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Seed abortion of 'Tosa-Buntan' pummelo pollinated with soft-X-irradiated pollens

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Abstract

Cross-pollination was performed with soft-X-irradiated hyuga-natsu pollens (1000 Gy) for 'Tosa-Buntan' pummelo (*Citrus grandis* (L.) Osbeck). This resulted in the transformation of large and complete seeds into small and empty ones (practically seedless). Although fruit set, fruit retention, total soluble solids content (TSS) and titratable acidity of the juice were not affected, decrement in the fruit size was observed. Two weeks after the pollination, endosperm cell division with free nuclei began in both the non-irradiated and irradiated pollen treatment conditions. Seven weeks after pollination, endosperm division with the cell wall occurred in the non-irradiated pollen treatment conditions; however, the endosperm development ceased in most ovules that underwent the irradiated pollen treatment, and the ovules remained in their free nuclear stage. The delayed degeneration of the ovules, following successful fertilization and commencement of endosperm cell division, allow these seedless fruits to be categorized as pseudo-parthenocarpic.

Keywords: *Citrus grandis*; Soft-X-ray; Pseudo-parthenocarpy

1. Introduction

'Tosa-Buntan' is one of the most popular domestic pummelo (*Citrus grandis* (L.) Osbeck) in Japan with fine aroma and taste; however, the fruit of this diploid cultivar contains usually numerous large seeds. Further, the seed formation is intensified by artificial pollination with pollens of other citrus; this is performed for the stabilization of the fruit set against its reproductive characteristics including high self-sterility and the absence of parthenocarpic ability.

Sugiyama and Morishita (2000a) developed a new method to produce seedless watermelon fruit in diploid cultivars by using pollen irradiated with 800 Gy of soft-X-ray by a irradiator. Prior to the innovation of this novel technique, seedless watermelons were mainly produced with triploid plants, which are obtained by crossing a diploid male with an autotetraploid female induced by colchicine treatment (Kihara and Nishiyama, 1947; Kihara, 1958; Terada and Masuda, 1943). The advantages of the method with soft-X-irradiated pollens include reduced process for triploid hybrid seed production and ease with regard to the direct application on current diploid cultivars without the breeding process.

Ohara et al. (2006) were the first to apply this method in 'Tosa-Buntan' pummelo. They demonstrated that cross-pollination through irradiation of hyuga-natsu (*Citrus tamurana* hort. ex Tanaka) pollens with 300–1000 Gy soft-X-rays caused the normal seeds to convert completely into empty seeds, which have only seed coats without inner tissues; a larger dose decreased the size of the empty seeds more effectively; however, it resulted in lower fruit retention, and the final fruit size was smaller.

The ovule pollinated with the irradiated pollen, which would become empty at harvest, must perform the role for fruit bearing during an early stage of fruit growth; this is due to the fact that production of true seedless fruits by inhibition of pollination are seldom in 'Tosa-Buntan' due to its poor parthenocarpic ability. It is necessary to elucidate the process of empty seed formation in order to understand the contribution and limitation of empty seeds to fruit bearing and growth and in

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order to improve this technique for the production of practically seedless pummelos.

In this study, we investigated the developmental processes of the embryo and endosperm of 'Tosa-Buntan' following pollination with soft-X-irradiated pollens.

2. Materials and methods

Between 26 April and 1 May 2006, the anther was collected from the flowers of hyuga-natsu that were planted in a greenhouse at Fruit Tree Experiment Station, Kochi Agricultural Research Center; subsequently, the anther was dehisced at a temperature of 28 °C for 24 h in a drying oven (SH-600, Yamato). The pollens were irradiated with 1000 Gy dose (15.0 Gy/min) of 10^4 to 10^5 eV-X-ray by a soft-X-irradiator (Soft X-ray Unit OM-B205, OHMIC) and preserved at −20 °C in a refrigerator until hand pollination was performed.

