgCl_2 , and 23% RH/5 °C conditions, respectively. An additional 54 h (72 h timepoint) of

moisture adjustment did not significantly change the MC of the pollen, resulting in average moisture contents of $5.68 \pm 0.75 \text{ g g}^{-1} \text{ dw}$, $4.80 \pm 0.41 \text{ g g}^{-1} \text{ dw}$, and $5.03 \pm 0.40 \text{ g g}^{-1} \text{ dw}$ for the $\text{Ca}(\text{NO}_3)_2$, MgCl_2 , and 23% RH/5 °C conditions, respectively (Table 1).

In vitro germination

Freshly harvested (Harvest 1) date palm pollen was used to identify in vitro germination conditions. The medium, rehydration conditions, and germination temperature were determined. Pollen germination percentages varied among cultivars and the five culture media that were tested. The highest pollen germination levels ($47 \pm 5\%$) were obtained for Medium B: Marquard medium with 15% sucrose. Medium D had $4 \pm 3\%$ average germination levels and no germination was observed for Medium E (Table 2).

The effect of the pollen rehydration conditions was measured by placing fresh pollen in a 100% RH environment over H_2O for 2 h or over copper sulfate (96% RH) for 3 h, both at 23 °C, followed by plating on Marquard medium (Medium B) and incubated at 23 °C overnight. Rehydration in a saturated H_2O environment resulted in an average 55% germination level, compared to an average of 48% for the

Table 1 Moisture content of date palm freshly 'Harvest 1' pollen after 18 h or 72 h of moisture adjustment over saturated salt solutions of $\text{Ca}(\text{NO}_3)_2$ or MgCl_2 at 23 °C or placement in a 23% RH/5 °C room. Moisture content is calculated on a dry weight basis

	Initial MC ($\text{g g}^{-1} \text{ dw} \pm \text{SE}$)	Moisture content after 18 h ($\text{g g}^{-1} \text{ dw} \pm \text{SE}$)			Moisture content after 72 h ($\text{g g}^{-1} \text{ dw} \pm \text{SE}$)		
		$\text{Ca}(\text{NO}_3)_2$	MgCl_2	23% RH/5 °C	$\text{Ca}(\text{NO}_3)_2$	MgCl_2	23% RH/5 °C
Dayri BC2	$21.73 \pm 1.44 \text{ a}$	$6.36 \pm 0.06 \text{ cde}$	$5.62 \pm 0.69 \text{ def}$	$5.18 \pm 0.07 \text{ def}$	$8.46 \pm 0.03 \text{ c}$	$6.13 \pm 0.28 \text{ cdef}$	$6.26 \pm 0.10 \text{ cde}$
Fard #4	$5.75 \pm 0.86 \text{ def}$	$5.48 \pm 0.83 \text{ def}$	$4.18 \pm 0.30 \text{ ef}$	$4.50 \pm 0.21 \text{ ef}$	$5.85 \pm 0.29 \text{ cdef}$	$5.00 \pm 0.11 \text{ def}$	$4.45 \pm 0.17 \text{ ef}$
Jarvis #1	$15.41 \pm 2.55 \text{ b}$	$7.62 \pm 0.34 \text{ cd}$	$4.83 \pm 0.21 \text{ ef}$	$4.84 \pm 0.51 \text{ ef}$	$4.70 \pm 0.06 \text{ ef}$	$4.35 \pm 0.10 \text{ ef}$	$5.45 \pm 0.12 \text{ def}$
Thoory BC3	$13.09 \pm 0.63 \text{ b}$	$5.67 \pm 0.61 \text{ def}$	$4.39 \pm 0.75 \text{ ef}$	$3.55 \pm 0.08 \text{ f}$	$5.27 \pm 0.35 \text{ def}$	$4.90 \pm 0.34 \text{ ef}$	$3.93 \pm 0.06 \text{ ef}$
Thoory X F1	$19.83 \pm 1.51 \text{ a}$	$5.97 \pm 0.16 \text{ cdef}$	$4.24 \pm 0.13 \text{ ef}$	$4.06 \pm 0.04 \text{ ef}$	$4.10 \pm 0.11 \text{ ef}$	$3.62 \pm 0.12 \text{ f}$	$5.07 \pm 0.77 \text{ def}$
Means	15.16 ± 2.80	6.22 ± 0.38	$4.65 \pm 0.27 \text{ def}$	4.43 ± 0.29	5.68 ± 0.75	4.80 ± 0.41	5.03 ± 0.40

