

## Optimizing Storage and *In vitro* Germination of Date Palm (*Phoenix dactylifera*) Pollen

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### ABSTRACT

Iran is one of the main date (*Phoenix dactylifera*) producing countries in the world. however, little information is available on the pollen viability and favorable storage conditions of Iranian male date cultivars. This study was conducted to estimate the best *in vitro* pollen germination of three male date palm cultivars, 'Ghanami', 'Samsmavi' and 'Gheibani' using various doses of boric acid (0, 50, 100 and 200 mg L<sup>-1</sup>), calcium nitrate (0, 200 and 300 mg L<sup>-1</sup>) and sucrose (15% and 25% (w/v)) in a Modified Brebaker and Kwack (MBK) medium. The results showed that the best germination was achieved with 50 mg L<sup>-1</sup> boric acid, 200 mg L<sup>-1</sup> calcium nitrate and 15% (w/v) sucrose. Once the optimal *in vitro* germination medium had been established, pollen viability (% germination) of these three cultivars stored for up to 200 days at room temperature (dark and light), in a refrigerator (4°C), freezer (-20°C) and in liquid nitrogen (-196°C) was compared. For all the tested cultivars, germination declined as storage temperature increased and, by 200 days, the highest survival rate was obtained with cryostored pollen. Long-term storage of date pollen using an ultra-low temperature (-196°C) can be used without any deteriorating effect on pollen viability, while room temperature storage significantly reduced pollen germination.

**Keywords:** Cryopreservation, *Phoenix dactylifera*, Pollen germination, Pollen storage.

### INTRODUCTION

Being a dioecious species in character, date palm sexes are borne by separate individuals. The unisexual flowers are pistillate and staminate in character, and so artificial pollination is an essential cultural practice to obtain satisfactory fruit set (Zaid, 1999). Pollen grains also have a direct effect on the size, shape, and weight, as well as time of ripening of the resulting fruit (DeMason and Sekhar, 1988). The emergence of many early inflorescences on female date palms before the opening of an adequate number of male spathes on

available male palms always results in a scarcity of pollen and growers have to use pollens of unknown sources which are characterized by low fertility and incompatibility. Consequently, storage of pollen within the pollination period (2-3 months) or from one season to another is a necessity (Mortazavi, 2003). Also, pollen storage is an important technique for germplasm conservation and breeding programs (Lora *et al.*, 2006). During the storage period, factors such as temperature and relative humidity (RH) have effect on pollen longevity.

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Pollen viability is generally considered to indicate the ability of the pollen grains to perform their function of delivering the sperm cells to the embryo sac following compatible pollination (Shivanna *et al.*, 1991). *In vitro* germination of pollen allows the measurement of its genuine aptitude to germinate outside any interaction between pollen and stigma. On the other hand, pollen capacity to fertilize the ovule and set the fruit is considered as a measure of natural aptitude (Boughediri and Bounaga, 1987) and identifying a suitable method is the key to a more accurate estimation of pollen viability (Rodriguez-Riano and Dafni, 2000). *In vitro* germination has been used as an important technique for testing pollen viability, but difficulties in establishing an optimal culture medium have been reported (Khatun and Flowers, 1995). Clear differences exist between species in the requirements in germination medium due to the different germination media offered by the different stigmata (Heslop-Harrison, 1987). Studies on the viability of stored date palm pollen go back to the 1930's. Albert (1930) showed that the germination percentage of date pollen stored for one year at 3.3 °C was greater than that of pollen stored at room temperature. Using modified Brewbaker and Kwak (1964) medium, Furr and Enriquez (1966) were able to obtain a high percentage of *in vitro* germination of date palm pollen. It is known that pollen germination and tube growth are significantly regulated by the transport of inorganic ions such as  $\text{Ca}^{2+}$  and  $\text{K}^{+}$  across the plasma membrane of the pollen (Taylor and Hepler, 1997). Boron is also an important element for pollen germination (Potts and Gore, 2000). Asif *et al.* (1983) showed that 100 ppm boric acid in the medium had the highest effect on date palm pollen germination.

