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Effect of storage temperature and duration on pollen viability and *in vitro* germination of seven pistachio cultivars

Abdallah Aldahadha[1]*, Nezar Samarah[2] and Ahmad Bataineh[1]

*[1]Field Crops Directorate, National Agricultural Research Center (NARC), P.O. Box 19831, Baqa, Jordan. [2]Department of Plant Production, Faculty of Agriculture, Jordan University of Science and Technology (JUST), P.O. Box 3030, 22110, Irbid, Jordan. *E-mail: abdallah.aldahadha@narc.gov.jo*

Abstract

The study was conducted to examine the effect of storage temperature and duration on the viability and *in vitro* germination of pollen grains of seven pistachio cultivars. Pollens were stored at room temperature (24±2 °C), refrigerator (4 °C) and freezer (-5 °C) for 0, 1, 2, 3 and 4 weeks. Pollen viability was estimated by using staining methods including tetrazolium test (TTC), iodine and potassium iodine (IKI) and safranin solutions, and using an *in vitro* pollen germination. The results showed that at all storage methods and durations, pollen viability and *in vitro* pollen germination were significantly the highest for Batouri and Ashouri cultivars and the lowest for Marawhi and Elemi cultivars. The highest pollen viability as estimated by safranin staining was attained when pollens were stored under freezer condition. However, pollen viability by TTC was the lowest at room temperature storage. In addition, *in vitro* pollen germination and viability significantly decreased as storage duration increased. This study revealed no differences between *in vitro* germination percentages in refrigerated and freezer stored pollen up to 2 weeks. Meanwhile, *in vitro* germination of room-stored pollen gradually decreased when storage duration increased. At the end of storage period, pollen viability was reduced slightly under freezer conditions, whereas, the reduction in viability was the highest for refrigerated and room-stored pollen with no differences between them. This study showed a significant interaction effect of cultivar x storage temperature x storage duration on pollen viability but not for *in vitro* pollen germination.

Key words: Pollen, storage, pistachio, cultivars, viability, germination

Introduction

The genus *Pistacia* is a member of the family Anacardiaceae (Ak *et al.*, 2016) which contains at least 13 species, including pistachio (*Pistacia vera* L.). Pistachio trees are dioecious which have the pistillate and staminate flowers on different trees (Acar *et al.*, 2010). Pistachio is wind-pollinated tree as the flowers have no petals to attract insects (Hosseini *et al.,* 2015). It is necessary to have enough male trees to insure adequate pollination and to get maximum nut production. Male and female trees are usually inter-planted in the orchard with one male to eight or eleven females depending on the orchard size (Bahramabadi *et al.,* 2018). Therefore, the amount of pollen produced in each cluster and germination rate of pollen must be high (Ak *et al.*, 2016). Pistachio trees are cultivated mostly in the Mediterranean regions of Europe, the Middle East and California (Vaknin *et al.*, 2002). In Jordan, pistachio production ranged between 10 tons in 1975 and 410 tons in 2017 (FAOSTAT, 2017).

Pistachio productivity is mainly affected by efficiency of pollen performance since the seed is the marketable product. Generally, there is a linear correlation between germinability and pollen viability (Stanley and Linskens, 1974). Therefore, *in vitro* pollen germination tests are required to determine the real amount of viability of fresh or stored pollen (Sulusoglu and Cavusoglu, 2014). If the conditions are not proper, viable pollen may not really germinate either *in vitro* or *in vivo* (Beyhan and Serdar, 2008). *In vitro* pollen germination is a convenient method as it can unravel both physiological and biochemical conditions, necessary for positive germination of pollen grains and growth of pollen tube and can be used for determining pollen vigor by observing the germination rate over a period of time or the length of pollen tubes (Shivanna and Ram, 1993). However, pollen grains of pistachio have been considered to be hard for *in vitro* germination (Golan-Goldhirsh *et al.,* 1991). Besides, pollen viability commonly refers to the ability to deliver efficient sperm cells to the embryo sac after compatible pollination (Shivanna and Ram, 1993). Usually, pollen quality is assessed on the basis of viability and vigor of pollen grains.

