Spanish Journal of Agricultural Research
18 (3), e0804, 10 pages (2020)
eISSN: 2171-9292
https://doi.org/10.5424/sjar/2020183-16626
Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA)

RESEARCH ARTICLE

OPEN ACCESS

Effect of freezing conservation time on loquat (*Eriobotrya japonica*) pollen germination

Roberto Beltrán (Beltrán, R)[1], Nuria Cebrián (Cebrián, N)[1], Carlos Zornoza (Zornoza, C)[2], Alfonso Garmendia (Garmendia, A)[3] and Hugo Merle (Merle, H)[1]

[1] *Universitat Politècnica de València, Dept. Ecosistemas Agroforestales. Camino de Vera s/n, 46022 Valencia, Spain.* [2] *S.A. Explotaciones Agrícolas Serrano (SAEAS). c/ En Sanz 5-1. 46001 Valencia, Spain.* [3] *Universitat Politècnica de València, Instituto Agroforestal Mediterráneo, 46022 Valencia, Spain.*

Abstract

Aim of study: Several studies point out that storage at -20 ºC is a suitable method for preserving pollen of many species in the long term. Part of those studies indicate the total storage time at which these conditions are optimal. However, we have found a lack of information about the freezing time conditions and incubation temperature of loquat pollen. The main objective of this study was to evaluate the effect of the -20 ºC conservation temperature on loquat (*Eriobotrya japonica* (Thunb.) Lindl.) pollen.

Area of study: The study was conducted in Montserrat (Valencia, Spain).

Material and methods: Loquat flowers were collected in November 2017 and stored at -20 ºC for three time periods: 4 (T1), 6 (T2) and 8 (T3) months. Subsequently, pollen grains were incubated at different temperatures for 72 h. We analyzed (i) the effect of freezing conservation time; (ii) the effect of incubation temperature on germination; (iii) the interaction between these two factors.

Main results: T1 showed higher germination percentage and tube length values (mean and maximum) than T2 and T3. The highest germination percentage (52.77%) was detected for T1 at an incubation temperature of 25 ºC. The interaction between freezing time and incubation temperature showed more consistent results for T1 than for T2 and T3.

Research highlights: This suggests that storing at -20 ºC for more than 4 months affects pollen grain and reduces germination and pollen growth. Therefore, -20 ºC loquat pollen storage should not exceed 4 months.

Additional key words: pollen germination rate; pollen tube length; fruit tree; Rosaceae; pollen conservation.

Authors' contributions: Conceived, designed and performed the experiments: RB, NC, AG and HM. Analyzed the data: RB, GA and HM. Contributed reagents/materials/analysis tools: AG and CZ. Wrote the paper: RB. All authors read and approved the final manuscript

Citation: Beltrán, R; Cebrián, N; Zornoza, C; Garmendia, A; Merle, H (2020). Effect of freezing conservation time on loquat (*Eriobotrya japonica*) pollen germination. Spanish Journal of Agricultural Research, Volume 18, Issue 3, e0804. https://doi.org/10.5424/sjar/2020183-16626

Received: 02 Mar 2020. **Accepted:** 01 Oct 2020.

Funding agencies/institutions	Project / Grant
Asociación Club de Variedades Vegetales Protegidas, as a part of a project undertaken with the *Universitat Politècnica de València*	UPV-20190822

Competing interests: The authors have declared that no competing interests exist.

Correspondence should be addressed to Roberto Beltrán: robelmar@upvnet.upv.es

Introduction

Loquat (*Eriobotrya japonica* (Thunb.) Lindl.) is a subtropical evergreen tree that originates from China (Blasco *et al.*, 2016). This species was introduced in Europe in 1784 when several plants were acquired by the National Garden, Paris (Sharpe, 2010). It commenced to be cultivated at the beginning of 19th century. Throughout that century, loquat orchards extended to several European countries and the United States (Agustí, 2010). Nowadays, it is cultivated mainly in subtropical climate countries. The world's main loquat producers are China, Japan and Spain (Caballero & Fernández, 2002). *E. japonica* belongs to the *Maloideae* subfamily of *Rosaceae*. One of the main characteristics of this species is that the flowering season takes place in autumn in Mediterranean countries, unlike other *Rosaceae* species (Agustí, 2010). Loquat trees can withstand low temperatures of up to -12 ºC (Freihat *et al.*, 2008). The optimum temperature for its development ranges from 20 ºC to 30 ºC, depending on variety. Likewise, it has been observed that temperatures above 35 ºC can be unfavorable for its plant growth (Freihat *et al.*, 2008).

The pollination and fertilization processes of *E. japonica* are similar to other *Rosaceae* species. Some studies cite loquat as a self-compatible species (Cuevas *et al.*, 2003; Freihat *et al.*, 2008), but the study performed by Sharafi *et al.* (2011) reported most loquat cultivars with gametophytic self-incompatibility. Several studies consider cross-pollination in loquat to be an essential factor for high yields (Freihat *et al.*, 2008; Sharafi *et al.*, 2011). It has been specifically proven that fruit set and fruit size depend on reproduction and pollination processes (Yang *et al.*, 2012). After fecundation, several seeds degenerate and only 3 or 4 seeds reach the mature state (Qin *et al.*, 2008). Cuevas *et al.* (2003) indicated the importance of pollinators activity on loquat cross-pollination. However, pollination is also associated with the formation of more seeds, which results in reduced fruit quality (Yang *et al.*, 2012). Loquat pollination studies remain fundamental to improve the main crop variables.