The experiment was conducted at the experimental orchard at Kochi University where 32 'Tosa-Buntan' pummelo trees (24 years old) were cultured. Four blocks comprising 8 trees were arranged randomly. Pollination was performed from 9 to 14 May 2006. Twenty-four raceme inflorescences in each tree, whose top flowers had already bloomed while the others remained in the balloon stage in the clusters, were selected and labelled for pollination treatments. All the petals and stamens of the 3rd or the 4th flower from the top of the cluster were removed with a pincette; the other flowers were thinned manually. Additionally, all the other flower clusters that were placed on the same bearing shoot of the emasculated flower were pinched. Immediately after emasculation was performed, the non-irradiated and irradiated pollens were hand-pollinated (eight flowers each) with a drawing brush; subsequently, all the flowers including the eight non-pollinated flowers were covered with small paraffin bags in order to avoid pollination by insects.

For each treatment, one of eight labelled fruits from each tree was measured fruit retention rate and fruit diameter every week; further, the fruits harvested on 20 December 2006 were examined seed number, fruit weight, rind weight, total soluble solids content (TSS) and titratable acidity of juice. TSS was estimated as Brix value determined by a refractometer (PR-100, Atago Co.), and titratable acidity titrated with NaOH was represented as mass of citric acid in 100 mL juice. Every data was analyzed for statistical significance by *t*-test.

One fruit of other labelled fruits in each treatment was sampled for tissue observation at 1, 2, 4, 7 and 10 weeks after pollination, if it remained avoiding physiological fruit drop; samples were preserved in FAA solution (5% formaldehyde, 5% acetic acid, 45% ethanol). The fruits were fixed in a mixture of ethanol and acetic acid (3:1, v/v) for 12 h and dehydrated in graded *n*-butanol series (*n*-butanol:ethanol:water in ratios of 20:50:30 for 24 h; 35:50:15 for 1 h; 55:40:5 for 1 h; 75:25:0 for 1 h; and 100:0:0 for 5 h and 100:0:0 for 24 h (v/v/v)). The samples were placed in a mixture of paraffin and *n*-butanol (1:1, v/v) at 58 °C, and the paraffin was concentrated by allowing evaporation of *n*-butanol for 3 days. Using a rotary microtome (Yamato Kohki Co.), sample sections (10 μm thick) were obtained by cutting the fruits in parallel with the fruit axis; subsequently, the sections were stained with safranin and fastgreen to enable observation under a light microscope (BX-50, Olympus Co.).

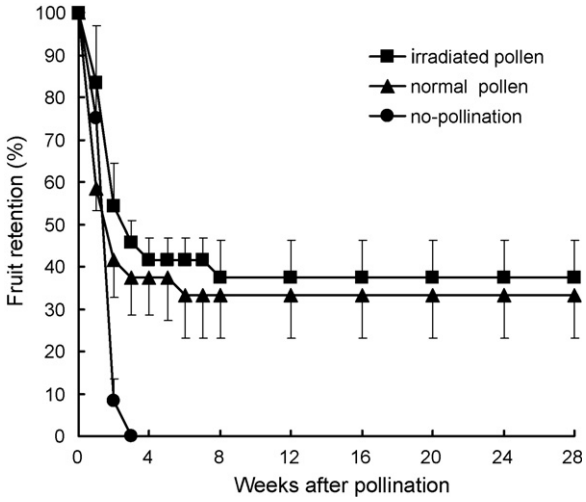

Fig. 1. Retention rates of the cross-pollinated fruits obtained with irradiated and non-irradiated pollens and non-pollinated ovaries. Values are means of four replications and bars indicate standard error.

