Effect of Pollen Behavior on Seedless Fruit Production of Mutant SoE Mandarin

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Abstract. Character improvement of citrus could be done by mutation breeding in which one of the purposes is to obtain seedless fruit. One of the mechanisms of seedlessness is through low viability and germination rate of pollen. This study was aimed to know the behavior of pollen of Mutant SoE Mandarin and its effect on seedless character of the fruit. The research was done in 2015-2016 in Laboratory of ICSFRI. Plant materials used were flowers derived from Mutant plants of SoE Mandarin, which were grown from irradiated budwoods of SoE Mandarin. The irradiated buds were grafted onto 6 months-old rootstocks of Japanese Citroen and maintained in pots in Tlekung Experimental Field ICSFRI. For controls, flowers from local Mandarin (SoE and Batu 55) varieties which were available during the study were used. The pollen were observed on the viability using acetocarmine 1% and successful pollination of flowers. The result showed that there was clearly different pollen viability of the mutant and control plants hence the different seed numbers. Mutant SoE has 36.7% viability and average number of seeds of 2.3 while SoE Mandarin has 98.94% viability and average number of seeds of 11.6. Observation on pollination showed that most pollens were arrested on stigma and less or no pollen found in ovaries of both self-pollinated flowers of Mutant SoE and cross-pollinated flowers of Mutant SoE x Batu 55. This assumed to be the reason of the low number of seeds on mutant fruits.

1. Introduction
Citrus is one of the most cultivated fruits in the world with a total production reached 139 million tons in the year 2018 [1]. Of the number, fifty four percent is oranges, twenty five percent comprises of mandarins and tangerines, fourteen percent is lemon and lime, and seven percent is grapefruit including pummelo. Total production in 2018 was nine percent lower than the production in the year 2017 which calculated around 146 million tons.

Citrus fruits have an important role in our daily life as food, source of nutrients and healthy substances [2]. Consumers show concern for these attributes when buying citrus fresh fruit [3] i.e. appearance, freshness, taste, size of the fruit, easy peeling, number of seeds, juiciness, and price. These attributes reflect consumer preferences that could be approached by variety improvement. One of the methods for variety improvement to obtain superior citrus fruit quality is through mutation breeding. By mutation breeding, some characters of citrus varieties were improved, for example seedless character in

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sweet orange (\textit{Citrus sinensis} (L.) OSB.), flesh color in grapefruit (\textit{C. paradisi} Macf.) and disease tolerance in lemon (\textit{C. limon} (L.) Burm f.) (Anderson, 2000; Deng, 2000, cited by Bermejo et al. [4]).

In citrus, seedless character is desirable since it offers efficiency in consumption for customers and is of great value especially for processing industry [5]. Seedless fruit in citrus could be obtained via parthenocarpy in the absence of fertilization, embryo sac abortion, polyplody, male sterility, adverse climate, hormone application (reviewed by Bender et al. [6]), also by crossing to obtain triploid hybrid, endosperm culture and irradiation [7].

Gamma rays is one of the mutagens frequently used in mutation breeding [8]. Study by Bermejo et al. [9] showed that mutations with gamma rays was effective to reduce the number of seeds from 9.03 to 0.23 - 2.47 per fruit in Murcott cultivar clones. Khalil et al. [10] and Goldenberg et al. [11]) both obtained mandarin mutant with less seed using gamma-ray irradiation in their respective studies. Kafa et al. [12] suggested that gamma ray radiation in the mandarin patch 'Nova' and 'Robinson' caused a reduction in seeds in mutants.

SoE Mandarin (\textit{Citrus reticulata}, Blanco) is one of the prime varieties of citrus in Indonesia which is characterized by its bright reddish orange peel which is difficult to find in other local mandarin. This type of citrus is preferred by consumers due to its attractive peel color [13]. To further improve its fruit quality, SoE Mandarin was used in this mutation breeding program which is intended to obtain seedless fruit while still keeping the genetic background. The purpose of this study was to know the behavior of pollen from Mutant SoE Mandarin resulted from gamma rays radiation.

2. 