The longevity of pollen is defined as the retaining viability of stored pollen after long-term preservation (Vaknin and Eisikowitch, 2000). There has been a frequent concern in evolving consistent approaches for both short- and long-term storage of pistachio pollen (Ateyyeh, 2012). Such techniques would be essential for storing pollen to be used in breeding programs (Vithanage and Alexander, 1985) and supplemental pollination programs for pistachio production which involves adequate quantities of pollen collection and then storage for short (hours to weeks) or long (months to years) durations for retaining pollen viability (Shivanna and Sawhney, 1997). In general, short-term storage is achieved in experiments for genetics and breeding programs, but long-term storage is targeted for genetic conservation (Engelmann, 2004). Furthermore, pollen viability is affected by storage conditions such as temperature and relative humidity (Deng and Harbaugh 2004) and affected

by age of pollen, physiological state of flower, and pollen water content (Soares *et al.,* 2008). The overall aim of this study was to investigate the effect of storage method and duration on pollen viability as estimated by using three staining tests for seven pistachio cultivars compared to *in vitro* pollen germination.

Materials and methods

Plant material and location: The research was carried out at Maru Agricultural Research Station (MARS) on seven pistachio cultivars: Lazaourdi, Nab-El Jamal, Boundiki, Batouri, Marawhi, Aschouri, and Elemi. All pistachio cultivars have been grown since 1984 and originally imported from Syria. MARS is located in Irbid at 32° 33′N latitude, 35° 51′ E longitude and 589 m above sea level (Al-Ghzawi *et al.,* 2018). Maru has usual Mediterranean climate conditions with hot and dry summer. This location signifies an intermediate drought area with a mean annual rainfall of around 380 mm. *In vitro* pollen germination and viability tests were undertaken at the biotechnology lab of the National Agricultural Research Center (NARC), Baqa'a, Jordan.

Pollen collection: Pollen grains of each cultivar were collected in Spring (22-26 April, 2019) from flowers having the same phenological stage (anthesis) from various inflorescences. Pistachio clusters of each cultivar were extracted by shaking in a square glass tray. The pollens were then collected carefully to avoid contamination. Subsequently, pollens of each cultivar were kept in closed plastic vials to be used later on.

Pollen storage: For each pistachio cultivar, samples of pollen were stored in small plastic vials at three storage temperatures: room temperature (24 ± 2 °C), refrigerator at 4 °C, and freezer at -5 °C. Pollen viability and *in vitro* pollen germination were tested after 0, 1, 2, 3, and 4 weeks of anthesis under each storage temperatures (room, refrigerator and freezer).

In vitro **pollen germination test**: To test the percentage of *in vitro* pollen germination, a medium was prepared by containing 1 % agar, 15 % sucrose and 100 ppm boric acid (H_3BO_3). Afterwards, pollen grains were spread on the medium and incubated in a chamber room at 24 °C. After 24 hours of incubation, pollen grains from each sample (total 105 samples for each cultivar, storage method and storage duration) were counted using a light microscope to estimate the percentage of pollen germination. For each sample, 3 replicates (Petri-dishes) were used. Pollen was considered to be germinated if the developed pollen tube was beyond (2-3 times) of its diameter. The germination percentage of pollen was calculated using the formula below:

$$\text{Pollen germination (\%)} = \frac{\text{Number of germinated pollen grains}}{\text{Number of incubated pollen grains}} \times 100$$

Pollen viability tests: Pollen viability was tested using three staining techniques: Tetrazolium test or TTC (2,3,5-Triphenyl tetrazolium chloride), IKI (iodine + potassium iodide) and safranin solution. For TTC test, pollen viability was assessed using 1 % TTC and 60 % sucrose. TTC-sucrose solution was stored in a brown glass bottle in a refrigerator. One drop of solution was placed onto a micro-slide then a small amount of pollen was suspended in the drop and cover glass was placed onto the micro-slide, wrapped with aluminum foil and incubated in a chamber room at 28 °C for 60 minutes. Pollen grains stained orange or bright red color were considered as viable. To prepare IKI solution, 1 g of potassium iodide (KI) + 0.5 g of iodine (I) were dissolved in 100 mL distilled water. The pollen viability counts were made five minutes after pollen grains were placed on IKI solution under light microscope. Pollen grains stained dark red or brown color were counted as viable. Safranin solution was prepared by dissolving 1 g of safranin in 40 mL of 95 % alcohol and then adding 60 mL distilled water to make 100 mL stock solution. The staining solution was prepared by mixing 20 mL from safranin solution with 40 mL glycerol and 20 mL distilled water. The counts were made one hour after pollen grains placed on staining solution using light microscope. Pollens from each cultivar were counted using a light microscope to estimate the percentage of pollen viability. In each viability test, 3 replicates (micro-slides) were used for each sample. Pollen viability percentage was calculated using the formula:

$$\text{Pollen viability (\%)} = \frac{\text{Number of viable pollen grains}}{\text{Number of incubated pollen grains}} \times 100$$

Experimental design and statistical analysis: The experiment was performed in a factorial design with seven pistachio cultivars, three storage methods and five storage durations to investigate pollen viability and *in vitro* pollen germination. There were three replicates for each cultivar and storage treatment. Data was analysed by factorial ANOVA using SAS program. When there were significant interactions among cultivar, storage conditions and durations, one-way ANOVA was used to separate means using Least Significant Difference (LSD) at $P \leq 0.05$.

