Int.J.Curr.Microbiol.App.Sci (2017) *6*(5): 892-902

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 6 Number 5 (2017) pp. 892-902**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2017.605.099

Influence of Storage Duration and Storage Temperature on In-Vitro Pollen Germination of Citrus Species

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A B S T R A C T

The aim of present study was to access the pollen in- vitro germination of pollen grains of five citrus species belonging to the family Rutaceae viz., mandarin (*citrus. reticulata* Blanco) sweet orange (*C. sinensis* L. Osbeck) grapefruit (*C. paradisi* Macf.) pummelo (*C. maxima* Burm. Merr.) and tangelo *(C. reticulata* × *C. maxima* or × *C. paradise)* under low temperature storage conditions during year 2014. Pollen germination were checked up to 48 weeks, stored at different temperatures like 4ºC, -20ºC, and -196ºC. Pollen stored at low temperature (-196℃ and -20℃) showed better germination percentage as compared to pollen stored at 4^0C and room temperature. The cultivar Mosambi had maximum average germination percentage when pollens were stored at -196℃ (70.8 %,) -20℃ (66.3 %,) which was significantly higher than all other genotypes and followed by W.Murcott -196℃ (60.9%) -20℃ (48.5%). Minimum germination percentage was found in Marsh Seedless stored at -196℃ (21.7%) -20℃ (12.5%). The results indicate that pollen collected and stored at sub-zero temperatures from early blooming citrus varieties can be stored for very long period without any appreciable loss of germination. The stored pollen can be used along the whole blooming season for hybridization programmes by fruit breeders.

Keywords

Citrus, In- vitro, Pollen, Storage, Temperature, Species and Germination.

Article Info

Accepted:
04 April 2017
Available Online:
10 May 2017

Introduction

Citrus is one of the world's important fruit crop which is grown between latitude 35°N~35°S. Citrus comprises around 60 species, most of which are cultivated throughout the tropics and subtropics. They are indigenous in some parts of India, China, Northern Australia and New Caledonia (Harley *et al.,* 2006). Citrus in India is grown in 1.07 million ha area with a total production of 11.14 million tonnes. The most important commercial citrus groups or cultivars in India are the mandarin (Citrus *reticulata* Blanco) sharing 41% followed by sweet orange (Citrus *sinensis* Osbeck) 23% and acid lime (Citrus *aurintifolia* Swingle), 23% respectively (NHB, 2014).

One of the routine tasks in plant evolutionary biology, pollination biology and crop breeding research is determining total pollen grains per flower and proportion of viable pollen (Kevan, & Husband, 2005 and Fang, Turner, Yan, Li, & Siddique, 2010). The development of reliable methods for determining the functional quality of pollen helps in monitoring pollen vigour during storage, genetics and pollen-stigma interaction studies, crop improvement and

breeding, and incompatibility and fertility studies (Dafni, 1992). The quality of pollen is assessed on the basis of viability and vigour of the pollen grain. Pollen vigour refers to the speed of germination of pollen grains and the rate of pollen tube growth (Ottaviano and Mulcahy, 1989).

In-vitro pollen germination tests have been used to determine the germination percentage of pollen and can also be used for assessing pollen vigour by monitoring the rate of germination over a period of time or the length of pollen tubes (Shivanna and Mohan Ram, 1993). Pollen which could not germinate usually shows poor tube growth which is likely to be ineffective in causing fertilization. Several methods of pollen storage have been tried of which the most important factors are controlled temperature and humidity Zhang (2002), Bomben *et al*., (2006), Song & Tachibana (2007) and Dutta *et al*., (2013). Different investigators have same consensus that low temperature and humidity are the two major influencing factors in storage of pollen grains for a long period of time (Mesejo *et al*., 2006; Janick *et al*., 2010). Pollen grains can germinate and grow readily in water or in sugar medium, their growth can be accelerated by adding vitamins and microelements such as boron, magnesium, calcium, potassium etc. Pollen physiology especially germination and viability has received considerable attention for its application in plant breeding, conservation, adaptation and understanding of the physiological behaviour of the fertilizing pollen grains. There are several reports on pollen germination and viability of different taxa (Dafni & Firmage, 2000; Vaknin *et al*., 2003; Ateyyeh, 2005; Bermejo *et al*., 2011 and Khan *et al*., 2013) with varied aims and objectives. Nair (1964) correlated pollen morphology of Vitis vinifera with physiological potential by studying pollen germination in different types. Mesejo *et al*., (2006) found out the inhibitory effect of $CuSO_4$ on In vitro pollen germination of "Manderin" and after 8 hrs of incubation the development of pollen tube was stopped.