Materials and Method

The research was done in Plant Breeding Laboratory of Indonesian Citrus and Subtropical Fruits Research Institute (ICSFRI), Batu East Java in 2015 - 2016. Plant materials used were flowers derived from Mutant plants of SoE Mandarin, which were grown from irradiated budwoods of SoE Mandarin. The irradiated buds were grafted onto 6 months-old rootstocks of Japanese Citroen and maintained in pots in Tlekung Experimental Field ICSFRI. For controls, flowers from local Mandarin (SoE and Batu 55) varieties which were available during study were used. SoE mandarin was used since it was the parent of the mutant plant. Batu 55 was used because the plants are abundant in the area and its pollen is greatly potential as pollen sources in typical citrus orchard in this area.

To observe the pollen behavior there were two activities done:

1. \textit{Identification of Pollen Viability}

The viability of the pollen was identified in the following way:

Ten flowers harvested from Mutant plants and controls and then placed at room temperature. When stamen broke, the pollen grains were collected in a vial and given 2-3 drops of 1% acetocarmine solution and let immersed for three hours. After that, the pollens were spread on slides and observed under microscope with 20-40x magnification. Lastly, the percentage of viable pollen was calculated.

2. \textit{Pollen Behavior after Pollination}

Observation of pollen behavior after pollination was done through these treatments:

(a). Cross-pollination between Mutant plants as male parent and local mandarin plants as female parent (Batu 55 Mandarin). The cross pollination was conducted as follows: pollens for pollination were harvested before blooming from 20-40 flowers from. The crowns and pistils were removed and let dry at room temperature for 24 hours. The pollen obtained immediately used for crossing. Meanwhile, flowers used for female parent were castrated and then pollinated with pollen from male parent which collected the day before. The pollinated flowers were covered with paper bag and labeled.

(b). Self-pollination of Mutant plants. Self-pollination was done in a similar way of cross-pollination except that the donor pollen came from the same plants.

(c). Negative control was used to see whether fruits could be produced without pollination. 10 flowers from Mutant plants were castrated, sealed with paper bag and left without pollination from other pollen.

(d). Open-pollination. This treatment was done to see the potential of Mutant plants to produce seedless fruits under common practices of citrus cultivation in Indonesia (natural pollination).
Several flowers from treatments a and b were harvested and observed at 3-4 days after pollination (DAP) using SEM which was done in Biology Laboratory of State University Malang. The rest of the flowers maintained until 4 months after anthesis. The fruits were then harvested (10 fruits per accession/variety), opened and number of seeds per fruits were counted.

3. Results and Discussions

3.1. Pollen Viability

Observation on pollen viability showed the pollen viability of mutant plant showed reduction greatly compare to its parent (SoE) or Batu 55 Mandarin (Table 1). In fruits resulted from open-pollination, the number of seeds of SoE and Batu 55 Mandarin showed their typical number of seeds while on the other hand, the mutant has lower number of seeds.

Table 1. Pollen viability and seed number of two local Mandarins and Mutant SoE

| Observation | SoE  | Batu 55 | Mutant SoE |
|-------------|------|---------|------------|
| 1           | 97.22| 97.79   | 54.88      |
| 2           | 100.00| 97.01   | 28.57      |
| 3           | 99.60| 96.03   | 26.65      |
| Average viability (%) | 98.94 | 96.94 | 36.70*     |
| Average number of seed/fruit op (normal + abortus) | 11.6 + 0.3 | 15.4 + 3 | 2.3 + 1.6 |

* significantly different with t test, α 5%; op: open-pollinated

Figure 1. Pollen from SoE Mandarin, before (a) and after staining (b) compare with pollen from Mutant SoE before (c) and after staining (d), 20x magnification.
Normal pollen for *Citrus reticulata* has typical circular-elliptical shape [14] which can be seen in pollen from SoE Mandarin before staining. These pollens also have uniform shape and size in general. On the other hand, pollen from Mutant SoE has slightly bigger size and some of them change in shape, shrinking and smaller in size. Staining treatment showed further that the changing in shape and size affect the viability. As seen in figure 1, normal pollens were viable showing full red color while those changing in shape were not. Mutant SoE has a lot of pollen with abnormal shape, crook and smaller in size compare to those of SoE (control). As reviewed by Kundu et al. [15], irradiation may reduce the citrus pollen capability to interconvert carbohydrate reserve. This further affects the state of cytoplasmic water resulted in dehydrated pollens causing them to look shrunken or necrotic. This resulted in meiosis abnormality or changes in pollen properties, consequently reducing pollen viability. These effects could be the reasons of low viability of Mutant SoE.

