

## Effect of Low Temperature and Cryopreservation on *In vitro* Pollen Germination of Some Olive Cultivars

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**Abstract:** Pollen viability and its germination capability for fruit set in olive are essential. In this study, the viability of stored olive pollens cultivars i.e., Teffahi, Eggizi shami, Wardan and Eggizi oshem at 4, -20 and -80°C was assessed through evaluation of their germination percentage using medium consists of 10 % sucrose, 100 mg/l boric acid and 1 % agar. The germination percentage of pollen exceeded 86 % for all cultivars and 87 % for all temperatures after 3 months of storage. Following 12 months of storage at 4°C, germination percentage decreased to 12.50 % for Teffahi, 45.38 % for Eggizi shami, 37.08 % for Wardan, with no germination observed for Eggizi oshem cultivar. Storage pollen at sub-freezing temperatures -20°C differed significantly in their tolerance to low temperature, the highest germination percentage of pollen above 71 % was noticed with Eggizi shami cultivar, decreased to 58.89 % for Wardan followed by 47.41 % for Teffahi and 18.18 % for Eggizi oshem. However, germination percentage of pollen was 56.33 % for Eggizi shami cultivar storage at -80°C and decreased to 46.28 %, 45.04 % for Teffahi and Wardan without significant differences between them. While, Eggizi oshem cultivar recorded the lowest germination percentage (11.98 %) of pollen. Regarding storage at -196°C (cryopreservation), the highest germination percentage (91.87 %) was found in Eggizi Shami followed by Wardan and Eggizi oshem (90.21, 90.35 %). While, Teffahi recorded the lowest germination percentage (88.42 %) of pollen.

**Key words:** Cryopreservation • *In vitro* germination • Low temperature • Olive • Pollen

### INTRODUCTION

The olive (*Olea europaea* L.) is one of the oldest and most important crops grown in the Mediterranean basin and its cultivation is concentrated in this area [1]. However, it is now cultivated in many other countries of the world on a commercial scale and as a consequence the demand for olive trees is increasing [2].

Long term storage of viable pollen is important for bank germplasm constitution so that it is possible to preserve resources that can be used in breeding programs, biotechnologies and genetic engineering [3-5]. Pollen storage is important for germplasm conservation, exchanges and handling and it enhances improved breeding efficiency [6]. The application of pollen storage as an integrated method for the long-term conservation of olive genetic resources and for use in olive improvement programs holds great promise.

*In vitro* germination has been used as an important technique for testing pollen viability, but difficulties in establishing an optimal culture medium [7].

Storage of pollen under low temperatures as a means of plant genetic resources conservation has been widely discussed by many authors for various species [8-11].

The objective of this study was to evaluate the viability and shelf life of pollen grains of some important olive cultivars stored at low temperature conditions.

### MATERIALS AND METHODS

Single male flowers picked from inflorescences at the end of March 2014 of Egyptian olive cultivars named Teffahi, Eggizi shami, Wardan and Eggizi oshem were used as experimental materials. Anthers were selected just before dehiscence. Undehisced anthers were dried at room temperature for 24 hours in petri dishes to induce dehiscence. Pollen grains were collected and divided into 3 samples from these anthers and treated as follows:

- Pollen grains were sprinkled in a fine layer over the surface of the growing medium and incubated for 24 hours in dark at room temperature

into petri dishes filled with 10 ml medium consists of 10 % sucrose, 100 mg/l boric acid and 1 % agar (Control).

- Pollen grains were placed in a 1.8 ml cryotubes and immersed into liquid nitrogen (LN<sub>2</sub>) at -196°C for 1 hour, then thawing was done for 1 min. at 40° C in a water bath and cultured on a previous medium.
- Pollen grains were placed in polypropylene vials and stored into refrigerator at 4°C, freezer at -20 and -80°C for (3, 6, 9 and 12) months. Pollen viability was tested at 3 month intervals using germination on a previous medium.

Fresh pollen was compared after 24 hours of incubation on pollen germination medium at room temperature.

The experiments were repeated four times and were arranged in a completely randomized design for each cultivar.

Duncan's multiple range test at 5 % level was used to verify the differences between means of the treatments in all the experiments [12].

## RESULTS AND DISCUSSION

**Effect of Desiccation on Pollen Germination:** Data presented in Table (1) demonstrate that the highest germination percentage of pollen after cultured on medium containing 10% sucrose, 100 mg/l boric acid and 1% agar was noticed with Eggizi shami cultivar (99.1 %) compared to the other cultivars with significant differences among them, followed by Wardan and Eggizi oshem (98.88, 98.65 %) without significant difference between them. While, Teffahi cultivar recorded the lowest germination percentage (98.25 %).

