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The mechanism of seedlessness in watermelon generated using soft-X-ray irradiated pollen

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A new variety of seedless watermelon, with high sugar content, good taste, and easy storage has been developed using scanning electron microscopy (SEM) fluorescence staining, paraffin and 4',6-diamidino-2-phenylindole (DAPI) staining. The study investigated the mechanism behind the production of seedless watermelon after soft X-ray irradiation. The results showed that soft X-ray irradiation did not damage the cell walls of the watermelon pollen, and leading to normal pollination and fertilization. However, the chromosomal double helix of the watermelon pollen were damaged, thereby inhibiting embryotic developmental processes, leading to abortion of the embryo and degeneration of endosperm, which lead to the production of seedless watermelon. In conclusion, artificial pollination of pollen after soft X-ray irradiation can produce no seed watermelon, and the sugar content is higher than that of the seed watermelon.

Key word: Watermelon, X-ray, seedless.

INTRODUCTION

Watermelon (*Citrullus lanatus*) is a dicotyledonous flowering plant belonging to Cucurbitaceae with origins in Africa. As a new variety of *C. lanatus*, seedless watermelon is characterized by high sugar content, good taste, and easy storage. Producers and consumers find it attractive on the fruit market for its high quality, convenient eating, resistance against disease, waterlogging tolerance, high and stable yield, tolerance of storage and transportation, and other advantages (Akutsu and Sugiyama, 2008; Hassell and Schultheis, 2007). The cultivation area of Seedless watermelon was 230,000 hm^2 across China in 2009, with 80% being cultivated in the humid regions to the south of the Yangtze River and 90% in the regions to the South of the Yellow River (Liu, 2010). In many regions, the yield of *C. lanatus* with seeds is 2,000 to 2,500 Kg for every 667 m^2, while the yield of seedless watermelon can reach 3,000 to 5,000 Kg (Liu et al., 2006). The demand for seedless watermelon is rapidly increasing on the market of China and the United States (Liu, 2010; Schultheis et al., 2007; Yang et al., 2012), leading to the need for research into new methods for obtaining seedless watermelon.

Terada and Masuda (1943) could produce seedless watermelons by first generating tetraploid plant using colchicine, then hybridizing with diploid watermelon as the male parent, and finally obtaining triploid seedless

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watermelon by exploiting the high sterility of the triploid (Akutsu and Sugiyama, 2008; Eisho et al., 2002). However, compared with the diploid, triploid seedless watermelon had disadvantages such as high breeding cost, low germination rate, late ripeness, poor fruit quality, long production cycle (Akutsu and Sugiyama, 2008). Therefore, its cultivation area is gradually decreasing. In recent years, Sugiyama and Morishita, 2000 and Sugiyama et al. (2002) have discovered that seedless watermelon can be obtained by irradiating pollen from male flowers of diploids with 600 Gy soft X-rays and then fertilizing female flowers with the irradiated pollen. This technique can be used for producing seedless watermelon but has only been applied for a short time and its mechanism is still not clear. In this study, we research the mechanism of producing seedless watermelon in this way in order to provide a theoretical basis for the technique, and to facilitate further exploration of new methods for producing seedless watermelon.

MATERIALS AND METHODS

Huaian Academy of Agricultural Science bred new varieties of watermelon 'Huaime'. Non-irradiated pollen of diploid 'Huaime' and pollen irradiated by soft X-rays (600 Gy) were used in the experiment.

Observation of pollen by scanning electron microscope

Differences between irradiated pollen and control pollen exine were observed with a scanning electron microscope. Dried pollen were placed on the platform of a Philips SEM-505 scanning electron microscope that had been prepared with Mayer's albumn (50 ml glycerol + 50 ml egg white) (Of. 2009) and sprayed with an ion sputter coater. Pollens were then observed and photographed.

