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Optimization of *invitro* Pollen Storage Conditions in Seeded and Low Seeded Citrus Genotypes

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A B S T R A C T

In vitro pollen storage study was conducted using pollens of three seeded citrus genotypes viz., 'Mexican lime',' W.Murcott' and 'Mosambi' and five seedless citrus 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), refrigerator 4°C, freezer (-20°C), freeze drier (-80°C). *In vitro* pollen viability capacity was tested with acetocarmine stain (2%). Among all the tested sucrose concentration (0, 5, 10,15,20,25 %) for *in vitro* pollen germination 15 % sucrose concentration showed highest pollen germination. Result showed a significant differences in pollen viability and germination under different storage temperature conditions. The pooled data revealed that, among seeded genotype, W. Murcott showed maximum mean viability and germination percentage (67.86 % and 60.88 %, respectively) after 48 weeks of storage at -80°C storage temperature and minimum was observed in Mexican lime (46.57 % and 33.71, respectively). Whereas, in low seeded genotype, Mukaku Kishu had maximum mean pollen viability and germination (71.52 % and 64.07 %, respectively) after 48 weeks of storage at -80^{0}C storage temperature, and minimum was observed in Jaffa (39.36 % and 28.08 %, respectively)**.** The results indicate that freeze drier storage temperature (-80°C) recorded best in terms of retaining pollen viability and germination in both the seeded and low seeded genotypes. However, a progressive declining rate in pollen viability and germination was observed with increase in duration at all the storage temperatures, reaching a minimum at 48 weeks of after storage. However, reduction in pollen storage ability was highest at room temperature and 4°C. Pollen grains stored at low temperatures (-80°C and -20°C) showed good viability and germination percentage over pollen stored at room temperature and 4°C.

Introduction

Citrus is one of the most significant and predominant crops grown worldwide, with immense nutritional, medicinal and economic values. It belongs to the family Rutaceae with most of species being diploid in nature. In India, citrus covers an area of 10.5 lakh ha and it is the third most important fruit crop with an annual production of about 14.0 million tons (Anonymous, 2020a). However, the pace of expansion is not so fast as expected and the major reason behind this is non availability of suitable varieties for the citrus growing regions. To overcome this problem many breeding programmes has been initiated at several research stations. Hybridization between the citrus species is often prevented because of pre zygotic barriers such as non synchronous flowering, gametic incompatibilities between citrus species etc. Under such circumstances pollen storage is the suitable technique to overcome the hybridization barriers between parent species grown at different regions separated by spatial and geographical distribution. Further, long term storage of pollen grains is useful means for conservation of gene pool and to establish pollen banks where pollen grains of a desired species can be preserve as genetic resource material available for breeding programmes. Pollen viability, germination and pollen tube growth are pre requisite for fertilization process, formation of seed and fruit (Zheng *et al.,* 2019). Various studies on pollen storage conditions were carried out and indicates similar consensus that, low temperature can store pollen grains for a long period of time in different fruit crops viz., in almond (Martinez *et al.,* 2000), in strawberry (Aslantus and Pirlak, 2002), in sweet cherry (Alburquerque *et al.,* 2007), in Mango (Khan and Perveen, 2009), in pear (Bhat *et al.,* 2012) and in citrus (Lora *et al.,* 2006; Khan and Perveen, 2014; Kundu *et al.,* 2014 and Ahmed *et al.,* 2017). Similarly, Anushma *et al.,* (2018) examined pollen viability and germination in date palm stored at 4°C and -20°C and -196°C and observed that pollen stored sub zero temperature (-196°C) showed better germination percentage over pollen stored at 4°C. In recent years, physiology of pollens particularly germination and viability has gained enormous focus for the breeding purpose, conservation, and adaptation (Khan and Perveen, 2014). Pollen scarcity in citrus genotypes during pollination due to differences in their blooming period which leads to poor fruit set has urged its storage to overcome all the above difficulties.

Materials and Methods

Pollens from three seeded (W. Murcott, Mexican lime and Mosambi) and five low seeded citrus genotypes (Jaffa, Hamlin, Clementine, Mukaku Kishu and Lisbon lemon) were used in this study. These genotypes were maintained at Punjab Agricultural University, Dr J. C. Bakhshi Regional Research Station, Abohar, Punjab. The experiment was carried out during 2019 and 2020. Pollens after dehiscence from these flowers were collected in vials (1.5 ml) and stored under different temperature conditions viz. room temperature (in anhydrous calcium chloride) (control), refrigerator 4^0C, freezer (-20^0C), freeze drier (-80^0C). For sub-zero temperatures (-20℃ and -80^0C). For *in vitro* pollen viability tests, aceto-carmine (2 %). Deeply stained and non-shrivelled pollen grains were counted as viable, whereas shrivelled, irregular and non-stained were counted as non viable. For *in vitro* germination test, pollens of seeded and low seeded genotypes were assessed at different sucrose concentrations i.e. 0, 5, 10,15,20,25 % using hanging drop method. The prepared slides were then keep in petri-dishes lining with moist filter paper to maintain high humidity (70-80 % R.H.). Pollen was considered as germinated when pollen tube length grows at least two times longer than its diameter. Pollen germination was observed after 24 hours of incubation at 22±2.

Experimental Design and Statistical Analysis

For evaluation of pollen viability and germination slides were prepared with three replications, and three fields per slides (with atleast 200 pollen grains in each fields) were recorded for each genotypes. The experiment was carried out as Completely

Randomized Design (CRD). The slides were then observed under Olympus Magnus MLX-B Plus binocular digital microscope at monthly intervals. "The data pertaining to the viability and germination was expressed in percentage. Statistical analysis were performed on pooled data for (2019 and 2020) with SAS 9.4 statistical software (SAS Inst. Inc., Cary, N.C., U.S.A). With a Least significant difference ($P \leq 0.05$) among the genotypes mean.

