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Seedless fruit pummelo induced by Gamma Ray irradiation: Fruit morphological characters and stability evaluation

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Abstract. *Mariana BD, Arisah H, Yenni, Selvawajayanti M. 2018. Seedless fruit pummelo induced by Gamma Ray irradiation: Fruit morphological characters and stability evaluation. Biodiversitas 19: 706-711.* Pummelo is one of native citrus crops found in South East Asia including Indonesia. It is well known due to its big size and distinct taste compared to other citrus fruits. However, it contains many seeds, which may reduce the market demand and customer preferences. Therefore, the objective of this study was to generate seedless citrus by mutation techniques using gamma-ray radiation. The mutant induction of Pamelo Nambangan using gamma-ray radiation was conducted in 2003 using bud wood as plant materials. The bud wood was then irradiated with the dosage of 20, 40, and 60 Gy. The buds then grafted to Japansche Citroen rootstocks and maintained properly until the fruits could be observed. After three years of selection based on performance and fruit evaluation on MV2 generation, a mutant plant derived from 20 Gy irradiation treatments were obtained with improved character on the number of seeds. Pamelo Nambangan has more than 40 seeds/fruit and the mutant has less than 10 seeds/fruit on average. In the seedless mutant, it was observed that the mutant has embryo sac abortion leading to seedless fruit and low pollen viability (7.7%). The mutant has been released in 2016 as a new seedless pummelo variety under the name of 'Pamindo Agrihorti'.

Keywords: Mutation breeding, pummelo, seedlessness

INTRODUCTION

Pummelo [*Citrus maxima* (Burm.) Mer.] is one of the native citrus species in South East Asia (Wu et al. 2014). Pummelo has easily recognized due to its unique morphological characteristics such as broad leave, large flower, and big fruit (Susandarini et al. 2013). The fruit is usually consumed fresh with the taste flavor is a combination of various level of sweet, acid and bitter. Furthermore, it has nutritional value and antioxidant compounds (Toh et al. 2013; Sing et al. 2015). Pummelo contains a source of flavonoids, mainly naringin, which is responsible for its bitter taste (Chaiwong and Theppakorn 2010; Shah et al. 2013).

Indonesia has many local varieties of pummelo. Up to 2015, 17 varieties of pummelo collected from the indigenous population in several regions in Indonesia have been released (balitjestro.litbang.pertanian.go.id). Of the 17 varieties, the most cultivated variety of pummelo is 'Pamelo Nambangan' due to its long shelf life, good taste, and seedless potency (Pangestuti et al. 2004). For fresh consumption, seedless fruit is necessary since it is easy and convenient to be consumed (Qin et al. 2015). Seedless means no seed, traces of aborted seed or the reduced number of seeds (Varoquaux et al. 2000). Pummelo fruit containing less than 10 seeds/ fruit is considered seedless (Susanto et al. 2011).

One of methods to improve the fruit quality of 'Pamelo Nambangan', especially the seedless potency, is breeding program using induced mutation by gamma-ray irradiation. It has been reported as a successful tool to reduce seed in citrus particularly from the ones with seedy character (Bermejo et al. 2012). Khalil et al. (2011) and Goldenberg et al. (2014) obtained mandarin mutant with less seed using gamma-ray irradiation in their respective studies. In addition, mutation breeding could also improve other characters in citrus such as flesh color and disease tolerance (Jain 2010).

Most citrus varieties are propagated vegetatively by grafting, thus the materials used for irradiation are usually budwoods, which are then grafted on rootstocks (Khalil et al. 2011; Goldenberg et al. 2014; Montañola 2015; Kafa 2015). In addition, citrus is highly heterozygous plant having long juvenile phase and mostly polyembryogeny (Novelli et al. 2006; Gulsen et al. 2010), which makes it complicated to use the seeds as materials for irradiation treatment.

There are many possible factors inducing seedless fruits, namely female or male sterility, defective ovules and embryo-sac abortion, self-incompatibility, polyploidy, certain environmental condition and application of gibberellins (Vardi et al. 2008; Bermejo et al. 2011). For pummelo, self-incompatibility has been observed as one of factors causing seedless fruit (Yamamoto et al. 2006; Ngo et al. 2010). This study presents the progress of mutation breeding program on pummelo, emphasizing the current result on fruit evaluations for seedlessness.