Pollen viability may decrease quickly depending upon the storage conditions. Long-term storage of pollen has been demonstrated for many plant species. A low temperature is an important condition but the temperature recommended depends on

the species (Lee *et al.*, 1985; Hanna and Towill, 1995). Boughediri and Bounaga (1991) found that storing date palm pollen in a desiccator containing anhydrous calcium chloride and placed in 4°C for 230 days provided satisfactory maintenance of pollen viability.

Boughediri *et al.* (1995) determined that freeze-drying is the optimal condition for maintaining long term viability of date palm pollen. Engelmann (1997) reported that the ideal method for long-term storage of pollen is cryopreservation; this refers to storage at ultra-low temperatures such as liquid nitrogen (-196°C) that arrests all cellular activities. Cryopreservation has been used as an effective method for long-term storage of oil palm pollen (Tandon *et al.*, 2007). Liquid nitrogen treated date palm pollen was found to be capable of pollinating and setting fruit in mature trees of Deglet-Noor cultivar (Tisserat *et al.*, 1985).

On the basis of FAO reports, Iran is one of the leading countries in date production and export in the world (Anonymous, 2007) but unfortunately, there are few reports available on the Iranian male date cultivars, their pollen viability and longevity. The objectives of this investigation were (1) the optimization of an *in vitro* germination protocol to effectively detect viable date pollen and (2) to compare the effect of different storage conditions on date palm pollen longevity.

## MATERIALS AND METHODS

### Plant Material Used

Date palm pollen used in this study was obtained from trees grown at the Date Palm Institute collection orchard, Ahvaz, Iran. The orchards (31°21' N, 48°40' E) were situated 15 m above sea level. The spathes of three commercial Iranian male date cultivars, namely '*Ghanami*', '*Samsmavi*' and '*Gheibani*' were obtained in February 2001 from male palms of same age of about

15 years which had been subjected to the same agricultural practices.

### Pollen Collection and Processing

The spathes of each male cultivar were taken to the traditional drying room (25-30°C and 30-40% RH) as soon as they cracked. After 48 hours, extraction of pollen grains was carried out by the traditional hand method, and the pollens were then shaken out as the anther dehisced and spread on large sheets of paper. Pollen grains of 5-6 male spathes were mixed together to minimize variations that might have existed between the pollens of those spathes.

### *In vitro* Pollen Germination

In the first experiment, to determine the effects of various chemicals on *in vitro* pollen germination, a factorial experiment involving two sucrose concentrations (15% and 25% w/v), four boric acid concentrations (0, 50, 100 and 200 mg L<sup>-1</sup>) and three CaNO<sub>3</sub> concentrations (0, 200 and 300 mg L<sup>-1</sup>), i.e. a total of 24 different germination media, was performed. The basic medium to which the various concentrations of chemicals were added, was prepared following the procedure of Brebaker and Kwack, 1964 by adding 1% agar, 200 mg L<sup>-1</sup> MgSO<sub>4</sub> and 100 mg L<sup>-1</sup> KNO<sub>3</sub>, then boiled for an hour. For each treatment, 10 ml of medium were poured into each plastic Petri dish (90 mm diameter). Treatments were replicated three times and in each treatment, pollens were uniformly sown on the medium with a fine brush and forceps. All Petri dishes were incubated at 30°C for 12 hours, then the growth of pollen tubes was stopped by spraying a 45% acetic acid solution on the medium (Al-Helal *et al.*, 1988). Random counts of 400 pollen grains were made in each replication under 40X magnification using Olympus BX41 microscope.

### Pollen Storage Treatments

In the second experiment, to discover favorable storage conditions for pollen of 'Ghanami', 'Samsmavi' and 'Gheibani' cvs., the collected pollen grains were placed in small glass vials with stoppers. The vials (3 replications) were subjected to the following regimes: room temperature (dark and light), refrigerator (4°C) and freezer (-20°C) and liquid nitrogen (-196°C). Also about 10 g of pollen grains were treated with liquid nitrogen (-196°C) by wrapping them within pieces of aluminum foil and then immersing them in a thermos of liquid nitrogen. After 40 and 200 days of storage, pollens of each storage regime were subjected to viability tests using the technique based on the results of the first experiment.