Sharafi *et al.* (2011) conducted an *in vitro* germination study of loquat pollen, where pollen was incubated only at 22 ºC for 1 day. Instead, pollen germination studies at several temperatures in other *Rosaceae* species can be frequently found. Among them, the studies of Vasilakakis & Porlingis (1984) in *Pyrus communis* L., Egea *et al.* (1992) in *Prunus armeniaca* L., Hedhly *et al.* (2004) in *Prunus avium* (L.) L., Hedhly *et al.* (2005) in *Prunus persica* (L.) Batsch, and Sorkheh *et al.* (2011) in *Prunus dulcis* (Mill.) D.A.Webb, stand out. Recently, the effect of temperature on the pollen germination of several *Rosaceae* species has been published (Beltrán *et al.*, 2019). In these studies, the maximum pollen germination and maximum pollen tube length values were achieved by incubating pollen grains at 20 ºC.

Pollen germination studies are also available at different temperatures in species from other families, such as those published by Kakani *et al.* (2002) in groundnut (*Arachis hypogaea* L.), Reddy & Kakani (2007) in *Capsicum* spp., and Acar & Kakani (2010) in Pistacia spp. In these cases, the optimum temperature for pollen tube growth ranged between 20 ºC and 30 ºC.

Other loquat pollen germination studies have focused on stigma receptivity duration and the time at which pollen tubes reach the ovule but have left aside the temperature at which these processes occur (Qin *et al.*, 2008). In most studies conducted with loquat pollen or anthers, pollen was either used fresh or stored in a refrigerator at 0-4 ºC for a short period of time.

Fresh pollen has been used to study the relation between loquat pollen tube length and genomic characterization (Carrera *et al.*, 2009), the effect of rain on loquat pollen adhesion to stigma (Yang *et al.*, 2011), and induced parthenogenesis on loquat (Blasco *et al.*, 2016). Qin *et al.* (2008) stored loquat pollen at -20 ºC in their *in situ* pollen study, and then used that pollen for the cross-pollination of loquat trees.

Several studies report pollen germination capability after a certain period at low temperature. Weinbaum *et al.* (1984) stored pollen of *Prunus dulcis* and *P. persica* at -20 ºC until further use and did not observe loss of germination rates. Some studies conducted on pollen grains of *Solanum melongena* L. pointed out that storage at temperatures below -20 ºC for 48 weeks provided better germination results than those obtained with fresh pollen (Khan & Perveen, 2006). Similar results are reported for the same authors in *Citrullus lanatus* L. (Khan & Perveen, 2010), *Lagenaria siceraria* (Molina) Standley (Khan & Perveen, 2011) and five citrus species (Khan & Perveen, 2014).

Although the results obtained in other species indicate that long-term freezing is a suitable pollen conservation method, information about long-term loquat pollen preservation by freezing and loquat pollen germination at different temperatures is scarce.

Hence the aim of this study was to: (i) evaluate the germination rates of loquat pollen stored at -20 ºC depending on freezing times; (ii) analyze these germination rates depending on incubation temperatures; and (iii) check the interaction between both these factors.

Materials and methods

Loquat flowers were collected in November 2017 in an orchard located in the municipal district of Montserrat (Province of Valencia, Spain; Latitude: N 39.359629, Longitude: E -0.547494, Altitude: 153 m). The site's climate is cold semiarid, BSk in the Köppen & Geiger (1936) classification, with an average temperature of 16.8 ºC and an average rainfall of 432 mm. The employed loquat variety was 'Algerie' and trees were 10 years old. Fifty flowers per tree from five trees were taken at anthesis. All the samples were kept in bags and stored in a freezer at -20 ºC for 4 (T1 or treatment 1), 6 (T2 or treatment 2) and 8 (T3 or treatment 3) months. Samples were placed inside a humid chamber at 4 ºC for 2 h before extracting pollen to achieve its pre-hydration (Mesejo *et al.*, 2006). Three or four anthers were taken, and pollen grains were extracted using binocular lenses and placed in 5 mL of modified BK medium containing 100 g L^{-1} sucrose, 0.1 g L^{-1} H_3BO_3, 0.3 g L^{-1} Ca $(NO_3)_2$, 0.1 g L^{-1} KNO_3 and 10 g L^{-1} agarose (Brewbaker & Kwak, 1963) to induce their germination on 90-mm Petri dishes. These dishes were incubated for 72 h in the dark at: 5, 10, 15, 20, 25 and 30 ºC (Hedhly *et al.*, 2004; Beltrán *et al.*, 2019). The pollen germination percentage, the average pollen tube length and the maximum pollen tube length were calculated for each treatment (number of freezing months) and temperature. Pollen was considered germinated when the pollen tube length exceeded the diameter of its pollen grain. Pollen tube length was measured as the ratio to pollen diameter. These variables were measured for the first 100

pollen grains observed on each dish. If there were only a few grains, the variables were calculated for the total number of grains.

All the statistical analyses were done using R (R Core Team, 2017) and RStudio (RStudio Team, 2016). The ANOVAs, Kruskal-Wallis rank sum and Tukey post hoc tests were used to make comparisons between treatments (temperature and freezing times) using the "agricolae" package (Mendiburu, 2019). When significant differences were found, Levene's test and eta-squared statistics were calculated to assess the homogeneity of variances and the size effect in the ANOVA, respectively. Normality of residuals was tested by the Shapiro-Wilk test and by looking at the density curves.

Results

Effect of freezing time on pollen germination

The highest values of pollen germination percentage, average pollen tube length and maximum pollen tube length were obtained at T1 (4-month frozen pollen; Fig. 1, Table 1). The average germination percentage for all the temperatures at T1 was 27.64%. The mean pollen tube length and the maximum pollen tube length were 1.83 and 2.47, respectively (ratio of tube length to pollen diameter).