MATERIALS AND METHODS

Plant materials and irradiation treatments

The research was started in 2003 with bud woods of 'Pamelo Nambangan' as plant material, which was then irradiated with dosage of 20, 40 and 60 Gy using Cobalt-60 source in Center for Application of Isotopes and Radiation Technology, Pasar Jumat, Jakarta. The buds were then grafted onto 6 months-old rootstocks of Japanese Citroen and maintained in pots in Tlekung Experimental Field, Batu, East Java located at 950 m above sea level (as described previously by Sutarto et al. 2009). Only 24 plants derived from 20 Gy treatment was survived while irradiated plants with the dosage of 40 and 60 Gy failed to grow at early development. To grow optimally, the survived plants were regrafted (MV2) then transferred to the field in Kraton Experimental Field, Pasuruan, East Java, which is located at low land, 5 m above sea level.

Branch and fruit evaluation

The survived plants started to bear fruits in the field in late 2012. To avoid the risk of chimera, every bearing-fruit-branch was labeled with a unique number for documentation and the ones with seedless fruits (≤ 10 seeds/fruit) were counted and observed for further evaluation. 15-20 fruits aged 25-27 weeks after anthesis were observed for phenotypical characteristics including fruit size and weight, peel thickness, juice volume, taste, total soluble solid and total acid. Fruits sizes were measured by tape; fruit weights were measured using electronic (digital) balance, and fruit peel thickness was measured using calipers. Fruit juice was extracted from the pulp by straining through a muslin cloth, which was then measured using a cylinder measurement. The total soluble solid content of fruits was observed using a hand-held refractometer. Juice acidity was estimated by titration using 0.1 N NaOH and phenolphthalein as an indicator.

Pollen observation in relation to seedless characters

Initial study on the mechanism of seedless was done through observations on flower characteristic and pollen viability. Flower characteristics were observed under a light microscope, Olympus SMZ16. Pollen viability was observed using staining method with 1% acetocarmine. Pollens from 10 flowers of each selected mutant and flowers of Pamelo Nambangan as control were collected and put into vials containing 1% acetocarmine solution. The pollen was then immersed in acetocarmine for 3 hours. After immersion process, pollens were distributed on glass slide and observed using light microscope Nikon R2. Pollens with abnormal shape and light or half coloring were considered unstained while pollens with intact exine and fully colored were classified as stained pollens. Data were collected from ten different fields of views. Percent viability was counted as the percentage of viable pollen in comparison to the total analyzed pollen grains x100.

RESULTS AND DISCUSSION

Branch and fruit observation

The result of branch observation of 22 pummelo mutants was presented in Table 1. In this study, the branch observation of mutant number 6 and 10 was excluded due to poor plant performances. Of all pummelo mutants, it shows that the branch of mutants no. 4, 17 and 22 had a high percentage of seedless branches. Interestingly, mutant number 4 had the highest branch number of all observed mutants. In addition, it also had a better performance on plant vigor compared to all mutant plants although this needed to be further observed comprehensively.

The stability of seedless characters of fruits was further observed in 2014 due to the period of fruit production of pummelo which needs much longer time. Table 2 shows the result of fruit observation of mutant plant no. 4. The most notable difference between the parent and mutant was the number of seed along with flesh color. It shows that the seed number of mutant plant significantly reduced compared to that of the parent. Another important character to value the citrus quality is the juiciness, which is mostly indicated by the volume of juice. Mutant plant no. 4 shows higher volume of juice compared to that in Pamelo Nambangan. However, the fruit appearance between mutant plant and parent in term of the fruit shape had no difference, showing a combination of spheroid and pyriform shape. The difference between them was shown prominently when the fruit was cut, indicating the less seed contained and more intense red color of flesh of the mutant fruit compared to that of parent (Figure 1).