### Statistical Analysis

The experimental design used for both experiments was a completely randomized design with three replications. To normalize data distribution, the values for the proportion of pollen germination were angular-transformed before analysis. Data were subjected to analysis of variance, using the MSTATC statistical package (MSTATC, Michigan State University, East Lansing, MI) and the levels of significance determined by the *F*-test. Comparison of the means was performed using the least significant difference (LSD) at  $P \leq 0.01$ . Standard errors are provided where appropriate.

## RESULTS

### Optimizing *In vitro* Germination Medium

Variance analysis of the initial factorial experiment indicated significant differences in pollen germination associated with the sucrose, boric acid, and calcium nitrate treatments (Table 1). Concerning the effects of various chemicals on the studied trait the data presented in Table 2 indicated that

**Table 1.** Analysis of variance for transformed values of percentage germination of fresh pollen, used in the determination of an optimal *in vitro* germination medium.

Source of variation	d.f.	Mean square	F-value	P-value
Cultivar	2	11165.19	6177.14	< 0.01
Sucrose	1	4152.77	2297.52	< 0.01
Cultivar×Sucrose	2	87.06	48.16	< 0.01
Boric acid	3	105.05	58.12	< 0.01
Cultivar×Boric acid	6	28.99	16.04	< 0.01
Sucrose×Boric acid	3	148.15	81.97	< 0.01
Cultivar×Sucrose×Boric acid	6	12.01	6.64	< 0.01
Calcium nitrate	2	705.14	390.11	< 0.01
Cultivar×Calcium nitrate	4	113.79	62.95	< 0.01
Sucrose×Calcium nitrate	2	72.18	39.93	< 0.01
Sucrose×Sucrose×Calcium nitrate	4	40.85	22.59	< 0.01
Boric acid×Calcium nitrate	6	51.29	28.37	< 0.01
Cultivar×Boric acid×Calcium nitrate	12	19.21	10.63	< 0.01
Sucrose×Boric acid×Calcium nitrate	6	144.09	79.72	< 0.01
Cultivar×Sucrose×Boric acid×Calcium nitrate	12	14.41	7.97	< 0.01
Error	144	1.81		

pollen germination could be beneficially influenced by the addition of such chemicals to the culture medium. Near-optimum concentrations of sucrose (15% w/v), boric acid (50 mg L<sup>-1</sup>), and calcium nitrate (200 mg L<sup>-1</sup>) were identified. According to our results, pollen germination varied from one cultivar to another and these differences were statistically significant ( $P \leq 0.01$ ). ‘*Samsmavi*’ and ‘*Ghanami*’ cultivars had the highest and lowest viability with 81.8% and 58.4% pollen germination respectively (Data

not shown).

#### Optimizing Pollen Storage Regimes

There was a significant effect of storage duration on pollen germination ( $P \leq 0.01$ ), as well as a significant overall effect of cultivar on pollen germination (Table 3). There was, however, a significant cultivar-by-storage time interaction and a significant treatment-by-storage time interaction after 200 days of storage among the five different regimes. This was in consistent with reports for other species such as cherimoya (Lora *et al.*,

**Table 2-** The effect of sucrose, boric acid, and calcium nitrate concentrations on pollen germination of three male date cvs. (Mean±SE).