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 pooled data revealed that viability of pollen grains was more than 68 percent in three seeded genotypes, after fresh collection in laboratory with maximum viability in W. Murcott (85.40), followed by Mosambi (82.26) and minimum in Mexican lime (69.19) (Plate 1). Yamamoto *et al.*, (2006) reported a similar pollen fertility percentage of fresh pollen i.e. (89.40%) in Clementine (*Citrus clementina* hort. ex Tanaka), (95.40 %) in Kinokuni (Kishu) (*C. kinokuni* hort. ex Tanaka) and 64.00 % pollen fertility in doubled-diploid Mexican lime (Rouiss *et al.,* 2018). It was observed that pollens stored at room temperature had greater loss in their viability in comparison to other storage temperatures. However, viability was recorded maximum at sub zero storage temperatures (-20^0C and -80^0C) for the entire storage duration with the highest mean value in genotype W.Murcott (56.10 %) and lowest in Mexican lime (38.44 %). Overall, a decreasing trend was recorded in pollen viability with the increase in storage time and inverse relation was observed for the pollen viability and their extent of storage. In W.Murcott, maximum viability was observed at -80℃ (76.73%) and minimum viability (7.00%) was observed at room temperature (control) (Table 1). Maximum viability loss of stored pollens was recorded at room temperature where (5.65 %) viability was recorded after four weeks of storage duration. While, 85.40 to 49.00, 62.72 and 67.86 percent loss in viability was found at 4^0C,-20^0C and -80^0C, respectively (Figure 1, 2 and 3). Their interaction was also found significant. Similarly in Mosambi and Mexican lime, mean viability varied from 6.65 to 72.96 percent and 5.49 to 58.24 percent under varying storage temperatures with a maximum value under freeze drier i.e. -80^0C (72.96 and 58.24 %). Minimum viability of pollens were recorded at room temperature (6.65 and 5.49 %). It was observed that, with the increasing in storage duration its viability also reduced under different storage temperatures. While, lesser loss in viability was observed at -80^0C (82.26-62.95 % and 69.19 - 46.52 %) in both above genotypes.

Thus all the seeded genotypes responded differently for the pollen viability under storage conditions. In this experiment, pollens stored under freeze drier (-20℃ and -89℃) showed gradual decrease in viability percentage and it may be due to the regular freezing and thawing of pollens. Furthermore, intracellular ice formation causing cell death could be the major factor that leads to the loss in viability of pollens (Bhat *et al.,* 2012). Similar variations in pollen viability percentage at different storage temperatures was reported by Bhat *et al.,* (2012) in three cultivars of pear cultivars and maximum pollen viability percentage at -20^0C (67.40%) and -196℃ (68.06%) was recorded in cultivar Patharnakh. However, loss in pollen viability at room temperature was maximum in 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 up to 48 weeks under varying storage conditions viz., room temperature (control), 4^0C,-20^0C,-80^0C (Figure 4, 5 and 6). The result obtained showed that viability of pollens in all the five low seeded genotypes was more than 64 percent on first day of collection in laboratory with highest viability observed in Mukaku Kishu (88.50%), followed by Clementine (87.38%), Lisbon lemon (83.34%), Hamlin (81.49%) and minimum in

Jaffa (65.83) genotypes (Plate 2). Among all the storage temperatures, pollen stored at room temperature possess maximum loss in viability. While, sub zero storage temperatures (-20^{0}C and -80^{0}C) showed highest average viability percent in Mukaku Kishu (59.64 %) and lowest in Jaffa (33.90 %) genotype.

However, declining trend was observed for the viability of pollens with an increase in storage duration, viability and duration of storage are inversely related to each other. Our results are in agreement with the findings of Sharafi and Bahmani (2010) in loquat crop. Room temperature exhibited more loss in pollen viability after four weeks of storage ranged from 7.50 to 1.07 among different cultivars and maximum in Mukaku Kishu and minimum in Jaffa. After 48th weeks of storage duration the average viability of pollens were reduced from 88.50 to 48.99 percent 87.38 to 47.48, 83.34 to 45.62, 81.49 to 36.45and 65.83 to 20.44 percent in Mukaku Kishu, Clementine, Lisbon lemon, Hamlin and Jaffa, respectively. With the increase in storage duration viability of pollens were reduced under all the storage temperatures, however gradual loss was observed in freeze drier (-20^{0}C,-80^{0}C). However, loss in viability was recorded minimum at freeze drier -80^{0}C in all the varieties. Similar results were reported in sweet cherry by Alburquerque *et al.,* (2007), six sweet cherry cultivars viz., Brooks, Cristobalina, Marvin, New Star, Ruby and Somerset were evaluated for pollen viability, stored at 4°C and -20°C and viability was decreased after 15 or 30 days of storage.

While, pollen stored at low temperature (-20°C) remained viable for a year in sweet cherry cultivar Cristobalina cultivar in particular exhibited highest pollen germination up to 60 percent at -20°C temperatures. Present results were also in agreement with the findings of Salles *et al.,* (2007) in citrus, Thaipong *et al.,* (2008) in grapes, Bhat *et al.,* (2012) in pear, Mesnoua *et al.,* (2018) in date palm and Chander *et al.,* (2019) in sugar apple, respectively. Our results clearly indicated that it is feasible to store pollen grains of citrus genotypes at sub zero temperatures i.e. -20^{0}C and -80^{0}C with considerable viability for long duration.

In vitro pollen germination of fresh pollen for seeded and low seeded genotypes were evaluated at different sucrose concentration and in water solution (control). It was observed that among all the sucrose concentrations the best germination of pollen was recorded in 15 percent sucrose solution in all the seeded and low seeded genotypes, followed by 20 % sucrose solution. The results of this experiment revealed that all the genotypes showed good germination percentage at 15 percent sucrose concentrations and minimum in control treatment. Result revealed that germination percentage in seeded genotypes at 15 % sucrose solution was ranged from 58.91 to 78.20 % in Mexican lime and W.Murcott genotype (Plate 3).