The In vitro pollen germination of Citrus maxima and *C. paradisi* was suppressed by adding olive oil to a medium containing 0.8% agar, 10% sucrose and 50 ppm Citric acid, but the germination was significantly increased in *C. maxima* in a medium having 0.8% agar and 20% sucrose (Ateyyah, 2005). Pollen viability and germination of these five Citrus species was assessed to develop a pollen bank from where desired pollen may be available for hybridization programmes. Present study was planned to access the in vitro pollen germination under different low temperature storage conditions.

Materials and Methods

Pollen from five citrus species viz. mandarin (*citrus. reticulata* Blanco) (Daisy, W.Murcott and Kinnow) sweet orange (*C. sinensis* L. Osbeck) (Jaffa, Mosambi and Itaboria) grapefruit (*C. paradisi* Macf.) (Foster, Star Ruby and Marsh Seedless) pummelo (*C. maxima* Burm. Merr.) (White Pummelo, Pink Pummelo and Devanpalli) and tangelo *(C. reticulata* × *C. maxima* or ×*C. paradise)* (Minneola and Pearl) were collected from old orchard Punjab Agricultural University Ludhiana, Punjab for the use of this study. Flowers from male parents were collected at pre blooming stage (balloon stage) and were allowed to shed the pollen in shade for 3-4 hours under 100 watt lamps. Immediately after anther dehiscence, pollen were collected in vials and subjected to different storage conditions viz. room temperature (in anhydrous calcium chloride), 4℃, -20℃ and cryogenic storage in liquid nitrogen (-196℃).

For in Vitro germination, pollen grains were germinated in 15 percent sucrose solution at

weekly intervals. For this 1-2 drops of sucrose solution were placed in the cavity of a cavity slide and pollen grains were dusted over it by camel brush. The slides were covered with cover slip and edges were smeared by molten wax and slide was inverted instantly so as to form a hanging drop on the cover slip. The cavity slides were then placed in petridishes containing moist filter paper to ensure uniform and high relative humidity. Pollen tube growth was assessed for each genotype under microscope after 24 hrs of incubation at 22±2℃. The pollen grains having pollen tube at least two times longer than pollen diameter were considered to be germinated. Pollen germination was observed under digital microscope. Data were collected on four Petri dishes with at least 200 pollen grains in each one.

The experiment was carried out as completely randomized design (CRD). Data was analyzed using SAS software (SAS 9.3) and compression of means was carried out with Duncan's multiple range tests, considering $p \leq 0.05$ as the level of significance.

Results and Discussion

Pollen Germination

Sweet orange (*C. sinensis* L. Osbeck) species showed 49.2% to 80.2% (Table:-1 and Fig:-1) germination of fresh pollen with maximum in Mosambi (80.2%) followed by Jaffa (65.1%) and minimum in Itaboria with (49.2%). After 48 weeks of storage results obtained revealed that maximum average germination from 4th to 48th was noted as was noted as 70.8% in mosambi (which was significantly higher than all other genotypes $p \leq 0.05$) at freeze temperature (-196^{0}C) and minimum as 28.1% in Itaboria at refrigerated temperature (4^{0}C). The duration starts form 4 weeks to 48 weeks i.e. for whole one year and at 4 weeks the germination was found maximum in Mosambi 80.5% at freeze temperature (-196^{0}C) and after 48 weeks of storage the germination was found 58.2% where as Itaboria at 4 weeks shows lowest germination of 40.5% at refrigerated temperature (4^{0}C) and after 48 weeks germination was found 17.5% which is lowest among all sweet orange groups.

Mandarin (*C. reticulate* Blanco) species showed 69.7% to 75.5% (Table:-2 and Fig:-2) germination of fresh pollen with maximum in kinnow (75.5%) followed by Murcott (72.6%) and minimum in Daisy with (69.7%). After 48 weeks of storage results obtained revealed that maximum average germination from 4th to 48th was noted as 61.2% in kinnow (which was significantly higher than all other genotypes $p \leq 0.05$) at freeze temperature (-196^{0}C) and minimum as 44.7% in Daisy at refrigerated temperature (4^{0}C). The duration starts form 4 weeks to 48 weeks i.e. for whole one year and at 4 weeks the germination was found maximum in Kinnow 75.3% at freeze temperature (-196^{0}C) and after 48 weeks of storage the germination was found 52.3% where as daisy at 4 weeks shows lowest germination of 60.5% at refrigerated temperature (4^{0}C) and after 48 weeks germination was found 28.5% which is lowest among all mandarin groups.