Pollen germination on stigma and growth in style

Pollen germination on the stigma and pollen tube growth were observed after pollinating diploid watermelon with irradiated and control pollen. Fresh styles and ovaries were picked at 2, 4, 8, and 24 h after pollination, and immediately placed in FAA fixative that is composed of 5 ml Formalin, 5 ml Acetic acid and 90 ml 70% Alcohol for 5 days. The styles and ovaries were then clarified for 24 h with transparent solution (alcohol mixed with an equal volume of xylene), and washed five times with distilled water, and stained for 24 h with aniline blue (0.1 g water-soluble aniline blue in 0.1 mol/L K_3PO_4). Photographs and observations of flattened styles and ovaries were taken with a fluorescence microscope (OLYMPUS BX43) to compare stigma germination and pollen tube growth behaviors.

The embryo sac and fertilization process were observed via a paraffin fixing method

Embryos of different days (days 1-7, 12, and 15) were washed with water and cut into 2 × 4 mm cuboids. These were section and examine using the paraffin method of Randolph (1935) (Randolph, 2009).

Differences in chromosomes were observed via DAPI staining

One mg of 4', 6-diamidino-2-phenylindole (DAPI) solution was dissolved in 3.6 ml 70% alcohol to obtain 1 M DAPI solution, and 50 ml of solution was prepared by adding 5 µL DAPI alcohol solution to PBS buffer solution. Pollen was cultured for 15 min in incubator at 22°C with 1/10 DAPI solution to pollen culture medium (Sucrose 15%, KNO_3 0.1%, $Ca(NO_3)_2$, H_3BO_3 0.01%, $MgSO_4$ 0.1%, pH 6.5). Cells were then washed twice with PBS buffer solution and chromosomes observed by fluorescence microscope (OLYMPUS BX43) with optical filter, with 360 mm excitation wavelength and 460 emission wavelength.

Data analysis

The software of Image J (Schneider et al., 2012) was used for measuring the fluorescence values of pollen chromosomes. Mean fluorescence values of chromosomes of *C. lanatus* pollen irradiated by soft X-rays and control pollen were compared and difference analysis of data (> 300 pollen was counted respectively) were calculated with Excel 2007.

RESULTS

Morphological comparison between irradiated pollen and control pollen

Under scanning electron microscope (Figure 1), ripe *C. lanatus* pollen is elliptical, with clearly defined irregular germination apertures, and a reticular texture on the exine with a tubercle-shaped substance in the grids. Side-by-side comparison between soft X-ray irradiated pollen and untreated pollen found no obvious differences, indicating that irradiation by soft X-rays does not directly influence pollen exine.

Pollen germination on stigma and growth in style

Soft X-rays were less detrimental to cell activity than hard X-rays but can still directly damage cells. In this study, pictures taken under fluorescence microscope show that both soft X-ray irradiated pollen and control pollen were able to germinate on stigma (Figure 2A and B) and enter the ovary (Figure 2C and D) through the style. Pollen tubes reached the ovule within 2 days, then entered embryo sac (Figure 2E and F), and released spermatoblasts. The results indicated that soft X-ray did not affect the viability of pollen.

Difference analysis of sac and fertilization process

Paraffin sectioning of the watermelon ovaries after pollination allowed observation of the embryo sac and fertilization 1 to 7 days after pollination. No readily apparent difference was observed between pollination by irradiated pollen and control pollen in embryo sac development and the fertilization process (Figure 3A and

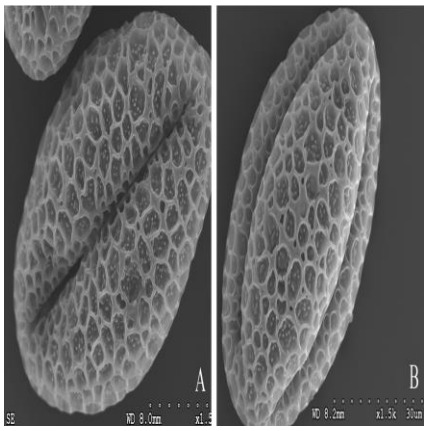

Figure 1. Photos of pollen by scanning electron microscopy. A. Control. B. Soft x-ray irradiated pollen treatment.