Germination was maximum in W.Murcott and Mexican lime seeded genotype, whereas very low pollen germination was recorded in control. Ateyyah (2005) studied inhibitory effect of certain substances such as olive oil to the media containing agar (0.8%), sucrose (10%), and 50 ppm citric acid in *Citrus maxima* and *Citrus paradisi*. Khan and Parveen (2014) reported that pollen germination in *various citrus species* showed better results with sucrose at 10 and 20 percent but occasionally 30 and 40 percent sucrose solutions also showed reasonably good results in *Citrus.* Citrus pollen grains can easily germinate and grow in sucrose solution and their growth can be accelerated by adding vitamins and microelements.

Data pertaining to *in vitro* pollen germination of fresh pollens in different low seeded genotypes of citrus genotypes is presented in (Table 4 and Plate 4). The result revealed that pollen germination was ranged from 55.58 to 83.16 % at 15 percent sucrose concentrations in seeded genotypes. Maximum in Mukaku Kishu (83.16 %) and minimum (55.58 %) in Jaffa. Soares *et al.,* (2013); Kundu *et al.,* (2014); Shekari *et al.,* (2016), agreed that *in vitro* germination is more reliable since staining methods can overestimate the viability of pollens. On the

other hand, the longevity of pollen, considered as the period of time during which pollen maintains its viability, that is, the capacity for germination and fertilization, depend highly on genotype and species and storage conditions Dafni and Firmage (2000).

Studies have been carried out to determine the viability and longevity of citrus pollen (Khan and Perveen, 2014; Kundu *et al.,* 2014; Baswal *et al.,* 2015; Demir *et al.,* 2015; Ahmed *et al.,* 2017). Pollen germination of diploid and doubled diploid "Clemenules" Clementine was recorded as 80.7 % and 55.8 % under *in vitro* and *in vivo* conditions (Lora *et al.,* 2022). Short term storage of pollen facilitates the breeder with viable pollen within a flowering season and allows pollination of a late emerging flower with an earlier flowering genotype (Chaudhury *et al.,* 2010; Dutta *et al.,* 2013 and Mishra and Shivanna, 1982).

Pollen germination in seeded citrus genotypes

Data pertaining to pollen germination in seeded genotype under different storage condition is presented in Table 5. And an attempt has been made to compare the germination capacity of three seeded citrus genotype up to 48 weeks under different storage temperatures such as refrigerators 4^0C, freezer (-20^0C), freeze drier (-80^0C) and room temperature (control). Both freezer and freeze dried condition showed better results.

The results showed that 15% solutions was suitable medium for pollen germination of citrus genotypes. The results on pollen germination revealed that highest germination was recorded from freshly collected pollens and W.Murcott (79.76 %) showed maximum germination followed by Mosambi (75.84%), and minimum was recorded in Mexican lime (60.90 %) (Table 5) (Figure 7, 8 and 9). Among different storage temperatures, pollen stored at room temperature showed maximum loss in germination. While, maximum germination percent was recorded at freeze drier (-80^0C) for the entire storage period with the highest mean germination percent in genotype W.Murcott (70.11 %) and lowest in Mexican lime (46.51 %). Pollen germination showed decreasing trend with increase in storage period thus, it was observed that germination and duration had inverse relation. Our results coincide with those obtained by Lora *et al.,* (2006) for cherimoya**,** Weatherhead *et al.,* (2006) for potato pollen, Gomes *et al.,* (2003) for onion and Sharafi and Bahmani (2010) for loquat.

The germination showed a decreasing trend with increase in storage period and thus observed an inverse relation between germination and storage duration. Loss in germination was highest in pollen stored at room temperature, which showed no germination after four weeks. After 48^{th} weeks of storage the mean germination was declined from 79.76 to 37.50, 75.84 to 31.60 and 60.90 to 17.45 percent in W.Murcott, Mosambi and Mexican lime, respectively. Mosambi, mean germination varied from 5.83 to 62.20 percent at all the storage temperatures with maximum germination percent at -80^0C (70.31 %). Lowest germination was recorded at room temperature (5.83) i.e. control condition.

Whereas, minimum loss was observed at freeze drier -80^0C (75.84 to 62.20 %) (Fig 9), followed by -20℃ (75.84 to 60.65%) (Fig 8) and 4℃ (75.84 to 47.53%) (Fig 7), respectively. Furthermore, loss in germination was maximum at room temperature (75.84 to 5.83 %) from 1^{st} day of storage to 48^{th} weeks of storage.

Similar trend for germination was observed in genotype Mexican lime, as that in W.Murcott and Mosambi. Room temperature possess maximum (4.68%) germination while -80^0C exhibited minimum value for average loss of germination. The average germination was loss from 60.90 to 30.23 per cent from 1^{st} day of storage to 48^{th} weeks after storage (Table 5). Similar variations in pollens storage capacities were also observed by (Martinez-Gomez *et al.,* 2001) in almond and (Aslantus and Pirlak, 2002) in strawberry. There are several other reports on pollen germination and viability in other taxa (Zeng-Yu-Wang *et al.,* 2004; Khan and Perveen, 2006).