Table 1. Number of branches in relation to seedless fruit characters observed in 22 Pummelo mutant accessions from 2012-2013

Code of mutant accession	Total number of branch	Number of branch with seedless fruits	% Seedless branch
1	26	0	0
2	32	0	0
3	34	0	0
4	64	62	96.88
5	45	0	0
7	34	0	0
8	29	0	0
9	41	0	0
11	33	31	93.94
12	53	0	0
13	48	7	14.58
14	25	4	16
15	9	2	22.22
16	9	0	0
17	42	41	97.62
18	16	0	0
19	10	0	0
20	20	0	0
21	27	0	0
22	23	23	100
23	34	0	0
24	11	0	0
Control (Pamelo Nambangan)	37	0	0

The morphological performances of flower and pollen exhibiting seedless fruit characteristics

The flower characteristics of mutant plant number 4 and control was observed by cutting **horizontally** the ovary of open-pollinated flowers aged approximately seven to ten days after anthesis. It shows that both Pamelo Nambangan and the mutant share similar characteristics of flower (Figure 2. A). The anther dehisces before the flower opens in balloon stage. The stigma is located slightly higher than the anthers and it is already receptive before anthesis. It means that the stigma could be pollinated only when the flower opens since it needs either wind or insects to help the pollination process. Ovary transverse section (Figure 2.B) shows locules, which will further develop fruit segments, and ovules, which later develop seeds. The number of seed per fruit segment could be counted by observing the number of ovule per locule. The locule with shrunk embryo sac, meaning no ovule observed, will later develop into fruit segment without seed. On the other hand, locule with a number of ovules will become fruit segment with several seeds (Figure 2.C).

Of the pollen performances, it shows that the viability percentage of Mutant no. 4 was much lower (7.73) than that of control (96.51). The number of pollen was significantly different with the pollen viability between Pamelo Nambangan and Mutant no. 4. From the pollen shape, it shows that most pollens of Pamelo Nambangan mostly had the same size. Most pollens showed deep red colored indicating its viability. In contrast, most pollen of Mutant no. 4 was not colored, had faint or half colored and varied in size and shape. Most of stained pollens of mutant plant number 4 had bigger size than non-stained ones (Figure 3).

Table 2. Several fruit characteristics of mutant plant no. 4 in two different years compared to control (Pamelo Nambangan)

Characters	Pamelo Nambangan*	Mutant plant no. 4	
		2013	2014
Fruit diameter (cm)	49.41	45.83	48.84
Fruit height (cm)	48.16	44.70	47.9
Fruit weight (g)	1139	1025	1019
Fruit segment	15	12	12
Peel thickness (cm)	2.01	1.63	2.36
Total soluble solid (^{o}brix)	10.1	9.7	9.5
Total acid (%)	3.5	2.8	1.9
Number of seed	41.2	6.2	4.1
Volume of juice (mL/100g)	42.3	56.75	58.83
Taste	Sweet, slightly acid	Sweet, slightly acid, slightly bitter	Sweet, slightly acid, slightly bitter
Flesh color	Pink	Red	Red

