Watermelon (Citrullus lanatus) Seed Formation by Pollination with Normal Pollen Following Pollination with Soft X-ray Irradiated Pollen

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Watermelon (*Citrullus lanatus*) Seed Formation by Pollination with Normal Pollen Following Pollination with Soft X-ray Irradiated Pollen

Keita SUGIYAMA [1], Daisuke KAMI [1] and Takato MURO [2]

Ⅰ. Introduction

Almost all seedless watermelon (*Citrullus lanatus*) is produced by a triploid technique by which diploid pollen is used to pollinate a pistillate flowers (KIHARA, 1951; TERADA and MASUDA, 1943). Another method for producing seedless watermelon is by pollinating with soft X-ray irradiated pollen (SUGIYAMA and MORISHITA, 2000). Using soft X-ray irradiated pollen (partially inactivated pollen; PI-Pollen) has an advantage because seedless watermelons can be produced with ordinary cultivation methods since they are diploid plants; however, the production of seedless watermelon by PI-Pollen is time-consuming. In addition to making the PI-Pollen, pistillate flowers must be covered with a bag before flowering, the flowers must be artificially pollinated and the pistillate flower must be covered after pollination.

The first problem in this new method is that a large amount of PI-Pollen must be produced prior to pollination for the mass production of seedless watermelon. To overcome this issue, we developed a pollen storage method for watermelon (AKUTSU and SUGIYAMA, 2008), allowing us to offer the farmer PI-Pollen in large amounts. The second problem is the need to cover pistillate flowers with a bag before anthesis to prevent normal pollination by insects. To solve this problem, we tested pollinating flower buds in the afternoon prior to anthesis and found equivalent fruit set rates to flowers pollinated on the day of anthesis (SUGIYAMA and AKUTSU, 2010). We thought that this bud pollination method was sufficiently effective to omit covering the pistillate flower with a bag; however, even if pollinating pistillate flowers with PI-Pollen before anthesis, we found normal seeds in the fruit.

Our goal in this paper was to verify the effectiveness of the bud pollination method. That is, we tested the efficacy of the bud pollination method to block normal pollen carried by insects. After pollination of PI-Pollen on the day before anthesis, normal pollen was used to pollinate flowers on the day of anthesis. We investigated the relationship between number of normal seeds and the timing of using normal pollen after pollination with PI-Pollen. The difference in pollen tube elongation into the ovaries for normal pollen and PI-Pollen was also investigated. In addition, the distribution of normal seeds in the mature fruits produced by the use of normal pollen after pollination with PI-Pollen was determined.

Ⅱ. Materials and Methods

1. Normal seed formation by pollinating with normal pollen after pollination with PI-Pollen

Watermelon cultivar 'Fujihikari TR' (a monoecious plant) seeds were sown in pots on June 22, 2008 in a glasshouse, and seedlings with five to six leaves were transplanted 50 cm apart in two beds (2.3 m X 25 m) in a greenhouse on

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1) NARO Hokkaido Agricultural Research Center, Lowland Farming Research Division
2) NARO Hokkaido Agricultural Research Center, Upland Farming Research Division

July 18. The bed was covered with black and gray polyethylene film and fertilized with a pre-plant broadcast application of N-10 P-10 K-10 (kg/ha) before transplanting. Plants were topped at the five-leaf stage, and three lateral vines were allowed to grow. Pistillate flowers were pollinated starting at about the 15th node of the lateral branches. Experiments were performed at the National Agriculture and Food Research Organization Hokkaido Agricultural Research Center in Sapporo, Japan.

Pollen from watermelon was collected by cutting the anthers from the male flowers on the day of anthesis 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 Co., Ltd.) at 15.0 Gy/min. Irradiated pollen (PI-Pollen) was packed with a vacuum packaging machine ('TOSPACK' V-380G, TOSEI, Shizuoka, Japan), under vacuum for storage at -25℃. Normal pollen was collected on packed in paraffin paper and was packed with a vacuum packaging machine, under vacuum for storage at -25℃. Pollen was stored no longer than one week prior to use in pollinating pistillate flowers of 'Fujihikari TR'.

After pollination with PI-Pollen on the day before anthesis, normal pollen was used to pollinate flowers 14, 16, 20 and 24 h later. Prepared pollen was applied to the stigmas with a brush. After pollination, pistillate flowers were covered with cellophane bags to prevent contact with insect-transferred pollen.