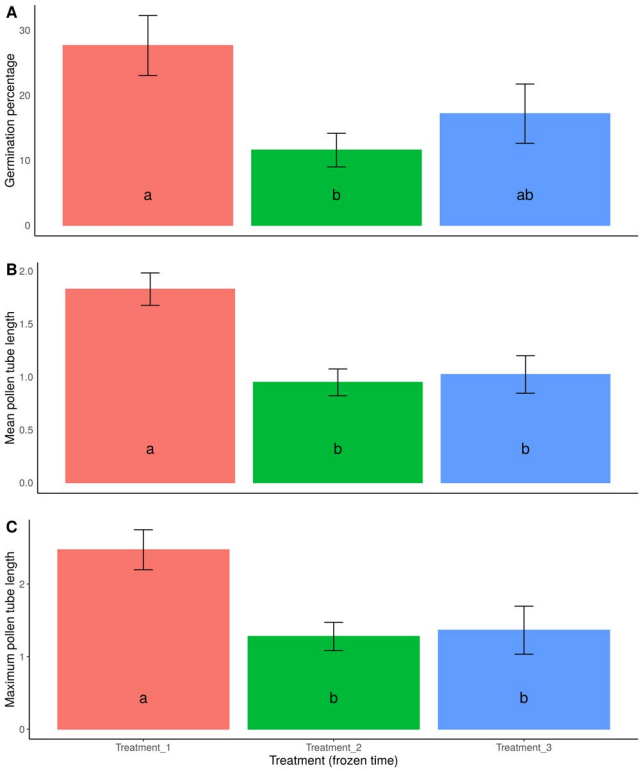

Figure 1. Barplots for the effect of treatment (freezing time) on the pollen germination variables. Different letters mean significant differences in the Kruskal-Wallis tests by ranks.

The lowest values for these variables were obtained at T2 (6-month frozen pollen). At T2, the average pollen germination was 11.57%. The values observed for the longest freezing time (T3: 8-month frozen pollen) were slightly higher than those observed at T2. Significant differences were observed for the germination percentage between T1 and T2, but not at T3. All the differences observed for pollen tube length between T1 and the other two treatments were significant (see Table 1).

Effect of incubation temperature on pollen germination

The highest germination percentages and mean pollen tube length values were obtained at 25 ºC, while the minimum pollen germination (6.09%) was observed at 5 ºC. A gradual increase in the pollen germination percentage was noted between 10 ºC and 25 ºC, although differences were not significant, except for those between 5 ºC and 20 ºC (Fig. 2, Table 2). The highest mean pollen tube length was obtained at 20 ºC (1.62) while the longest pollen tube value was found at 10 ºC (2.55). However, no significant differences were observed between pollen tube length at different temperatures, which was probably due to the remarkably wide variability of pollen tube lengths.

Interaction between freezing time and incubation temperature

T1 showed the most consistent germination pattern. Significant differences between the two highest incubation temperatures and the lower temperatures were recorded only for T1 (Fig. 3). In this case, the highest germination percentage was observed at 25 ºC (52.77%), while the lowest one was noted at 5 ºC (4.4%). The germination percentage clearly increased with the rise in the incubation temperatures for T1. For T2 and T3, no significant differences in the germination percentage appeared among the incubation temperatures.

No significant differences were found among the temperatures at T1 and T2 for the mean pollen tube length, but significant differences were found between 10 ºC and the two highest temperatures obtained for T3 (Fig. 4). Finally, the maximum pollen tube length did not show any significant differences for T1, but several significant differences appeared among the incubation temperatures for T2 and T3 (Fig. 5). Germination failed for incubation temperatures 15 ºC and 20 ºC at T3.

Discussion

The highest pollen germination rates were reached between 20 ºC and 25 ºC for all the tested freezing times

Table 1. Kruskal-Wallis (KW) for the effect of treatment (freezing time) on the germination percentage, mean pollen tube length and maximum pollen tube length values. T1: 4 months stored at -20 ºC. T2: 6 months stored at -20 ºC. T3: 8 months stored at -20 ºC.

Variable: germination percentage

Block	N	Mean	KW	SD	SE	Skew	Kurtosis	Shapiro
T1	18	27.64	a	19.53	4.60	0.372	-1.40	0.05
T2	18	11.57	b	10.97	2.59	0.99	-0.38	0.00
T3	11	17.17	ab	15.12	4.56	0.98	0.87	0.37

	DF	Sum Sq	Mean Sq	F value	Pr(>F)	eta.sq	Levene	Shapiro
Block	2	2372.29	1186.14	4.83	0.01	0.18	0.02	NA
Residuals	44	10812.59	245.74	NA	NA	NA	NA	0.02

Variable: mean pollen tube length

Block	N	Mean	KW	SD	SE	Skew	Kurtosis	Shapiro
T1	18	1.83	a	0.65	0.15	0.14	-1.44	0.05
T2	18	0.95	b	0.54	0.13	-0.45	0.31	0.04
T3	11	1.02	b	0.59	0.18	-0.64	0.72	0.10

	DF	Sum Sq	Mean Sq	F value	Pr(>F)	eta.sq	Levene	Shapiro
Block	2	8.11	4.05	11.49	0.00	0.34	0.17	NA
Residuals	44	15.52	0.35	NA	NA	NA	NA	0.06

Variable: maximum pollen tube length

Block	N	Mean	KW	SD	SE	Skew	Kurtosis	Shapiro
T1	18	2.47	a	1.17	0.28	0.42	-0.38	0.10
T2	18	1.28	b	0.83	0.19	0.02	-0.28	0.18
T3	11	1.36	b	1.10	0.33	1.26	2.86	0.08

	DF	Sum Sq	Mean Sq	F value	Pr(>F)	eta.sq	Levene	Shapiro
Block	2	15.04	7.52	7.06	0.00	0.24	0.30	NA
Residuals	*44*	*46.89*	*1.06*	*NA*	*NA*	*NA*	*NA*	*0.02*

Different letters on KW mean significant differences for alpha = 0.05. SD: standard deviation. NA: not available.