	Sucrose		Boric acid		Calcium nitrate	
	Concentration (%)	Germination <sup>a</sup> (%)	Concentration (mg/l)	Germination (%)	Concentration (mg l <sup>-1</sup> )	Germination (%)
Ghanami	15 %	63.6±0.83 <sup>a</sup>	0	59.2±0.41 <sup>b</sup>	0	59.52±0.43 <sup>b</sup>
	25 %	53.2±0.27 <sup>b</sup>	50	60.5±0.12 <sup>a</sup>	200	63.07±1.15 <sup>a</sup>
			100	58.0±0.52 <sup>c</sup>	300	52.71±0.25 <sup>c</sup>
			200	56.0±0.89 <sup>d</sup>		
Gheibani	15 %	82.2±0.71 <sup>a</sup>	0	75.9±0.74 <sup>c</sup>	0	77.37±0.20 <sup>b</sup>
	25 %	72.6±0.33 <sup>b</sup>	50	80.5±0.52 <sup>a</sup>	200	80.05±0.70 <sup>a</sup>
			100	77.3±1.01 <sup>b</sup>	300	74.87±0.20 <sup>c</sup>
			200	76.0±0.40 <sup>c</sup>		
Samsmavi	15 %	85.0±0.17 <sup>a</sup>	0	82.2±0.20 <sup>a</sup>	0	80.31±0.39 <sup>bc</sup>
	25 %	79.1±0.78 <sup>b</sup>	50	81.9±0.23 <sup>a</sup>	200	84.26±0.14 <sup>a</sup>
			100	82.7±0.16 <sup>a</sup>	300	81.06±0.45 <sup>b</sup>
			200	80.7±0.54 <sup>b</sup>		

<sup>a</sup> Means of pollen germination followed by different letters are significantly different ( $P \leq 0.01$ ).

**Table 3.** Analysis of covariance for transformed values of percentage germination of pollen used in the determination of temperature regimes for optimal pollen storage.

Source of variation	d.f.	Mean square	F-value	P-value
Cultivar	2	4367.51	1289.57	< 0.01
Treatment	4	5350.66	1579.86	< 0.01
Cultivar×Treatment	8	30.39	8.97	<0.01
Error	30	3.38		
Time	2	18619.74	4701.05	< 0.01
Cultivar×Time	4	111.85	111.85	< 0.01
Treatment×Time	8	3490.49	3490.49	< 0.01
Cultivar×Treatment×Time	16	71.51	71.51	< 0.01
Error	60			

2006), oil palm (Tandon *et al.*, 2007) and sweet cherry (Albuquerque *et al.*, 2007).

Based on the results shown in the Table 4, the germination percentage showed similar changes for all three studied cultivars. Fresh date palm pollen stored at room temperature showed that pollen germination decreased to 51.36, 74.29 and 75.94% for 'Ghanami', 'Gheibani' and 'Samsmavi', respectively after 40 days (data not shown) and no pollen germination was obtained 200 days after storage, also no significant differences were observed between pollen stored in both dark and light conditions. Pollen grains stored in the refrigerator (4°C) showed an acceptable viability after 200 days, with 54.9, 71.6 and 77.8% for cvs. 'Ghanami', 'Gheibani' and 'Samsmavi', respectively. Storage in the freezer kept pollen grain viability significantly higher than in the refrigerator and the differences were highest for Gheibani cultivar under freezer conditions. Generally, pollen grains from all cultivars appeared to give the best response when they were stored in liquid nitrogen and pollen stored in this temperature had lost

only about 2% of its viability after 200 days of storage.

## DISCUSSION

These differences between pollen germination in the cultivars studied could be due to the variability in their genetic make up. The results were shown that regardless of orchardists interest in 'Ghanami' as a very popular male cultivar in the south of Iran, this cultivar had the lowest viability. It seems that pollen of this cultivar has the highest compatibility with the main female cultivars such as 'Estamiran' and 'Barhee' regardless of its viability.

According to the present findings, a medium containing 15% (w/v) sucrose, 50 mg L<sup>-1</sup> boric acid and 200 mg L<sup>-1</sup> calcium nitrate on the basis of Brewbaker and Kwack medium appeared to be optimal for *in vitro* germination of fresh date palm pollen of all the cultivars used in the study. This medium was chosen and used for the

**Table 4.** The effect of storage conditionS on the mean pollen germination (%) of three male date cvs.

Storage conditions	Cultivars		
	Ghanami	Gheibani	Samsmavi
Room temperature, Light	37.5 <sup>d</sup> *	52.0 <sup>d</sup>	53.7 <sup>d</sup>
Room temperature, Dark	38.2 <sup>d</sup>	51.5 <sup>d</sup>	53.3 <sup>d</sup>
Refrigerator (4 °C)	54.9 <sup>c</sup>	71.6 <sup>c</sup>	77.8 <sup>c</sup>
Freezer (-20 °C)	59.6 <sup>b</sup>	78.4 <sup>b</sup>	80.2 <sup>b</sup>
Liquid Nitrogen (-196 °C)	67.1 <sup>a</sup>	80.9 <sup>a</sup>	83.9 <sup>a</sup>

\* Means of pollen germination followed by different letters are significantly different (P< 0.01).



second experiment. A sucrose concentration of 25% had an inhibitory effect on pollen germination and some pollen burst was observed at this concentration.