Fruits were selectively thinned, leaving one fruit per plant to mature for each treatment. Mature fruits were harvested about 40 days after pollination. Fruit weight, shape, rind thickness, flesh color, soluble solids (Brix) and the number of normal seeds were recorded for each fruit. The fruit shape index (l:w) was expressed as the ratio of length from the peduncle to the blossom end to the equatorial diameter. Mature harvested watermelons were cut in half, and the flesh color was measured with a colorimeter (a*: hue relates to the red (+60) – green (-60) color axes. NF 333, NIPPON DENSHOKU INDUSTRIES Co., Ltd. Tokyo, Japan). The number of normal seeds produced was recorded for each treatment.

Each treatment was arranged in a randomized block design with three replications and one block had four or five plants. The fruits of the same treatment were treated as one group in data analysis. The mean differences between normal fruit and seedless fruit were determined by the *t* test. The Tukey-Kramer multiple-range test ($p<0.05$) was used to test differences between treatment means.

2. Normal seed formation and distribution of normal seeds in the fruit by pollinating with normal pollen after pollination with PI-Pollen

Watermelon cultivar 'Fujihikari TR' seeds were sown in pots on June 10, 2009 in a glasshouse, and seedlings with five to six leaves were transplanted 50 cm apart in two beds (2.3 m X 25 m) in a greenhouse on July 6. Other cultivation conditions and pollination methods were identical to those in Ⅱ-1.

After pollination with PI-Pollen on the day before anthesis, normal pollen was used to pollinate flowers 24 and 30 h later. Pollination with PI-Pollen was performed at noon or 4 AM on the day before anthesis.

Mature fruits were harvested about 40 days after pollination. The fruit was cut in half equatorially, and the number of normal seeds on the lower portion (on blossom end side) and on the upper portion (on the stem end side) was recorded.

Each treatment was arranged in a randomized design with five single-plant replicates. The mean differences between normal fruit and seedless fruit were determined by the *t* test. The Tukey-Kramer multiple-range test ($p<0.05$) was used to test differences between treatment means.

3. Difference in pollen tube elongation after pollination with normal pollen and PI-Pollen

Plant cultivation was the same as Ⅱ-1. The treatment was as follows: 1) normal pollen or PI-Pollen was used to pollinate flowers at 8 AM on the day before anthesis; 2) normal pollen or PI-Pollen was used to pollinate flowers at 4 PM on the day before anthesis; 3) normal pollen or PI-Pollen was used to pollinate flowers at 8 AM on the day of anthesis.

To observe the elongation of pollen tubes, three pistillate flowers from each treatment were collected after pollination and rapidly fixed with an acetic acid-ethanol (1:3) solution. The ovary was cut in one of the sections as indicated in Fig. 1. Thin cross-sections of the ovaries in each position were soaked in 1N sodium hydroxide for 10 min, washed and stained with 0.5% aniline blue until the tissues were stained.

Ⅲ. Results

1. Normal seed formation by pollinating with normal pollen after pollination with PI-Pollen

When normal pollen only was used to pollinate pistillate flowers on the day of anthesis at 8 AM, there were about 560 normal seeds in the fruit (Table 1). When only PI-Pollen was used to pollinate on the day of anthesis or the day before anthesis, normal seeds were not found in the fruit. The number of normal seeds was about 150 when normal pollen was used to pollinate flowers 14 h after pollination with PI-Pollen. When normal pollen was used to pollinate flowers 16 h after pollination with PI-Pollen, there were about 350 normal seeds, which was the peak value. When normal pollen was used to pollinate flowers at 20 h or 24 h after pollination with PI-Pollen, about 200 normal seeds or 160 normal seeds were formed respectively. Thus, normal seed formation was inhibited by pollination with PI-Pollen before pollination with normal pollen; however, normal seeds were formed when normal pollen was used for pollination even 24 h after pollination with PI-Pollen.

Fruit weight of plants pollinated with normal pollen on the day of anthesis at 8 AM or PI-Pollen on the day of anthesis at 8 AM was lower than that of when normal pollen was used to pollinate flowers at 14 h or 16 h after pollination with PI-Pollen. Although fruit thickness of plants pollinated with normal pollen on the day of anthesis at 8 AM or PI-Pollen on the day of anthesis at 8 AM tended to be thinner than for other treatment, there was no consistent relationship with pollination time of normal pollen after pollination of PI-Pollen (Table 2). There was no significant difference in fruit shape, color or Brix among the treatments.

