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Storage conditions of soft X-ray irradiated pollen for producing seedless watermelons

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Abstract: A safe and efficient method for preserving viable soft X-ray-irradiated pollen for the production of seedless watermelon (*Citrullus lanatus* L.) from diploid plants was tested by packing the pollen under vacuum, O_2, CO_2 or N_2 gas at 25°C, 4°C or -25°C. Pollen germination rates decreased most rapidly with storage at 25°C and slowest with storage at -25°C. Oxygen as a storage gas was not good for storage of pollen, but pollen stored in N_2 or CO_2 gave good germination. Pollen stored at -25°C for 90 days germinated, but pollen stored at 4°C for 90 days did not, and N_2 storage tended to result in higher fruit set than vacuum storage. Fruit set was significantly affected by pollen storage conditions, with N_2 storage being more effective than vacuum storage at 4°C. Storage at -25°C produced little difference in fruit set between vacuum and N_2 storage. Thus, temperature was the major factor for maintaining viable and effective pollen, and the use of N_2 gas was an effective adjunct. Fruit quality was not significantly affected by storage parameters in this experiment.

1. Introduction

Generally, seedless watermelons are produced from triploid plants that are the product of crosses between tetraploid and diploid plants (Terada and Masuda, 1943; Kihara and Nishiyama, 1947; Kihara, 1951). Diploid seedless watermelons have also been produced by pollination with soft X-ray irradiated pollen (Sugiyama and Morishita, 2000; Sugiyama *et al*., 2002 a). Using irradiated pollen is advantageous because seedless watermelons can be produced with ordinary cultivation methods due to the use of diploid plants. However, mass production of seedless watermelon seed by this method will require the production, irradiation, preservation and storage of a lot of pollen. Pollen viability after freezing and low temperature storage at low relative humidity has been reported

in many plant species (Holman and Brubaker, 1926; McGuire, 1952; King, 1961; Fatmer and Barnett, 1974; Nath and Anderson, 1975; Anderson *et al.*, 1978). Miyaji and Shirazawa (1977) reported that the best storage conditions for watermelon pollen are 5°C at 20 to 40% humidity, which extended the viability of pollen to 60 days on germination medium, but did not investigate fruit set. Watermelon pollen had fruit setting ability for 13 days when stored at 5°C under dry conditions (silica gel) (Araki *et al*., 1987). Pollen stored under temperature and humidity control, tend to have rapid germination rate decreases (Miyaji and Shirazawa, 1977; Araki *et al*., 1987; Sugiyama *et al*., 1998). Watermelon pollen storage in organic solvents has also been investigated (Kodani and Omura, 1981; Shimizu, 1983; Sugiyama *et al.*, 2002 b). Among the organic solvents, ethyl acetate and ethyl ether were the most suitable for watermelon pollen storage (Shimizu, 1983). Pollen stored in ethyl acetate at -20°C had a germination rate of over 40% for one month on germination medium (Morishita *et al.*, 2000), and fruit set was observed up to 79 days of storage in ethyl acetate at -20°C (Sugiyama *et al.*, 2002 b). These methods are effective for watermelon pollen, but are not suitable for long-term storage because of a decrease in vigor. Also, it is very difficult for farmers to recover and use pollen that has been stored in organic solvents. N_2, CO_2 and/or O_2 gas have been used as a general storage medium in the food industry, but there are few reports using gas for pollen storage. In this study, we describe effective new pollen storage method using atmospheric gases.

2. Materials and Methods

Storage conditions for watermelon pollen

Pollen from watermelon cultivar Green seeded was collected in October 2005 by cutting the anthers from the flowers and shaking the contents through a stainless steel filter into a stainless steel cup. Collected pollen was packed in paraffin paper and irradiated with 600 Gy soft X-ray (Soft X-ray Unit OM-60R, OHMIC Ltd.) at 14.5 Gy·min^{-1}. Irradiated pollen aliquots of about 1 g each were packed with a vacuum packaging machine ('TOSPACK' V-380G, TOSEI, Shizuoka, Japan), in air or under vacuum storage at 25°C, 4°C or -25°C for storage over 35 days. A second similar storage test was initiated in May 2007 to investigate effect of atmospheric gases on storage. Irradiated pollen aliquots of about 3 g each were packed with a vacuum packaging machine under N_2, O_2, CO_2, air or vacuum for storage at 25°C for 1-6 days.