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	75.08	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 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≤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			

Table.3 Effect of different sucrose concentration on *in vitro* germination of fresh pollen in seeded citrus 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≤0.05)	2.23	3.312	2.79	2.69

Table.4 Effect of different sucrose concentration on *in vitro* germination of fresh pollen in low seeded citrus genotypes

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

Fig.1 Variation in pollen viability percent in seeded citrus genotypes at 4^0C

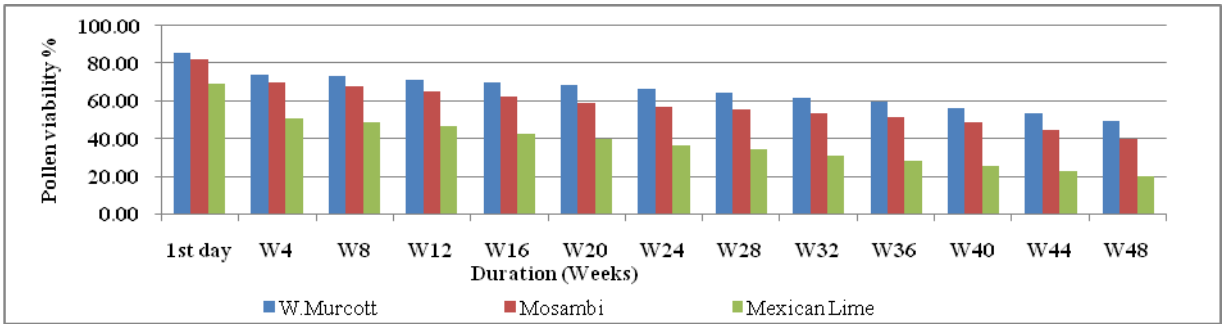


Table.5 Effect of different storage temperatures on *in vitro* pollen germination in seeded citrus genotypes

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	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	

Fig.2 Variation in pollen viability percent in seeded citrus genotypes at -20^{0}C

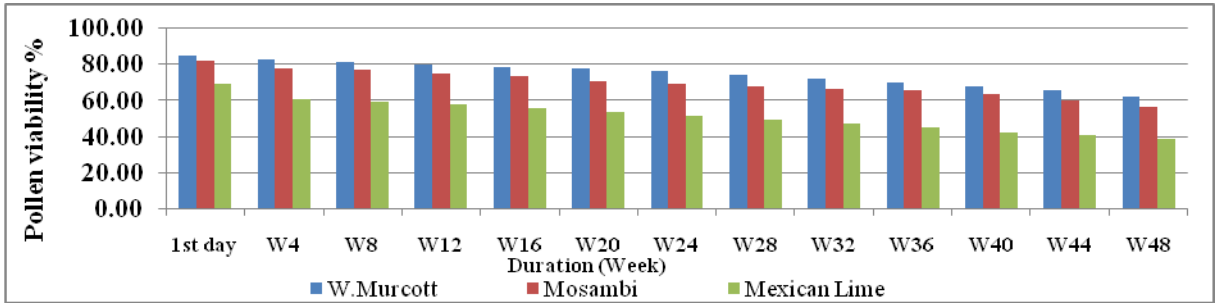


Table.6 Effect of different storage temperatures on *in vitro* pollen germination in low seeded citrus genotypes

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	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	52.54	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			

Fig.3 Variation in pollen viability percent in seeded citrus genotypes at -80^{0}C

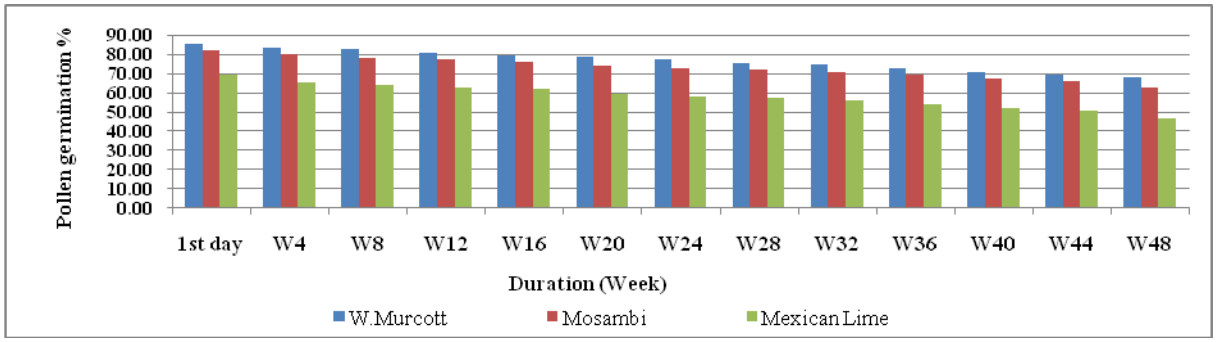


Fig.4 Variation in pollen viability percent in low seeded citrus genotypes at 4^{0}C

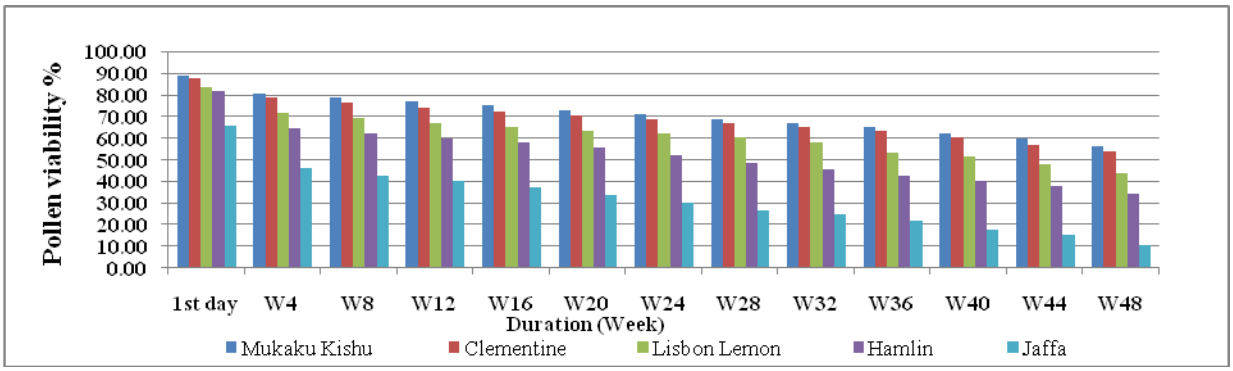


Fig.5 Variation in pollen viability percent in low seeded citrus genotypes at -20^{0}C