3. Results

Three weeks after pollination, the physiological drop of flowers and fruit was occurred; all the non-pollinated ovaries were dropped during this period (Fig. 1). Four weeks after pollination, the decrease in the fruit number by physiological fruit drop plateaued in both the non-irradiated and irradiated pollen treatment conditions. Further, the fruit retentions at harvest were 33% and 38% when compared with that of the pollinated flowers in the non-irradiated and irradiated pollen treatment conditions, respectively. The increase in the transverse diameter of the fruits in the irradiated pollen treatment group was lower when compared with that observed in the non-irradiated pollen treatment group (Fig. 2).

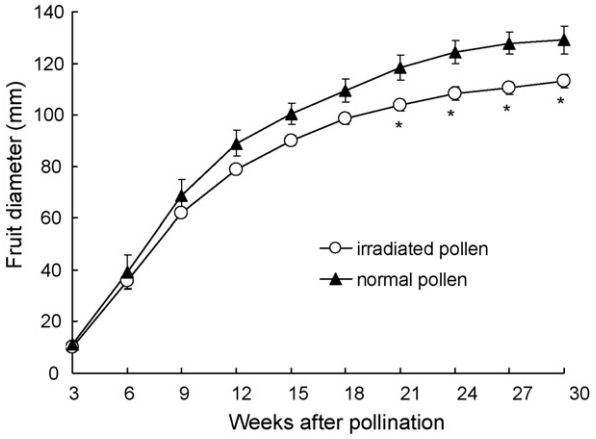

Fig. 2. The time-course of changes in the transverse diameter of fruits obtained from pollination with irradiated and non-irradiated pollens. Values are means of four replications and bars indicate standard error. Asterisks show the presence of statistical difference to non-irradiated treatment determined by *t*-test.

Table 1
Effect of pollination with irradiated and normal pollen on the number of seeds in the harvested fruit of 'Tosa-Buntan' pummelo

Materials used for pollination	Seed number per fruit				Total number of seeds weight (g)
	Perfect	Empty (large)	Empty (small)	Total	
Irradiated pollen	0.6 ± 0.5[a]	23.6 ± 7.7	66.4 ± 11.2	90.6 ± 16.7	1.1 ± 0.1
Normal pollen	74.5 ± 15.3	5.0 ± 1.5	21.2 ± 3.9	100.7 ± 12.9	22.1 ± 5.0
Significance[b]	**	**	**	N.S.	**

[a] Mean ± S.E. ($n = 4$).
[b] N.S. and ** indicate non-significant and significant differences at $P = 0.01$ by t-test, respectively.

Upon investigation of the harvested mature fruit, 74% of the total seeds were large and consisted complete cotyldones in the non-irradiated pollen treatment group (Table 1); on the other hand, more than 99% of the seeds in the irradiated pollen treatment group showed growth abortion to form thin and empty seeds (Fig. 3). Further, 89% of the seeds were smaller than 8 mm in longitudinal length. The total seed numbers for both the treatment conditions were identical (approximately 100) (Table 1). The fruit weight and size under normal pollen treatment condition were obviously larger than those in the irradiated pollen treatment condition; however, no differences were observed with regard to the flesh/whole-fruit ratio (w/w), TSS and titratable acidity of juice between the normal and irradiated pollen treatment conditions (Table 2).

One week after pollination, the synergid and antipodal cells remained in the embryo sac (Fig. 4A). Two weeks after pollination, endosperm cell division with free nuclei occurred in the ovules in both the treatment groups (Fig. 4B and C), thereby indicating successful fertilization; on the contrary, at the same time, the infertile embryo sac structure with distinct synergid and antipodal cells remained in the non-pollinated ovules (Fig. 4D).

Four weeks after the pollination, endosperm cell division with free nuclei was more prominent in the ovules in both the treatment conditions (Fig. 5A and B); however, the initial process of degeneration of the endosperm was evident in some ovules pollinated with irradiated pollens (Fig. 5C).

