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Optimization of *in vitro* pollen storage conditions in seeded and low-seeded citrus genotypes

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ABSTRACT

***An in vitro* pollen storage study was conducted using pollen from three seeded citrus plant genotypes, viz., 'Mexican lime', 'W'. Murcott' and 'Mosambi' and five seedlesscitrus genotypes, viz., 'Lisbon lemon', 'Jaffa', 'Clementine', 'Hamlin' and 'Mukaku Kishu'. Pollen viability and germination percentage were evaluated at different storage temperature treatments, i.e., at room temperature (in anhydrous calcium chloride) (control), in a refrigerator at 4°C, in a freezer (-20°C), and in a freeze drier (-80°C). The viability of the pollen plants was tested with an acetocarmine stain (2%). Among all the tested sucrose concentrations (0, 5, 10, 15, 20, and 25%) for *in vitro* pollen germination, the 15% sucrose concentration had the highest effect on pollen germination. The results showed significant differences in pollen viability and germination under different storage temperature conditions. The pooled data revealed that, among the seeded genotypes, W. Murcotts showed the maximum mean viability and germination percentage (67.86% and 60.88%, respectively) after 48 weeks of storage at -80°C, and the minimum values were observed for Mexican lime (46.57% and 33.71%, respectively). However, in the low-seeded genotype, Mukaku Kishu had the maximum mean pollen viability and germination (71.52% and 64.07%, respectively) after 48 weeks of storage at -80°C, and the lowest values were observed in Jaffa (39.36% and 28.08%, respectively). The results indicate that the freeze drier storage temperature (-80°C) had the greatest effect on retaining pollen viability and germination in both the seeded and low-seeded genotypes. However, a progressive decrease in pollen viability and germination rate was observed with increasing duration at all storage temperatures, reaching a minimum at 48 weeks after storage. However, the reduction in pollen storage ability was greatest at room temperature and 40°C. Pollen grains stored at low temperatures (-80°C and -20°C) showed good viability and germination percentage compared with those stored at room temperature and 4°C.**

Introduction

Citrus plants are among the most significant and predominant crops grown worldwide and have immense nutritional, medicinal and economic value. It belongs to the family Rutaceae, and most of its species are diploid in nature. In India, citrus covers an area of 10.5 lakhha, and it is the third most important fruit crop, with an annual production of approximately 14.0 million tons. However, the pace of expansion is not as fast as expected, and the major reason behind this is the lack of availability of suitable varieties for citrus growing regions. To overcome this problem, many breeding programmes have been initiated at several research stations. Hybridization between citrus species is often prevented by prezygotic barriers, such as nonsynchronous flowering and gametic

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incompatibilities between citrus species. Under such circumstances, pollen storage is a suitable technique for overcoming hybridization barriers between parent species grown in different regions separated by spatial and geographical distribution. Furthermore, long-term storage of pollen grains is useful for conserving the gene pool and establishing pollen banks where pollen grains of a desired species can be preserved as genetic resource material for breeding programmes. Zheng *et al.,* 2019 revealed that pollen viability, germination and pollen tube growth are prerequisites for the fertilization process and for the formation of seeds and fruits. Several studies on pollen storage conditions have been carried out, and similar results have shown that low temperature can store pollen grains for a long period of time in different fruit crops, for example, in almond (Martinez *et al.* 2001), in strawberry (Aslantus and Pirlak 2002), in sweet cherry (Alburquerque *et al.* 2007), in mango (Khan and Perveen 2006, in pear (Bhat *et al*. 2012) and in citrus (Lora *et al.* 2006, Khan and Perveen 2006, Kundu *et al*. 2014 and Ahmed *et al*. 2017). Similarly, workers examined pollen viability and germination in date palm stored at 4°C, -20°C and -196°C and observed that pollen stored at subzero temperatures (-196°C) had a greater percentage of germination than pollen stored at 4°C. In recent years, the physiology of pollen, particularly its germination and viability, has gained enormous attention for breeding purposes, conservation, and adaptation (Khan and Perveen 2006). The scarcity of pollen in citrus genotypes during pollination due to differences in their blooming period, which leads to poor fruit set, has led to increased storage of pollen to overcome all the above difficulties.

Material and Methods

Pollens from three-seeded (W. Murcott, Mexican lime and Mosambi) and five low-seeded citrus plant species (Jaffa, Hamlin, Clementine, Mukaku Kishu and Lisbon lemon) were used in this study. These genotypes were maintained at Punjab Agricultural University, Dr. J. C. BakhshiRegional Research Station, Abohar, Punjab. The experiment was carried out during 2019 and 2020. After dehiscence, pollen from these flowers was collected in vials (1.5 ml) and stored under different temperature conditions, viz. room temperature (in anhydrous calcium chloride) (control), a refrigerator at 4°C, a freezer at -20°C, and a freeze drier at -80°C. For subzero temperatures (-20°C and -80°C). For *in vitro* pollen viability tests, aceto-carmine was used (2%). Deeply stained and nonshriveled pollen grains were considered viable, whereas shriveled, irregular and nonstained pollen grains were considered nonviable. For the *in vitro* germination test, pollens of seeded and low-seeded genotypes were assessed at different sucrose concentrations, i.e., 0, 5, 10, 15, and 20.25%, using the hanging drop method (Vasil, 1964). The prepared slides were then kept in petri dishes lined with moist filter paper to maintain high humidity (70-80% R.H.). Pollen was considered germinated when the pollen tube length was at least two times greater than its diameter. Pollen germination was observed after 24 hours of incubation at 22±2°C.

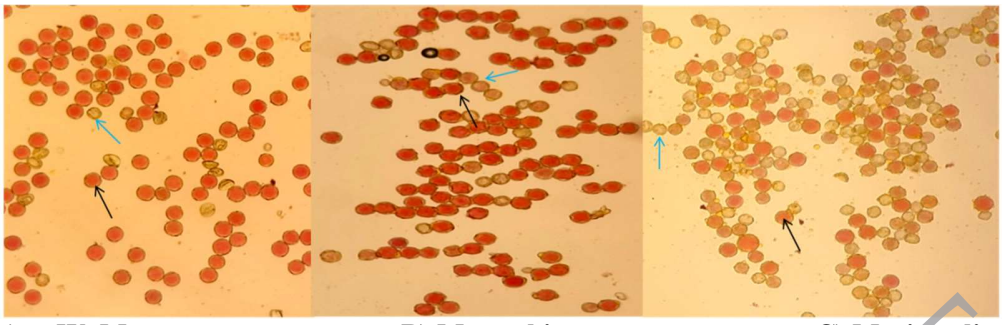
Experimental design and statistical analysis

For evaluation of pollen viability and germination, slides were prepared with three replications, and three fields per slide (with at least 200 pollen grains in each field) were recorded for each genotype. The experiment was carried out in accordance with a completely randomized design (CRD). The slides were then observed under an Olympus Magnus MLX-B Plus binocular digital microscope at monthly intervals." The data pertaining to viability and germination are expressed as percentages. All the statistical analyses were performed on pooled data from 2019 to 2020 with SAS 9.4 statistical software (SAS Inst. Inc., Cary, N.C., U.S.A.). The means of the genotypes were least significantly different ($P \leq 0.05$).