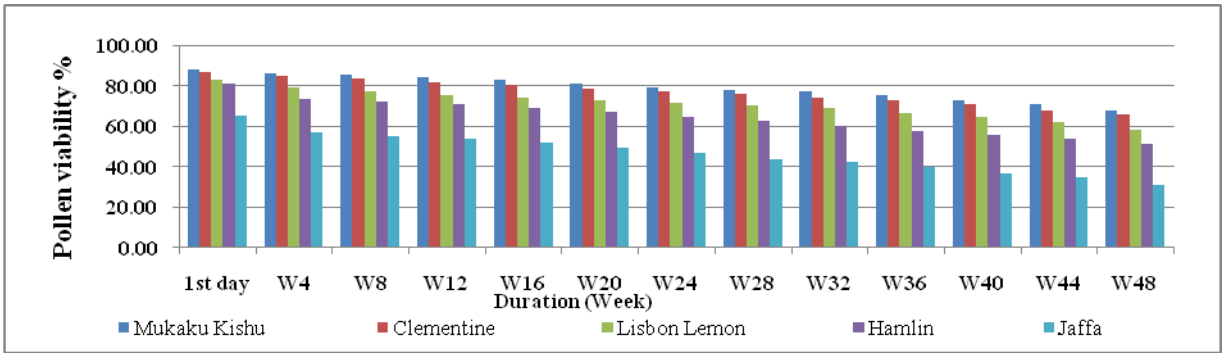


Fig.6 Variation in pollen viability percent in low seeded citrus genotypes at -80^{0}C