Means with the same letters were not significantly different at $P \leq 0.05$

Table 2 In vitro germination of date palm freshly 'Harvest 1' pollen rehydrated over H_2O for 2 h, plated on five different culture media (A–E; see text) and incubated at 23 °C overnight

	Medium A (% $\pm \text{SE}$)	Medium B (% $\pm \text{SE}$)	Medium C (% $\pm \text{SE}$)	Medium D (% $\pm \text{SE}$)	Medium E (% $\pm \text{SE}$)
Dayri BC2	$7 \pm 2 \text{ b}$	$33 \pm 3 \text{ a}$	$37 \pm 2 \text{ a}$	$3 \pm 2 \text{ b}$	$0 \pm 0 \text{ b}$
Fard #4	$23 \pm 1 \text{ b}$	$49 \pm 3 \text{ a}$	$48 \pm 1 \text{ a}$	$14 \pm 3 \text{ b}$	$0 \pm 0 \text{ c}$
Jarvis #1	$23 \pm 2 \text{ b}$	$40 \pm 3 \text{ a}$	$26 \pm 2 \text{ b}$	$0 \pm 0 \text{ c}$	$0 \pm 0 \text{ c}$
Thoory BC3	$30 \pm 0 \text{ b}$	$60 \pm 2 \text{ a}$	$58 \pm 2 \text{ a}$	$0 \pm 0 \text{ c}$	$0 \pm 0 \text{ c}$
Thoory X F1	$13 \pm 0 \text{ b}$	$51 \pm 3 \text{ a}$	$47 \pm 1 \text{ a}$	$2 \pm 1 \text{ c}$	$0 \pm 0 \text{ c}$
Means	$19 \pm 4 \text{ b}$	$47 \pm 5 \text{ a}$	$43 \pm 5 \text{ a}$	$4 \pm 3 \text{ bc}$	$0 \pm 0 \text{ c}$

Means within rows with the same letters were not significantly different at $P \leq 0.05$

Table 3 In vitro germination of date palm freshly ‘Harvest 1’ pollen rehydrated over H₂O for 2 h or CuSO₄·5H₂O for 3 h at 23 °C, plated on Medium B (Marquard medium) and incubated at 23 °C overnight

	H ₂ O (% ± SE)	CuSO ₄ ·5H ₂ O (% ± SE)
Dayri BC2	45 ± 2a	34 ± 1b
Fard #4	57 ± 2a	53 ± 2a
Jarvis #1	46 ± 3a	36 ± 2a
Thoory BC3	61 ± 2a	64 ± 4a
Thoory X F1	64 ± 4a	54 ± 2a
Means	55 ± 4a	48 ± 6a

Means within rows with the same letters were not significantly different at $P \leq 0.05$

Table 4 In vitro germination of date palm cultivars Fard #4 and Jarvis #1 freshly ‘Harvest 1’ pollen rehydrated over H₂O for 2 h, plated in Medium B (Marquard medium) and incubated at 23 or 30 °C overnight

	23 °C (% ± SE)	30 °C (% ± SE)
Fard #4	46 ± 3a	33 ± 2b
Jarvis #1	57 ± 2a	45 ± 1b
Means	52 ± 6a	39 ± 6b

Means within rows with the same letters were not significantly different at $P \leq 0.05$

CuSO₄·5H₂O rehydration, with no statistical significance between the conditions (Table 3). Pollen was placed over a saturated H₂O environment for 2 h for all subsequent viability assessments.