Results and Discussion

Pollen viability in seeded citrus genotypes

Pollen viability was assessed upto 48 weeks duration under varying storage conditions viz., room temperature (control), refrigerator 4^0C, freezer (-20^0C), freeze drier (-80^0C) in seeded genotypes. The results of the pooled data revealed that the viability of the pollen grains was more than 68% for the three seeded genotypes after fresh collection in the laboratory, with the maximum viability occurring in W. Murcott (85.40), followed by Mosambi (82.26) and the minimum viability occurring in Mexican lime (69.19) (Plate 1). Yamamoto *et al.* (2006)



A) W. Murcott **B) Mosambi** **C) Mexican lime**

Plate 1. *In vitro* viability of fresh pollens in seeded citrus genotypes. (Black represents viable pollen, and blue represents nonviable pollen.)

reported a similar pollen fertility percentage of fresh pollen, i.e., 89.40%, in clementines (*Citrus clementina* hort.). ex Tanaka), (95.40%) and Kinokuni (Kishu) (*C. kinokuni* hort. ex Tanaka) and 64.00% pollen fertility in doubled-diploid *Mexican lime (*Rouisset *al* 2018). It was observed that the viability of pollen stored at room temperature decreased more than that of pollen stored at other temperatures. However, the greatest viability was recorded at subzero storage temperatures (-20°C and -80°C) for the entire storage duration, with the highest mean value occurring in the W. Murcott genotype (56.10%), and the lowest in the Mexican lime genotype (38.44%). Overall, a decreasing trend was recorded in pollen viability with increasing storage time, and an inverse relationship was observed for pollen viability and extent of storage. In W. Murcott, the maximum viability was observed at -80°C (76.73%), and the minimum viability (7.00%) was observed at room temperature (control) (Table 1). The maximum viability loss of the stored pollen was recorded at room temperature, where 5.65% viability was recorded after four weeks of storage. However, 85.40 to 49.00, 62.72 and 67.86% losses in viability were found at 4^0C, 20°C and -80°C, respectively (Figure 1, 2 and 3).

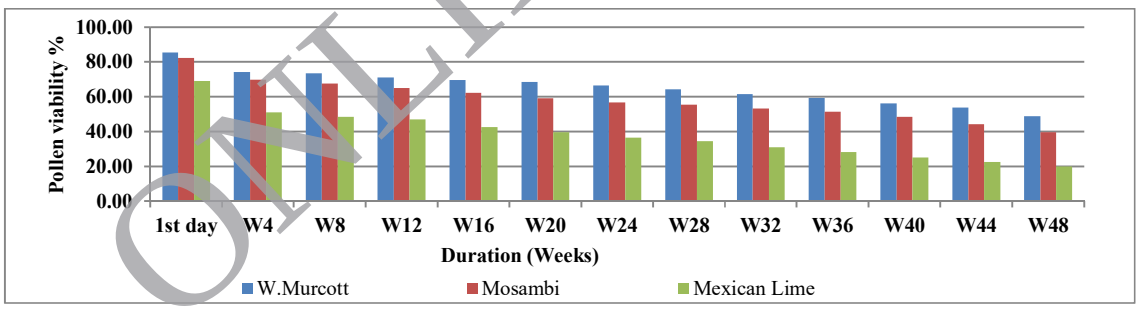


Figure 1: Variation in pollen viability percentage in seeded citrus genotypes at 4°C

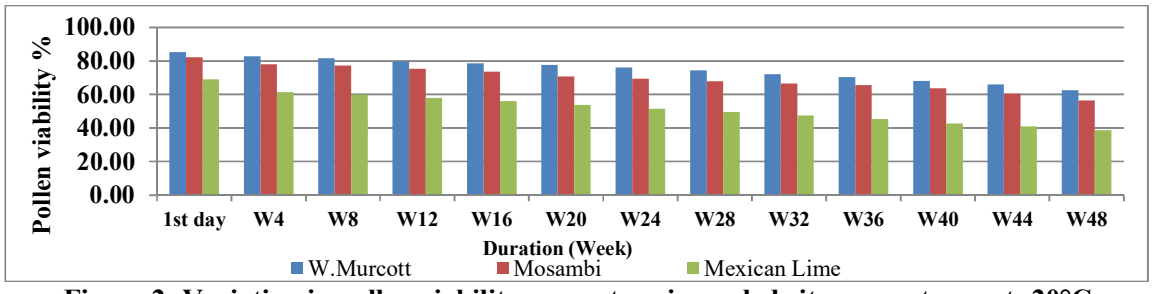


Figure 2: Variation in pollen viability percentage in seeded citrus genotypes at -20°C

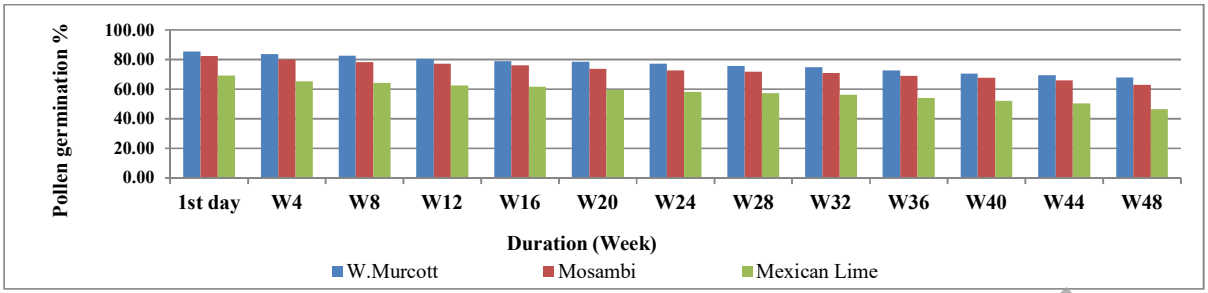


Figure 3: Variation in pollen viability percentage in seeded citrus genotypes at -80°C

Their interaction was also found to be significant. Similarly, in Mosambi and Mexican lime, the mean viability varied from 6.65 to 72.96% and 5.49 to 58.24%, respectively, under varying storage temperatures, with a maximum value occurring under freeze drier conditions, i.e., -80°C (72.96 and 58.24%). The minimum viability of the pollens was recorded at room temperature (6.65 and 5.49%). With increasing storage duration, the viability of the plants also decreased at the different storage temperatures. However, a decreased loss in viability was observed at -80°C (82.26-62.95% and 69.19-46.52%) for both of the above genotypes. Thus, all the seeded plant genotypes responded differently to pollen viability during storage. In this experiment, pollen stored under freeze drying conditions (-20°C and -89°C) gradually decreased in viability, possibly due to the regular freezing and thawing of pollen. Furthermore, cell death caused by intracellular ice formation could be the major factor leading to a decrease in the viability of pollen (Bhat *et al* 2012). Similar variations in pollen viability percentage at different storage temperatures were reported by Bhat *et al.* (2012) for three cultivars of pear cultivars, and maximum pollen viability percentages at -20°C (67.40%) and -196°C (68.06%) were recorded for the cultivar Patharnakh. However, the loss in pollen viability at room temperature was greatest for all the seeded genotypes, and viability was completely lost after four weeks of storage (Table 1).