Results

Main effect of pistachio cultivars: The main effect of pistachio cultivars on pollen viability tests and *in vitro* pollen germination is summarized in Table 1. Pollen viability of pistachio cultivars ranged from 47.2 to 61 % for all tests while *in vitro* pollen germination varied between 37.4 and 42.9 %. Pollen viability by all tests and *in vitro* pollen germination were significantly the highest for Batouri and Ashouri cultivars. Pistachio cultivars Nab El-Jamal, Marawhi and Elemi had significantly low pollen viability by TTC, while Marawhi and Elemi cultivars had significantly the lowest pollen viability by IKI and *in vitro* pollen germination. The lowest pollen viability by safranin was significantly found only in Elemi cultivar. The other pistachio cultivars were categorized as medium for pollen viability and *in vitro* pollen germination (Table 1).

Table 1. Means for pollen viability by TTC, IKI, or safranin and *in vitro* pollen germination for seven cultivars of pistachio averaged across three storage methods and five storage durations

Cultivars	TTC (%)	IKI (%)	Safranin (%)	*In vitro* germination (%)
Lazourdi	50.0b	51.6b	53.0b	39.1bc
Nab El-Jamal	48.2c	51.2b	52.7b	39.3bc
Boundiki	50.2b	51.6b	52.0bc	40.1b
Batouri	59.5a	59.8a	60.2a	42.9a
Marawhi	47.2c	48.5c	51.4c	38.4cd
Ashouri	58.7a	59.8a	61.0a	42.9a
Elemi	48.3c	49.2c	49.9d	37.4d
LSD (P=0.05)	1.4	1.3	1.1	1.3

Main effect of storage methods: Table 2 summarized the main effect of storage conditions or temperatures on pollen viability and *in vitro* pollen germination for all pistachio cultivars with different storage durations. The results showed that as storage

Table 2. Means for pollen viability by TTC, IKI, or safranin and *in vitro* pollen germination for three storage methods averaged across seven cultivars and five storage durations

Storage conditions	TTC (%)	IKI (%)	Safranin (%)	*In vitro* germination (%)
Room temperature	37.6c	39.9c	40.9c	28.4c
Refrigerated	52.6b	53.3b	54.0b	43.4b
Freezer	65.0a	66.1a	68.1a	48.3a
LSD (P=0.05)	0.9	0.8	0.7	0.8

Table 3. Means for pollen viability by TTC, IKI, or safranin and *in vitro* pollen germination for five storage durations averaged across seven cultivars and three storage methods

Storage durations (week)	TTC (%)	IKI (%)	Safranin (%)	*In vitro* germination (%)
0	75.0a	77.0a	79.0a	71.4a
1	61.0b	62.8b	64.0b	56.9b
2	52.8c	54.5c	55.0c	47.3c
3	42.2d	43.1d	44.2d	19.8d
4	27.7e	28.2e	29.5e	4.7e
LSD (P=0.05)	1.2	1.1	1.0	1.1

temperatures increased, pollen viability and *in vitro* pollen germination decreased. The highest pollen viability was attained by safranin test (68.1 %) when pollen stored under freezer condition. However, pollen viability by TTC was the lowest (37.6 %) among all viability tests under room temperature storage. On the other hand, *in vitro* pollen germination was the lowest (28.4 %) and highest (48.3 %) under room temperature and freezer storage, respectively. In addition, pollen viability by TTC, IKI and safranin were reduced under refrigerated condition by 12.4, 12.8 and 14.1 %, respectively when compared with frozen stored pollen. However, *in vitro* germination of refrigerated pollen decreased by 4.9 % (Table 2).