(Fig. 2, Table 2). Several studies have also shown that the optimum germination temperature of loquat pollen fluctuates within this range. Qin *et al.* (2008) and Demirkeser *et al.* (2007) obtained their highest germination and tube length values at 20 ºC. The study conducted by Sharafi *et al.* (2011) in Iran with several loquat genotypes also obtained the highest pollen germination percentages at 22 ºC in some genotypes. Several studies carried out on other *Rosaceae* species have also indicated that the optimal temperature range for pollen germination oscillate between 20 ºC and 25 ºC. For example, Weinbaum *et al.* (1984) noticed that the maximal pollen germination percentage for *P. persica* was set at 23 ºC in a cold sensitivity study, while Hedhly *et al.* (2004) obtained the highest pollen germination rate in two *P. avium* varieties at 20 ºC. These same authors indicated that the pollen germination rates for two *P. persica* cultivars were also optimal at 20 ºC (Hedhly *et al.*, 2005). Sorkheh *et al.* (2018) demonstrated that the optimal temperature for pollen germination in several Iran-native almond genotypes fell within the 20-25 ºC range. In a recent study, Beltrán *et al.* (2019) pointed out that the highest pollen germination percentages for *Cydonia oblonga* Mill., *P. avium*, *Prunus domestica* L., *P. dulcis*, *P. persica* and *P. communis* were close to 20 ºC.

No clear pattern was observed for pollen tube length in relation to the six tested incubation temperatures (Figs. 3-5). This result could be related to the prolonged freezing time at -20 ºC. T1 showed longer tube length compared to T2 and T3. In any case, we should consider that

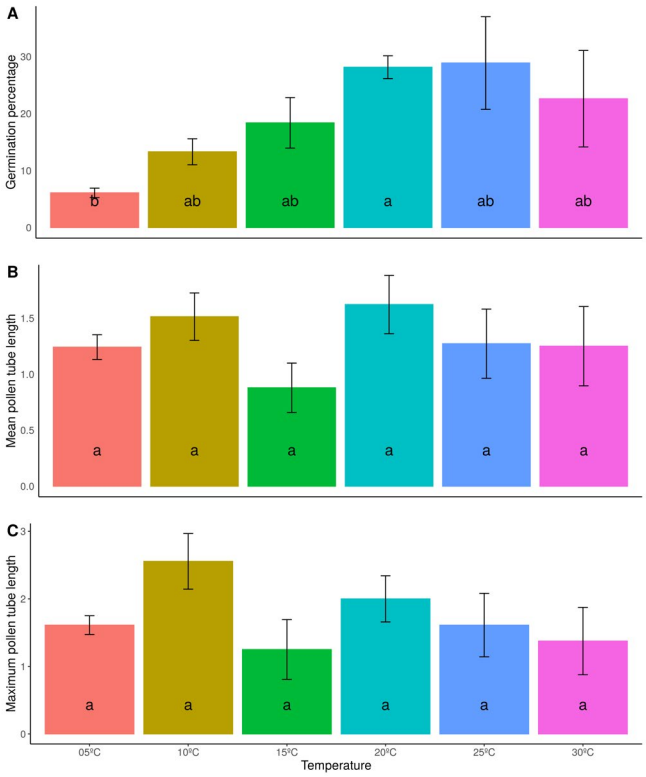

Figure 2. Barplots for the effect of temperature on the pollen germination variables. Different letters mean significant differences in the Kruskal-Wallis test by ranks.

several loquat genotypes have shown a wide variability of pollen tube length values (Sharafi *et al.*, 2011). In this study, significant differences were found in pollen tube length between genotypes, but the highest average pollen tube length value was given at 20 ºC. This result coincides with the pollen tube lengths observed in loquat pollen by Demirkeser *et al.* (2007). In other *Rosaceae* species, the results were similar. Sorkheh *et al.* (2018) obtained the longest pollen tube length values for several almond genotypes between 20 ºC and 30 ºC. In *Prunus cerasus* L., the maximal pollen tube length was detected between 15 ºC and 20 ºC (Cerović & Ružić, 1992), and an identical result was reported years later in several *P. armeniaca* cultivars (Pirlak, 2002).

There is very little information available in the literature about the long-term viability of loquat pollen stored at low temperatures. Some studies have shown that using pollen stored between 0 and 4 ºC is another effective way to handle loquat pollen (Germanà *et al.*, 2006; Sharafi *et al.*, 2011). Likewise, other studies have worked with loquat pollen stored in freezers at -20 ºC. Qin *et al.* (2008) demonstrated that loquat pollen can be stored at this temperature for up to 3 years with no loss of its total germination capacity, and the long-term stored pollen was used to carry out the cross-pollination of loquat trees. However, our results revealed that germinability loss in these cases would be high. Similar studies are found on pollen germination capability in other species. In this sense, Khan & Perveen (2014) carried out a study with five species of the genus Citrus, where the germination of pollen stored at 4 ºC was compared to pollen frozen at different temperatures (-20, -30 and -60 ºC). Freezing at -60 ºC was found to be the best method to conserve pollen. Similar conclusions have been drawn by other studies previously conducted by the same authors for different species, like *S. melongena* (Khan & Perveen, 2006), *C. lanatus* (Khan & Perveen, 2010) and *L. siceraria* (Khan & Perveen, 2011). In all these species, freezing at -20 ºC or -30 ºC did not completely reduce germination. Therefore, the authors recommended freezing as a suitable method for long-term pollen preservation. Another study indicated that deep-frozen pollen (-196 ºC) gave better germination rates than fresh pollen in sweet orange (*Citrus sinensis* (L.) Osbeck), mandarin (*Citrus reticulata* Blanco) and other citrus fruits (Ahmed *et al.*, 2017).