Grape fruit (*C. paradisi* Macf.) species showed 30.5% to 42.6% (Table:-3 and Fig:-3) germination of fresh pollen with maximum in Star Ruby (42.6%) followed by Foster (38.5%) and minimum in Marsh Seedless with (30.5%). After 48 weeks of storage results obtained revealed that maximum average germination from 4th to 48th was noted as 33.3% in Star Ruby (which was significantly higher than all other genotypes $p \leq 0.05$) at freeze temperature (-196^{0}C) and minimum as 12.5% in Marsh Seedless at refrigerated temperature (4^{0}C). The duration starts form 4 weeks to 48 Marsh Seedless weeks i.e. for whole one year and at 4 weeks the germination was found maximum

in Star Ruby (42.0%) at freeze temperature (-196^{0}C) and after 48 weeks of storage the germination was found 23.0% where as Marsh Seedless at 4 weeks shows lowest germination of 20.0% at refrigerated temperature (4^{0}C) and after 48 weeks germination was found 5.0% which is lowest among all grapefruit groups.

Pummelo (*C. maxima* Burm. Merr.) species showed 60.8% to 45.7% (Table:-4 and Fig:-4) germination of fresh pollen with maximum in Devanpalli (60.8%) followed by White Pummelo (49.4%) and minimum in Pink Pummelo with (45.7%). After 48 weeks of storage results obtained revealed that maximum average germination from 4th to 48th was noted as 48.8% in devanpalli (which was significantly higher than all other genotypes $p \leq 0.05$) at freeze temperature (-196^{0}C) and minimum as 23.4% in Pink Pummelo at refrigerated temperature (4^{0}C). The duration starts form 4 weeks to 48 Marsh Seedless weeks i.e. for whole one year and at 4 weeks the germination was found maximum in devanpalli (60.0%) at freeze temperature (-196^{0}C) and after 48 weeks of storage the germination was found 40.0% where as Pink Pummelo at 4 weeks shows lowest germination of 36.4% at refrigerated temperature (4^{0}C) and after 48 weeks germination was found 12.4% which is lowest among all pummelo groups.

Tangelo *(C. reticulata* × *C. maxima* or × *C. paradise)* species showed 64.6% to 67.5% (Table:-5 and Fig:-5) germination of fresh pollen with maximum in Minneola (67.5%) and minimum in Pearl with (64.6%). After 48 weeks of storage results obtained revealed that maximum average germination from 4th to 48th was noted as 58.3% in Minneola (which was significantly higher than all other genotypes $p \leq 0.05$) at freeze temperature (-196^{0}C) and minimum as 42.2% in Pearl at refrigerated temperature (4^{0}C). The duration starts form 4 weeks to 48 weeks i.e. for whole one year and at 4 weeks the germination was found maximum in Minneola (67.4%) at freeze temperature (-196^{0}C) and after 48 weeks of storage the germination was found 49.5% where as Pearl at 4 weeks shows lowest germination of 57.0% at refrigerated temperature (4^{0}C) and after 48 weeks germination was found 24.5% which is lowest among all tangelo groups.

Pollen germination was examined up to 48 weeks in different storage conditions viz. 4 ^{0}C, -20 ^{0}C and -196 ^{0}C (Table and Figure). The results obtained revealed that pollen germination was more than 30.00 per cent in all the fourteen cultivars immediately after gathering in laboratory with maximum in Mosambi (80.2%) (which was significantly higher than all other genotypes $p \leq 0.05$) followed by Kinnow (75.5%) and minimum in Marsh Seedless (30.5 %). While comparing different storage temperatures, the viability was lost immediately after storage in case of room temperature. However, maximum viability was observed at sub zero storage temperatures (-196℃) during the whole period of storage duration with highest average pollen viability was noted in cultivar Mosambi (75.7 %) (Table:-6) at freeze temperature (-196^{0}C) from 4th to 48th week (which was significantly higher than all other genotypes $p \leq 0.05$) and lowest in Marsh seedless (25.7%) at refrigerated temperature (4^{0}C). Maximum pollen germination among all genotypes was found in Mosambi (80.5%) at freeze temperature (-196^{0}C) at 4th week of storage and minimum was found in Marsh Seedless (5.0%) at refrigerated temperature (4^{0}C) on 48th week of storage. Fresh pollens have more germination than the stored one. Eti (1991); Parfitt and Almedhi (1984); Seiheimer and Stover (1982) have indicated that germination percentage vary significantly according to fruit species or cultivars.