Main effect of storage duration: The main effect of storage duration on pollen viability and *in vitro* pollen germination for seven pistachio cultivars under different storage conditions is presented in Table 3. The findings revealed that there was a decrease in pollen viability and *in vitro* germination when storage periods increased. The highest pollen viability was recorded in safranin test (79 %) for fresh (non-stored) (0 week) pollen. However, pollen viability by TTC was the lowest (27.7 %) when pollen stored for 4 weeks. The pollen germination percentage was significantly higher (66.7 %) for 0 week compared with those stored for 4 weeks (Table 3).

Storage condition x duration interaction: There was a significant ($P < 0.01$) storage condition × duration interaction effect on percentage of pollen germination and pollen viability by different tests for all pistachio cultivars (Fig. 1). There were no differences between *in vitro* germination percentages for pollen stored under refrigerated and freezer conditions up to 2 weeks. After that, *in vitro* pollen germination reduced sharply to 29.7 and 21.3 % for pollen stored under freezer and refrigerator conditions for 3 weeks, respectively. After 4 weeks, freezer-stored pollen retained 14.2 % of its germinability while room- and refrigerator-stored pollen did not germinate. Furthermore, *in vitro* germination of room-stored pollen was steadily reduced as pollen duration increased. The highest difference in germination (38.7

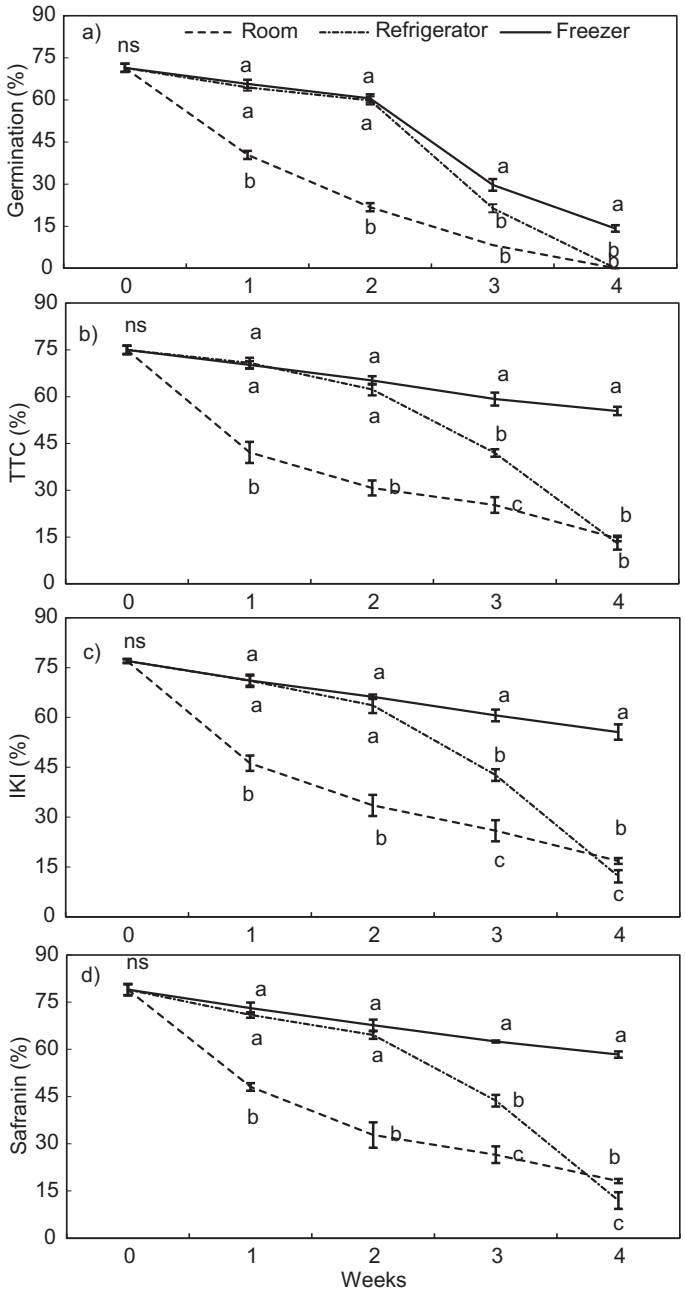

Fig. 1. Effect of storage methods and durations on (a) *in vitro* pollen germination, (b) pollen viability by TTC, (c) pollen viability by IKI, and (d) pollen viability by safranin for average of all pistachio cultivars. Bars represent the standard error of the mean. Mean values with the same letters are not significantly different by Least Significantly Difference ($P \leq 0.05$).

%) between freezer- and room-stored pollen was at 2 weeks of storage (Fig. 1a).