The higher mean pollen germination rates were close to 28% (Table 2). Sharafi *et al.* (2011) found a great variability in pollen germination rates among twenty loquat genotypes, recording the highest pollen germination rate close to 95% while the lowest one remained at 15%. Another study conducted by Reig *et al.* (2014) obtained 72% of pollen germination in loquat cv. 'Algerie' non treated (control) pollen. We found that pollen germination percentages went down below 15% at 5 ºC and 10 ºC. No other studies for *E. japonica* pollen germination at temperatures below 10 ºC have been reported. More studies conducted on pollen germination with other *Rosaceae* species under cold conditions indicate that these temperatures do not favor pollen grain germination. Some studies highlight that low temperatures lead to poor pollen growth in *P. avium* and *P. communis* (Sanzol & Herrero, 2001). Likewise, a study on several Citrus species reports no pollen growth at 10 ºC (Distefano *et al.*, 2012).

The herein obtained pollen germination percentages were lower than 60% at all the tested temperatures. Sharafi (2011) indicated germination percentages between 30% and 40% for some stone fruit cultivars after incubating pollen at 24 ºC. One study has reported a germination percentage below 50% in *P. armeniaca, P. avium* and *P. cerasus* after running germination tests on sucrose media (Bolat & Pirlak, 1999). Germination percentages between 20% and 60% have been published for *Gossypium* pollen germination tests (Kakani *et al.*, 2005). Towil (2010) indicated that pollen quality loss could occur after long-term storage between -10 ºC and -20 ºC in some species. Although many studies have emphasized that freezing can be a suitable method to preserve pollen, they have also observed how different freezing temperatures and times can modify germination patterns. In a study about *Malus pumila* L., the germination capability of pollen was lower at -20 ºC than at

Table 2. Kruskal-Wallis (KW) for the effect of incubation temperature on the germination percentage, mean pollen tube length and maximum pollen tube length values.

Variable: germination percentage								
Temperature	**N**	**Mean**	**KW**	**SD**	**SE**	**Skew**	**Kurtosis**	**Shapiro**
05ºC	9	6.09	b	2.48	0.83	0.65	-1.02	0.19
10ºC	9	13.30	ab	6.85	2.23	0.99	-0.47	0.06
15ºC	6	18.38	ab	10.85	4.43	-0.88	1.03	0.75
20ºC	6	28.15	a	4.91	2.00	-0.97	0.28	0.59
25ºC	9	28.89	ab	24.39	8.13	-0.05	-2.09	0.10
30ºC	8	22.62	ab	23.94	8.46	0.54	-1.98	0.05
	DF	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**	**eta.sq**	**Levene**	**Shapiro**
Temperature	5	3281.56	656.31	2.72	0.03	0.25	0	NA
Residuals	41	9903.32	241.54	NA	NA	NA	NA	0.08
Variable: mean pollen tube length								
Temperature	**N**	**Mean**	**KW**	**SD**	**SE**	**Skew**	**Kurtosis**	**Shapiro**
05ºC	9	1.24	a	0.33	0.11	1.75	3.16	0.01
10ºC	9	1.51	a	0.63	0.21	1.92	3.86	0.01
15ºC	6	0.88	a	0.54	0.22	-0.94	-0.36	0.20
20ºC	6	1.62	a	0.64	0.26	0.34	-2.29	0.17
25ºC	9	1.27	a	0.93	0.31	-0.11	-1.19	0.26
30ºC	8	1.25	a	1.00	0.35	0.09	-1.39	0.26
	DF	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**	**eta.sq**	**Levene**	**Shapiro**
Temperature	5	2.15	0.43	0.82	0.54	0.09	0.03	NA
Residuals	41	21.47	0.52	NA	NA	NA	NA	0.07
Variable: maximum pollen tube length								
Temperature	**N**	**Mean**	**KW**	**SD**	**SE**	**Skew**	**Kurtosis**	**Shapiro**
05ºC	9	1.61	a	0.42	0.14	-0.50	-1.28	0.03
10ºC	9	2.56	a	1.24	0.41	1.11	0.76	0.06
15ºC	6	1.25	a	1.08	0.44	0.79	0.07	0.66
20ºC	6	2.00	a	0.84	0.34	0.38	-1.79	0.25
25ºC	9	1.61	a	1.41	0.47	0.57	-0.91	0.23
30ºC	8	1.37	a	1.41	0.50	1.16	0.48	0.04
	DF	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**	**eta.sq**	**Levene**	**Shapiro**
Temperature	5	9.19	1.84	1.43	0.23	0.15	0.10	NA
Residuals	41	52.75	1.29	NA	NA	NA	NA	0.00

Different letters on KW mean significant differences for alpha = 0.05. SD: standard deviation. NA: not available.

-60 ºC (Perveen & Khan, 2008). Seyrek *et al.* (2016) reported that pollen germination percentage and pollen tube length values in *Actinidia eriantha* Benth were clearly lower after 1 year of freezing at -20 ºC compared to 6-month freezing at the same temperature. Our results revealed loquat pollen quality loss after increasing freezing times as prolonging freezing times lowered the mean values of the three studied variables.