**Effect of Different Low Temperatures on Pollen Germination after Storage for Three Months:** Results in Table (2) show the effect of low temperatures (4, -20 and -80°C) on germination percentage of pollens of four cultivars of olive cultured on medium containing 10 % sucrose, 100 mg/l boric acid and 1 % agar after 3 months of storage.

Concerning the effect of different temperatures on pollen germination after storage at 3 months results illustrated that the highest germination percentage (90.34 %) was recorded with 4°C and then decreased gradually by decreasing the temperature from 4, -20 to -80°C as the germination percentage were 90.34 %, 89.41 % and 87.07 % respectively without significant differences among them.

Table 1: Effect of desiccation on pollen germination percentage after 24 hours maintenance at room temperature of four olive cultivars

Cultivars	Pollen germination (%)	
Teffahi	98.25	B
Eggizi shami	99.01	A
Wardan	98.88	AB
Eggizi oshem	98.65	AB

Mean followed by the similar letter (s) are not significantly different by Duncan test ( $P \leq 0.05$ ).

With respect to the varietal differences regarding pollen germination after storage 3 months results cleared that Teffahi cultivar recorded the highest germination percentage of pollens (91.58 %) without significant differences among all cultivars, followed by germination percentage of cultivars Eggizi shami, Wardan and Eggizi oshem (89.59 %, 87.97 % and 86.62 % respectively).

With regard to the effect of interaction between different temperatures and the examined cultivars, results showed that all pollens conserved at all temperatures under investigation for 3 months registered high germination percentages. Teffahi cultivar showed the highest germination percentage 92.74 %, 91.09 % and 90.92 % with 4, -20 and -80°C respectively, 90.28 % with Eggizi shami at -80°C. These germination percentages decreased without significant differences to 89.66 %, 89.18 % and 89.80 % when pollens conserved at 4°C with Eggizi shami, Wardan and Eggizi oshem cultivars respectively, 89.94 %, 88.83 % and 87.77 % when pollens were conserved at -20°C with Eggizi oshem, Eggizi shami and Wardan cultivars respectively. The lowest germination percentage 86.96 % and 80.10 % were recorded when pollens were conserved at -80°C with Wardan and Eggizi oshem cultivars, respectively.

**Effect of Different Low Temperatures on Pollen Germination after Storage for Six Months:** Results in Table (3) clear the effect of low temperatures (4, -20 and -80°C) on germination percentage of pollens of four olive cultivars cultured on medium containing 10 % sucrose, 100 mg/l boric acid and 1 % agar after 6 months of storage. Concerning to the effect of different temperatures on pollen germination after storage 6 months results illustrated that the highest germination percentage (81.49 %) was noted with -20°C decreased to 78.53 % and 78.07 % with temperature 4 and -80°C respectively, without significant differences among them.

With respect to the effect of different cultivars on pollen germination after storage 6 months results appeared that Eggizi shami cultivar showed the highest germination percentage of pollens (85,75 %)

Table 2: Effect of different low temperatures on pollen germination percentage after storage for three months of four olive cultivars

Cultivars	Pollen germination (%)						Mean	
	4°C		-20°C		-80°C			
Teffahi	92.74	a	91.09	a	90.92	a	91.58	A
Eggizi shami	89.66	a	88.83	a	90.28	a	89.59	A
Wardan	89.18	a	87.77	a	86.96	a	87.97	A
Eggizi oshem	89.80	a	89.94	a	80.10	a	86.62	A
Mean	90.34	A	89.41	A	87.07	A		

Mean followed by the similar letter(s) are not significantly different by Duncan test ( $P \leq 0.05$ ).

Table 3: Effect of different low temperatures on pollen germination percentage after storage for six months of four olive cultivars

Cultivars	Pollen germination (%)						Mean	
	4°C		-20°C		-80°C			
Teffahi	78.68	abc	73.44	bc	77.74	abc	76.62	A
Eggizi shami	81.23	abc	87.13	ab	88.89	a	85.75	A
Wardan	77.60	abc	76.88	abc	78.53	abc	77.67	A
Eggizi oshem	76.62	abc	88.53	a	67.12	c	77.42	A
Mean	78.53	A	81.49	A	78.07	A		

Mean followed by the similar letter(s) are not significantly different by Duncan test ( $P \leq 0.05$ ).