Seven weeks after pollination, most of endosperm cells that were partitioned with cell wall emerged in the ovules in the non-irradiated pollen treatment group (Fig. 5D), while no ovule in the irradiated pollen treatment achieved the stage of endosperm cell division with the cell wall. Additionally, the

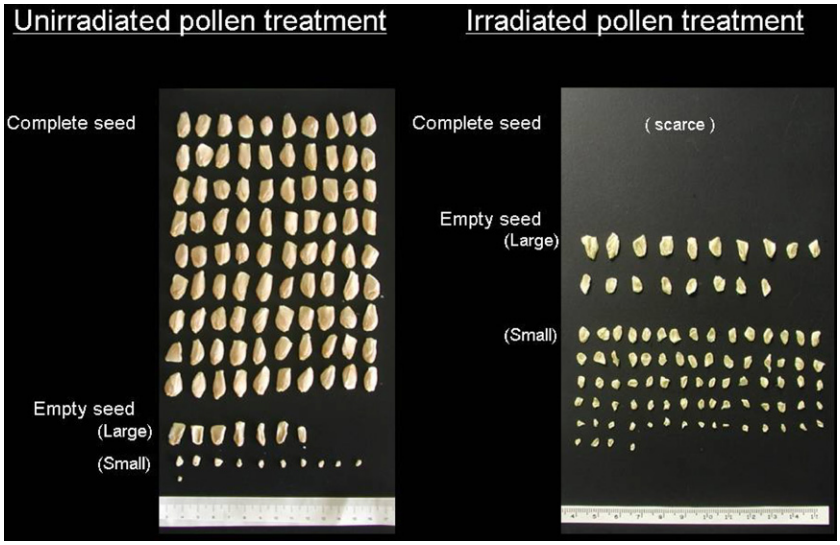

Fig. 3. Seeds excised from the cross-pollinated fruits obtained from irradiated and non-irradiated pollens.

Table 2
Effect of pollination with irradiated and normal pollen on the quality of the harvested fruit of 'Tosa-Buntan' pummelo

Materials for pollination	Fruit weight (g)	Fruit diameter (mm)		Flesh/whole fruit (w/w)	TSS content (Brix °)	Titratable acidity (citric acid g/100 mL)
		Transverse	Longitudinal			
Irradiated pollen	475 ± 34[a]	114 ± 3	99 ± 4	0.55	10.3 ± 0.3	1.22 ± 0.10
Normal pollen	740 ± 77	130 ± 5	111 ± 4	0.57	10.5 ± 0.2	1.11 ± 0.05
Significance[b]	**	*	*	N.S.	N.S.	N.S.

[a] Mean ± S.E. ($n = 4$).
[b] N.S., * and ** indicate non-significant, significant differences at $P = 0.05$ and $P = 0.01$ by t-test, respectively.