Pollen viability in low-seeded citrus genotypes

Pollen viability was assessed for up to 48 weeks under various storage conditions, viz., room temperature (control), 4^0C, 20°C, and 800°C (Table 2 and Figure 4, 5 and 6). The results showed that the viability of the pollens of all five low-seeded plant genotypes exceeded 64% on the first day of collection in the laboratory, with the highest viability observed in Mukaku Kishu (88.50%), followed by Clementine (87.38%), Lisbon lemon (83.34%), and Hamlin (81.49%), and the lowest viability observed in Jaffa (65.83) (Plate 2). Among all the storage temperatures, the pollen stored at room temperature exhibited the greatest decrease in viability. However, subzero storage temperatures (-20°C and -80°C) had the highest average viability percentage in Mukaku Kishu (59.64%) and the lowest in the Jaffa (33.90%) genotype. However, a decreasing trend was observed for the viability of pollen with increasing storage duration, and the viability and duration of storage were inversely related to each other. Our results are in agreement with the findings of Sharafi and Bahmani (2010) for loquat plants. After four weeks of storage, the pollen viability decreased more in the room temperature treatment group, ranging from 7.50 to 1.07 among the different cultivars; the maximum loss occurred in Mukaku Kishu, and the minimum loss occurred in Jaffa. After the 48th week of storage, the average viability of the pollens decreased from 88.50 to 48.99%, 87.38 to 47.48%, 83.34 to 45.62%, 81.49 to 36.45% and 65.83 to 20.44% in Mukaku Kishu, Clementine, Lisbon lemon, Hamlin and Jaffa, respectively. With increasing storage duration, the viability of the pollens decreased at all storage temperatures; however, a gradual decrease in viability was observed in the freeze drier (-20°C, -80°C). However, a minimum loss in viability was recorded at a freeze drier temperature of -80°C for all the varieties. Similar results were reported for sweet cherry by Alburquerque *et al.* (2007). The viability of six sweet cherry cultivars, viz., Brooks, Cristobalina, Marvin, New Star, Ruby and Somerset, stored at 4°C and -20°C decreased after 15 or 30 days of storage.

Table 1: Effect of different storage temperatures on *in vitro* pollen viability in seeded citrus genotypes

Duration	W.Murcott					Mosambi					Mexican Lime				
	Room Temp.	4°C	-20°C	-80°C	Mean	Room Temp.	4°C	-20°C	-80°C	Mean	Room Temp.	4°C	-20°C	-80°C	Mean
1st day	85.40	-	-	-	85.40	82.26	-	-	-	82.26	69.19	-	-	-	69.19
W4	5.65	74.21	82.7	83.66	61.55	4.24	69.94	78.15	80.09	58.11	2.14	51.01	61.29	65.30	44.93
W8	0	73.46	81.68	82.50	59.41	0	67.47	77.30	78.24	55.75	0	48.36	59.86	64.18	43.10
W12	0	71.19	79.86	80.44	57.87	0	65.04	75.30	77.27	54.40	0	46.88	58.11	62.53	41.88
W16	0	69.69	78.69	79.00	56.84	0	62.30	73.62	76.17	53.02	0	42.72	56.09	61.71	40.13
W20	0	68.42	77.76	78.42	56.15	0	59.20	70.90	73.67	50.94	0	39.56	53.77	59.46	38.20
W24	0	66.44	76.26	77.17	54.97	0	56.84	69.58	72.71	49.78	0	36.59	51.58	58.20	36.59
W28	0	64.27	74.31	75.61	53.55	0	55.30	67.89	71.80	48.75	0	34.34	49.62	57.36	35.33
W32	0	61.46	72.08	74.72	52.06	0	53.21	66.57	70.90	47.67	0	30.93	47.43	56.17	33.63
W36	0	59.28	70.44	72.61	50.58	0	51.44	65.67	69.04	46.54	0	28.23	45.37	54.02	31.91
W40	0	56.28	68.04	70.53	48.71	0	48.30	63.85	67.48	44.91	0	25.07	42.82	52.06	29.99
W44	0	53.70	66.11	69.53	47.33	0	44.20	60.42	65.84	42.61	0	22.69	40.89	50.35	28.48
W48	0	49.00	62.72	67.86	44.90	0	39.54	56.59	62.95	39.77	0	19.91	38.67	46.57	26.29
Mean	7.00	65.60	75.03	76.73	56.10	6.65	58.08	69.85	72.96	51.89	5.49	38.11	51.90	58.24	38.44
L.S.D. (p≤0.05)	Temp			0.24		Temp			0.35		Temp			0.37	
	Duration			0.51		Duration			0.72		Duration			0.76	
	Temp x Duration			0.88		Temp x Duration			1.25		Temp x Duration			1.32	

Table 2: Effect of different storage temperatures on *in vitro* pollen viability in seeded citrus plant genotypes

Duration	Mukaku Kishu					Clementine					Lisbon Lemon					Hamlin					Jaffa				
	Room Temp	4°C	-20°C	-80°C	Mean	Room Temp	4°C	-20°C	-80°C	Mean	Room Temp	4°C	-20°C	-80°C	Mean	Room Temp	4°C	-20°C	-80°C	Mean	Room Temp	4°C	-20°C	-80°C	Mean
1st day	88.50	-	-	-	88.50	87.38	-	-	-	87.38	83.34	-	-	-	83.34	81.49	-	-	-	81.49	65.83	-	-	-	65.83
W4	7.50	80.70	86.68	87.21	65.52	6.54	78.71	85.53	85.86	64.16	5.18	71.75	79.89	81.36	59.55	3.47	64.29	74.17	78.41	55.08	1.07	46.36	57.57	58.85	40.96
W8	0	78.71	85.74	86.09	62.63	0	76.07	83.88	84.92	61.22	0	69.50	77.97	80.02	56.87	0	62.26	72.66	77.43	53.09	0	42.44	55.37	57.98	38.95
W12	0	76.75	84.77	85.58	61.77	0	73.93	82.17	83.89	60.00	0	66.65	75.72	78.86	55.31	0	60.05	71.14	75.50	51.67	0	40.06	53.89	56.56	37.63
W16	0	74.96	83.14	83.57	60.42	0	72.11	80.61	82.77	58.87	0	65.22	74.63	77.86	54.43	0	58.25	69.62	74.53	50.60	0	37.36	51.98	54.97	36.08
W20	0	72.89	81.37	82.71	59.24	0	70.57	79.18	81.22	57.74	0	63.60	73.42	76.11	53.28	0	55.67	67.68	72.42	48.94	0	33.87	49.91	53.66	34.36
W24	0	71.01	79.7	81.48	58.05	0	68.73	77.70	79.37	56.45	0	61.91	71.72	75.00	52.16	0	52.06	65.1	70.89	47.01	0	30.12	46.97	52.65	32.44
W28	0	68.89	78.42	79.59	56.72	0	67.04	76.62	78.78	55.61	0	60.07	70.94	73.61	51.15	0	48.74	62.85	68.91	45.13	0	26.65	43.72	50.87	30.31
W32	0	66.69	77.77	78.60	55.77	0	64.82	74.84	77.60	54.31	0	58.13	69.17	72.48	49.95	0	45.54	60.47	67.2	43.30	0	24.67	42.47	48.75	28.97
W36	0	64.96	76.06	76.32	54.34	0	63.12	73.49	75.80	53.10	0	53.44	67.12	70.91	47.87	0	42.56	57.94	65.52	41.50	0	21.96	40.18	46.49	27.16
W40	0	62.20	73.27	74.59	52.51	0	60.63	71.43	73.60	51.41	0	51.28	65.02	68.90	46.30	0	40.29	56.14	63.70	40.03	0	17.86	37.20	43.82	24.72
W44	0	59.65	71.22	72.43	50.83	0	57.04	68.32	72.11	49.37	0	48.13	62.43	66.97	44.38	0	37.76	54.02	62.04	38.45	0	15.27	34.74	41.39	22.85
W48	0	56.15	68.29	71.52	48.99	0	53.72	66.20	69.99	47.48	0	43.54	58.55	64.39	41.62	0	34.25	51.86	59.68	36.45	0	10.92	31.46	39.36	20.44
Mean	7.38	70.93	79.61	80.63	59.64	7.22	68.77	77.50	79.50	58.25	6.81	61.27	71.53	74.60	53.55	6.54	52.55	65.01	70.59	48.67	5.15	31.80	47.02	51.63	33.90
L.S.D. ($p \leq 0.05$)	Temp		0.29			Temp		0.24			Temp		0.35			Temp		0.39			Temp		0.40		
	Duration		0.60			Duration		0.50			Duration		0.72			Duration		0.81			Duration		0.82		
	Temp x Duration		1.04			Temp x Duration		0.87			Temp x Duration		1.25			Temp x Duration		1.39			Temp x Duration		1.43		