Table 1. Seed formation by pollination with normal pollen after pollination with PI-Pollen.

Time of pollination		Number of fruit	The time from A to B (hour)	No. of normal seed per fruit
A: PI-Pollen[1)]	B: Normal pollen			
One day pre-anthesis	Anthesis			
—	8 AM	13	—	557.3±26.1 d[2)]
12 PM (twelve noon)	12 PM (twelve noon)	13	24	163.9±39.4 ab
4 PM (16:00)	—	13	—	0.0 a
4 PM (16:00)	6 AM	13	14	148.4±39.2 ab
4 PM (16:00)	8 AM	13	16	351.7±63.4 c
4 PM (16:00)	12 PM (twelve noon)	12	20	200.6±39.1 bc
8 AM (Anthesis)	—	7	—	0.0 a

1) Parthially inactivated pollen.
2) Means followed by the same letter are not significantly different at the 5% level by Tukey-kramer's multiple-range test.

Table 2. Fruit quality of watermelon produced by different pollinetion methods.

Time of pollination				Fruit weight	Fruit shape[1]	Rind thickness	Flesh color[2]	Brix
A: PI-Pollen[3]	B: Normal pollen	The time from A to B	Number of fruit	(kg)	(l:w)	(mm)		(%)
One day pre-anthesis	Anthesis	(hour)						
—	8 AM	—	13	5.8 a[4]	1.07a	16.0 a	19.1a	12.1a
12 PM (twelve noon)	12 PM (twelve noon)	24	13	6.9 ab	1.15a	18.3 b	19.3a	12.2a
4 PM (16:00)	—	—	13	6.3 ab	1.14a	18.1 b	20.0a	12.4a
4 PM (16:00)	6 AM	14	13	7.1 b	1.12a	18.2 b	20.1a	12.5a
4 PM (16:00)	8 AM	16	13	7.1 b	1.09a	17.6 ab	19.1a	12.6a
4 PM (16:00)	12 PM (twelve noon)	20	12	6.6 ab	1.07a	17.5 ab	18.9a	12.6a
8 AM (Anthesis)	—	—	7	5.5 a	1.07a	15.4 a	18.5a	11.9a

1) Length/width
2) a*: Hue relates to red (+60) – green (–60) color axes.
3) Parthially inactivated pollen
4) Means followed by the same letter are not significantly different at the 5% level by Tukey-kramer's multiple-range test.

2. Normal seed formation and distribution of normal seeds in the fruit by pollinating with normal pollen after pollination with PI-Pollen

When normal pollen only was used to pollinate flowers at noon on the day of anthesis, 500 normal seeds were produced (Table 3). When only PI-Pollen was used to pollinate flowers at noon on the day before anthesis, normal seeds were not observed. The number of normal seeds was decreased by pollinating with normal pollen after pollinating with PI-Pollen as well as Ⅱ-1. A bout 106 normal seeds were obtained in the case of pollinating of normal pollen at 24 h (12 PM) after pollination of PI-Pollen. And about 43 normal seeds developed for plants whose flowers were pollinated with normal pollen 30 h after pollination of PI-Pollen.

As for the distribution of normal seeds in the fruits from all treatments, there were more normal seeds on the blossom end side (upper portion) of the fruit than on the stem end side (lower portion). When only normal pollen (control) was used for pollination, about 60% of the total normal seeds were in the upper portion, and the remaining 40%

Table 3. Seed formation and distribution of seeds in the fruit resulting from pollination with normal pollen after pollination with PI-Pollen.