Note: *Based on the variety release document of Pamelo Nambangan

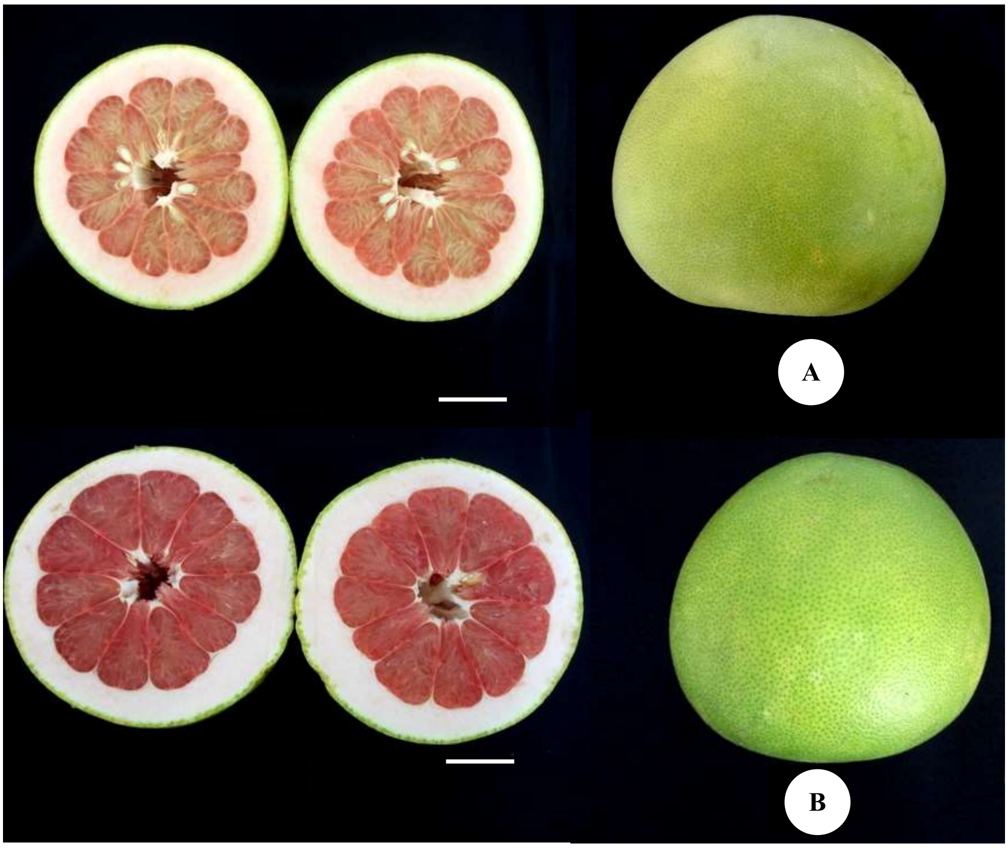

Figure 1. Transverse section of fruit of Pamelo Nambangan (A) and mutant plant no. 4 (B). Bar = 5 cm

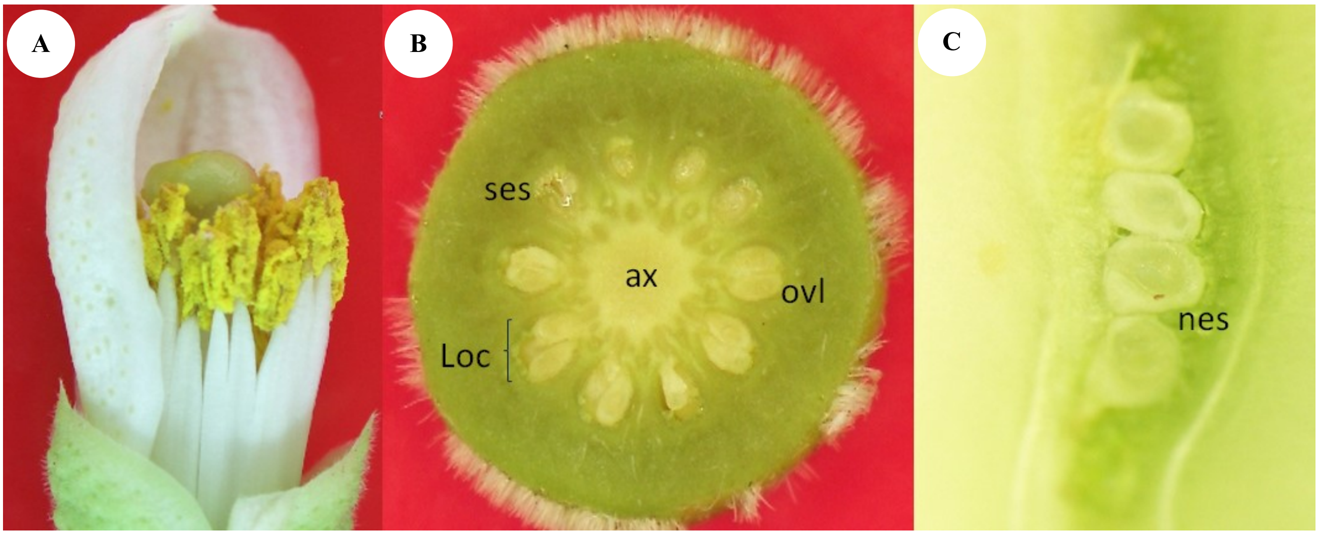

Figure 2. Flower and ovary cut. A. A fresh balloon-stage flower, B. Transverse section of ovary from Mutant no. 4, C. Longitudinal section of locule from flower of Pamelo Nambangan, Abbreviation: shrunk embryo sac (ses), normal embryo sac (nes), axis (ax), locule (loc), ovule (ovl)