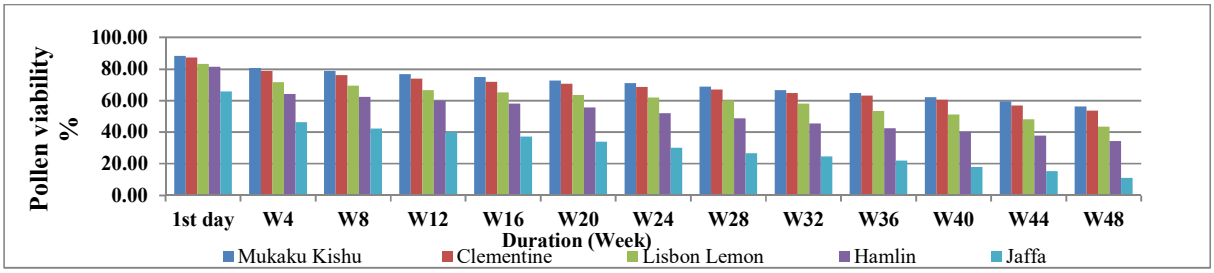


Figure 4: Variation in pollen viability percentage in low-seeded citrus genotypes at 4°C

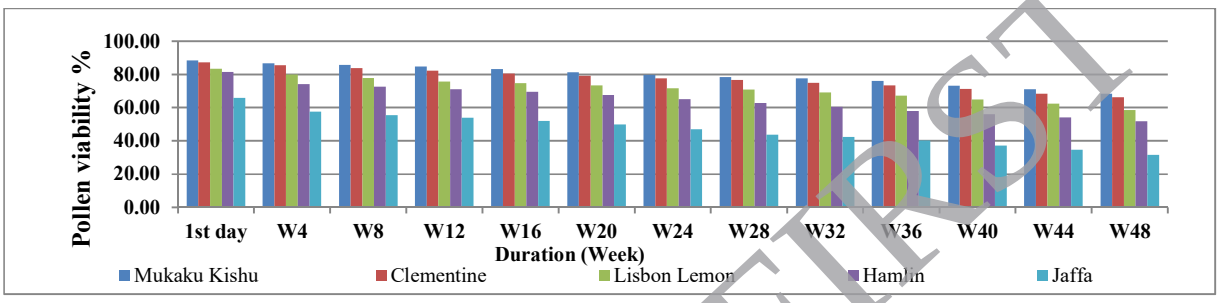


Figure 5: Variation in pollen viability percentage in low-seeded citrus genotypes at -20°C

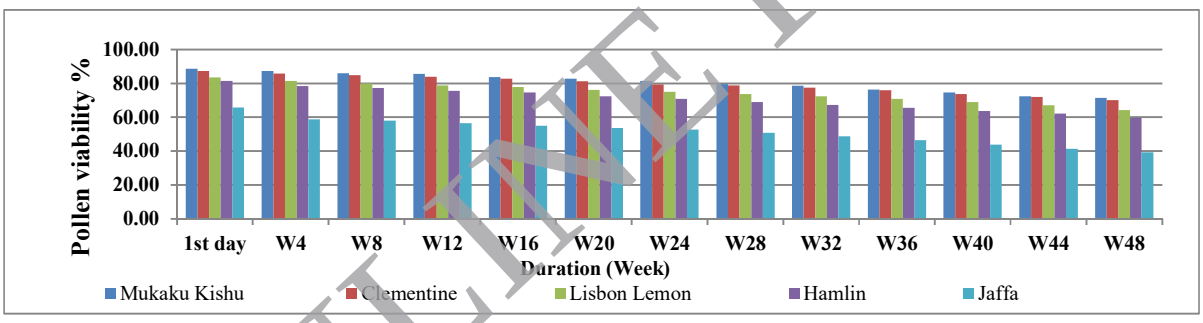
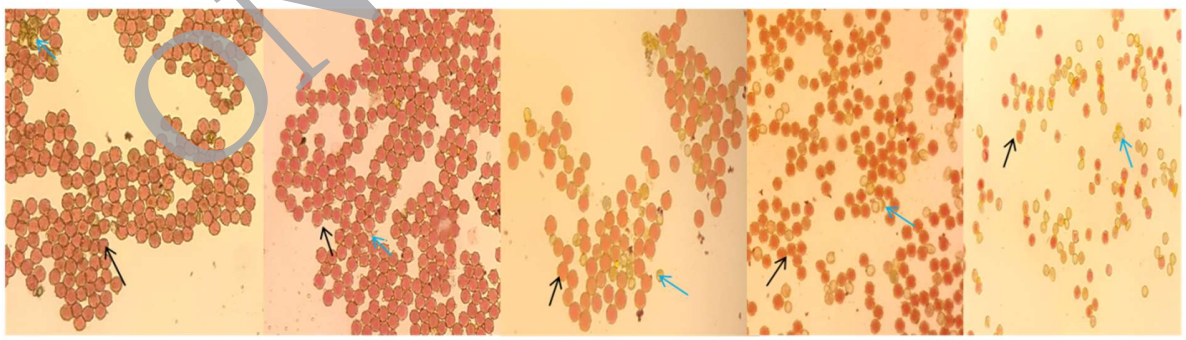


Figure 6: Variation in pollen viability percentage in low-seeded citrus genotypes at -80°C



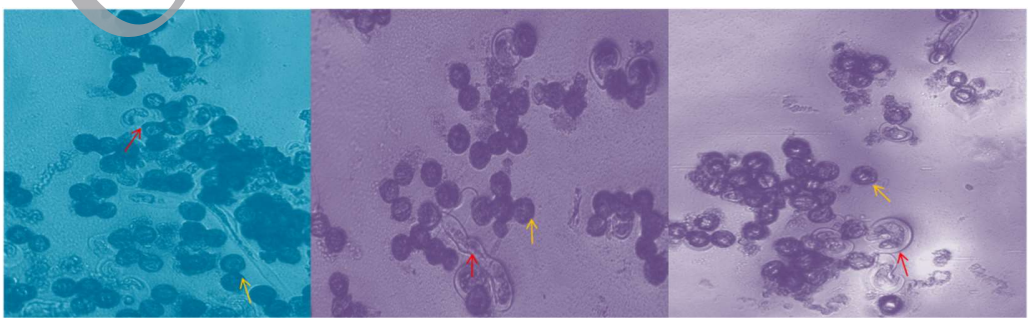
A) Mukaku Kishu B) Clementine C) Lisbon lemon D) Hamlin E) Jaffa

Plate 2. *In vitro* viability of fresh pollens in low-seeded citrus genotypes. (Black represents viable pollen, and blue represents nonviable pollen.)