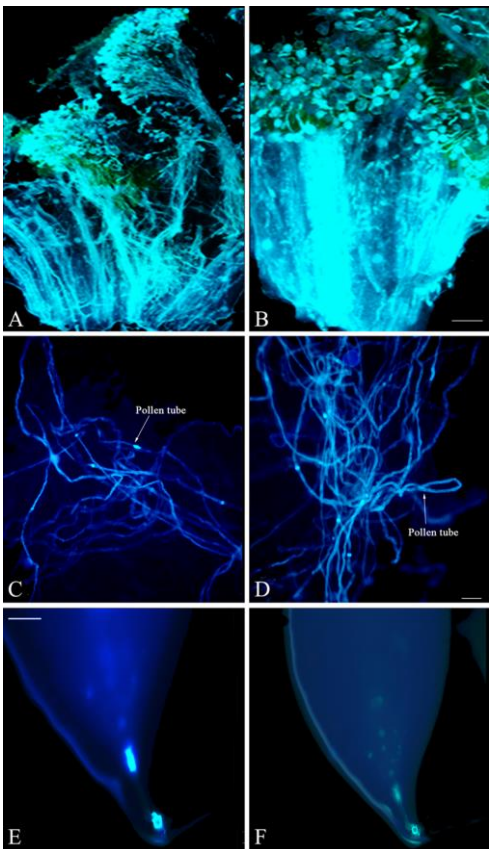

Figure 2. Pollen geminated and grew normally on stigma, entered into ovary, and then entered into embryo from micropyle. Controls are A, C, E. Soft x-ray irradiated pollen treatment is B, D, F. Scale bar is 1 mm in A, B, C, D and 200 µm in E, F.

B). Observation of embryo sac and fertilization 12 days after pollination showed many ovules in embryo sac, but with different levels of development. Formation of the embryo sac could not be observed in most *C. lanatus* pollinated by pollen. Amongst the few ovules that had formed in the embryo sac, the rate of abortion was very high. The embryo sacs were hollow and arrested growth of the ovule nucleolus affects the growth of the embryo sac. A black deteriorating structure was observed in the embryo sac but there was no fixed structure, then the embryo degrades and the ovule of the degrading embryo also does not develop. The endosperm degenerates (Figure 3A and B) and cannot develop into impervious seed of normal size as that of control *C. lanatus* (Figure 4A to C).

Difference comparison of *C. lanatus* pollen chromosome

DAPI can directly penetrate the cell membrane and bind to double-chain DNA in nuclei to show fluorescence over 20 times stronger than DAPI by itself. The fluorescence value of *C. lanatus* pollen chromosomes irradiated by soft X-rays is 107.05 while that of the control is 213.70. This indicates that pollen chromosome structure irradiated by soft X-rays and its DNA structure are damaged (Figure 5).

DISCUSSION

In this study, pollen irradiated by 600 Gy soft X-rays was used to fertilize diploid *C. lanatus* to produce seedless watermelon. No normal seeds were observed in the fruit but some abortive seeds were still present. Seedless watermelon produced by pollination with soft X-ray irradiated pollen were the same as the control with seeds in relation to size, shape, pulp color, skin thickness, and sugar content. In general, the quality is not influenced. Sugar content in some seedless watermelons even tends to be higher (Table 1). Sugiyama and Morishita (2000) fertilized normal diploid *C. lanatus* with irradiated pollen and abortive seeds were formed. This embryological research indicates that double fertilization still occurs after pollination and the embryo develops normally into a globular embryo, but then stops development and degenerates. Ovules of the degenerating embryos also do not develop, and so are unable to become impervious seeds of normal size but instead become abortive seeds that are smaller than normal seeds. Seedless mechanism of a mandarin cultivar 'Wuzishatangju' (*Citrus reticulata* Blanco) indicated that the activities of the male gamete and the fertility of the embryo sac were functioning normally with no embryo abortion during embryonic development. Gametophytic self-incompatibility (SI) caused seedlessness by blocking fertilization in the ovary (Ye et al., 2009). These are consistent with our result from observing *C. lanatus* pulp and the fertilization