Freezing allows pollen to be preserved in the long term with no loss of total germination capability. In our study, loquat pollen germinated after 8 months of freezing, but with a lower germination percentage. For T1, the pollen germination pattern corresponded to that observed in previous studies conducted with loquat and other *Rosaceae* and fruit tree species. However, different and more erratic patterns appeared for pollen

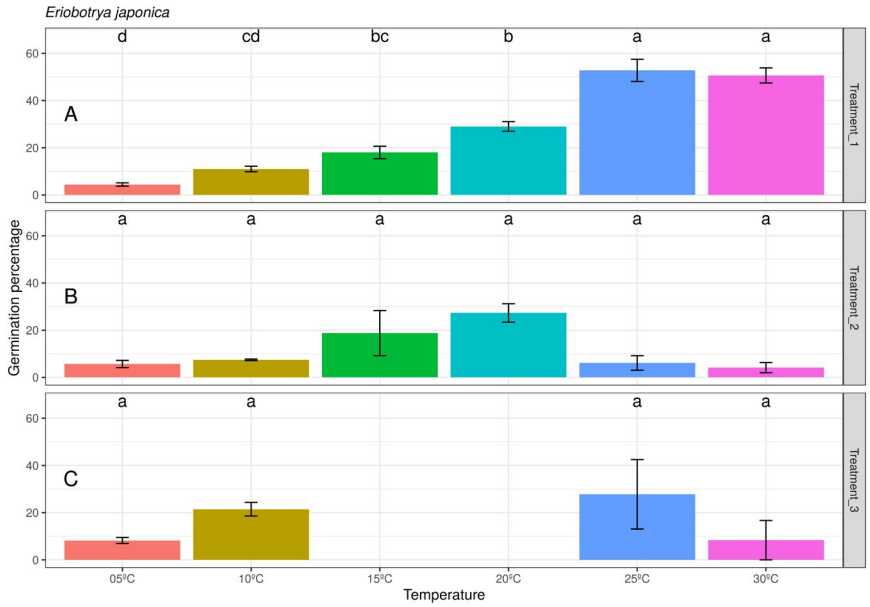

Figure 3. Effect of temperature on the pollen germination percentage for the three treatments (freezing times). Different letters mean significant differences in the Kruskal-Wallis test by ranks.

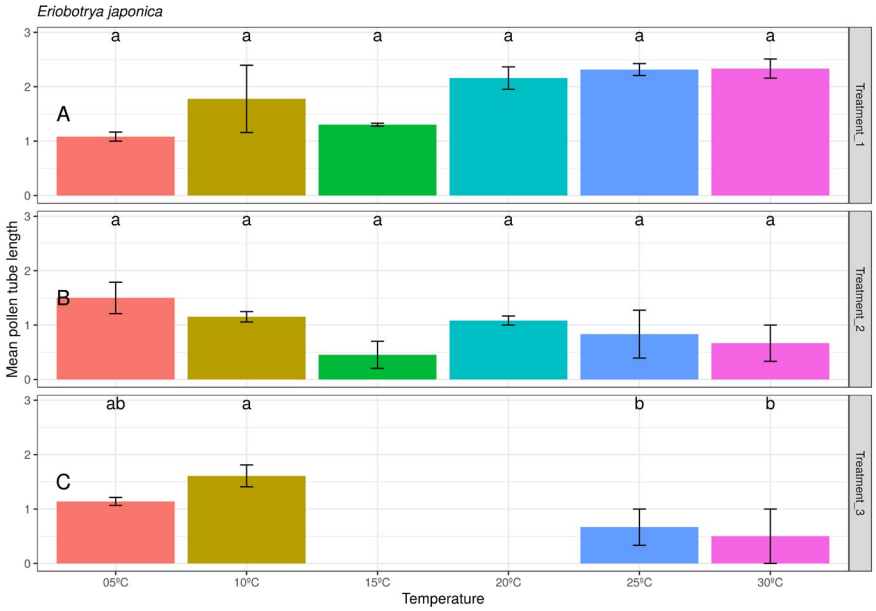

Figure 4. Effect of temperature on the mean pollen tube length for the three treatments (freezing times). Different letters mean significant differences in the Kruskal-Wallis test by ranks.

germination, tube length and maximum tube length at T2 and T3.

In conclusion, the results showed that storing loquat pollen at -20 ºC for 4 months preserves pollen and allows its posterior germination. Germination percentages above 50% were observed at 25 ºC and 30 ºC for incubations after 4 months of freezing (T1). The mean pollen tube length and maximum pollen tube length values showed no significant differences among the incubation temperatures for T1. Instead no significant differences were observed among the incubation temperatures after 6 months of freezing (T2 and T3). The germination capability of the loquat pollen stored at -20 ºC for 8 months was the lowest, and no pollen grain germinated at 15 ºC and 20 ºC. After 4 months of freezing time at -20 ºC, the germination rates of loquat pollen did not follow a clear pattern. Accordingly, long-term freezing at -20 ºC can modify pollen germination. These results indicate that studies into the effect of temperature on pollen

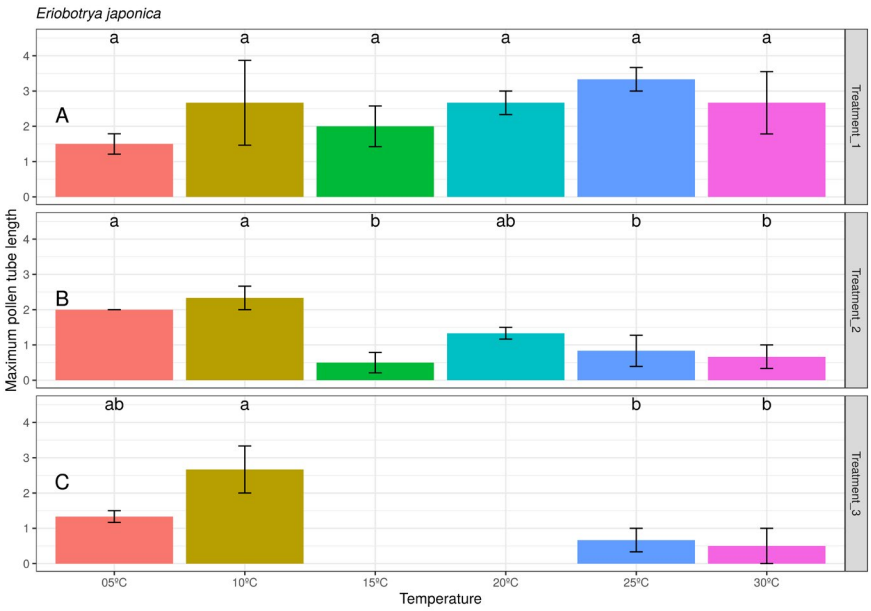

Figure 5. Effect of temperature on the maximum pollen tube length for the three treatments (freezing times). Different letters mean significant differences in the Kruskal-Wallis test by ranks.

germination or pollen tube growth should be carried out using fresh pollen or short-term freezing to avoid any possible adverse effects on pollen caused by prolonged storage at low temperatures.