However, pollen stored at low temperature (-20°C) remained viable for one year; in particular, the sweet cherry cultivar Cristobalina exhibited the highest pollen germination, up to 60%, at -20°C. These results are also in agreement with the findings of Salles *et al*. (2007) for citrus *plants,* Thaipong *et al.* (2008) for grape plants, Bhat *et al.* (2012) for pear plants, for date palm plants and Chander *et al.* (2019) for sugar apple plants. Our results clearly indicated that it is feasible to store pollen grains of citrus genotypes at subzero temperatures, i.e., -20°C and -80°C, with considerable viability for long durations. *In vitro* pollen germination of fresh pollen from seeded and low-seeded plants was evaluated at different sucrose concentrations and in water (control). Among all the sucrose concentrations, the best germination of pollen was recorded in 15% sucrose solution for all the seeded and low-seeded genotypes, followed by 20% sucrose solution (Table 3). The results of this experiment revealed that all the genotypes exhibited good germination at 15% sucrose and that the germination percentage was minimal in the control treatment. The results revealed that the percentage of germinated plants in the Mexican lime and W. Murcott genotypes ranged from 58.91 to 78.20% after treatment with 15% sucrose solution (Plate 3). Germination was greatest in the W. Murcott and Mexican lime-seeded genotypes, whereas very low pollen germination was recorded in the control. Ateyyah (2005) studied the inhibitory effect of certain substances, such as olive oil, on media containing agar (0.8%), sucrose (10%), and 50 ppm citric acid in *Citrus maxima* and *Citrus paradisi*. Khan and Parveen (2006) reported that pollen germination in *various Citrus species* was improved by sucrose at 10 and 20%, but occasionally, 30 and 40% sucrose solutions also produced reasonably good results in *Citrus plants.* Citrus pollen grains can easily germinate and grow in sucrose solution, and their growth can be accelerated by the addition of vitamins and microelements.

Table 3: Effect of different sucrose concentrations on the *in vitro* germination of fresh pollen from seeded citrus plant genotypes

Sucrose Concentration	Genotypes			
	Mexican Lime	W.Murcott	Mosambi	Mean
0 (control)	9.84	21.24	14.49	15.19
5	22.18	34.99	28.96	28.71
10	43.72	58.83	52.42	51.66
15	58.91	78.20	74.59	70.57
20	51.33	62.97	58.61	57.64
25	32.61	47.84	37.25	39.23
Mean	36.43	50.68	44.39	43.83
L.S.D. ($p \leq 0.05$)	2.23	3.312	2.79	2.69



A) W. Murcott **B) Mosambi** **C) Mexiacn lime**

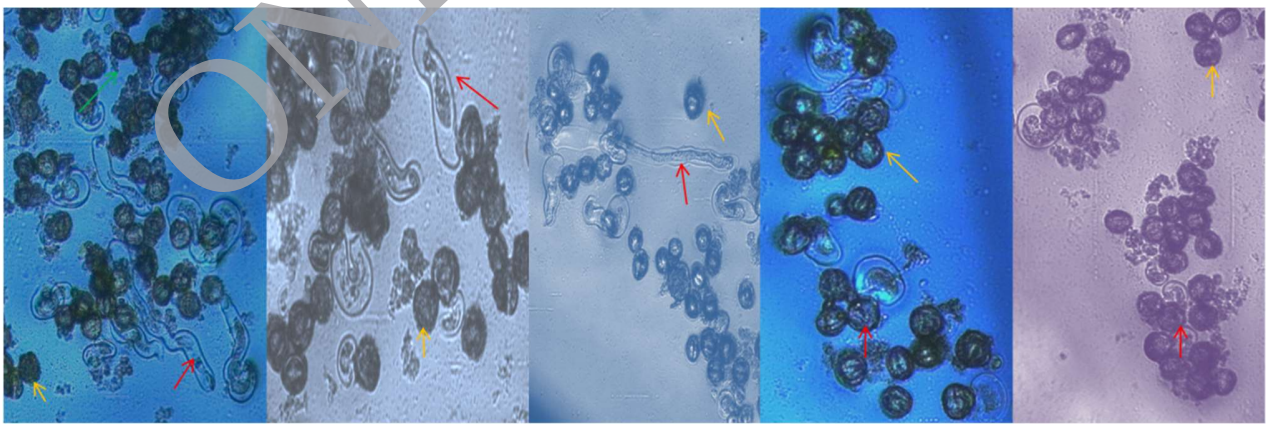
Plate 3. *In vitro* germination of fresh pollen from low-seeded citrus plants was assessed via digital microscopy. Red represents germinated pollen, and yellow represents nongerminated pollen grains

The seeded genotypes of the citrus plants are presented in Table 4 and Plate 4. The results revealed that pollen germination ranged from 55.58 to 83.16% at 15% sucrose concentration in the seeded genotypes. The maximum was in Mukaku Kishu (83.16%), and the minimum was in Jaffa (55.58%). Kundu *et al.* (2014) agreed that *in vitro* germination is more reliable since staining methods can overestimate the viability of pollen. On the other hand, the longevity of pollen, considered the period during which pollen maintains its viability, that is, the capacity for germination and fertilization, strongly depends on the genotype, species and storage conditions. Studies have been carried out to determine the viability and longevity of citrus pollen (Khan and Perveen, 2006, Kundu *et al* 2014; Ahmed *et al* 2017). The pollen germination rate of diploid and doubled diploid "Clemenules" Clementines was 80.7% and 55.8%, respectively, under *in vitro* and *in vivo* conditions (Lora *et al* 2022). Short-term pollen storage facilitates the development of viable pollen within a flowering season and allows the pollination of a late emerging flower with an earlier flowering genotype (Chaudhury *et al* 2010, Dutta *et al* 2013 and Mishra and Shivanna, 1982).

Table 4. Effects of different sucrose concentrations on the *in vitro* germination of fresh pollen from low-seeded citrus plants

Sucrose concentration	Genotypes					
	Clementine	Jaffa	Hamlin	Kishu	Lisbon Lemon	Mean
0 (control)	24.15	8.28	12.49	25.91	17.37	17.64
5	41.01	20.55	26.56	38.62	30.76	31.50
10	62.92	37.32	47.38	60.79	56.91	53.06
15	82.25	55.58	69.63	83.16	75.48	73.24
20	69.16	44.72	53.35	66.95	61.99	59.23
25	53.12	28.49	35.82	48.29	41.56	41.46
Mean	55.89	32.48	40.87	53.5	47.34	46.02
L.S.D. (p≤0.05)	3.52	2.11	2.62	3.34	3.17	3.02

Data pertaining to *in vitro* pollen germination of fresh pollen from different low



A) Mukaku Kishu **B) Clementine** **C) Lisbon lemon** **D) Hamlin** **E) Jaffa**

Plate 4. *In vitro* germination of fresh pollens in low seeded citrus genotypes. Red represents germinated pollen, and yellow represents nongerminated pollen grains

Pollen germination in seeded citrus genotypes

The data pertaining to pollen germination in the seeded genotype under different storage conditions are presented in Table 5. An attempt has been made to compare the germination capacity of three-seeded citrus genotypes for up to 48 weeks under different storage temperatures, such as a 4^0C freezer (-20°C), a freeze drier (-80°C) and a room temperature (control). Both the frozen and freeze-dried samples showed better results. The results showed that 15% solution solution was a suitable medium for pollen germination of citrus plants. The results of pollen germination revealed that the highest germination was recorded for freshly collected pollens, and W. Murcott (79.76%) exhibited maximum germination, followed by Mosambi (75.84%), while the lowest germination was recorded for Mexican lime (60.90%) (Table 5) (Figure 7, 8 and 9). Among the different storage temperatures, the pollen stored at room temperature exhibited the greatest loss during germination. The maximum germination percentage was recorded for the freeze-dried plants (-80°C) for the entire storage period, with the highest mean germination percentage occurring in the W. Murcott genotype (70.11%) and the lowest in the Mexican lime genotype (46.51%). Pollen germination decreased with increasing storage time; thus, germination and duration exhibited an inverse relationship.