Time of pollination		The time from A to B	Number of fruit	Fruit weight	Total seed number per fruit	No. of normal seed in different portion	Portion	Seed ratio of upper potion and lower portion in the fruit
A: PI-Pollen[1]	B: Normal pollen							
One day pre-anthesis	Anthesis	(hour)		(kg)				(%)
—	12 PM (twelve noon)	—	5	5.3a[2]	499.5 ± 47.4a	291.8 ± 17.0	upper[3]	58.4
						207.8 ± 32.1	lower[4]	41.6
12 PM (twelve noon)	12 PM (twelve noon)	24	5	4.1a	105.8 ± 41.3b	69.4 ± 24.4	upper	65.6
						36.4 ± 17.5	lower	34.4
12 PM (twelve noon)	6 PM (18:00)	30	5	4.2a	43.0 ± 14.9b	34.6 ± 11.0	upper	80.5
						8.4 ± 4.1	lower	19.5
12 PM (twelve noon)	—	—	5	5.3a	0.0	0.0	upper	-
						0.0	lower	-
4 PM (16:00)	4 PM (16:00)	24	5	5.3a	71.0 ± 22.1b	53.8 ± 15.3	upper	75.7
						17.3 ± 8.7	lower	24.3

1) Parthially inactivated pollen.
2) Means followed by the same letter are not significantly different at the 5% level by Tukey-kramer's multiple-range test.
3) The portion from the blossom end to the equatoial position of the fruit.
4) The portion from the stem end to the equatoial position of the fruit.

were in the lower portion. Having a higher percentage of normal seeds in the upper portion is a result similar to the case of using normal pollen after pollination with PI-Pollen. Additional increases in the percentage of normal seeds in the upper portion of the fruit were measured when normal pollen was used after even longer time periods after pollination with PI-Pollen; 80.5% of the normal seeds were in the upper portion when normal pollen was used 30 h after PI-Pollen.

There was no significant difference in the fruit weight among the treatments.

3. Difference in pollen tube elongation after pollination with normal pollen and PI-Pollen

The elongation of pollen tubes from PI-Pollen used for pollination on the day of anthesis tended to be faster than those from normal pollen used for pollination one day pre-anthesis (Table 4). The growth of PI-Pollen tubes 48 h after pollination was similar to the growth of normal pollen tubes at 24 h after pollination. When PI-Pollen was used to pollinate flowers at 8 AM one day prior to anthesis, only a few pollen tubes elongated and they were quite short. When normal pollen and PI-Pollen were used to pollinate flowers one day pre-anthesis, pollen tubes at 24 after pollination did not penetrate the micropyles. At 48 h since pollination with normal pollen one day pre-anthesis, pollen tubes were observed in the lower portion of the ovary. PI-Pollen tubes were observed in the upper portion of the ovary at 48 h after pollination one day pre-anthesis.

When normal pollen and PI-Pollen were used to pollinate flowers at 8 AM on the day of anthesis, the penetration speed of the pollen tubes was fast; at 24 h after pollination tubes had penetrated into the micropyles. In normal pollen, tubes penetrated into the micropyle in the middle portion of the ovary at 24 h after pollination and penetrated into the micropyle in the lower portion of the ovary at 48 h after pollination. On the other hand, in PI-

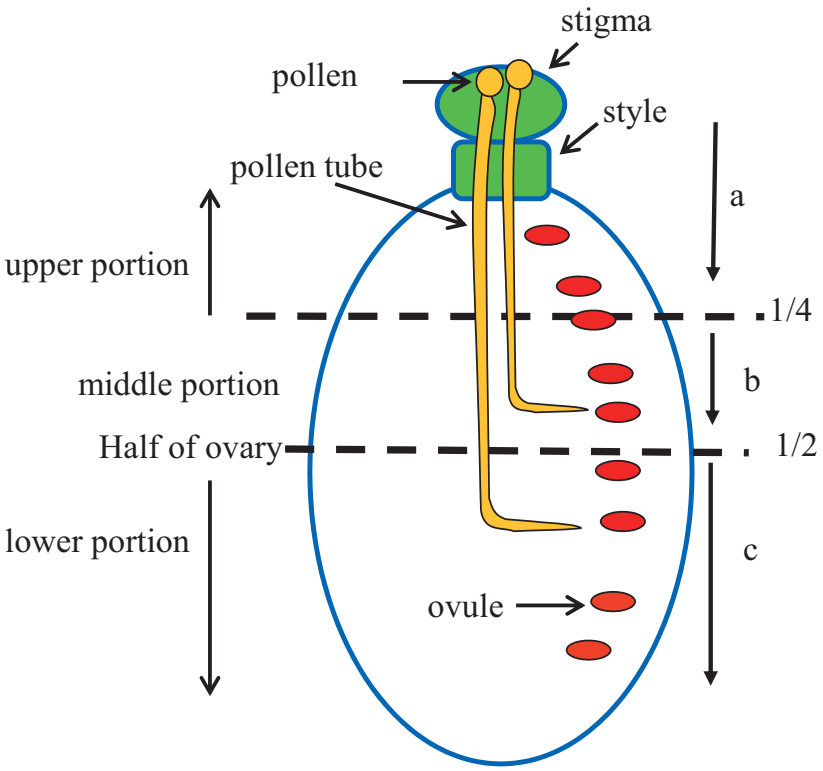

Fig. 1. Position of pollen tubes in an ovary after pollination

a: upper portion, b: middle portion, c: lower portion.