Table 4: Effect of different low temperatures on pollen germination percentage after storage for nine months of four olive cultivars

Cultivars	Pollen germination (%)						Mean	
	4°C		-20°C		-80°C			
Teffahi	64.33	ab	56.55	ab	55.42	ab	58.77	A
Eggizi shami	59.09	ab	78.52	a	82.61	a	73.41	A
Wardan	59.69	ab	72.26	a	70.24	a	67.39	A
Eggizi oshem	39.65	b	80.45	a	35.77	b	51.96	A
Mean	55.69	A	71.95	A	61.01	A		

Mean followed by the similar letter(s) are not significantly different by Duncan test ( $P \leq 0.05$ ).

without significant differences among cultivars, followed by germination percentage of cultivars Wardan, Eggizi oshem and Teffahi (77.67 %, 77.42 % and 76.62 % respectively).

With regard to the effect of interaction between different temperatures and the cultivars on pollen germination after 6 months of storage, results showed that the highest germination percentage (88.89 % and 88.53 %) were found with pollen stored at -80°C in Eggizi shami cultivar and pollen stored at -20°C in Eggizi oshem cultivar without significant differences in-between, followed with significant difference by 87.13 % of pollen of Eggizi shami cultivar conserved at -20°C. This germination percentage decreased without significant differences to 81.23 %, 78.68 %, 77.60 % and 76.62% on pollen of Eggizi shami, Teffahi, Wardan and Eggizi oshem cultivars respectively, with pollen conserved at 4°C, 76.88 % for Wardan pollen cultivar conserved at -20°C, 78.53 % and 77.74 % for pollen of Wardan and Teffahi cultivars conserved at -80°C,

followed by 73.44 % for Teffahi pollen conserved at -20°C. On contrast, the lowest germination percentage (67.12 %) was noticed with pollens of Eggizi oshem conserved at -80°C.

**Effect of Different Low Temperatures on Pollen Germination after Storage for Nine Months:** Results in Table (4) indicate the effect of low temperatures (4,-20 and -80°C) and the four olive cultivars on germination percentage of pollens cultured on medium containing 10 % sucrose, 100 mg/l boric acid and 1% agar after 9 months of storage.

Concerning to the effect of different temperatures on pollen germination after storage 9 months results illustrated that the highest germination percentage (71.95 %) was cleared with -20°C and decreased to 61.01 % and 55.69 % with temperature -80 and 4°C respectively, without significant differences among the temperatures storage.

Table 5: Effect of different low temperatures on pollen germination percentage after storage for twelve months of four olive cultivars

Cultivars	Pollen germination (%)						Mean	
	4°C		-20°C		-80°C			
Teffahi	12.50	d	47.41	b	46.28	b	35.40	B
Eggizi shami	45.38	b	71.61	a	56.33	ab	57.77	A
Wardan	37.08	bc	58.89	ab	45.04	b	47.00	AB
Eggizi oshem	0.00	d	18.18	cd	11.98	d	10.06	C
Mean	23.74	B	49.02	A	39.91	AB		

Mean followed by the similar letter(s) are not significantly different by Duncan test ( $P \leq 0.05$ ).

Table 6: Effect of cryopreservation on pollen germination percentage after storage at -196°C of four olive cultivars

Cultivars	Pollen germination (%)	
Teffahi	88.42	C
Eggizi shami	91.87	A
Wardan	90.21	B
Eggizi oshem	90.35	B

Mean followed by the similar letter(s) are not significantly different by Duncan test ( $P \leq 0.05$ ).

With respect to the effect of different cultivars on pollen germination after storage 9 months results declared that Eggizi shami cultivar recorded the highest germination percentage of pollens (73.41 %) without significant differences among the cultivars, followed by germination percentage on cultivars Wardan, Teffahi and Eggizi oshem (76.39 %, 58.77 % and 51.96 % respectively).

Concerning the effect of interaction between temperature storage and the different cultivars after 9 months, results indicated that the highest germination percentage (82.61 %) was recorded in Eggizi shami cultivar decreased to 70.24 % in Wardan with pollen storage at -80°C, 78.52 %, 72.26 % and 80.45 % for pollens of Eggizi shami, Wardan and Eggizi oshem cultivars stored at -20°C without significant differences among them, followed with significant difference by 64.33 % with pollen of Teffahi, the same germination percentage (59.09 %) was noticed on pollens of Eggizi shami and Wardan conserved at 4°C, 56.55 % and 55.42 % for pollens germination with Teffahi cultivar conserved at -20°C and -80°C respectively without differences among them. The lowest germination percentages (39.65 %, 35.77 %) were noticed with pollens of Eggizi oshem conserved at 4 and -80°C respectively without significant differences between them.

**Effect of Different Low Temperatures on Pollen Germination after Storage for Twelve Months:** Results in Table (5) reveal the effect of low temperatures (4, -20 and -80°C) and different cultivars on germination percentage of pollens cultured on medium containing 10 % sucrose, 100 mg/l boric acid and 1 % agar after 12 months of storage.