Table.1 pollen germination % of sweet orange (C. sinensis L. Osbeck) varieties

	Mosambi			Jaffa			Itaboria		
Duration (weeks)	4°C	-20°C	-196°C	4°C	-20°C	-196°C	4°C	-20°C	-196°C
W4	70.4	76.3	80.5	55.3	61.0	65.1	40.5	46.0	49.5
W8	64.4	72.0	77.3	50.2	60.0	64.4	35.4	45.0	47.4
W12	63.1	71.0	76.2	49.4	58.0	63.2	34.1	43.7	46.3
W16	62.4	70.0	75.1	48.3	57.0	61.2	32.4	42.0	44.4
W20	60.5	68.0	74.2	46.3	55.0	60.3	30.5	40.0	43.3
W24	58.5	67.0	73.3	45.5	54.0	58.4	29.2	39.0	41.5
W28	56.4	64.7	71.3	43.3	52.0	57.1	27.3	37.7	40.4
W32	53.4	64.0	69.2	42.4	49.0	55.1	25.3	36.0	39.4
W36	51.2	62.0	67.3	40.5	47.0	54.4	23.4	34.0	38.3
W40	50.1	63.0	64.3	39.1	45.0	51.4	21.2	31.7	37.1
W44	48.4	61.0	62.3	37.4	43.0	49.4	20.1	31.0	35.4
W48	46.4	56.0	58.2	35.5	40.0	47.1	17.5	28.0	32.6
LSD ($p \leq 0.05$)	LSD (Variety) =1.01 LSD (Week) =2.03 LSD (Temperature) =1.01			LSD (Variety x week) =3.5(NS) LSD (Variety x Temperature) =1.76(NS) LSD (Week x Temperature) =3.51(NS) LSD (Variety x week x Temperature) =6.09(NS)					

Fresh pollen Germination (%) at room temperature	65.1	80.2	49.2
After one week pollen germination % at room temperature	00.00	00.00	00.00

Table.2 pollen germination % of Mandrin (*C. reticulate* Blanco) varieties

	Kinnow			Daisy			W. Murcoutt		
Duration (weeks)	4°C	-20°C	-196°C	4°C	-20°C	-196°C	4°C	-20°C	-196°C
W4	62.4	68.0	75.3	60.5	64.3	69.0	63.3	65.3	72.0
W8	57.3	63.0	67.4	53.4	60.5	66.0	56.4	63.4	67.3
W12	56.4	62.0	66.3	52.3	59.4	65.0	55.3	62.3	66.0
W16	54.4	58.0	63.4	51.3	57.4	64.0	54.2	58.3	64.0
W20	53.5	55.0	62.5	49.5	54.4	63.0	52.3	55.5	63.0
W24	50.5	51.0	60.4	46.6	50.5	61.0	50.4	50.5	62.0
W28	47.4	47.0	59.4	43.2	46.5	60.0	48.6	47.3	60.0
W32	43.4	45.0	58.2	40.5	43.5	59.0	46.5	45.5	59.0
W36	40.5	43.0	57.4	38.5	41.5	57.0	42.3	43.5	57.0
W40	39.4	42.0	56.4	36.5	39.5	55.0	40.5	40.4	56.0
W44	36.4	40.0	55.1	35.3	36.5	54.0	39.4	39.5	55.0
W48	30.4	34.0	52.3	28.5	30.5	50.0	32.4	35.5	50.0
LSD ($p \leq 0.05$)	LSD (Variety) =1.18 LSD (Week) =2.35 LSD (Temperature) =1.77			LSD (Variety x week) =4.08(NS) LSD (Variety x Temperature) =2.04(NS) LSD (Week x Temperature) =4.08 (NS) LSD (Variety x week x Temperature) =7.06					

Fresh pollen Germination (%) at room temperature	75.5	69.7	72.6
After one week pollen germination % at room temperature	00.00	00.00	00.00