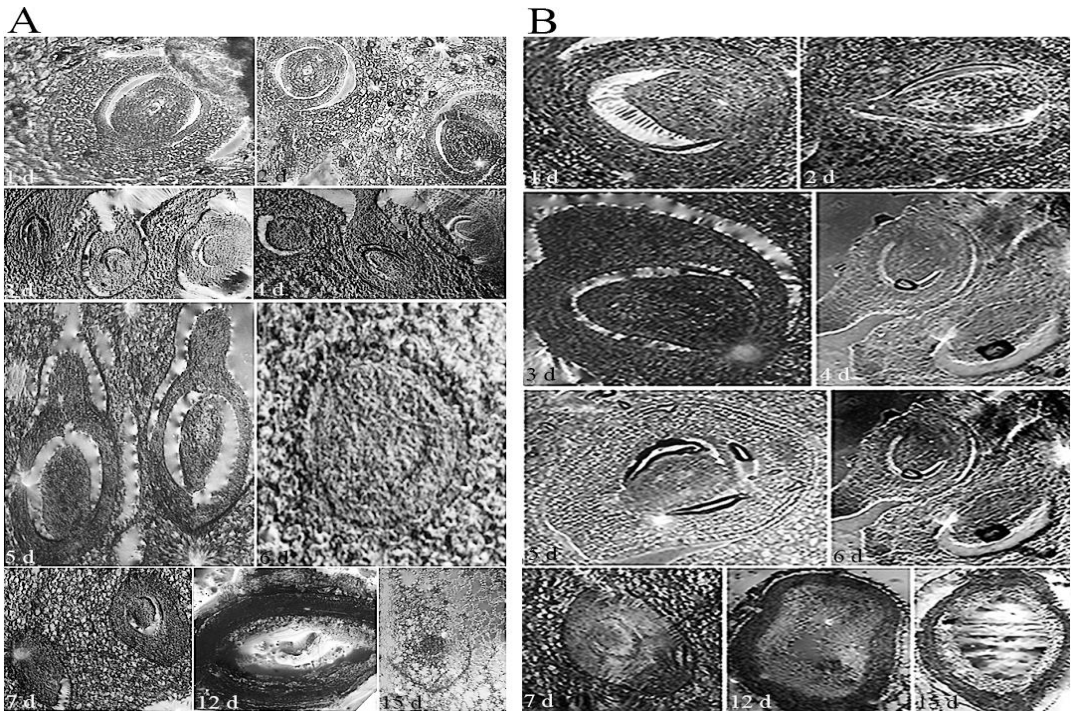

Figure 3. Paraffin sections of the watermelon ovary on days 1 to 15 after pollination. A. Control. B. Soft x-ray irradiated pollen treatment.

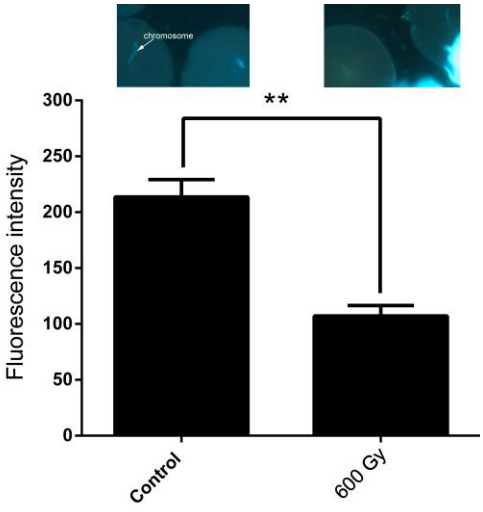

Figure 4. Watermelon which growing 25 days. The control and soft X-ray treatment of pollen at the comparison of the normal seeds of two varieties. Inner pictures were seeds. **, Highly significant (P <0.01).