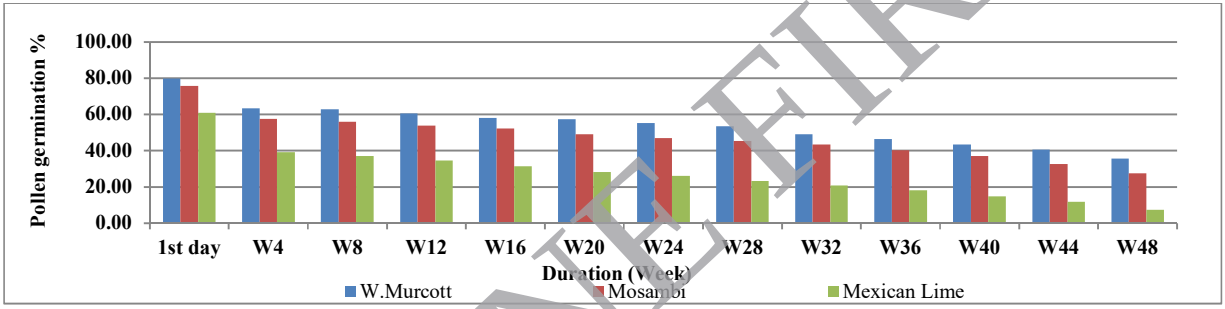


Figure 7: Variation in pollen germination in seeded citrus genotypes at 4°C

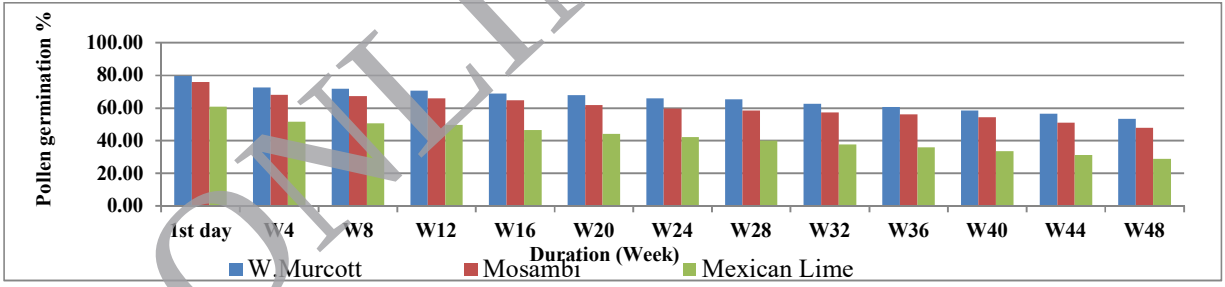


Figure 8: Variation in pollen germination in seeded citrus genotypes at -20°C

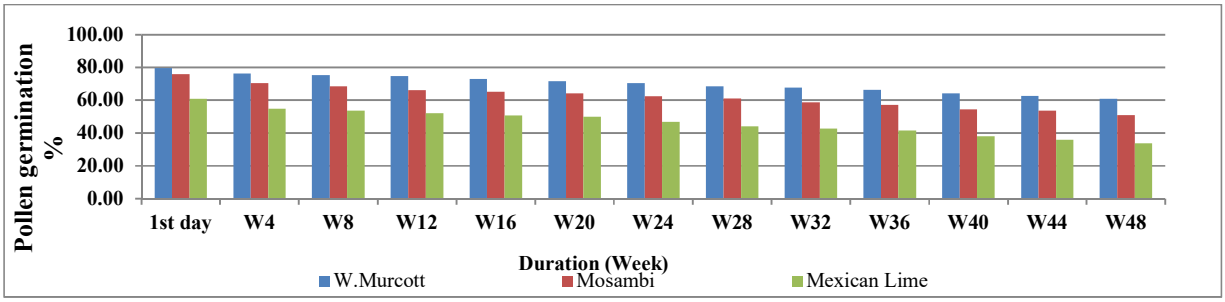


Figure 9: Variation in pollen germination in seeded citrus genotypes at -80°C

Table 5: Effect of different storage temperatures on *in vitro* pollen germination in seeded citrus plant genotypes

Duration	W.Murcott					Mosambi					Mexican Lime				
	Room Temp.	4°C	-20°C	-80°C	Mean	Room Temp.	4°C	-20°C	-80°C	Mean	Room Temp.	4°C	-20°C	-80°C	Mean
1st day	79.76	-	-	-	79.76	75.84	-	-	-	75.84	60.90	-	-	-	60.90
W4	0	63.52	72.70	76.25	53.11	0	57.68	68.05	70.31	49.00	0	39.25	51.63	54.76	36.41
W8	0	62.85	71.70	75.32	52.46	0	55.85	67.32	68.47	47.90	0	37.05	50.49	53.69	35.31
W12	0	60.50	70.60	74.65	51.43	0	53.92	65.98	66.09	46.50	0	34.63	49.61	52.00	34.06
W16	0	58.21	68.80	73.04	50.00	0	52.23	64.64	65.13	45.50	0	31.47	46.59	50.75	32.20
W20	0	57.42	68.00	71.69	49.27	0	49.06	61.89	64.19	43.80	0	28.26	44.22	49.97	30.61
W24	0	55.26	66.10	70.56	47.99	0	46.96	59.74	62.42	42.30	0	26.08	42.11	46.69	28.72
W28	0	53.42	65.40	68.48	46.83	0	45.34	58.65	61.11	41.30	0	23.18	40.04	44.07	26.82
W32	0	49.08	62.50	67.61	44.80	0	43.54	57.15	58.78	39.90	0	20.74	37.79	42.70	25.31
W36	0	46.53	60.60	66.41	43.39	0	40.31	56.12	57.21	38.40	0	18.25	35.87	41.36	23.87
W40	0	43.43	58.50	64.25	41.54	0	36.92	54.4	54.36	36.40	0	14.83	33.52	38.03	21.59
W44	0	40.64	56.50	62.53	39.91	0	32.74	50.89	53.64	34.30	0	11.90	31.30	35.99	19.80
W48	0	35.65	53.50	60.88	37.50	0	27.48	47.81	51.01	31.60	0	7.32	28.77	33.71	17.45
Mean	6.14	54.33	65.70	70.11	49.08	5.83	47.53	60.65	62.20	44.10	4.68	27.22	42.52	46.51	30.23
L.S.D. (p≤0.05)	Temp			0.44		Temp			0.70		Temp			0.44	
	Duration			0.93		Duration			1.46		Duration			0.92	
	Temp x Duration			1.60		Temp x Duration			2.53		Temp x Duration			1.60	