Table.3 Pollen germination % of Grapefruit (*C. paradisi* Macf.) varieties

	Foster			Star Ruby			Marsh Seedless		
Duration (weeks)	4°C	-20°C	-196°C	4°C	-20°C	-196°C	4°C	-20°C	-196°C
W4	30.2	35.0	38.6	31.7	38.0	42.0	20.0	26.5	30.5
W8	26.5	32.0	35.5	28.0	35.0	40.0	18.0	24.6	27.4
W12	25.4	31.0	34.3	27.0	34.0	39.0	17.0	22.5	26.2
W16	24.2	30.0	32.5	26.0	33.0	38.0	16.0	21.2	25.3
W20	23.1	29.0	31.3	23.0	32.0	37.0	15.0	19.5	23.6
W24	22.5	26.0	29.4	21.0	28.0	35.0	13.0	17.5	22.2
W28	21.1	24.0	28.1	20.3	26.0	32.0	12.3	16.2	21.3
W32	19.4	23.3	26.4	19.0	25.0	30.7	10.0	14.4	20.4
W36	17.2	21.0	25.4	17.0	23.7	30.0	9.0	13.2	19.2
W40	15.3	20.0	23.4	14.7	21.0	27.0	8.0	12.1	17.5
W44	13.4	18.0	22.1	13.7	20.0	26.0	7.0	11.3	15.4
W48	10.6	15.0	20.5	10.0	16.0	23.0	5.0	8.5	11.4
LSD ($p \leq 0.05$)	LSD (Variety) =1.24 LSD (Week) =2.48 LSD (Temperature) =1.24			LSD (Variety x week) =4.29(NS) LSD (Variety x Temperature) =2.15(NS) LSD (Week x Temperature) =4.29 (NS) LSD (Variety x week x Temperature) =7.43					

Fresh pollen Germination (%) at room temperature	38.5	42.6	30.5
After one week pollen germination % at room temperature	00.00	00.00	00.00

Table.4 Pollen germination % of Pummelo (*C. maxima* Burm. Merr.) varieties

	Pink Pumello			White Pumello			Devanpalli		
Duration (weeks)	4°C	-20°C	-196°C	4°C	-20°C	-196°C	4°C	-20°C	-196°C
W4	36.4	42.3	45.0	40.4	46.5	49.0	50.4	55.3	60.0
W8	30.5	40.3	43.0	32.5	42.4	46.0	43.4	52.4	55.0
W12	29.2	39.4	42.0	31.5	41.2	45.0	41.4	51.2	54.0
W16	28.5	38.4	41.0	29.2	40.5	43.0	40.4	50.3	53.0
W20	26.5	36.3	40.0	28.2	39.4	42.0	39.1	49.2	50.0
W24	23.5	34.1	38.0	26.5	37.1	40.0	38.3	47.5	49.0
W28	21.4	33.5	37.0	25.3	36.5	39.0	35.3	45.5	48.0
W32	20.2	31.4	35.0	24.2	35.1	38.0	33.5	43.6	46.0
W36	19.1	30.3	33.0	23.3	32.4	36.0	31.3	42.4	45.0
W40	17.4	29.5	32.0	21.6	30.6	35.0	30.5	40.5	44.0
W44	15.4	27.4	29.7	20.5	29.2	33.0	28.5	39.5	42.0
W48	12.4	25.4	27.0	17.5	26.5	30.0	25.4	35.5	40.0
LSD ($p \leq 0.05$)	LSD (Variety) =1.16 LSD (Week) =2.31 LSD (Temperature) =1.16			LSD (Variety x week) =4.00(NS) LSD (Variety x Temperature) =2.00(NS) LSD (Week x Temperature) =4.00 LSD (Variety x week x Temperature) =6.93					

Fresh pollen Germination (%) at room temperature	49.4	45.7	60.8
After one week pollen germination % at room temperature	00.00	00.00	00.00

Table.5 Pollen germination % of Tangelo (*C. reticulata* × *C. maxima* or × *C. paradise*) varieties

	Minneola			Pearl		
Duration (weeks)	4°C	-20°C	-196°C	4°C	-20°C	-196°C
W4	58.6	62.0	67.4	57.0	60.0	64.0
W8	54.4	60.0	64.5	51.0	58.0	61.0
W12	53.4	59.0	63.4	50.0	57.0	60.0
W16	52.3	57.0	62.2	49.0	55.0	59.0
W20	50.4	53.0	60.5	48.0	53.0	58.0
W24	46.5	49.0	59.4	45.0	51.0	55.0
W28	40.3	47.0	57.3	42.0	49.0	53.0
W32	38.5	45.0	55.5	39.0	47.0	52.0
W36	36.3	43.0	54.3	36.0	46.0	50.0
W40	33.4	40.0	53.3	33.0	42.0	49.0
W44	30.3	38.0	52.2	30.0	39.0	47.0
W48	24.5	31.0	49.5	26.0	35.0	42.0
LSD (p≤0.05)	LSD (Variety) =1.14 LSD (Week) =2.79 LSD (Temperature) =1.39			LSD (Variety x week) =3.94(NS) LSD (Variety x Temperature) =1.97 LSD (Week x Temperature) =4.83 LSD (Variety x week x Temperature) =6.83(NS)		