### 3.2. Pollen Behavior after Pollination

Behavior of pollen after pollination is summarized in figure 2. Abnormal pollen is difficult to germinate due to the decline in viability and fertility which then hamper their further move inside the style to reach the ovary. Pollen mostly found on the stigma and seemed they were arrested there. Further progress to the ovary was hard to be observed both in self-pollination of Mutant SoE and in cross-pollination Mutant SoE x Batu 55 either in 3 or 4 days after pollination. Only few pollens were observed in the style and much lesser in the ovary. In fact, pollens that could reach ovary were only observed in the self-pollination flowers both in 3 and 4 days treatment. Also, it was found that pollen already started to germinate in the third day in the ovary of the flowers of those treatment. In cross pollination flowers, it was more difficult to find any pollen in style and ovary section.

In flowering plants, pollen germination in vivo occurs in the stigma and then pollen tube grows straight to the ovary [16]. When pollens landing on stigma, the pollen will enlarge because it absorbs liquid on the surface of the pistil. The inner lining (intin) along with the protoplasm of the pollen will protrude to form a tube through the aperture, commonly called the germ pore. The tube will extend and find a way through the tissues on stigma and style until it enters the embryo sac inside the ovule. In the embryo sac, precisely in synergid cells, pollen tubes will break and free the nucleus sperm. If there are no obstacles, then the fertilization process will occur [17]. Thus, any defect on pollen and obstruction cause by mutation during this whole process may affect the fertilization, hence, reduce the chance to form seed.

Study by Distefano et al. [17] showed that in pollination with normal citrus pollen, about 75% pollen which accounted for about 100 pollen grains, could germinate on the stigmatic surface and could reach the upper part of the style during 48 hours after pollination. The number decrease to 8-12 grains when pollen tube reached the base of the style. Yamamoto et al. [18] used the number of pollen tube present in the upper, middle, and basal portions of the style and their growth behavior 6-8 days after pollination to determine incompatibility or compatibility of citrus pollination. Other studies on citrus pollination also showed the probability that there are more development could be observed after 8 days [19][20]. Unfortunately, we could not confirm this in this study due to shortage of flower material for further observation. Therefore, it is difficult to conclude what kind of mechanism is used in the process of seed formation which resulted in seedless fruit apart from the low viability of pollen from mutant plant. More study is needed to have a better understanding on the process.

Our finding regarding low viability pollen caused by mutation which resulted in seedless fruit is in line with the result of other studies such as study by Bermejo et al. [4] on parthenocarpic ‘Moncada’ mandarin resulted from gamma irradiation. In their study, the viability decreases to lower than ten percent compare to more than eighty percent in control plants. This confirm that irradiation is an important method to obtain citrus seedless cultivar from seedy ones which is not only observed on mandarin [10], [11] but also confirm in other types of citrus such as pummelo [21], orange [22], and lemon [23].
Figure 2. Pollen behavior after pollination. A: Mutant SoE self-pollinated 3 DAP; B: Mutant SoE x Batu 55 3 DAP; C: Mutant SoE self-pollinated 4 DAP; D: Mutant SoE x Batu 55 4 DAP; St: Stigma; S: Style; O: Ovary; circle: pollen; arrow: germinated pollen.

4. Conclusions
Mutation has prominent effect on pollen viability of Mutant SoE which affects the number of the seed on fruits. Mutant SoE has 36.7% viability and average number of seeds of 2.3 while SoE Mandarin has 98.94% viability and average number of seeds of 11.6. Observation on pollination showed that most pollens were arrested on stigma and less or no pollen found in ovaries of both self-pollinated flowers of
Mutant SoE and cross-pollinated flowers of Mutant SoE x Batu 55. This assumed to be the reason of the low number of seeds on mutant fruits.

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