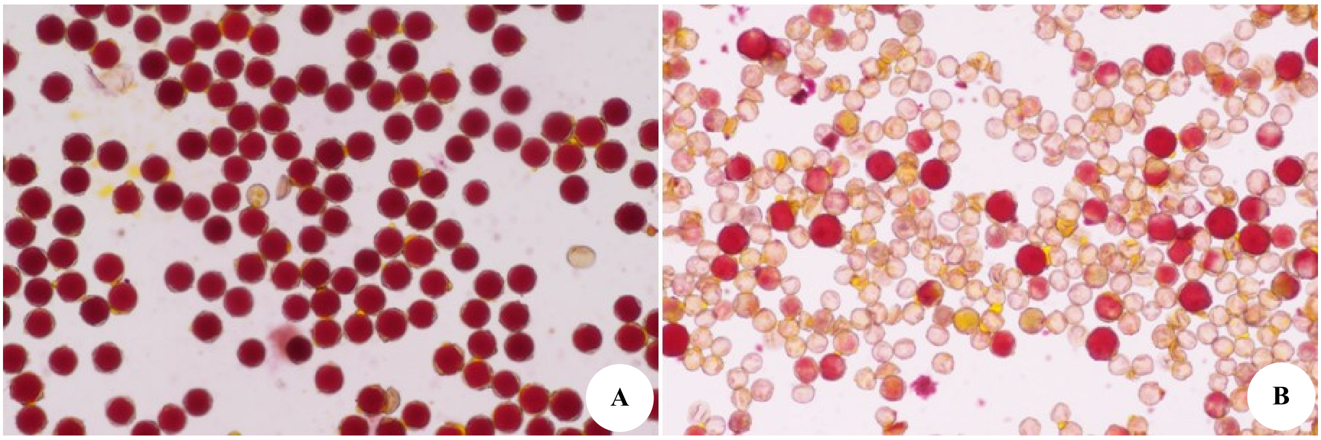

Figure 3. Pollen staining of Pamelo Nambangan (A) and mutant no. 4 (B)

Discussion

In this study, branch evaluation for the seedless character is one of the crucial steps in the selection program. Since our irradiated material was a bud, chimera may be one of the concerns when growing the bud to an intact plant. By doing branch selection, we expected that the branch will be developed from a single mutant cell, thus the stable and uniform performance of plants could be observed in the whole plants.

Fruit shape of mutant accession and Pamelo Nambangan is a combination between spheroid and pyriform in general. However, we also found some of the fruits of mutant have pyriform shape especially at the beginning of fruit observation when the plant has just started produces fruits in the field. According to Susanto et al. (2011) seedless pummelo fruits usually have pyriform shape, but they also found a variety of spheroid-pyriform shape with seedless fruit (Bali Merah 2). Furthermore, they suggest that fruit shape could change due to environment factor in different observations of an accession.

For fresh consumption, seed number and juiciness along with other sensory attributes such as flavor (sweetness, acidity) are important in Mandarin citrus due to consumer preferences (Houqe et al. 2011). These attributes were also important in pummelo citrus, since it is mostly consumed in fresh condition. However, the study of consumers' preferences of pummelo in Indonesia has not been done, yet. Susanto et al. (2011) observed that the number of seed in Pamelo Nambangan is between 1 to 67 seeds/fruit while in the mutant plants, it was ranging from 27 to 66 seeds/fruit. On the other hand, Mutant plant no. 4 showed much less range, from 0 to 11 seeds/fruit. This result was in line with other studies on the effect of gamma rays in citrus. On lemon, Gulsen et al. (2007) obtained most seedless fruit from 50 Gray treatment while Spiegel-Roy et al. (2007) successfully obtained seedless fruit with

13.3 Gray. On mandarin, Kafa et al. (2015) obtained most mutant plants with seedless fruit with 30 Gray while Montañola et al. (2015) obtained seedless fruit with 40-50 Gray. On pummelo, on the other hand, there was only one seedless pummelo plant obtained from 95 Gray in a study by Somsri et al. (2008). It seems that the dosage they used for pummelo in their study is too high which resulted in fewer plants survived. Overall, since the result of the studies were varied, it is difficult to make a general conclusion regarding the best dosage of gamma-ray irradiation for specific citrus species. Nevertheless, based on the result of this study and the aforementioned studies, dosage between 13-50 could be used for citrus in general.