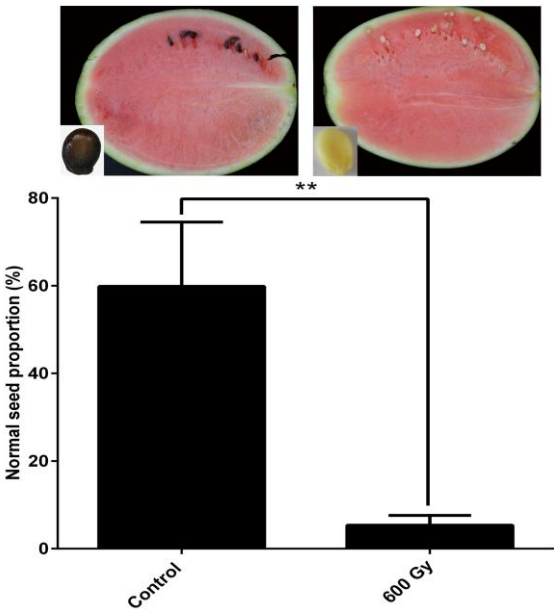

Figure 5. Pollen chromosome fluorescence value comparison. Inset pictures show watermelon pollen chromosomes after DAPI staining. **Highly significant (P <0.01).

process in the current study. Hu et al. (2007) found that *Citrus suavissima* Hort. Tanaka is seedless because pollen mutates during development and becomes inactive so that it cannot normally germinate on stigma. However, the research in this paper shows that *C. lanatus* does not become seedless due to pollen inactivity. Irradiation of *C. lanatus* pollen by soft X-rays damages the DNA double-helix structure of pollen, which can cause death of cells.

Table 1. Compared treatment with control on four main indicators.

Variety	Treatments	Central sugar concentration (%)	Edge sugar concentration (%)	Lycopene (μg/mg)	Citrulline (mg/100 g)
Huaimi	Control	9.93±0.29	8.87±0.33	1088.62±99.76	158.29±9.98
	600 Gy	11.43±0.23	10.07±0.54	1190.13±26.30	183.74±7.89

* All values are means +/- SD (n=18).

However, a low dosage of soft X-rays only slightly damages DNA structure and does not cause pollen death. Nevertheless, the change in DNA double-helix structure may influence chromosome pairing so that *C. lanatus* becomes seedless.

When researching changes in hormone content of ovule and sarcocarp of fertile and abortive Chinese dates, Luo et al. (2000) explored whether abortive embryos before and after stone hardening stage are directly related to hormone concentration in the embryo and sarcocarp. Chinese dates with abortive embryos contain clearly higher IAA, GA_3, and ZT, for example in sarcocarp than embryo in stone. Sarcocarp of seedless fruit contains more hormone than that of fruit with seeds. Thus, the sarcocarp is better able to grow and compete for nutrition than immature embryos, so embryos become abortive and fruits become seedless. According to research conducted by forerunners, the formation of seedless fruit is related to the development process of embryo sac. Nevertheless, it remains to be further researched whether seedless watermelon produced after soft X-ray irradiation is related to abortive embryos caused by differences of hormone content in the sarcocarp and immature embryo.

Conclusion

The research results of this paper show that irradiation of *C. lanatus* pollen by soft X-rays does not influence *C. lanatus* pollen exine, both soft X-rays irradiated pollen and control pollen can germinate on stigma, enter ovary through style, and the pollination and fertilization processes are also normal. However, irradiation of *C. lanatus* pollen by soft X-rays damages DNA double-helix structure of pollen chromosome so that *C. lanatus* embryo sac is blocked during development and the embryo gets aborted halfway. Then the ovules do not develop either, and are unable to become impervious seeds of normal size but become smaller abortive seeds. In this way, seedless watermelon is produced.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGMENT

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