References

Acar I, Kakani VG, 2010. The effects of temperature on *in vitro* pollen germination and pollen tube growth of Pistacia spp. Sci Hort 125 (4): 569-572. https://doi.org/10.1016/j.scienta.2010.04.040

Agustí M, 2010. Fruticultura. Mundi-Prensa Libros. Madrid, Spain. 507 pp.

Ahmed S, Rattanpal HS, Ahmad E, Singh G, 2017. Influence of storage duration and storage temperature on in-vitro pollen germination of Citrus species. Int J Curr Microbiol Appl Sci 6 (5): 892-902. https://doi.org/10.20546/ijcmas.2017.605.099

Beltrán R, Valls A, Cebrián N, Zornoza C, García Breijo F, Reig Armiñana J, Garmendia A, Merle H, 2019. Effect of temperature on pollen germination for several *Rosaceae* species: influence of freezing conservation time on germination patterns. Peer J 7: e8195. https://doi.org/10.7717/peerj.8195

Blasco M, Badenes ML, Naval MM, 2016. Induced parthenogenesis by gamma-irradiated pollen in loquat for haploid production. Breed Sci 66 (4): 606-612. https://doi.org/10.1270/jsbbs.16021

Bolat İ, Pirlak L, 1999. An investigation on pollen viability, germination and tube growth in some stone fruits. Turk J Agric For 23 (4): 383-388.

Brewbaker JL, Kwack BH, 1963. The essential role of calcium ion in pollen germination and pollen tube growth. Am J Bot 50: 859-865. https://doi.org/10.1002/j.1537-2197.1963.tb06564.x

Caballero P, Fernández MA, 2002. Loquat, production and market. Opt Med 58: 11-20.

Carrera L, Sanzol J, Herrero M, Hormaza JI, 2009. Genomic characterization of self-incompatibility ribonucleases (S-RNases) in loquat (*Eriobotrya japonica* Lindl.) (*Rosaceae*, Pyrinae). Mol Breeding 23 (4): 539. https://doi.org/10.1007/s11032-008-9254-7

Cerović R, Ružić D, 1992. Pollen tube growth in sour cherry (*Prunus cerasus* L.) at different temperatures. J Hortic Sci 67 (3): 333-340. https://doi.org/10.1080/00221589.1992.11516256

Cuevas J, Hueso JJ, Puertas M, 2003. Pollination requirements of loquat (*Eriobotrya japonica* Lindl.), cv.Algerie'. Fruits 58 (3): 157-165. https://doi.org/10.1051/fruits:2003004

Demirkeser TH, Caliskan O, Polat AA, Ozgen M, Serce S, 2007. Effect of natural lipid on pollen germination and pollen tube growth on loquat. Asian J Plant Sci 6 (2): 304-307. https://doi.org/10.3923/ajps.2007.304.307

Distefano G, Hedhly A, Las Casas G, La Malfa S, Herrero M, Gentile A, 2012. Male–female interaction and temperature variation affect pollen performance in Citrus. Sci Hort 140: 1-7. https://doi.org/10.1016/j.scienta.2012.03.011

Egea J, Burgos L, Zoroa N, Egea L, 1992. Influence of temperature on the *in vitro* germination of pollen of apricot (*Prunus armeniaca* L.). J Hortic Sci 67 (2): 247-250. https://doi.org/10.1080/00221589.1992.11516244

Freihat NM, Al-Ghzawi AAM, Zaitoun S, Alqudah A, 2008. Fruit set and quality of loquats (*Eriobotrya japonica*) as effected by pollinations under sub-humid Mediterranean. Sci Hort 117 (1): 58-62. https://doi.org/10.1016/j.scienta.2008.03.012

Germanà MA, Chiancone B, Guarda NL, Testillano PS, Risueño MC, 2006. Development of multicellular pollen of *Eriobotrya japonica* Lindl. through anther culture. Plant Sci 171 (6): 718-725. https://doi.org/10.1016/j.plantsci.2006.07.005

Hedhly A, Hormaza JI, Herrero M, 2004. Effect of temperature on pollen tube kinetics and dynamics in sweet cherry *Prunus avium* (*Rosaceae*). Am J Bot 91 (4): 558-564. https://doi.org/10.3732/ajb.91.4.558

Hedhly A, Hormaza JI, Herrero M, 2005. The effect of temperature on pollen germination, pollen tube growth, and stigmatic receptivity in peach. Plant Biol 7: 476-483. https://doi.org/10.1055/s-2005-865850

Kakani VG, Prasad PVV, Craufurd PQ, Wheeler TR, 2002. Response of *in vitro* pollen germination and pollen tube growth of groundnut (*Arachis hypogaea* L.) genotypes to temperature. Plant Cell Environ 25 (12): 1651-1661. https://doi.org/10.1046/j.1365-3040.2002.00943.x

Kakani VG, Reddy KR, Koti S, Wallace TP, Prasad PVV, Reddy VR, Zhao D, 2005. Differences in *in vitro* pollen germination and pollen tube growth of cotton cultivars in response to high temperature. Ann Bot 96 (1): 59-67. https://doi.org/10.1093/aob/mci149

Khan SA, Perveen A, 2006. Germination capacity of stored pollen of *Solanum melongena* L. (Solanaceae) and their maintenance. Pak J Bot 38 (4): 917-920.