On the other hand, the interaction effect of storage condition and duration on the pollen viability by different tests of pistachio cultivars had similar trends (Fig. 1b-1d). Pollen viability percentage decreased slowly as storage duration increased under freezer conditions. However, the difference between viability percentage for freezer- and refrigerator-stored pollen began to rise obviously after 2 weeks of storage. Under room temperature, the viability percentage was reduced at maximum rate (approximately 50 %) when pollen stored for one week. However, there was no difference between viability percentages of refrigerator- and room-stored pollen at 4 weeks of storage. In addition, the results indicated a significantly higher viability

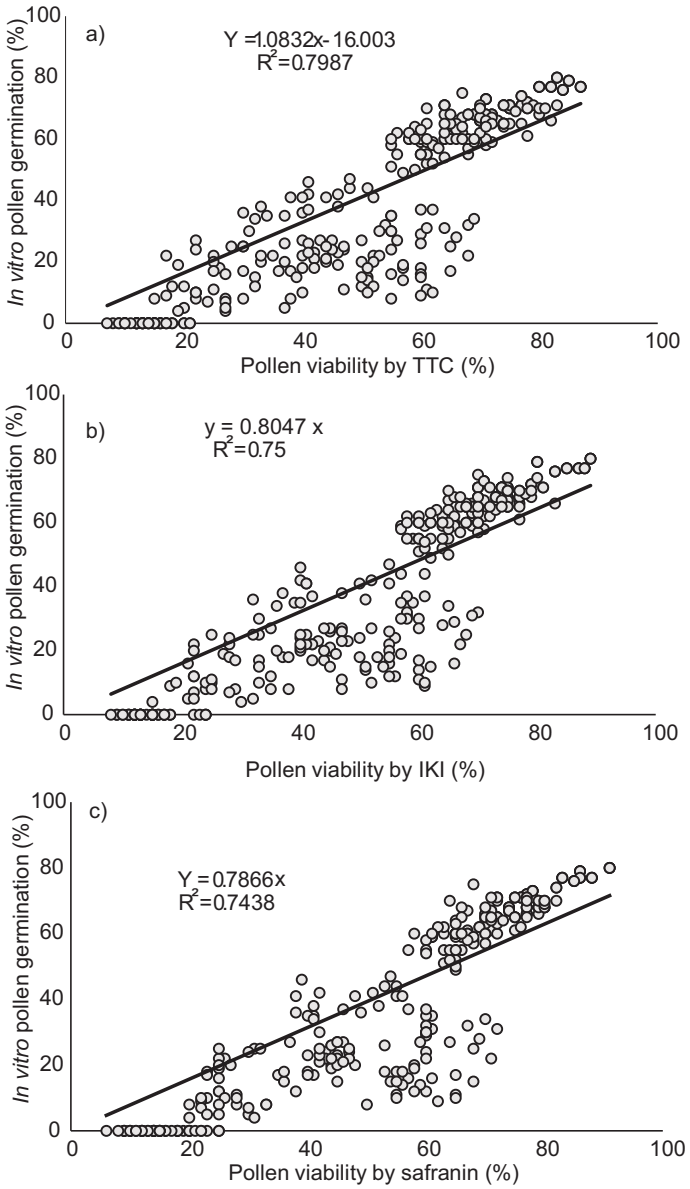

Fig. 2. Correlation between *in vitro* pollen germination and viability by (a) TTC, (b) IKI and (c) safranin tests (average of seven pistachio cultivars).

percentage for all freezer-stored pollen compared with those for room- and refrigerator-stored pollen at 3 and 4 weeks. Viability of frozen pollen by TTC, IKI and safranin was retained by 55.4, 55.6 and 58.3 %, respectively at 4 weeks of storage.

Correlation between *in vitro* pollen germination and viability tests: There was a significant (P<0.05) correlation between *in vitro* pollen germination and pollen viability in all tests (Fig. 2). The highest linear correlation was between *in vitro* germination and viability by TTC (R^2=0.80) for all cultivars under all storage conditions and durations (Fig. 2a). The correlations between *in vitro* germination and viability by IKI and safranin were 0.75 and 0.74, respectively (Fig. 2b and Fig. 2c).