Our results coincide with those obtained by Lora *et al.* (2006) for cherimoya pollen**,** Weatherhead *et al.* (2006) for potato pollen, Gomes *et al.* (2003) for onion and Sharafi and Bahmani (2010) for loquat. Germination decreased with increasing storage period, and an inverse relationship was thus observed between germination and storage duration. The loss in germination was greatest for pollen stored at room temperature, for which no germination occurred after four weeks. After the 48th week of storage, the mean germination decreased from 79.76 to 37.50%, 75.84 to 31.60% and 60.90 to 17.45% in W. Murcott, Mosambi and Mexican lime, respectively. In addition, for the Mosambi, the mean germination varied from 5.83 to 62.20% at all storage temperatures, with the maximum germination percentage occurring at -80°C (70.31%). The lowest germination density was recorded at room temperature (5.83), i.e., the control condition. However, the minimum loss was observed at freeze drier -80°C (75.84 to 62.20%) (Figure 9), followed by -20°C (75.84 to 60.65%) (Figure 8) and 4°C (75.84 to 47.53%) (Figure 7). Furthermore, the loss in germination maximum at room temperature (75.84 to 5.83%) occurred from the 1st day of storage to the 48th week of storage. A similar trend for germination was observed for the Mexican lime genotype as for the W. Murcott and Mosambi plants. At room temperature, the maximum germination percentage (4.68%) occurred, while at -80°C, the minimum average loss of germination occurred. The average germination loss ranged from 60.90 to 30.23% from the 1st day of storage to the 48th week after storage (Table 5). Similar variations in pollen storage capacity were also observed by Martinez-Gomez *et* al. (2001) for almond and by Aslantus and Pirlak (2002) for strawberry. There are several other reports on pollen germination and viability in other taxa (Zeng-Yu-Wang *et al* 2004, Khan and Perveen 2006).

Pollen germination in low-seeded citrus genotypes

The results revealed that, for all five low-seeded plant genotypes, the pollen germination percentage was more than 56%. The results showed that the most germination occurred in Mukaku Kishu (84.16%), followed by Clementine (82.83%), Lisbon lemon (76.73%), and Hamlin (70.99%), while the least germination occurred in Jaffa (57.07%) (Table 6) (Figure 10, 11 and 12). Among the various storage temperatures, room temperature had the fastest loss of pollen germination, and all the low-seeded plants could not germinate (0.00%) after one week at room temperature (Table 6). However, germination was greatest at subzero storage temperatures (-80°C) during the entire storage period, with the highest mean germination percentage occurring in the Mukaku Kishu genotype (74.22%) and the lowest occurring in the Jaffa genotype (41.83%) (Fig 12). Pollen germination in all the genotypes was initially high but decreased with increasing storage time, establishing an inverse relationship between germination and the duration of storage. The average mean germination percentage varied between 41.59% in Mukaku Kishu and 14.85% in Jaffa. All the other genotypes showed Maximim loss during germination at room temperature for all the genotypes, which ranged from 84.16 to 6.47, 82.83 to 6.37, 76.73 to 5.90, 70.99 to 5.46 and 57.07 to 4.39 for Mukaku Kishu, Clementine, Lisbon lemon, Hamlin and Jaffa, respectively (Table 6). The variation in pollen fertility among the genotypes is due to varietal differences (Alburquerque *et al.* 2007 and and Bahmani (2010).

Similar results were obtained by Martinez-Gomez *et al.* (2002), who reported that pollen germination lasted for one year at low storage temperatures (-20°C and -80°C). Robles-Gonzalez *et al.* (2019) reported that the longevity of pollen of Mexican lemon genotypes and Citrange C-35 remain stable for only 24 hours under room temperature storage conditions. Similar results were reported by Lora *et al.* (2006) in cherimoya plants under low temperature (-20°C, -80°C), where germination progressively declined with time of storage. In this study, pollen stored under freeze drier conditions (-20°C and -89°C) gradually decreased in germination percentage, possibly due to the regular freezing and thawing of pollen. Furthermore, intracellular ice formation and cell death could lead to a decrease in pollen germination. Similar variations in pollen germination percentage were reported by Alburquerque *et al.* (2007) in sweet cherry cultivars stored at 4°C and -20°C, and pollen germination was assessed for up to 365 days.

Table 6: Effect of different storage temperatures on *in vitro* pollen germination in low-seeded citrus plants

Duration	Mukaku Kishu					Clementine					Lisbon Lemon					Hamlin					Jaffa				
	Room Temp.	4°C	-20°C	-80°C	Mean	Room Temp.	4°C	-20°C	-80°C	Mean	Room Temp.	4°C	-20°C	-80°C	Mean	Room Temp.	4°C	-20°C	-80°C	Mean	Room Temp.	4°C	-20°C	-80°C	Mean
1st day	84.16	-	-	-	84.16	82.83	-	-	-	82.83	76.73	-	-	-	76.73	70.99	-	-	-	70.99	57.07	-	-	-	57.07
W4	0	70.86	78.00	80.70	57.39	0	66.92	75.87	79.14	55.48	0	60.79	69.03	73.00	50.70	0	53.40	64.55	65.27	45.8	0	35.41	47.13	50.53	33.27
W8	0	69.15	76.61	79.22	56.25	0	66.00	74.73	78.33	54.76	0	58.22	68.11	72.21	49.63	0	50.79	62.93	64.49	44.55	0	31.8	45.87	49.82	31.87
W12	0	66.72	75.03	78.33	55.02	0	64.95	73.86	76.76	53.89	0	55.45	66.92	71.68	48.51	0	49.23	61.48	63.12	43.46	0	30.01	44.76	47.53	30.58
W16	0	65.12	73.89	76.79	53.95	0	63.00	71.90	75.90	52.70	0	53.25	65.58	70.48	47.33	0	47.63	60.09	61.22	42.23	0	27.41	43.34	45.31	29.01
W20	0	62.70	71.77	75.85	52.58	0	60.49	70.14	74.82	51.36	0	52.34	64.41	68.49	46.36	0	44.93	57.98	60.00	40.73	0	25.04	41.89	43.56	27.62
W24	0	60.51	70.15	74.96	51.41	0	58.81	68.51	73.99	50.33	0	49.83	62.41	67.35	44.90	0	42.28	55.69	59.55	39.38	0	21.33	39.16	41.23	25.43
W28	0	58.98	69.00	73.50	50.37	0	54.61	67.20	72.79	48.65	0	48.38	61.58	66.23	44.05	0	39.12	53.14	57.05	37.33	0	19.33	36.90	40.19	24.11
W32	0	55.97	67.02	71.50	48.62	0	52.54	65.74	70.47	47.19	0	46.13	59.67	65.10	42.72	0	35.90	51.10	56.62	35.91	0	16.50	34.86	39.24	22.65
W36	0	53.94	65.66	70.08	47.42	0	50.13	63.64	69.04	45.70	0	42.76	57.52	63.38	40.91	0	32.09	49.45	55.00	34.14	0	14.88	32.79	36.42	21.02
W40	0	51.90	62.76	68.94	45.90	0	47.36	61.68	67.21	44.06	0	39.46	55.59	61.41	39.12	0	29.65	47.65	52.32	32.40	0	10.77	30.70	34.01	18.87
W44	0	48.35	59.42	66.79	43.64	0	44.53	59.74	65.10	42.34	0	36.22	52.99	59.03	37.06	0	25.59	44.60	49.97	30.04	0	8.00	27.59	30.73	16.58
W48	0	43.35	58.93	64.07	41.59	0	39.75	56.83	63.59	40.04	0	30.47	49.05	56.47	34.00	0	22.85	42.30	47.16	28.08	0	5.40	25.93	28.08	14.85
Mean	6.47	60.90	70.18	74.22	52.95	6.37	57.84	68.67	73.08	51.49	5.90	50.02	62.28	67.04	46.31	5.46	41.88	55.53	58.67	40.39	4.39	23.30	39.08	41.83	27.15
L.S.D. (p≤0.05)	Temp	0.40				Temp	0.42				Temp	0.44				Temp	0.33				Temp	0.43			
	Duration	0.83				Duration	0.88				Duration	0.91				Duration	0.69				Duration	0.89			
	Temp x Duration	1.43				Temp x Duration	1.52				Temp x Duration	1.57				Temp x Duration	1.12				Temp x Duration	1.54			