Several studies have examined the impact of boron on pollen germination and tube growth (Wang *et al.*, 2004; Ak *et al.*, 1995). A significant response in pollen germination was identified in the analysis of a broad range of boric acid treatments. In our study, date pollen germination was maximized on a medium containing 50 mg L<sup>-1</sup> boric acid whereas it was significantly inhibited at higher concentrations (200 mg L<sup>-1</sup>) of boric acid. Boron, which is provided by stigmas and styles, facilitates sugar uptake and has a role in pectin production in the pollen tube (Richards, 1986). It has an implication for the translocation and metabolism of carbohydrates, in the indole-acetic acid metabolism and it is required for the efficient operation of membrane transport systems (Shivana and Johri, 1985).

Presence of calcium nitrate in the medium also played a significant role. Pollen germination was significantly lower when no calcium nitrate was present in the medium or when there was an excessive amount of calcium nitrate (300 mg L<sup>-1</sup>). Pollen germination was maximized when the medium contained 200 mg L<sup>-1</sup> calcium nitrate (Table 1). Ca<sup>2+</sup> signals are thought to play an important role in plant growth and development, including key aspects of pollen tube growth and fertilization. The dynamics of a Ca<sup>2+</sup> signal are largely controlled by influx (through channels) and efflux (through pumps and antiporters) (Schiøtt *et al.*, 2004). The addition of calcium to the germination medium has been reported to increase fluorescence in the pollen tube tip. Lack of Ca<sup>2+</sup> in the growth medium results in morphological abnormalities such as coiling and tip swelling (Shivanna and Rangaswamy, 1992; Taylor and Hepler, 1997). *In vitro* germination assays revealed that a tip-localized intracellular Ca<sup>2+</sup> gradient, which

arises from the influx of Ca<sup>2+</sup> at the tube tip, is essential for pollen tube elongation (Holdaway-Clarke and Hepler, 2003). Calcium is also known to be involved in pectin synthesis and the control of osmotic conditions (Richards, 1986).

The second part of the study focused on determining suitable temperature regimes for the storage of pollen grains of the three date palm cvs. studied. From the results obtained, we can conclude that pollen conservation at -196°C can prove interesting for long-term storage such as conservation of date palm genetic resources. This method offers a suitable tool for storing pollen for hand pollination both for commercial fruit production as well as breeding programs. Our results coincide with those obtained by Lora *et al.* (2006) for cherimoya, Weatherhead *et al.* (2006) for potato pollen and Gomes *et al.* (2003) for onion. However, if liquid nitrogen is not available, pollen grains can be kept in the freezer or refrigerator for up to 200 days without too much decrease in viability.

As shown on Figure 1, the pollen grains of the cultivars studied stored in a freezer or in liquid nitrogen showed a slight increase in viability after 40 days of storage. This is somewhat unexpected since usually the decline in pollen germination increases throughout the period of storage. This phenomenon was also reported by Boughediri and Bounanga (1991) for some Algerian male date pollens after 50 days of storage in a freezer and dedicator. It can be surmised that some pollens can continue their development during storage and gradually turned into mature and viable pollens (Boughediri and Bounanga, 1991).

The results of this study corroborate the importance of the storage conditions of pollen grains when artificial pollination is a necessary and practical technique. However, more commonly the gap between pollen harvesting and deliverance may be days, weeks or months and during this time lag the pollen has to be stored in a good condition (Pinillos and Cuevas, 2008).