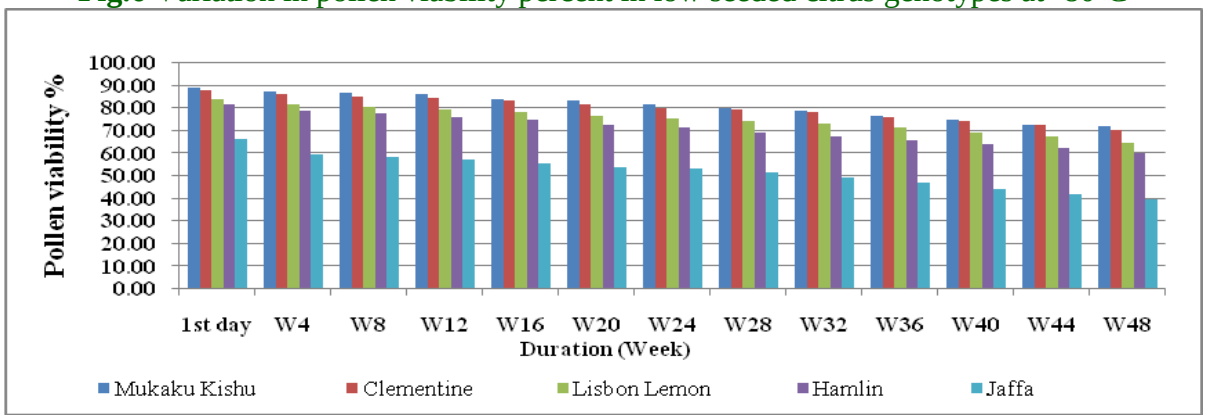


Fig.7 Variation in pollen germination in seeded citrus genotypes at 4^0C

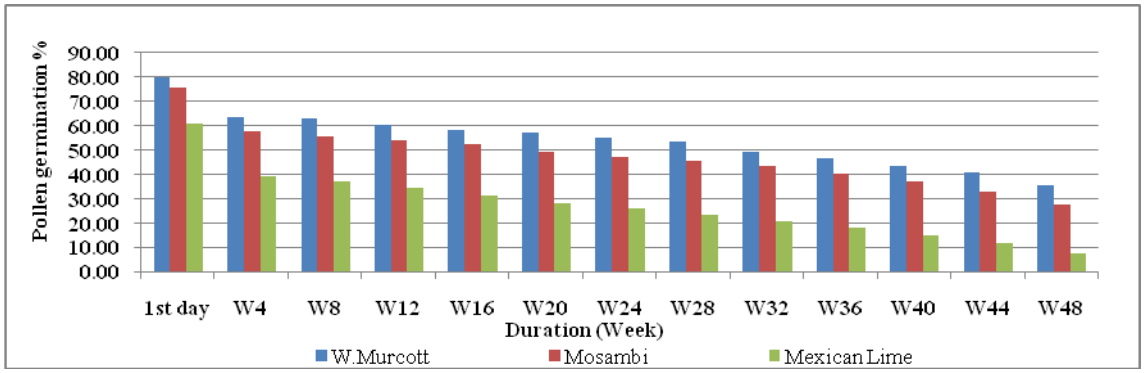


Fig.8 Variation in pollen germination in seeded citrus genotypes at -20^0C

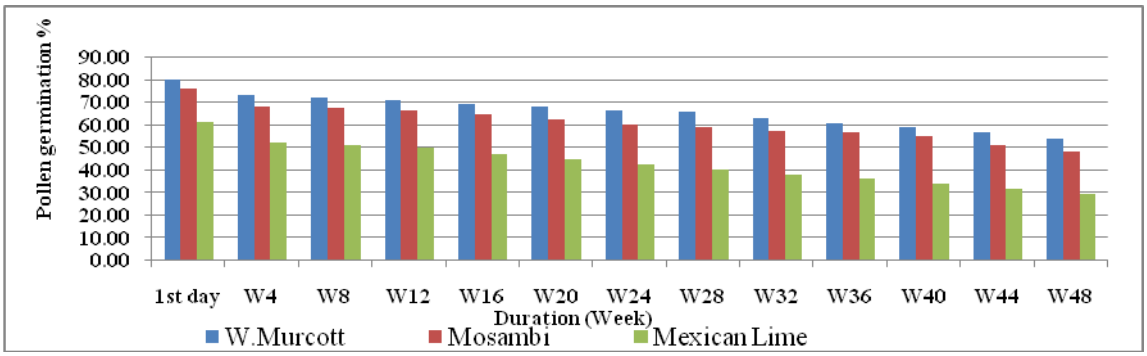


Fig.9 Variation in pollen germination in seeded citrus genotypes at -80^0C

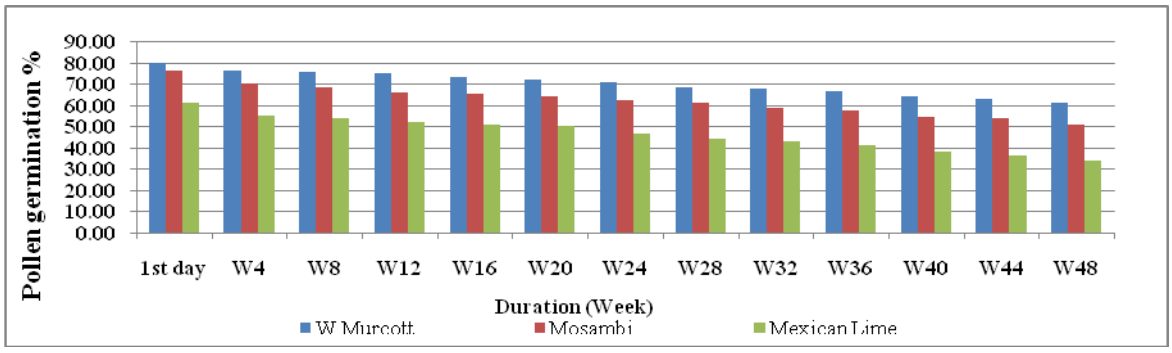


Fig.10 Variation in pollen germination percent in low seeded citrus genotypes at 4^0C

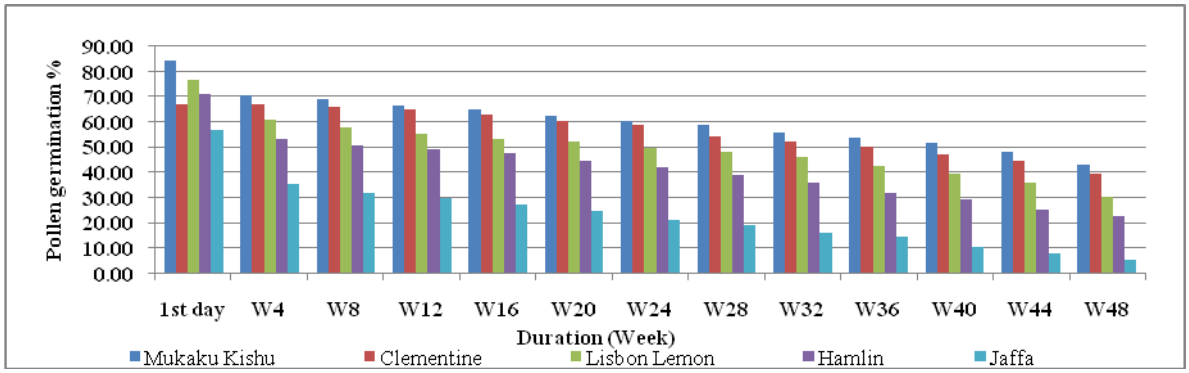


Fig.11 Variation in pollen germination percent in low seeded citrus genotypes at -20^0C

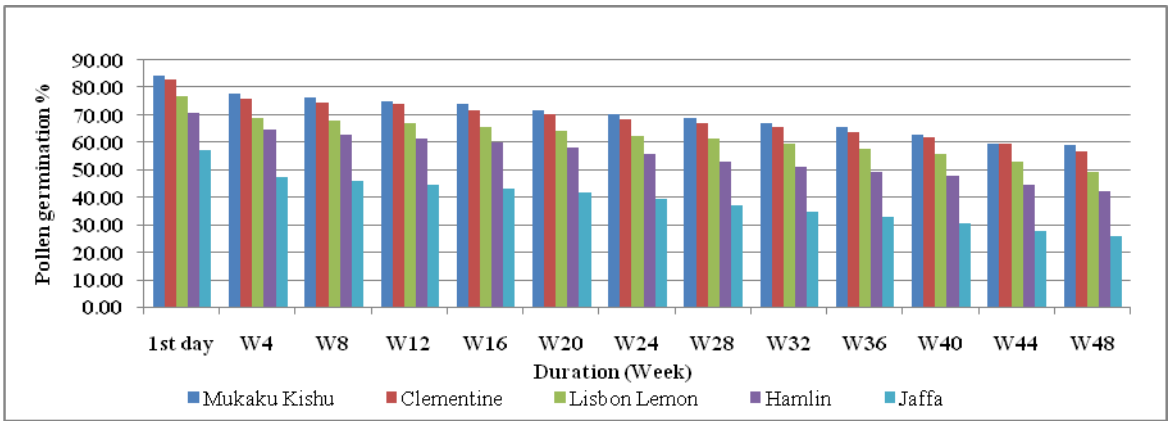


Fig.12 Variation in pollen germination percent in low seeded citrus genotypes at -80^0C