Viability of the stored pollen was judged on the basis of germination on an artificial medium consisting of 14% sucrose, 0.1% boric acid, and 1.5% agar (WAKO, Ltd, Osaka, Japan). Germination percentages were determined after 3 hour incubation at 25°C by counting random samples of 100 pollen or more for each storage condition.

The first test was replicated 8 times, and the second test was replicated 5 times. Tukey's multiple-range test ($p<0.05$) on statistical software (Statcel, oms-publishing, Saitama, Japan) was used to test differences between treatment means.

Fruit set ability of stored pollen, and fruits quality using stored pollen

Experiments were performed at the Hokkaido Research Center, Sapporo, Japan. During cultivation, greenhouse temperatures were recorded ('Ondotori', T&D Corporation, Nagano, Japan), and monthly mean temperatures were determined. The average temperatures during cultivation in the greenhouse were 25°C in July, 30.4°C in August, and 25.1°C in September. Watermelon cultivar Fujihikari TR seeds were sown in pots on June 15, 2006 in a greenhouse. Seedlings with 5 to 6 leaves were transplanted 50 cm apart in a bed (2.3 x 35 m) in a greenhouse on July 10, 2006. The bed was covered with black and gray polyethylene mulch and fertilized with 9N-9P-9K (in kg·ha^{-1}) before transplanting. Plants were topped at the five leaf stage and three lateral vines were allowed to grow. Male flowers were pollinated with a paint brash at about the 15th node of lateral branches. All treatments were arranged in a randomized complete-block design with four single plant replications. Four fruits were harvested for each treatment. Values were compared by Tukey's multiple-range test ($p<0.05$).

Source of pollen

Watermelon cultivar Green Seeded seeds were sown as a source of pollen for the storage experiments in February 2006, before 'Fujihikari TR' was sown. Male flowers of 'Green seeded' were harvested in the morning to obtain pollen that would be stored for 14, 28 and 90 days, from April through July. Collected pollen was irradiated with 600 Gy soft X-ray and packed with a vacuum packaging machine either under N_2 gas or vacuum storage at 4 or -25°C for 14, 28 or 90 days. Soft X-ray irradiated pollen processed on the morning of the experiment was

used as a non-storage control. The stored pollen was used to pollinate female flowers of 'Fujihikari TR'. Female flowers of 'Fujihikari TR' were bagged with cellophane before anthesis. Prepared irradiated pollen was applied with a brush. After pollination, female flowers were covered again with cellophane bags to prevent contact with insect-borne pollen for about 3 days. Pollination occurred during the period of August 2-6. One or two female flowers on each of the 3 lateral shoots were pollinated from each storage treatment, and fruit set was confirmed about 7 days later. The fruit set rate for each plant was calculated. Fruits were selectively thinned, leaving one fruit per plant to mature for each treatment. Mature fruits were harvested after about 42 days. After harvesting, fruit weight, shape, rind thickness, flesh color, sugar content (Brix) and number of empty seeds exceeding 6 mm in length were recorded for each fruit. Fruit shape index is expressed as the ratio of length from peduncle to blossom end, to equatorial diameter. Mature harvested watermelons were cut in half, and flesh color was measured with a colorimeter [a*= Hue relates to red (+60) - green (-60) color axes, Nihondenshokukogyo, Tokyo, Japan].

The germination ability of stored pollen was assayed as in experiment 1 before pollination (the period of August 2-6). Fruit set and pollen germination rates were compared by Tukey's multiple-range test ($p<0.05$).

3. Results and Discussion

Storage condition for watermelon pollen

Pollen germination rates decreased rapidly at 25°C to nearly zero after 7 days (Fig. 1). When stored in atmospheric air at 4°C, the germination rate was significantly lower than with other treatments (except at 25°C) after 21 days. After 35 days of storage under vacuum at 4°C, pollen viability was significantly lower than either treatment at -25°C ($p<0.05$). Thus, high temperature is not suitable for storage of pollen, and packing pollen under vacuum at 4°C prolonged viability.