Table 4. Position of pollen tube in normal pollen and PI-Pollen after pollination.

Type of pollen	One day pre-anthesis				Anthesis	
	8 AM		4 PM		8 AM	
	24 h[1)]	48 h.	24 h	48 h	24 h	48 h
Normal pollen	a[2)]	***b***[3)]-c	a-b	***b***-c	***b***-c	***c***
PI-Pollen	a	***a***	a	***a***-b	***a***-c	***b***-c

[1)] Time after pollination.
[2)] See Fig. 1.
[3)] Bold and italicized letters indicate that penetration of pollen tubes into the micropyle was observed.

Pollen, elongation of pollen tubes tended to be slower than for normal pollen tubes.

The above results indicate that the elongation speed of PI-Pollen tubes was slower than that of normal pollen tubes, and the position at 48 h after pollination of the PI-Pollen was close to the position at 24 h after pollination with normal pollen. Moreover, elongation of pollen tubes after pollination on the day of anthesis was faster than that of tubes from pollen used for pollination one day pre-anthesis.

Ⅳ. Discussion

Generally, fertilization of watermelon occurs between 24 h and 48 h after pollination with normal pollen (BUTTROSE and SEDGLEY, 1979; SUGIYAMA, 2001). We observed almost the same results in this test. Also, we reported that fruit was able to set when pollination occurred one day pre-anthesis (SUGIYAMA and AKUTSU, 2010). The fruit set rate of plants pollinated on the afternoon of the day before anthesis was almost the same as the fruit set rate of plants pollinated of the day of anthesis. To maximize the number of normal seeds, we presumed that it was necessary to use normal pollen as soon as possible after pollinating with PI-Pollen; however, more normal seeds were produced after pollination with normal pollen after 16 h than after 14 h. Anthesis began at about 6 AM, and the flowers were open completely at about 8 AM. Therefore, we hypothesized that the best time of the day for elongation of pollen tubes occurred from 8 to 10 AM to obtain the highest fruit set percentage (SUGIYAMA, 2001).

The number of normal seeds decreased by pollinating with PI-Pollen beforehand in all treatments in comparison with pollinations using normal pollen only. We hypothesized that PI-Pollen tubes would penetrate the ovules earlier than the pollen tubes from the later pollinated normal pollen and would disturb the normal pollen tubes.

However, normal seeds were resulted from pollinating with normal pollen even at 30 h after pollination with PI-Pollen. This result indicated that it was difficult to completely prevent later fertilization by normal pollen with early pollination of PI-Pollen. Therefore, prevention of pollination with normal pollen carried by insects is necessary for the production of seedless watermelon when using PI-Pollen.

Nearly all of the pollen tubes from the early PI-Pollen pollinations reached the nearest ovules; that is, ovules of the upper portion (blossom end side). Therefore, it must be a rare event that later pollinated pollen tubes can penetrate to the ovules of the blossom end side (upper portion); however, we obtained the opposite results. In the case of pollination with PI-Pollen at 4 PM one day pre-anthesis, pollen tubes reached the middle potion of the ovary at 48 h after pollination. On the other hand, in the case of pollination with normal pollen at 8 AM on the day of anthesis, 24 h after pollination the position of normal pollen tubes was at a slightly lower position (stem end side) of the ovary comparison with PI-Pollen tubes. Also,

we previously reported that fertilization by PI-Pollen was later than normal pollen (SUGIYAMA *et. al*, 2002) . Although it was obvious that normal pollen elongates fast we expected that PI-Pollens would occupy ovules at the blossom end. That is, many normal seeds in the lower portion of the fruit should be present if normal pollen can elongate ahead of PI-Pollen. As for the reason for the opposite outcome, we hypothesize that normal pollen tubes penetrated the ovules that the PI-Pollen tubes had not reached, or the timing for the arrival of normal pollen tubes to mature ovules was perfectly matched.