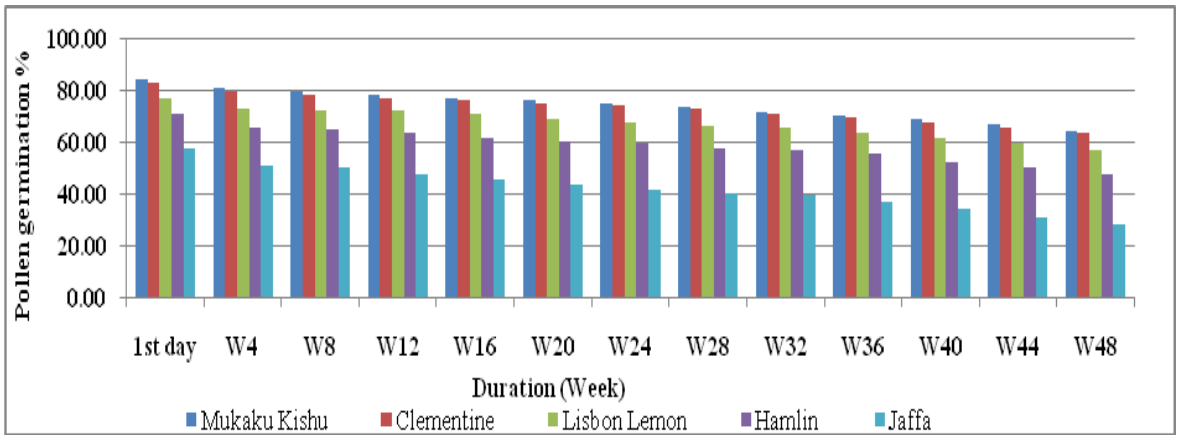


Plate.1 *In vitro* viability of fresh pollens in seeded citrus genotypes. (Black labell represents viable pollens and blue represents non-viable pollens

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

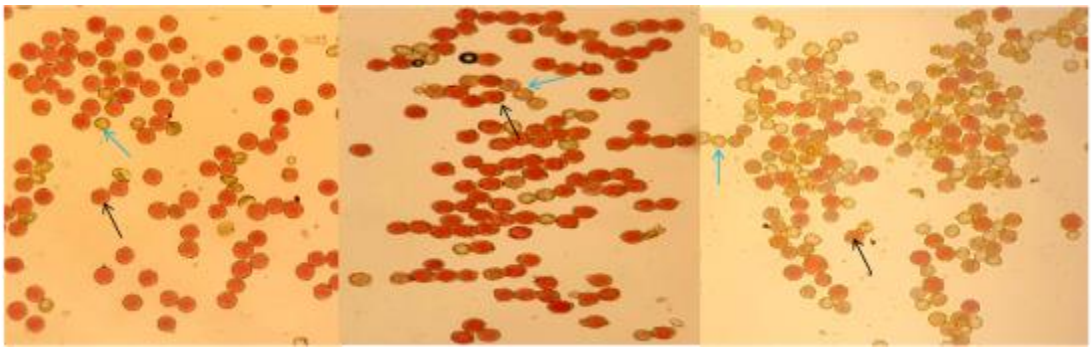


Plate.2 *In vitro* viability of fresh pollens in low seeded citrus genotypes. (Black labell represents viable pollens and blue represents non-viable pollens

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

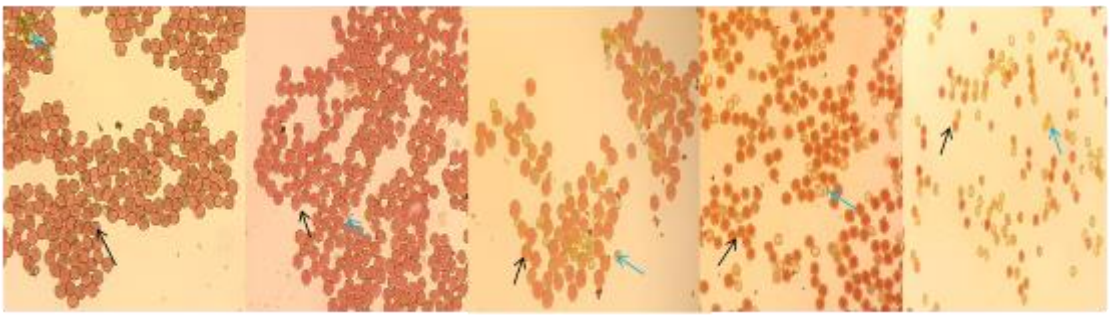


Plate.3 *In vitro* germination of fresh pollens in low seeded citrus genotypes with digital microscope. (Red labell represents germinated pollens and yellow represents non-germinated pollen grains.