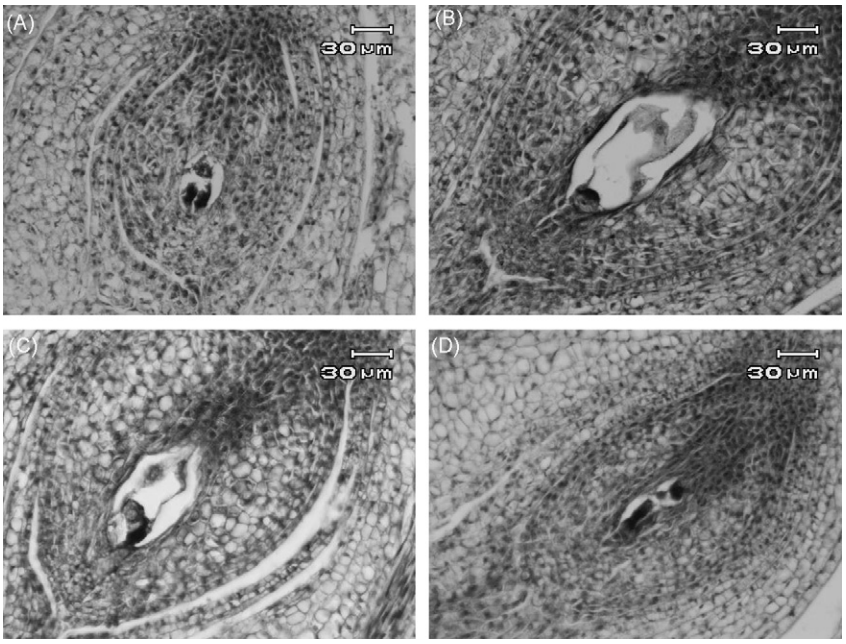

Fig. 4. Ovule development in pollinated and non-pollinated ovaries. (A) The embryo sac in an ovary pollinated with non-irradiated pollen at 1 week after treatment. (B and C) Endosperm division with free nuclei in fertilized ovules pollinated with non-irradiated (B) and irradiated pollen (C) at 2 weeks after treatment. (D) Embryo sac in a non-pollinated ovary contemporary with 2 weeks after pollination.

symptoms of degeneration of the embryo and endosperm in the irradiated pollen treatment group proceeded to the disappearance of nuclei and cavitation (Fig. 5E) and ovule collapse (Fig. 5F).

Ten weeks after pollination, the emergence of globular stage embryo (Fig. 5G), partially proceeding even to cotyledon formation (Fig. 5H), was observed in the non-irradiated pollen treatment group.

4. Discussion

In *Citrus*, there are a lot of varieties producing seedless fruit without fecundation; however 'Tosa-Buntan' requires cross-pollination with fertile pollen to set the fruit because of its high self-sterility and the absence of parthenocarpic ability (Yamamoto et al., 1995), resulting in numerous seeds formation.

Pollination with 1000 Gy soft-X-irradiated pollens did not obstruct fruit setting and retention (Fig. 1). This is unlike a preceding report (Ohara et al., 2006), which demonstrated that a larger dose of irradiation induced poorer fruit retention. The irradiated pollen treatment caused abortion in almost all seeds, which would complete with normal cotyldons if the ovules were fertilized with normal pollen (Table 1 and Fig. 3), like the findings of another previous study involving the production of seedless watermelons (Sugiyama and Morishita, 2000a; Sugiyama and Morishita, 2000b). The total number of seeds was not affected by the irradiated pollen treatment (Table 1); therefore, it is presumed that soft-X-ray irradiation does not affect fertilization itself.

There was no difference between the non-irradiated and irradiated pollen treatment conditions with regard to certain qualitative characteristics including flesh ratio, TSS and titratable acidity of juice. However, irradiated pollen treatment resulted in decreased fruit diameter and weight when compared with those observed in normal pollen treatment (Table 2); this was unlike that observed in watermelons, whose fruit weight was not affected by pollen irradiation (Sugiyama and Morishita, 2000a).

Two weeks after pollination, endosperm cell division with free nuclei was observed in a few ovules in both the treatment conditions (Fig. 4B and C). According to our observation, the initiation of endosperm cell division occurs at a time after fertilization has occurred; in the case of *Citrus*, this is considered to 7–15 days after pollination, as described in pummelos (Kitajima et al., 1997), *C. unshiu* and *C. natsudaidai* (Yang, 1968), and *C. tamurana* (Miwa, 1951). With regard to the ovules pollinated with irradiated pollens, successful fertilization and commencement of endosperm cell division, which were also found in the seedless watermelon ovules produced by the same method (Sugiyama et al., 2002), allow these seedless fruits to be categorized as pseudo-parthenocarpic, i.e. seedlessness due to delayed degeneration of embryo.

Iwamasa (1966) divided the possible sterility inducing seedlessness in *Citrus* into three classes: gametophyte sterility, self-incompatibility, and early embryo abortion. Most of seedlessness in parthenocarpic varieties are ascribable to male and/or female gametophyte abnormality; several instances are regarded as self-incompatibility type; however the pseudo-parthenocarpic embryo abortion has noted in few cases under natural condition (Iwamasa, 1966; Spiegel-Roy and Goldschmidt, 1996).