Fresh pollen Germination (%) at room temperature	67.5	64.6
After one week pollen germination % at room temperature	00.00	00.00

Table.6 Combined interaction of all varieties, weeks and temperature among each other and their values

LSD (p≤0.05)	LSD (Variety) =1.15 LSD (Week) =1.06 LSD (Temperature) =0.53	LSD (Variety x week) =3.96(NS) LSD (Variety x Temperature) =1.98 LSD (Week x Temperature) =1.83 LSD (Variety x week x Temperature) =6.85

Fig.1 Pollen germination % of sweet orange (C. sinensis L. Osbeck) varieties

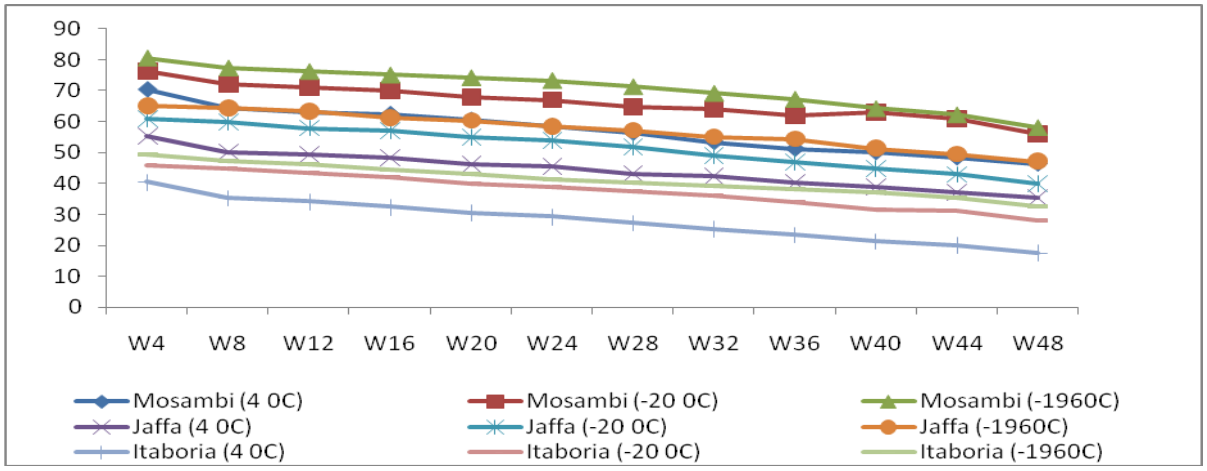


Fig.2 pollen germination % of Mandrin (*C. reticulate* Blanco) varieties

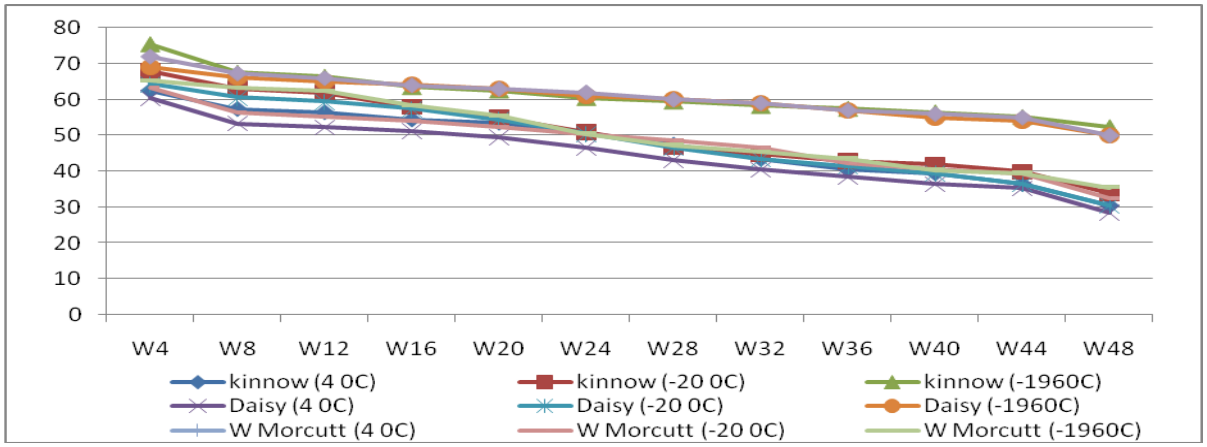


Fig.3 pollen germination % of Grapefruit (*C. paradisi* Macf.) varieties

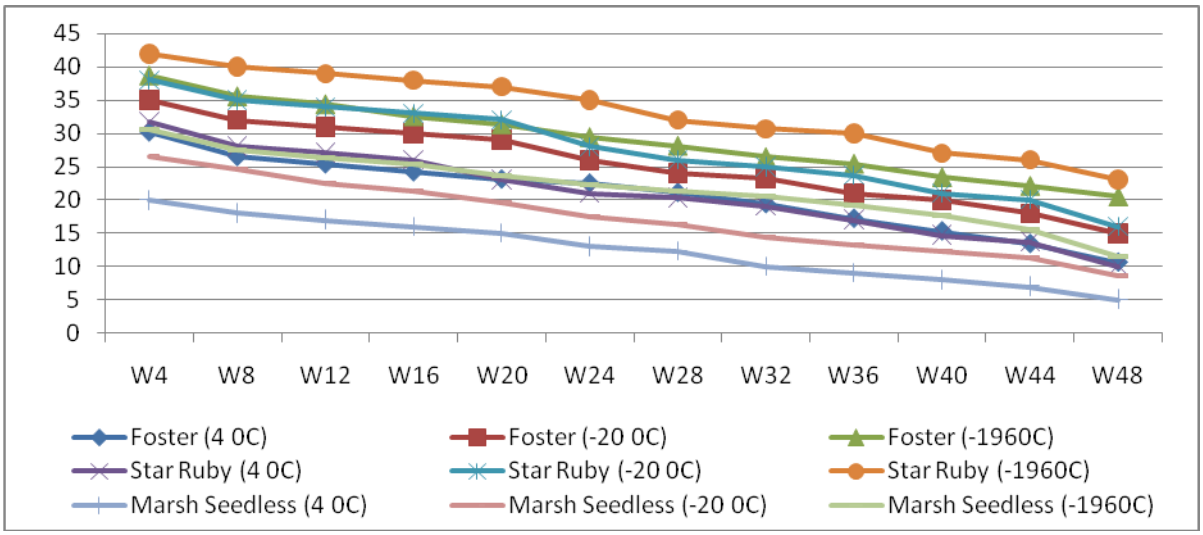


Fig.4 pollen germination % of Pummelo (*C. maxima* Burm. Merr.) varieties

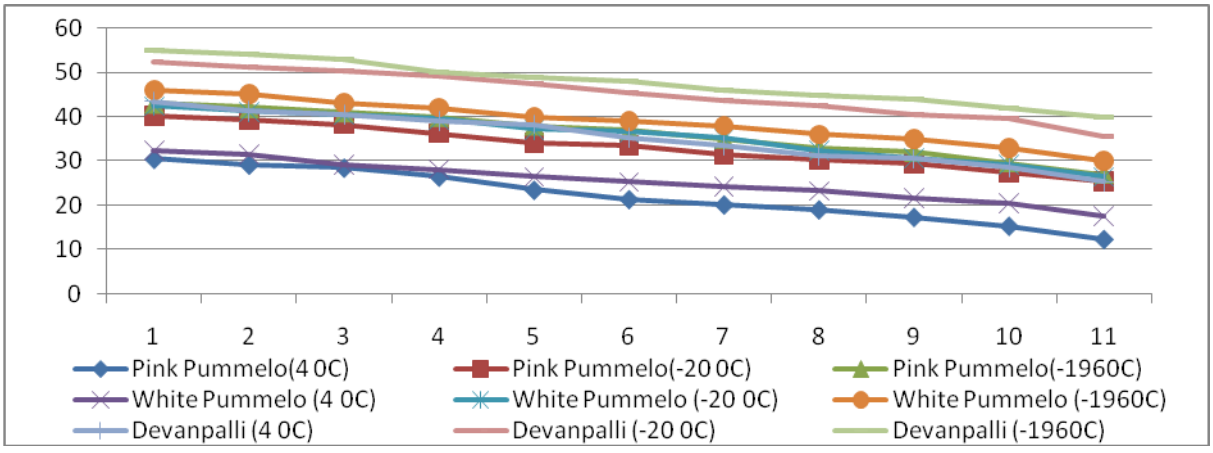
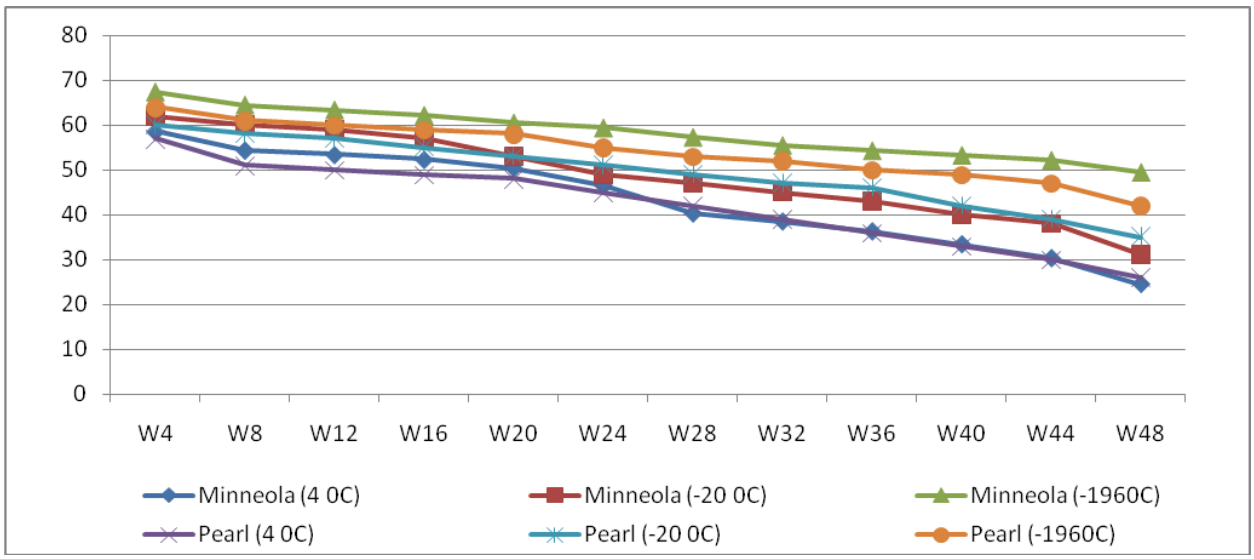