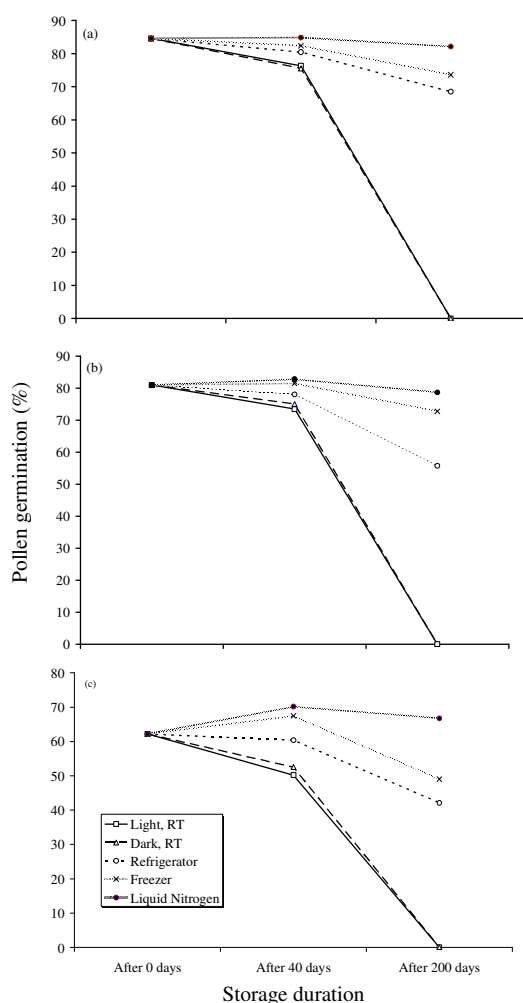


Figure 1. Effect of storage temperature over 200 days on in vitro pollen germination of cvs. (a) 'Ghanami' (b) 'Gheibani' and (c) 'Samsmavi'.

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## بررسی شرایط بهینه نگهداری و جوانه‌زنی درون‌شیشه‌ای گرده نخل خرما (*Phoenix dactylifera*)

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### چکیده

ایران یکی از مهم‌ترین کشورهای تولید کننده خرما (*Phoenix dactylifera*) در جهان است. اطلاعات اندکی در زمینه قوه نامیه و شرایط مناسب نگهداری ارقام خرمای نر ایران وجود دارد. این پژوهش به منظور بررسی شرایط بهینه جوانه‌زنی درون‌شیشه‌ای گرده سه رقم نر خرما، شامل "غنامی"، "سمسماوی" و "غیبانی" در غلظت‌های مختلف اسیدبوریک (۰، ۵۰، ۱۰۰ و ۲۰۰ میلی گرم در لیتر)، نیترات کلسیم (۰، ۲۰۰ و ۳۰۰ میلی گرم در لیتر) و ساکارز (نسبت وزن به حجم ۱۵٪ و ۲۵٪) بر اساس محیط تغییر یافته برویکر و کواک انجام گردید. نتایج نشان داد که بهترین جوانه‌زنی در شرایط ۵۰ میلی گرم در لیتر اسید بوریک، ۲۰۰ میلی گرم در لیتر نیترات کلسیم و ۱۵٪ ساکارز بدست آمد. پس از تعیین بهترین شرایط جوانه‌زنی، قوه نامیه (٪جوانه‌زنی) این سه رقم طی مدت زمان ۲۰۰ روز نگهداری در شرایط دمای اتاق (روشنایی و تاریکی)، یخچال (۴ °C)، فریزر (۱۸- °C) و نیتروژن مایع (۱۹۶- °C) مقایسه گردید. برای همه ارقام آزمایش شده، با بالاتر بودن دمای نگهداری جوانه‌زنی کاهش یافت و پس از ۲۰۰ روز گرده‌های قرار گرفته در شرایط نیتروژن مایع بیشترین بقا را داشتند و این شرایط را می‌توان بدون هر گونه اثرات سوء بر قوه نامیه برای نگهداری طولانی مدت دانه‌های گرده به کار برد در حالی که نگهداری در دمای اتاق به نحو چشمگیری قوه نامیه گرده‌ها را کاهش داد.