Khan SA, Perveen A, 2010. *In vitro* pollen germination capacity of *Citrullus lanatus* L. (Cucurbitaceae). Pak J Bot 42: 681-684.

Khan SA, Perveen A, 2011. Pollen germination capacity and viability in *Lagenaria siceraria* (Molina) Standley (Cucurbitaceae). Pak J Bot 43 (2): 827-830.

Khan SA, Perveen A, 2014. *In vitro* pollen germination of five citrus species. Pak J Bot 46 (3): 951-956.

Köppen W, Geiger R, 1936. Das Geographische System der Klimate. Borntraeger Science Publishers. Berlin

Mendiburu F, 2019. Agricolae: Statistical procedures for agricultural research. R package version 1.3-0. https://CRAN.R-project.org/package=agricolae.

Mesejo C, Martínez-Fuentes A, Reig C, Rivas F, Agustí M, 2006. The inhibitory effect of CuSO4 on *Citrus pollen* tuve growth and its application for the production of seedless fruit. Plant Sci 170: 37-43. https://doi.org/10.1016/j.plantsci.2005.07.023

Perveen A, Khan SA, 2008. Maintenance of pollen germination capacity of *Malus pumila* L. (*Rosaceae*). Pak J Bot 40 (3): 963-966.

Pirlak L, 2002. The effects of temperature on pollen germination and pollen tube growth of apricot and sweet cherry. Gartenbauwissenschaft 67 (2): 61-64.

Qin Y, Qun-Xian D, Yong-Qing W, Lu L, Yan F, Ying-Hong L, Lian T, Shi-Feng L, 2008. Study on characteristics of *in situ* pollen germination and pollen tube growth of loquat. Plant Sci Res 1 (3): 50-55.

R Core Team, 2017. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna.

RStudio Team, 2016. RStudio: Integrated Development for R. RStudio, Inc., Boston, MA, USA. http://www.rstudio.com/

Reddy KR, Kakani VG, 2007. Screening Capsicum species of different origins for high temperature tolerance by *in vitro* pollen germination and pollen tube length. Sci Hort 112 (2): 130-135. https://doi.org/10.1016/j.scienta.2006.12.014

Reig C, Mesejo C, Martínez-Fuentes A, Agustí M. 2014. Naphthaleneacetic acid impairs ovule fertilization and early embryo development in loquat (*Eriobotrya japonica* Lindl.). Sci Hort 165: 246-251. https://doi.org/10.1016/j.scienta.2013.11.030

Sanzol J, Herrero M, 2001. The "effective pollination period" in fruit trees. Sci Hort 90: 1-17. https://doi.org/10.1016/S0304-4238(00)00252-1

Seyrek UA, Qu X, Huang C, Tao J, Zhong M, Wu H, Xu X, 2016. Influence of storage time on pollen traits in *Actinidia eriantha*. Agric Sci 7 (6): 373. https://doi.org/10.4236/as.2016.76039

Sharafi Y, 2011. *In vitro* pollen germination in stone fruit tree of *Rosaceae* family. Afr J Agr Res 6 (28): 6021-6026. https://doi.org/10.5897/AJAR11.938

Sharafi Y, Motallebbi-Aza AR, Bahmani A, 2011. *In vitro* pollen germination, pollen tube growth and longevity in some genotypes of loquat (*Eriobotrya japonica* Lindl.). Afr J Biotechnol 10 (41): 8064-8069. https://doi.org/10.5897/AJB11.701

Sharpe RH, 2010. Loquat: botany and horticulture. Hortic Rev 23: 233-276. https://doi.org/10.1002/9780470650752.ch5

Sorkheh K, Shiran B, Rouhi V, Khodambashi M, Wolukau JN, Ercisli S, 2011. Response of *in vitro* pollen germination and pollen tube growth of almond (*Prunus dulcis* Mill.) to temperature, polyamines and polyamine synthesis inhibitor. Biochem Syst Ecol 39 (4-6): 749-757. https://doi.org/10.1016/j.bse.2011.06.015

Sorkheh K, Azimkhani R, Mehri N, Chaleshtori MH, Halász J, Ercisli S, Koubouris GC, 2018. Interactive effects of temperature and genotype on almond (*Prunus dulcis* L.) pollen germination and tube length.

Sci Hort 227: 162-168. https://doi.org/10.1016/j.scienta.2017.09.037

Towil LE, 2010. Long-term pollen storage. Plant Breed Rev 13: 179-207. https://doi.org/10.1002/9780470650059.ch5

Vasilakakis M, Porlingis IC, 1985. Effect of temperature on pollen germination, pollen tube growth, effective pollination period, and fruit set of pear. Hortscience 20: 733-735.

Weinbaum SA, Parfitt DE, Polito VS, 1984. Differential cold sensitivity of pollen grain germination in two *Prunus* species. Euphytica 33: 419-426. https://doi.org/10.1007/BF00021139

Yang Q, Fu Y, Wang YQ, Deng QX, Tao L, Yan J, Luo N, 2011. Effects of simulated rain on pollen-stigma adhesion and fertilisation in loquat (*Eriobotrya japonica* Lindl.). J Hortic Sci Biotech 86 (3): 221-224. https://doi.org/10.1080/14620316.2011.11512751

Yang Q, Wang YQ, Fu Y, Deng QX, Tao L, 2012. Effects of biological factors on fruit and seed set in loquat (*Eriobotrya japonica* Lindl.). Afr J Agr Res 7 (38): 5303-5311.