Discussion

Effect of storage methods on pollen viability and *in vitro* germination of seven pistachio cultivars was investigated in this research. Pollen grains of each cultivar were stored under three storage temperatures (-5, 4, and 24 °C) and tested for viability and germination at different storage durations (0, 1, 2, 3, and 4 weeks). The results indicated a significant interaction between cultivar x storage temperature x storage duration (data not shown) only for IKI and safranin, indicating that cultivars respond differently to storage treatments on pollen viability and germination. Similar results were obtained by Hechmi *et al.* (2015) for olive pollen viability.

Our results showed that viability and *in vitro* germination of pollen varied depending upon pistachio cultivar, pollen storage temperature and storage duration. The cultivar Batouri had significantly higher pollen viability and *in vitro* germination while cultivars Marawhi and Elemi had the lowest under all storage methods and durations. The differences between pollen germination and viability among studied cultivars could be due to the variability in their genetic make-up (Mortazavi, 2010). For all the cultivars, percentage of *in vitro* pollen germination was consistently lower compared to pollen viability tests. This can be due to the effect of different factors, which are hard to control such as pollen density, optimum culture media, and environmental requirement for each cultivar (Hechmi *et al.,* 2015). In this study, pollen viability was tested by using different staining methods including TTC, IKI and safranin to find out which method is more consistent. Pollen viability by safranin test was 3 % higher than those by TTC test for all pistachio cultivars. Also, Gunver-Dalkkilic and Dayi-Dogru (2011) found that the pollen viability by safranin in pistachio trees was 18 % more by TTC test. However, IKI staining method was more reliable for testing pollen viability (Du *et al.,* 2019).

The highest percentage of pollen viability and *in vitro* germination for all cultivars was in fresh pollen at 0 week of storage. There was a significant decrease in percentage of stored pollen viability and germination over the duration of storage. However, the reduction in pollen viability and germinability was significantly faster at room temperature compared to that recorded under refrigerator or freezer conditions. Pollen stored under freezer conditions had significantly higher germination and viability percentage for all cultivars over the duration of storage than those stored either under refrigerator or room temperature. Current study indicated that *in vitro* germination and viability of pollen stored in refrigerator and freezer had similar percentages during the first two weeks of storage and were significantly higher than those stored under room temperature. However, the percentage of pollen viability as estimated by *in vitro* germination and three staining tests at 3 and 4 weeks of storage was significantly higher for pollen stored at -5 °C than those stored at 4 °C and room temperature. Storing pollen at low temperatures and low moisture content usually extends pollen longevity (Dutta *et al.*, 2013). Polito and Luza (1988) perceived that 'Peters' pollen grains kept their germinability after 4 months when stored at -20 °C, but their germination ability was reduced severely after 12 months. Vaknin and Eisikowitch (2000) revealed that the pollen storage of pistachio at room temperatures for 7 days did not display any germination when tested directly. Pollen stored in the refrigerator at -4 °C retained viability for a week, while pollen stored under deep freezer (-20 °C) for 6 days was extremely and irreversibly lost germination (Vaknin and Eisikowitch, 2000). Cryopreserved pollen of pistachio could be stored for 4 weeks, while in refrigerator or freezer just for 2 weeks (Ateyyeh, 2012) which is sufficient for artificial cross-pollination purpose, if the

difference in flowering period between males and females did not exceed 2 weeks.

In this study, a significant and strong correlation between pollen viability and *in vitro* germination tests was determined. Similarly, a positive and highly significant correlation between different pollen viability staining tests and pollen germination test in *Momordica* species was reported (Rathod *et al.*, 2018). Pollen viability was correlated with *in vitro* pollen germination in *Banksia* and some other Proteaceae plants (Schori *et al.*, 1992). Viability as determined by TTC staining tests in cherry pollen did not show good correlation with the actual germination percentage (Sulusoglu and Cavusoglu, 2014). Parfitt and Ganeshan (1989) have established that the pollen stain tests (including TTC) were not reliable or consistent and were not positively correlated with *in vitro* germination tests.

In conclusion, the interaction effect of cultivar, storage method and duration was significant on viability as estimated by IKI and safranin. Pollen grains of Batouri and Ashouri cultivars retained their germination and viability under different storage temperatures and durations better than other cultivars. As storage duration increased, *in vitro* pollen germination and viability decreased. A short-term storage of pollen up to two weeks was best when pollens were stored in the refrigerator (4 °C) or in the freezer (-5 °C). For longer storage durations (3 and 4 weeks), the best storage method was the freezer. Pollen viability can be estimated by three chemical-staining tests, which were highly and positively correlated with *in vitro* pollen germination. Further research is needed to determine the correlation between pollen viability, fading of flowers, and fruit setting.

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