Germination levels of Fard #4 and Jarvis #1 pollen were assessed after fresh pollen was hydrated in a saturated H₂O (100% RH) environment for 2 h at 23 °C, then plated on Marquard medium (Medium B), and incubated in the dark at either 23 or 30 °C overnight. Higher levels of pollen germination were measured at the 23 °C incubation conditions (52 ± 6%) compared to 30 °C (39 ± 6%), so all subsequent assays were performed at 23 °C (Table 4).

Cryopreservation

Pollen from the five date palm cultivars, collected immediately (Harvest 1), 2 days (Harvest 2), and 4 days (Harvest 3) after spathe opening was equilibrated over saturated salt environments of Ca(NO₃)₂, MgCl₂, or at 23% RH/5 °C conditions and was kept at these conditions for up to 56 days prior to LNV exposure. Factorial models revealed that there were no significant differences among the in vitro germination levels of pollen with respect to the cultivar or the moisture equilibration conditions, hence these treatments

were combined (data not shown). Overall, pollen from Harvest 1, 2, and 3 responded similarly to extended storage, with declines in viability over time, described by regressions. Non LNV-exposed pollen viability dropped to 50% after 11, 14 and 7 days for Harvests 1, 2 and 3, respectively (Fig. 1a–c).

In vitro germination levels were compared for date palm pollen that was freshly harvested, – LNV and + LNV, and after 9 months of storage in LNV for each of the five cultivars (Fig. 2). Pollen samples from Harvest 1 were moisture-equilibrated using saturated salts of Ca(NO₃)₂ or MgCl₂, or at 23% RH/5 °C conditions. The equilibration conditions were not significant (data not shown) and were combined for graphical representation. Pollen viability did not decline after 9 months of LNV storage, and the highest levels of germination after 9 months were observed for Thoory F1 X (76 ± 1%), Thoory BC3 (74 ± 2%), and Fard #4 (64 ± 2%). Images of pollen grain in vitro germination for Dayri BC2, Fard #4, Jarvis #1, Thoory BC3 and Thoory X F1 after 9 months of LNV storage are shown in Fig. 3a–e.

Cooling and warming cycles

In vitro germination percentages were determined after pollen from each of the five date palm cultivars was cooled to LNV and then warmed to 23 °C up to 60 times. There were no significant responses among cultivars (data not shown). In vitro germination levels declined as the number of cycles increased, according to a quadratic regression, with a coefficient of determination (R^2) above 0.98. Viability was 20% after 60 cooling and warming cycles (Fig. 4).

Discussion

Date palm pollen is a valuable resource for conservation. The present study identified successful conditions for date palm pollen cryopreservation: pollen is harvested up to 4 days after spathe opening, adjusted to a moisture content between 4.4 and 6.2% (dry weight basis) and stored in LNV. For germination, pollen is warmed, rehydrated in a saturated H₂O environment for 2 h, and plated onto medium described by Marquard (1992). After an overnight incubation at 23 °C, pollen germination levels are measured. Date palm pollen is hardy because it can withstand nine months of cryogenic storage as well as multiple LNV cooling and warming cycles.

Desiccation-tolerant pollen can often be cryopreserved after air-drying (Franchi et al 2011); however, it is difficult to repeat “air-dried” conditions when the moisture content is not recorded in the literature. Publication of pollen moisture

Fig. 1 Viability (as assessed by *in vitro* germination) of date palm pollen harvested **a** 0 days (Harvest 1), **b** 2 days (Harvest 2) and **c** 4 days (Harvest 3), either with exposure to LNV (filled triangle) or without LNV (filled circle) for up to 56 days. Cultivar and moisture equilibration data were combined for the treatments. Error bars and regressions are provided