The number of normal seeds in the lower and upper portions of the ovary decreased after pollinating with PI-Pollen beforehand. When normal pollen was used to pollinate flowers on the day of anthesis at 6 PM after pollination with PI-Pollen one day pre-anthesis at 12 PM, the ratio of the number of normal seeds in the lower portion was very low. We assumed that PI-Pollen tubes blocked the pathway of normal pollen tubes in from the style to the upper portion of ovary. Also, perhaps the continued aging of the pistillate flower decreased the ability for fertilization to occur.

Fruit weigh of 'Fujihikari TR' is usually 5 to 7 kg in this cultivation season (SUGIYAMA, and AKUTSU, 2010) . In the experiments, the fruit was roughly normal size. Fruit weight of plants pollinated on the day of anthesis tended to be lower than plants pollinated one day pre-anthesis in Table 2, but there was no significant difference in fruit weight for plants pollinated on the day of anthesis in Table 3. Also, in our previous report, there was no effect of fruit weight on pollination time (SUGIYAMA, and AKUTSU, 2010) . Accordingly, the fruit weight had no relationship with the treatments. Other fruit characteristics had no relationship with the treatment used for pollination.

Ⅴ. Summary

We developed a method using soft X-ray irradiated pollen (partially inactivated pollen; PI-Pollen) to pollinate pistillate flowers of watermelon (*Citrullus lanatus*) prior to anthesis for producing seedless watermelon and hopefully prevent later normal pollination by insects. In this study, we have further characterized the efficacy of this method by pollinating pistillate flowers with PI-Pollen one day pre-anthesis and testing the effects of varying the timing of subsequent pollination with normal pollen. Normal seed formation was inhibited by pollination with PI-Pollen before pollination with normal pollen. However, normal seed was formed as a result of pollinating with normal pollen even at 30 h after pollination with PI-Pollen. The number of normal seeds on the blossom end side of the fruit (upper portion) was greater than on the stem end side (lower portion) in all treatments. The percentage of normal seeds in the upper portion of the fruit tended to increase as the duration increased between pollinating with normal pollen after first pollinating with PI-Pollen. The tubes of PI-Pollen pollinated to pistillate flower on the day before anthesis elongated more slowly than normal pollen tubes. In the case of pollination with normal pollen on the day of anthesis, 24 h after pollination the position of normal pollen tubes was at a lower position of the ovary comparison with PI-Pollen tubes. Therefore, for production seedless watermelon by IP-Pollen, it pollination by insects after bud pollination with PI-Pollen must be prevented.

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スイカにおける軟X線照射花粉の受粉後の普通花粉受粉による種子形成

杉山慶太[1)]・嘉見大助[1)]・室　崇人[2)]

摘　要

軟X線照射花粉（部分不活化花粉）を利用した種なしスイカを生産するにあたり、昆虫による普通花粉の受粉を防ぐために雌花に袋かけ等を行っている。我々はこの労力を削減するために、雌花が蕾である開花前日に部分不活化花粉を受粉する方法を開発した。これにより昆虫によって後から受粉された普通花粉の授精を妨げることが期待された。そこで、本研究では部分不活化花粉を開花前日に受粉することで、開花日における普通花粉の受粉による種子形成を妨げることが可能かどうか検討した。スイカ果実内の種子の数は、部分不活化花粉を普通花粉より先に受粉することで減少したが、部分不活化花粉を受粉した30時間後に、普通花粉が受粉された場合でも種子が形成された。果実内の種子は果実の中央部から花痕側の部分の方が、果梗側の部分よりも多かった。また、部分不活化花粉の受粉後に普通花粉を受粉するまでの時間が長くなるにしたがって、花痕部側における種子割合が多い傾向があった。部分不活化花粉の花粉管の伸長速度は普通花粉よりも遅く、部分不活化花粉を前日に受粉した場合よりも、開花当日に普通花粉を受粉した場合の方が早く子房の下部に到達した。以上のことから、部分不活化花粉による種なしスイカの生産には、開花前日の受粉であっても昆虫による普通花粉の受粉を防除する必要があった。

1）北海道農業研究センター　水田作研究領域
2）北海道農業研究センター　畑作研究領域