Concerning to the effect of different temperatures on pollen germination after storage 12 months results indicated that the highest germination percentage (49.02 %) showed with temperature of -20°C with significant differences among all temperatures. The germination percentage decreased gradually until reached to 39.91 % with temperature storage of -80°C. While, storage of 4°C showed the lowest germination percentage (23.74 %) of pollen.

With respect to the effect of different cultivars on pollen germination after storage 12 months results appeared that Eggizi shami cultivar showed the highest germination percentage (57.77 %) of pollens with significant differences among all cultivars and decreased to 47.00 % with Wardan cultivar, followed by germination percentage on cultivar Teffahi (35.40 %). While, Eggizi oshem showed the lowest germination percentage (10.06 %).

With regard to the effect of interaction between different temperatures and different cultivars after 12 months of storage, results proved that the highest germination percentage (71.61 %) was recorded in Eggizi shami cultivar with pollen storage at -20°C decreased to (58.89 %, 56.33 %) in Wardan cultivar with pollen storage at -20°C and Eggizi shami cultivar storage at -80°C respectively, without significant differences between them. Followed without significant differences by (47.41 %) for pollen Teffahi cultivar stored at -20°C, (46.28 %, 45.04 %) for pollen Teffahi and Wardan cultivars stored at -80°C respectively and (45.38 %) for pollen Eggizi shami cultivar stored at 4°C. Decreasing the germination percentage of pollen Wardan cultivar was recorded 37.08 % when pollen stored at 4°C with significant difference in-between with Eggizi oshem cultivar (18.18 %) when pollen stored at -20°C. The lowest germination percentage (12.50 %, 11.98 %) was noticed with Teffahi cultivar when pollens stored at 4°C and Eggizi oshem cultivar when pollen conserved at -80°C respectively without significant differences between them. While, pollen of Eggizi oshem cultivar cannot able to still alive when stored at 4°C.

**Effect of Cryopreservation on Pollen Germination Percentage of Four Olive Cultivars:** Results in Table (6) proved the effect of ultra-low temperature (-196°C) on germination percentage of pollens cultured on medium containing 10 % sucrose, 100 mg/l boric acid and 1 % agar for four cultivars.

Data presented in Table (6) demonstrated that the highest germination percentage (91.87 %) of cryopreserved pollens was noticed with Eggizi shami cultivar with significant differences among them, followed by Wardan and Eggizi oshem (90.21, 90.35 %) without significant difference between them. While, Teffahi cultivar recorded the lowest germination percentage (88.42 %).

These results are in agreement with those obtained by Borghazan *et al.* [13] who mentioned that the pollen grain viability of two kiwi varieties Tomuri and Matua decreased from 70 % to around 40 % stored at 4°C. After 120 days, about 35 % of the pollen grains were still viable if stored at -18°C, decreasing to 15 % after 240 days. After 365 days of storage, the pollen grains had completely lost the ability to germinate in culture medium. In cryopreservation, the pollen grain viability was maintained (~70 %) during the evaluated storage period. The storage conditions in liquid nitrogen (cryopreservation) maintained the viability of kiwi pollen grain for up to one year.

Pollens of 6 cherry laurel types and 2 sweet cherry cultivars was evaluated for germination following storage two years at room temperatures, in refrigerator (4°C) and in deep-freezer (-25°C) [14]. Germination was obtained in medium containing 0.5 % agar, 15 % sucrose and 5 ppm boric acid and tests were made at the end of 90, 180, 270 and 730 days. Cherry laurel pollens stored at room temperatures lost the germination ability faster than pollens stored at the 4 or -25°C but nearly half of them still showed germination after 90 days. Among the cherry laurel types, type 36 gave the highest respect to storage with the best performance after 730 days storage period at -25°C (59.62 %). While, type 25 pollens germination percentage was only 21.86 %. Sweet cherry cultivars were more sensitive to storage conditions and pollen viability was completely lost for 0900 Ziraat cultivar after 90 days storage at the room temperature. While, Prime Giant germination percentage was only 2.05 %. Germination of 0900 Ziraat and Prime Giant pollens stored at -25°C was 21.54 % and 33.50 % respectively, at the end of 730 days storage period.

Other study was achieved on storage of olive pollen grains. The results on *in vitro* of pollen grains germinability observed before and after long term storage

in liquid nitrogen showed highly significant responses among the 12 observed cultivars of olive [15]. Over all cultivars the observed average germinability on fresh and stored pollen grains was 57.7 and 20.8 %, respectively. A similar trend was observed by Ferri *et al.* [16] which reported *in vitro* germination rates of olive pollen grains stored 35 and 200 days at a temperature of -20°C.

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