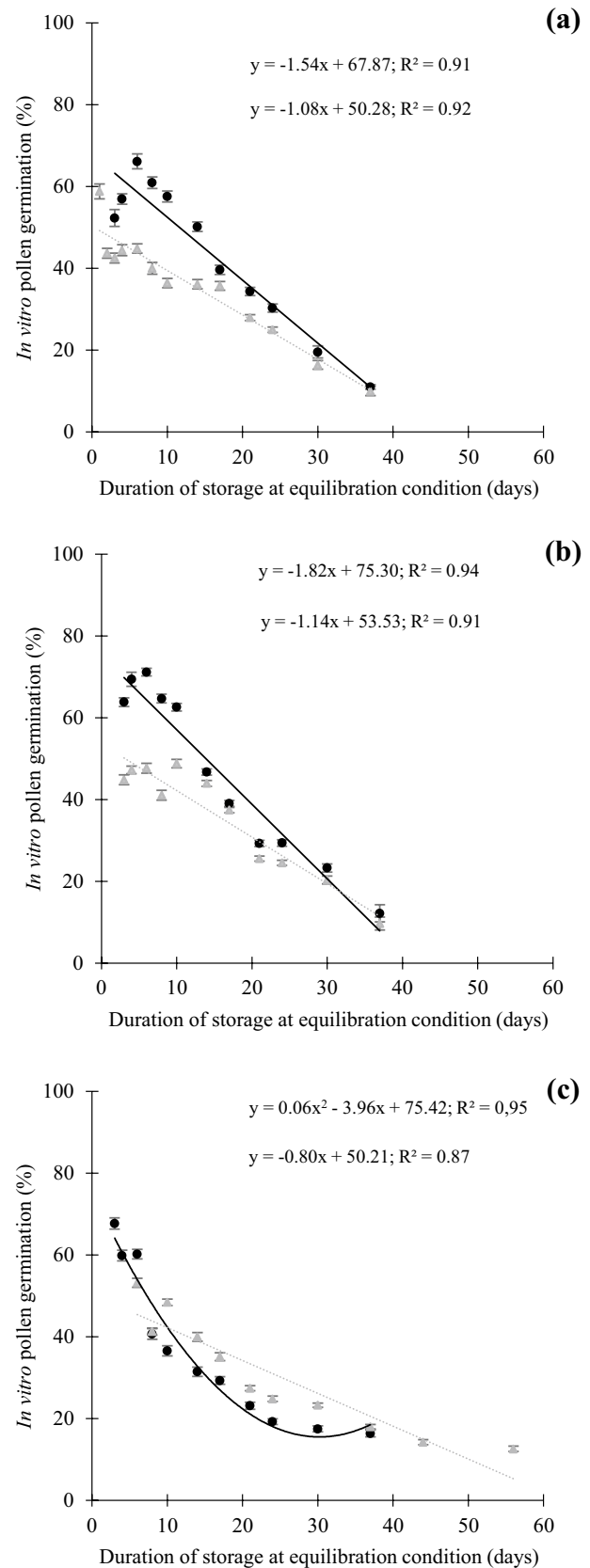
content after equilibration and before cryopreservation provides necessary information for successful experimental replication (Souza et al. 2018; da Silva et al. 2017). Herein, pollen was desiccated over saturated salts of $\text{Ca}(\text{NO}_3)_2$ at 23 °C, MgCl_2 at 23 °C, or in a room set at 23% RH/5 °C, with no significant differences among desiccation treatments after cryoexposure, as measured by *in vitro* germination.

Saturated salts solutions are well known to provide control of humidity (O'Brien 1948). In our studies, pollen desiccated for 18 h at 23 °C, over a saturated salt solution of MgCl_2 had a lower moisture content ($4.65 \pm 0.27\%$) than pollen desiccated over a saturated salt solution of $\text{Ca}(\text{NO}_3)_2$ ($6.22 \pm 0.38\%$). These results are in agreement with Connor and Towill (1993), who reported a similar moisture content value ($4.2 \pm 0.4\%$) for date palm pollen desiccated over saturated salts solutions of MgCl_2 . Some authors have used traditional drying rooms with temperatures between 25 and 30 °C for date palm pollen desiccation (Mortazavi et al. 2010; Maryam et al. 2015; Mesnoua et al. 2018a). In the current work, date palm pollen was placed in a reduced-temperature seed drying room set at 23% RH/5 °C and an average moisture content of $4.43 \pm 0.3\%$ was obtained. These conditions may be preferable for processing large quantities of pollen or for holding batches of pollen for up to about 2 weeks prior to LNV exposure, if processing delays are encountered.