The viability of watermelon pollen stored under vacuum or O_2 at 25°C decreased during storage to levels that make O_2 and vacuum conditions unacceptable as atmospheric media for pollen storage (Table 1). Pollen stored for 7 days under N_2 and CO_2 showed good germination, indicating that storage of soft X-ray irradiated watermelon pollen under N_2 or CO_2 would enhance its viability.

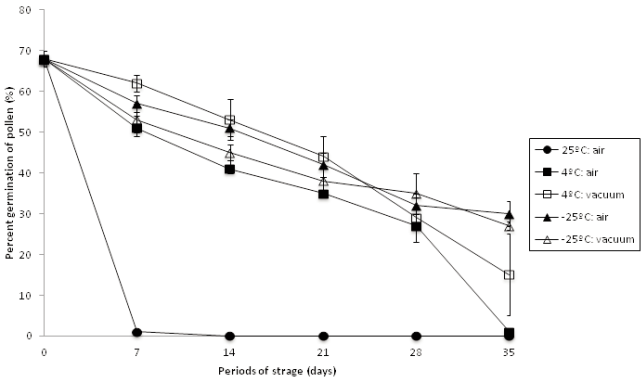

Fig. 1 - Germination of pollen after storage at different temperature and treatments. Bars indicate ± standard errors (n=8).

Table 1 - The differences of germination ability for cultured periods and treatments at 25°C

Treatments	Periods (days)				
	1	2	3	4	5
N_2	25.1 a [z]	21.2 a	16.5 a	13.8 a	7.9 a
O_2	11.2 b	4.7 b	0.0 d	0.0 c	0.0 b
CO_2	19.4 ab	17.6 ab	16.3 a	15.1 a	6.9 a
Vacuum	16.9 ab	10.9 b	2.0 c	1.7 b	0.0 b
air	20.3 ab	19.5 ab	9.3 b	3.3 b	0.2 b

[z] Means followed by the same letter are not significantly different at the 5% level by Tukey's multiple-range test.

Fruit set and quality produced from stored pollen

The ability of stored polled to germinate, and to produce agronomically acceptable fruit are two distinct qualities, both of which are necessary for the adoption of soft X-ray irradiated pollen on a commercial scale. There were observable differences in fruit set between pollen stored at 4°C and -25°C (Table 2). Lower temperatures resulted in good germination rates, and pollen which had been stored at -25°C had a longer shelf-life than at 4°C. Pollen stored at 4°C for

Table 2 - Relationship between storage condition and fruit set

Treatment	Storage		Pollination	Fruiting	Fruit set (%)
	Temperature (°C)	Period (days)			
Control		0	9	8	88.9
Nitrogen	4	14	17	17	100.0
Nitrogen	4	28	18	7	38.9
Nitrogen	4	90	16	0	0.0
Nitrogen	-25	14	11	10	90.9
Nitrogen	-25	28	16	14	87.5
Nitrogen	-25	90	16	15	93.8
Vacuum	4	14	17	11	64.7
Vacuum	4	28	7	0	0.0
Vacuum	4	90	13	0	0.0
Vacuum	-25	14	15	15	100.0
Vacuum	-25	28	15	14	93.3
Vacuum	-25	90	15	13	86.7

90 days did not set fruit whether or not it was stored under N_2 or vacuum. Although germination rates under N_2 and vacuum storage were almost the same, fruit set with pollen stored at 4°C for 14 or 28 days was more effective under N_2 than under vacuum. Pollen stored at 4°C for 28 days under N_2 had the ability to set fruit, but pollen stored at 4°C under vacuum did not. Snope and Ellison (1963) also reported that germination from freeze-dried pollen that had been stored under N_2 was got good. These results indicate that pollen vigor is maintained more effectively under N_2 than under vacuum. The percentage of germinating pollen stored at -25°C for 28 days was 24.8% and there was fruit set following hand pollination. However, the percentage of germinated pollen stored at 4°C for 28 days was 22.8%, but no fruit was set with this treatment. N_2 gas was an effective atmospheric medium for storage at 4°C in both tests. There were many reports that low temperature provides a good environment for stored pollen (Araki *et al*., 1987; Sugiyama *et al.*, 1998; Morishita *et al.*, 2000). Low temperature provides the major benefit, with storage in N_2 gas as an adjunct for pollen storage. Inert gases may be effective for the maintenance of pollen vitality because they may suppress metabolism of the pollen by displacing oxygen. The additive effect of N_2 with low temperature suggests that low metabolic rates are the key factor in maintaining pollen viability over time.