Non-pollinated flowers could mostly remain for 1–2 weeks and persist with their complete embryo sac structure (Fig. 4D);

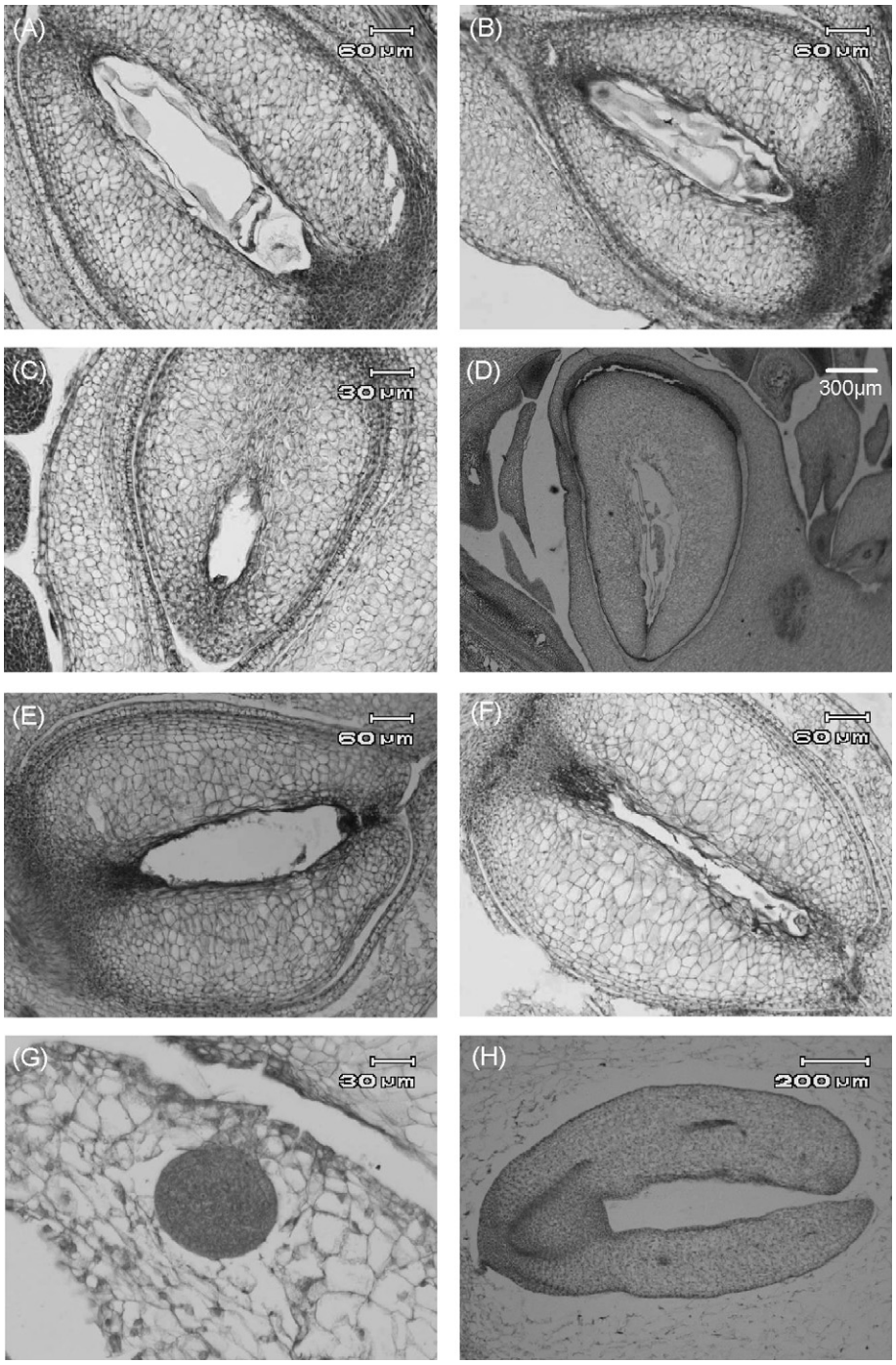

Fig. 5. Development and degeneration of ovule in ovaries pollinated with irradiated and non-irradiated pollens. (A and B) Endosperm division with free nuclei in a seed pollinated with non-irradiated (A) and irradiated pollen (B) at 4 weeks after treatment. (C) Symptoms of endosperm degeneration in a seed pollinated with irradiated pollen at 4 weeks after treatment. (D) Endosperm division with cell wall in a seed pollinated with non-irradiated pollen at 7 weeks after treatment. (E and F) Cavitary seed (E) and flat empty seed (F) in fruit pollinated with irradiated pollen at 7 weeks after treatment. (G and H) Globular stage (G) and torpid stage embryo (H) in seeds pollinated with non-irradiated pollen at 10 weeks after treatment.

subsequently, however, all of them dropped after 3 weeks of treatment; this was in accordance with the initiation of endosperm cell division that was observed in the non-irradiated and irradiated pollen treatment conditions. Therefore, it is likely that the established embryo and endosperm may generate certain physiological signals, which are related to the determination whether or not the ovule can be retained, as described by Kojima (1996).

Four weeks after pollination, free nuclear endosperm cell division was observed in more ovules in both the treatment conditions (Fig. 5A and B). Seven weeks after pollination, the cellular endosperm development was observed only in the

normally treated pollens (Fig. 5D); on the contrary, most ovules that were treated with the irradiated pollens demonstrated growth cessation and remained in the free nuclear endosperm stage (Fig. 5E), partially broke flatly with the disappearance of the endosperm tissue (Fig. 5F). Certain symptoms of endosperm degeneration were already evident as early as 4 weeks after pollination in the ovules treated with irradiated pollens (Fig. 5C). Ten weeks after pollination, globular embryo was first observed in the non-irradiated pollen treatment group (Fig. 5G).