Pollen must be rehydrated to a MC of about 20% (dw) prior to *in vitro* germination (Connor and Towill 1993). Controlled rehydration allows the dry pollen to uptake water gradually (Benson 2008) to prevent membrane damage (Conner 2011).

Date palm pollen *in vitro* germination varied among culture media and incubation temperature, which suggests some degree of genotype specificity. The best results were obtained for Medium B, which was Marquard with 15% sucrose at 23 °C for all cultivars, except for Dayri BC2. For Dayri BC2, Medium C (BK modified with $\text{Ca}(\text{NO}_3)_2 \cdot 2\text{H}_2\text{O}$ (0.200 g L^{-1}), H_3BO_3 (0.050 g L^{-1}), and 15% sucrose) exhibited a higher germination level.

The addition of nutrients, such as calcium nitrate and boric acid, may have positively influenced date palm pollen germination. Such elements play a key role on elongation of pollen tube growth (Brewbaker and Kwack 1964). Boron is known to improve sucrose uptake and takes part in pectin



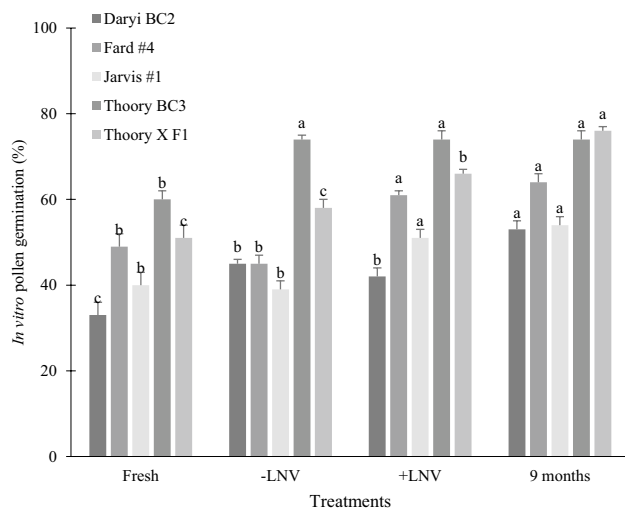


Fig. 2 Viability (as assessed by in vitro germination) of date palm pollen that was freshly harvested (fresh), – LNV, + LNV, and 9 months of + LNV storage. Data for moisture equilibration conditions of saturated salts of $\text{Ca}(\text{NO}_3)_2$, MgCl_2 , and 23% RH/5 °C were combined for data presentation. Means within bars for each cultivar among treatments with the same letter were not significantly different at $P \leq 0.05$

production (Wang et al. 2003). Calcium nitrate is involved in cell metabolism and maintenance of membrane integrity, permeability and orientation (Zheng et al. 2019). Sucrose promotes osmotic balance between pollen and culture media and acts as a source of energy (Sarkar et al. 2018). Mesnoui et al. (2018a, b) performed in vitro germination experiments for date palm pollen using culture media supplemented with 5% sucrose. Previous reports used sucrose concentrations of 5% to 25% (Mortazavi et al. 2010; Maryam et al. 2015). Contrary to that observed by Tisserat et al. (1983), the addition of EDTA in medium E might have inhibited date palm pollen tube growth in the present studies. Pollen from apple was also sensitive to this additive (Köpcke et al. 2002).

In general, pollen germinates well at 25 °C. Nevertheless, germination temperatures differ among genera/species and should be evaluated (Volk 2011). Herein, the highest in vitro germination levels were obtained for date palm pollen cultivars Fard #4 and Jarvis #1 incubated at 23 °C, which was the room temperature of the laboratory. Others found high levels of pollen germination at 23, 28 or 30 °C (Tisserat et al.

1983; Mortazavi et al. 2010; Maryam et al. 2015; Mesnoui et al. 2018a).