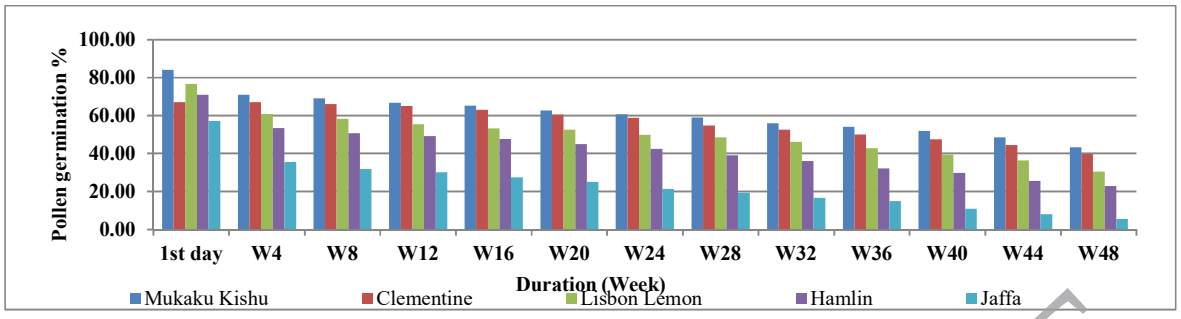


Figure 10: Variation in pollen germination percentage in low-seeded citrus genotypes at 4°C

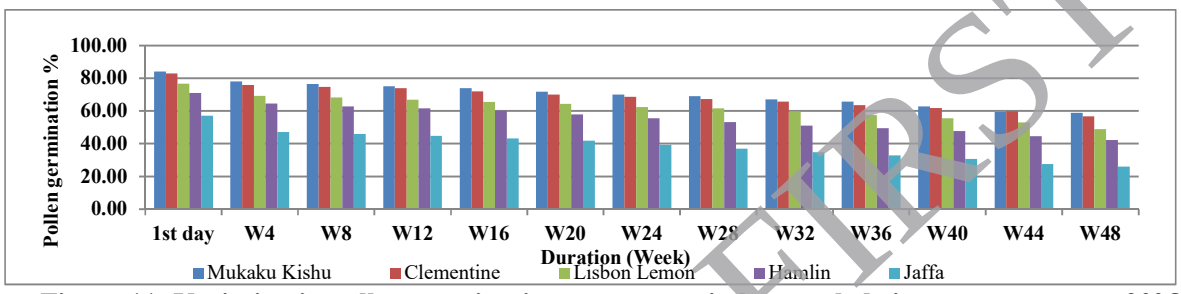


Figure 11: Variation in pollen germination percentage in low-seeded citrus genotypes at -20°C

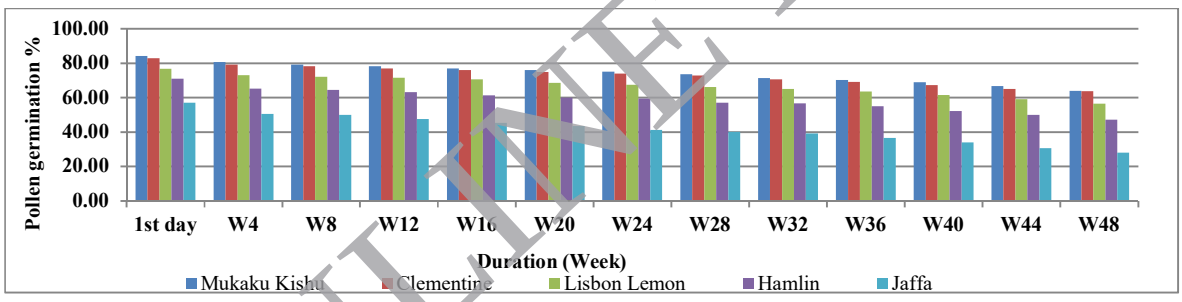


Figure 12: Variation in pollen germination percentage in low-seeded citrus genotypes at -80°C

The cultivar Cristobalina exhibited the highest degree of pollen germination (60%) at -20°C. Pollen germination in other cultivars varied from 36% to 44% at 4°C, and viability was maintained for sixty days; thereafter, a complete decrease in viability was observed. After a year of storage at subzero (-20°C), all the germinated pollen plants had the same percentage of germination as the control pollen plants. In a similar study, Anjum and Shaukat (2008) reported that freeze drying (-60°C) significantly affected the percentage of pollen germination in *Malus pumila* L. after 48 weeks of storage. However, Ahmed *et al.* (2017) recorded maximum pollen germination (80.2%) in Mosambi, and minimum pollen germination was recorded in Itaboria (49.2%) at -196^{0}C. They reported that pollen germinated for up to eight days; thereafter, no germination was observed at the fourth week of storage under room temperature conditions. Towil (2010) reported that pollen stored between −10°C and −20°C can be used to conserve material in the very long term, e.g., one to three years. However, this result should be qualified according to the species. Thus, the pollen of Citrus grandis (L.) Osbeck and Citrus medica L. maintained their germination capacity for three years at −20°C. However, the above-cited author indicated that the germination percentage of some Rosaceae plants, such as *Prunus domestica*, remained close to 60% after 2.5 years at −20°C, while *Prunus persica* achieved germination percentages higher than 65% with storage times between four and nine years at −20°C.

Conclusion

The results of this study indicated that among the different seeded and seedless citrus accessions we analyzed in our investigation, the pollen grains of W. Murcott (seeded) and Mukaku Kishu (low seeded) plants exhibited the highest viability and germination after 48 weeks of storage on a freeze-dryer (-80°C). At subzero temperatures, the pollen of all the genotypes showed a gradual decrease in viability and germination compared with those of the pollens stored at room temperature. Furthermore, freeze-dried pollens of citrus genotypes with different flowering times ensured their availability throughout the flowering season. These findings are valuable for overcoming prezygotic barriers in the hybridization of citrus genotypes. The results clearly indicate that it is feasible to store pollen grains from citrus plants at subzero temperatures for a long period without causing a considerable decrease in their storage ability. Pollen is a valuable genetic resource for conservation, and stored pollen can be utilized in citrus improvement programs.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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