Sugiyama et al. (2002) reported that embryonic development in the watermelon ovules that were treated with irradiated pollens continued up to the stage of globular embryo and occurred simultaneously with endosperm development with free nuclei for several days after pollination; subsequently, embryonic growth did not occur, and the embryos degenerated. However, no embryonic cell division was observed in our study.

Our results suggest that the crucial factor causing growth cessation of ovules pollinated with irradiated pollens was related to the abnormality in the endosperm during its conversion from the free nuclear stage into the cellular stage. This corresponds with certain anatomical aspects of ovules in 'Hiratanenashi' pseudo-parthenocarpic seedless persimmon as described by Sobajima et al. (1975), who demonstrated that abnormal endosperm division could have resulted in seed abortion of 'Hiratanenashi', because the embryo itself could be rescued by means of tissue culture. Zhuang et al. (1990a) proved that 'Hiratanenashi' persimmon is a natural triploid cultivar; subsequently, they insisted that the unbalanced chromosome numbers in the gametes must have led to the abnormal cell division and degeneration of the endosperm and embryo (Zhuang et al., 1990b). In the 'Tosa-Buntan' ovules that were pollinated with soft-X-irradiated pollens, it can be assumed that the incomplete zygote may have permitted endosperm cell division with free nuclei but not cellular endosperm division. Additionally, nonconformity of gametes in triploid plants due to the fragmentation of chromosome in polar nucleus by soft-X-irradiations would have occurred.

The decrement in the fruit size due to irradiated pollen treatment is an issue that must be resolved for the practical production of seedless fruit in 'Tosa-Buntan'. Therefore, it is necessary to develop newer techniques to facilitate fruit growth, for instance, by the application of plant growth regulators; additionally, it is necessary to explicate the hormonal effects on fruit growth not covered by degenerating seed in the future.

Acknowledgements

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