Date palm pollen from Harvests 1, 2 and 3 had similar germination levels for up to 56 days equilibration over saturated salts of $\text{Ca}(\text{NO}_3)_2$, MgCl_2 , and 23% RH/5 °C. All – LNV samples had viability levels of at least 50% for the first 2 weeks of storage at the equilibration conditions. Exposure to LNV dropped the viability levels compared to the – LNV samples; however, the viability did not further decline after extended duration of LNV storage. After 9 months of LNV storage, the viability levels significantly increased for LNV pollen for two of the five date palm cultivars. Anushma et al. (2018) also reported an increase in viability of date palm pollen after storage in liquid nitrogen. This increase in viability may be the result of slight variations in laboratory environmental conditions during the calendar year or as a result of unknown physiological responses. Overall, pollen viability levels were higher after the moisture content was adjusted. This may be because the freshly harvested pollen had not yet completely progressed through its physiological drying and maturation process at the time of first harvest. Mortazavi et al. (2010) and Anushma et al. (2018) reported viability levels of between 67 and 90% after LNV exposure.

Unlike other propagules (e.g. shoot tips, dormant buds), date palm pollen remained viable after many cycles of warming and cooling. This may be due to several structural adaptations, such as the capacity of the membranes to fold to minimize water loss. Once exposed to a humid environment, the process is reversed during rehydration (Katifori et al. 2010).

Successful conservation of date palm pollen is critical for both genebanks and breeding programs. Long-term preservation of pollen, the male gamete, does not effectively preserve the diversity of specific cultivars in genebank collections. In vitro plants and cryopreserved shoot tips are alternative methods in which date palm cultivars could be preserved. The development of successful shoot tip cryopreservation methods for date palm cultivars will be dependent upon having effective in vitro propagation systems. In vitro approaches for conservation must consider the possibility of somaclonal variation (Mirani et al. 2020) and may need to overcome the challenges associated with endophytic bacteria (Meziani et al. 2019).