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

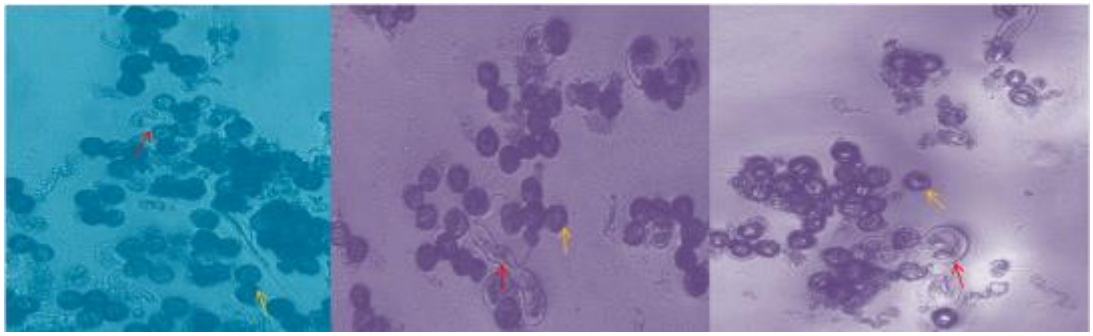
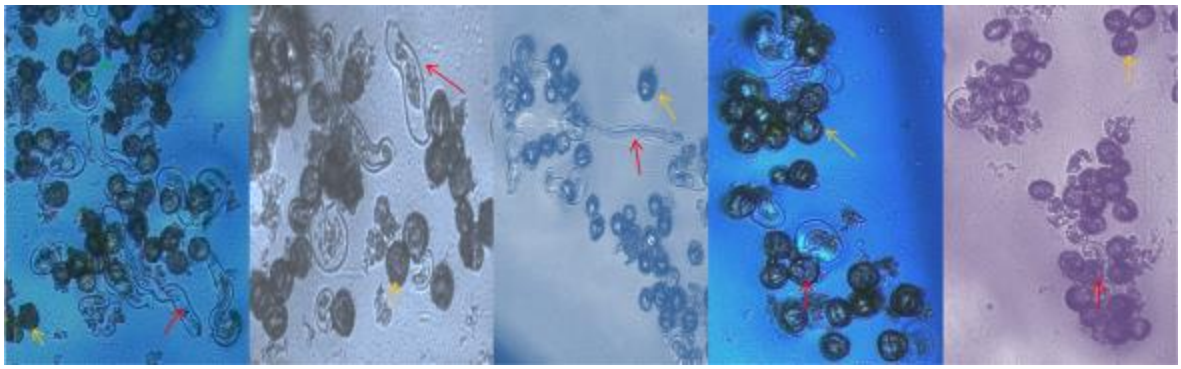


Plate.4 *In vitro* germination of fresh pollens in low seeded citrus genotypes. (Red labell represents germinated pollens and yellow represents non-germinated pollen grains.

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



Pollen germination in low seeded citrus genotypes

The result revealed that, in all the five low seeded genotypes pollen germination was more than 56 percent. Results showed that maximum germination was recorded in Mukaku Kishu (84.16 %), followed by Clementine (82.83 %), Lisbon lemon (76.73 %), Hamlin (70.99%) and minimum germination in Jaffa (57.07 %) (Table 6) (Figure 10, 11 and 12). Among storage temperatures room temperature recorded fastest loss in pollen germination and pollen could not germinate (0.00 %) after a week under room temperature conditions in all the low seeded genotypes (Table. 6). Whereas, germination was recorded maximum under sub zero storage temperatures (-80^0C) during the entire time of storage with highest mean germination percent in genotype Mukaku Kishu (74.22%) and lowest in genotype Jaffa (41.83 %) (Fig 12). Pollen germination in all the genotypes was initially high, but declined with the increase in storage time and establish an inverse relation between germination and time of storage. The average mean germination percentage varied between 41.59 in Mukaku Kishu to14.85 % in Jaffa. All other genotypes showed results in between maximum loss in germination was observed under room temperature in all the genotypes and ranged between (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 in Mukaku Kishu, Clementine, Lisbon lemon, Hamlin and Jaffa, respectively (Table 6). The variation in pollen fertility among the genotypes is due to varietal difference (Alburquerque *et al.*, 2007).

Similar results were obtained by Martinez-Gomez *et al.*, (2002) where pollen germination lasted for a year under low storage temperature (-20°C and -80°C). Robles-Gonzalez *et al.*, (2019) reported that the longevity of pollen in Mexican lemon genotypes and Citrange C-35 remain viable only for 24 hours under room temperature storage conditions. Similar results were reported by Lora *et al.*, (2006) in cherimoya under low temperature (-20°C, -80°C), where germination was progressively declined with time of storage. In this experiment, pollens stored under freeze drier (-20℃ and -89℃) showed gradual decrease in germination percent and it may be due to the regular freezing and thawing of pollens. Furthermore, intracellular ice formation, death of cell could have lead to the loss in pollen germination. Similar variations in pollen germination percent were reported by Alburquerque *et al.*, (2007) in sweet cherry cultivars under storage temperatures of (4°C and -20°C), pollen germination were assessed upto 365 days. Cultivar Cristobalina possess the highest pollen germination (60%) at -20°C. Pollen germination in other cultivars varied from 36 % to 44 % at 4°C and viability was lasted

for sixty days thereafter complete declining in viability was observed. After a year of storage at subzero (-20°C) pollen germination in all the cultivars had same percent germination as that of pollens under control storage. In a similar study, Anjum and Shaukat (2008) found that freeze drier (-60℃) storage conditions had significant result on pollen germination percentage in *Malus pumila* L., after 48 weeks of storage. Whereas, Ahmed *et al.,* (2017), recorded maximum pollen germination (80.2%) in Mosambi and minimum was recorded in Itaboria (49.2%) at (-196^{0}C). They reported that pollen showed germination upto only eight days thereafter, no germination was observed at fourth week of storage under room temperature conditions. Towil (2010) reported that pollen storage 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 species. Thus, the pollens 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, 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.

The results of this study indicate that, among different seeded and seedless citrus accessions we analyzed in our investigation, the pollen grains of W. Murcott (seeded) and Mukaku Kishu (low seeded) showed the highest viability and germination after 48 weeks of storage under freeze-drier (-80^{0}C) conditions. At sub zero temperature, pollens of all the genotypes showed a gradual loss in viability and germination over the pollens stored at room temperature. Furthermore, at room temperature, pollen grains of both seeded and seedless genotypes could not be stored for more than a week due to higher rate of loss in viability and germination abilities. Consequently, an ideal pollen storage condition in citrus genotypes has been optimised, which will encourage the preservation of pollens in elite citrus genotypes and permit the exchange of pollens between different breeding programmes. Furthermore, freeze dried pollens of citrus genotypes with different flowering times would ensure their availability throughout the flowering season. The findings are valuable for overcoming pre zygotic barriers in hybridization of citrus genotypes. Results clearly indicate that it is feasible to store pollen grains of citrus at sub- zero temperatures for a long period without showing considerable loss in their storage ability.

Acknowledgement

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