There was no significant difference in weight or shape between control fruit and fruit produced by pollen under any of the storage conditions (Table 3). Rind thickness and flesh color were also the same, except for fruits produced with pollen stored at -25°C for 90 or 14 days, respectively. Although the Brix of fruit from pollen stored at -25°C for 14 days was the lowest, there was no consistent relationship between storage conditions and Brix. Sugiyama and Morishita (2000) has observed that the number of empty seeds differs widely in individual fruits. In this experiment there were significant differences in the number of empty seeds, but there was no apparent relationship between empty seed numbers and storage conditions. Thus, there is no consistent relationship between pollen storage conditions and fruit quality. Further statistical analysis of the relationships between treatment, temperature and periods of pollen storage, was done for the survival of pollen, but there were no statistically significant differences.

Organic solvents are useful as a pollen storage method (Kodani and Omura, 1981). However, organic solvents carry the risk of environmental pollution and are difficult to use outside the laboratory. Storage of pollen at low temperatures and under N_2 eliminates the need for organic solvents, thus providing an eco-friendly storage solution which should be applicable for pollen export or import. Furthermore, pollen stored under N_2 at 25°C for 5 days had germination ability (Table 1) and then the temperature in the greenhouse was around 25-30°C, so it might be also no problem to bring the pollen from freezer to the greenhouse during pollination.

Table 3 - Fruit quality of seedless watermelon pollination with irradiated pollen in several conditions

Treatment	Storage		Fruit weight (kg)	Flesh shape [z]	Thickness of rind (mm)	Flesh color	Brix (%)	No. of empty seeds
	Temperature (°C)	Period (days)						
Control		0	5.5±1.1[y]	1.14	13.9±0.0 a [x]	21.4±1.1 a	10.3 a	96.5±12.1 b
Nitrogen	4	14	4.6±0.6	1.11	13.2±1.0 a	20.4±1.7 a	9.9 a	102.5±86.3 b
Nitrogen	4	28	4.9±0.5	1.12	13.5±0.2 a	23.9±2.8 a	9.7 b	104.5±17.6 b
Nitrogen	-25	14	5.1±1.0	1.11	12.2±1.0 a	18.4±3.6 b	9.6 bc	150.0±88.3 a
Nitrogen	-25	28	5.5±0.9	1.10	13.5±0.6 a	23.9±1.5 a	9.9 a	104.8±25.2 b
Nitrogen	-25	90	5.2±0.5	1.14	11.9±0.4 b	22.5±1.0 a	9.7 b	222.8±71.7 a
Vacuum	4	14	4.9±0.3	1.13	12.2±1.4 a	24.0±3.9 a	9.7 b	103.0±44.9 b
Vacuum	-25	14	5.4±0.5	1.10	12.7±1.9 a	20.6±2.5 a	10.2 a	134.8±21.1a
Vacuum	-25	28	5.1±1.5	1.15	12.3±1.6 a	21.0±1.9 a	10.1 a	158.5±81.0a
Vacuum	-25	90	5.7±0.4	1.17	12.5±0.6 a	22.5±0.6 a	10.0 a	127.0±24.5b

[z] Flesh shape is expressed as the ratio of height to width.

[y] Mean ± SE.

[x] Value within columns with the same letter are not significantly different (5% level).

Means followed by the same letter are not significantly different at the 5% level by Tukey's multiple-range test.

Plant breeders may also be able to overcome differences in crop or varietal flowering times by storing pollen with this method.

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