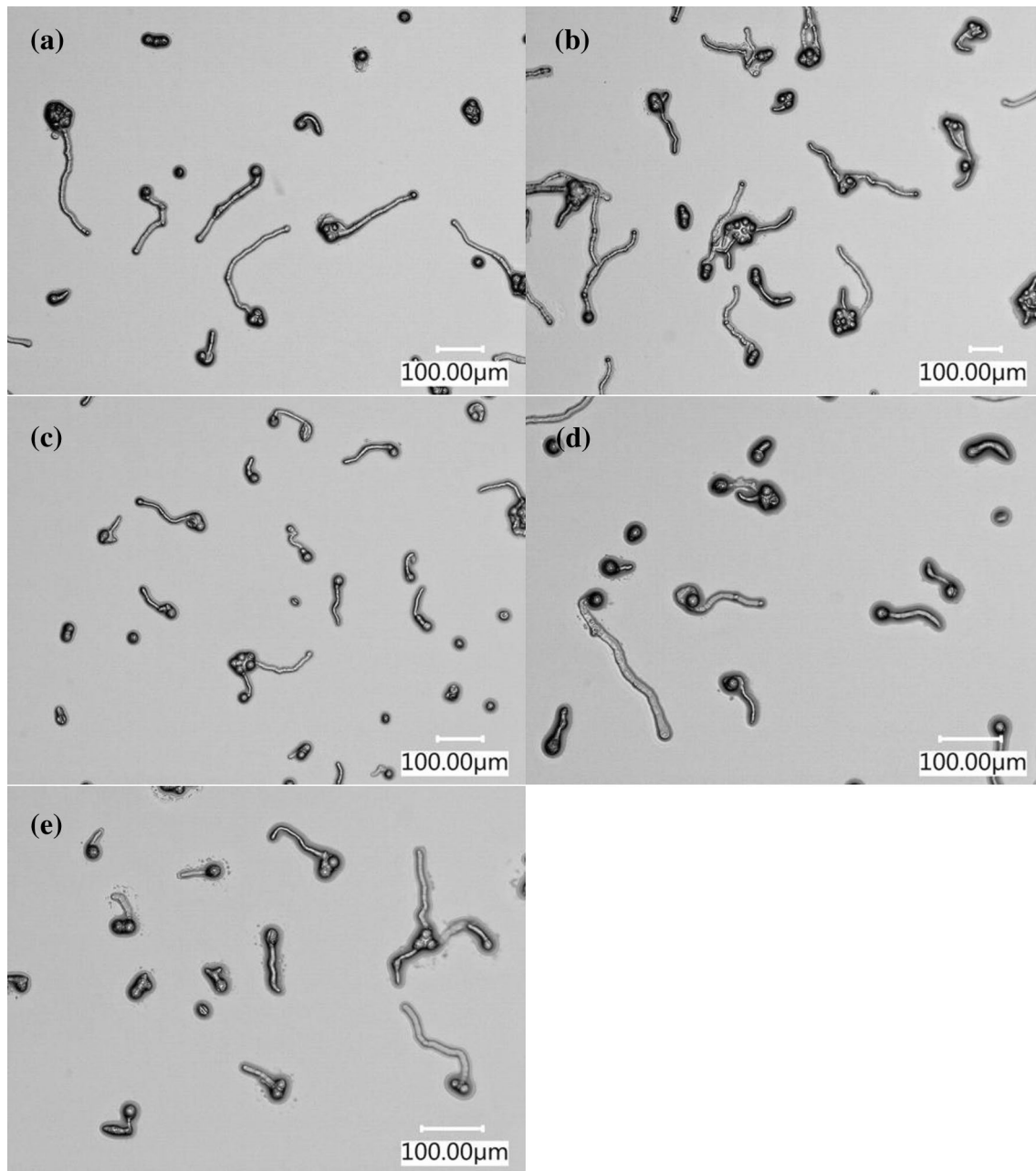


Fig. 3 Images showing in vitro germination of date palm pollen. Pollen was equilibrated for 1 day, stored in LNV for 9 months, rehydrated over H_2O for 2 h, and plated onto Medium B, and incubated

overnight for cultivars **a** Dayri BC2 ($Ca(NO_3)_2$ at 23 °C), **b** Fard #4 ($Ca(NO_3)_2$ at 23 °C), **c** Jarvis #1 ($Ca(NO_3)_2$ at 23 °C), **d** Thoory BC3 ($Ca(NO_3)_2$ at 23 °C) and **e** Thoory X F1 (23% RH/5 °C)

Date palm breeders need to store pollen to perform controlled crosses during the breeding season (Maryam et al. 2015; Mesnoua et al. 2018a). Our results suggest that cryopreservation actually improved viability and could perhaps be implemented in breeding programs to prolong duration of

storage across multiple seasons (Tisserat et al. 1983; Mortazavi et al. 2010). The methods presented can also be used to secure pollen at the NLGRP for long-term preservation of the 49 male trees in the NPGS date palm collection.

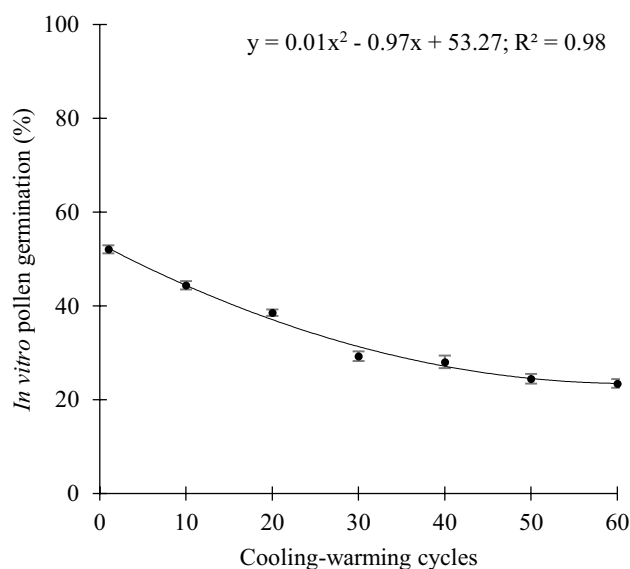


Fig. 4 Viability (as assessed by in vitro germination) of date palm pollen for up to 60 cooling-warming cycles in LNV. Cultivars and moisture-equilibrated conditions were combined for data presentation. Error bars and regression are provided

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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