Fig.5 pollen germination % of Tangelo (*C. reticulata* × *C. maxima* or × *C. paradise*) varieties



Higher values for pollen germination at low storage temperature and decline at high storage temperature were observed by several workers (Hanna and Towill, 1995; Perveen *et al*., 2007; Perveen and Khan, 2008). It is quite obvious that staining tests are not reliable measure of pollen viability as compared to in vitro pollen germination tests. Hence a combination of both viability and germination tests will provide a better understanding about the pollen behaviour. In the present study wide variations in viability and germination of pollen grains was observed with different storage temperatures and durations among different citrus cultivars. This variability may be due to pollen fertility, as a result of regular meiosis and activation of certain enzyme systems present in the pollen grain itself. Besides genotype and environmental interactions may also play an important role. This phenomenon indicates genetic differences among the genotypes which have been reported by many researchers in many of the fruit tree species and cultivars (Alburquerque *et al*., 2007; Sharafi *et al*., 2011). Anjum and Shaukat (2014) five *Citrus* species while studying pollen germination beyond 48 weeks in the refrigerator (4℃), freezer (-20℃, -30℃) and freeze drier (-60℃) the best method to maintain pollen seems to be freeze drier (-60℃) and the viability of stored pollen grains for a long period of time. Among five species Citrus aurantium, C. limon and C. sinensis showed high percentage of germination as compared to *C. reticulata* and *C. paradisi*i. The gradual loss of germination at low temperatures (-20℃ and -196℃) observed in the present study may be attributed to frequent freezing and thawing of pollen grains. Gubley *et al*., 2015 concluded that the pollen of pollen of 'Lamas' and 'Meyer' varieties of lemon had the highest *in vitro* germination with 39.77% and 39.04%, respectively. Furthermore low temperature might have lead to intracellular ice formation, cell death and thereby loss of germination. Our results clearly indicated that it is feasible to store pollen grains of citrus at sub zero temperatures without any significant loss in their viability and germinability, and they may be used effectively throughout the flowering season for assisted pollination so as to broaden the citrus genetic base.

Acknowledgements

This work was by financial supported

scholarships fellowship (DST INSPIRE "Innovation in Science Pursuit for Inspired Research"), Ministry of Science and Technology, Department of Science and Technology Technology Bhawan, New Mehrauli road New Delhi-110016

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How to cite this article:

Shahnawaz Ahmed, H.S. Rattanpal, Eajaz Ahmad and Gurteg Singh. 2017. Influence of Storage Duration and Storage Temperature on In- Vitro Pollen Germination of Citrus Species. *Int.J.Curr.Microbiol.App.Sci.* 6(5): 892-902. doi: https://doi.org/10.20546/ijcmas.2017.605.099