Since appearance is also an important attribute for consumers (Yosini 2011; Gao et al. 2011), the more intense color of fruit flesh will likely attract more customer's attention. In addition, red-flesh color may indicate that it contains healthier nutrients. A study by Liu et al. (2016) showed that red-fleshed pummelo has more lycopene and β-carotene than the pummelo variety with lighter color flesh. These carotenoids are crucial for health since they work as precursors of vitamin A, which is of importance to produce antioxidant activity and to protect against several serious diseases (Wang et al. 2011; Liu et al. 2016). Furthermore, the bitter taste may also be related to more flavonoids content. These healthy substances could be one of the selling points toward consumers especially those who interested in functional food. It is also important to note that most mutant characters are similar to the parent, confirming that the mutation is an effective approach to change one character while preserving the majority of the characters.

The flower characteristic for pummelo that we obtained in this study was quite unexpected since based on our observation on other citrus types, such as mandarin, anther dehiscence occurs after the flower opens. This unique character is clearly not an effect of induced mutation since the control plant as well as some other non-mutant accessions of pummelo in our germplasm collection also shows similar character. The finding regarding this is opposed to that of Hoque (2015) who studied in Bangladesh using ingenious varieties where anthesis of flowers firstly occurs, which were then followed by anther dehiscence about 10 hours later. Since it is obvious that both studies used different varieties, it is predicted that this flower characteristic is genotype dependence. In addition, based on our observation, most flowers of both Pamelo Nambangan and the mutant accession opened at 9-10 am. On the other hand, Hoque (2015) found that flowers commonly open around 3.30 to 4 am, and pollen dehisces in the afternoon. Most likely, besides genotype, type of climate also plays a role in the difference of flower anthesis/anther dehiscence characteristic.

The pollen viability test showed that mutant plant no. 4 had lower pollen viability compare to that of Pamelo Nambangan. Most non-viable pollens showed change in shape, either shrunken or smaller in size compared to that of normal pollen. Acetocarmine binds to the pollen nuclei and cytoplasm to a lesser degree. Non-stained pollen may have defects or abnormalities in its nuclei hence acetocarmine cannot bind to it. It is assumed that the effect of mutation caused abnormalities in pollen formation and its components which then affected the pollen viability. Low pollen viability means that it may not be able to germinate after pollination hence it will affect seed formation. Low pollen viability is one of many possible causes of seedless fruit (Khalil et al. 2011).

Studies by Ngo et al. (2010) and Yamamoto et al. (2006) on pummelo indicated that all varieties observed in their studies are all self-incompatible. There is a high probability that Pamelo Nambangan, which showed seedless potency, and mutant plant no. 4 showing consistent seedless character are both self-incompatible. The level of self-incompatibility for mutant accessions and the control, as well as other pummelo varieties in Indonesia, is not yet known. More researches are needed to find out the self-incompatibility character in pummelo varieties. The information on that character will give insight to the seedless potency of each variety.

Embryo sac abortion affects seedless fruit in several studies. A study in *Arabidopsis* showed that embryo sac formation is dependent on the development of normal ovule (Koltunow et al. 1995) since embryo and ovule development are related. We assumed that self-incompatibility, low pollen viability and embryo sac abortion are the main cause for the seedlessness of Mutant no. 4. However, the seedlessness mechanisms are still unclear. In addition, our attempt to study pollen fertility of mutant by conducting control pollination showed inconclusive result (data not shown). Therefore, in-depth study is needed to reveal the main cause and the mechanism of seedlessness of mutant plant no. 4.

This study showed that seedless pummelo could be obtained using mutation breeding using gamma-ray irradiation. The character is stable in two subsequent observations in different years. At the end of 2015, the Mutant no. 4 was proposed to be released as a new variety. After confirming the character stability and plant vigor observation, it was released in early 2016 as a new variety under the name of 'Pamindo Agrihorti'.

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