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Phenotypic Diversity of MV1 Generation Mutants of Terigas Orange Induced by Gamma-Ray Irradiation

Noor Farid*, Agus Sarjito, Zulfa Ulinnuha, Marya Ulfa

Department of Agrotechnology, Faculty of Agriculture, Universitas Jenderal Soedirman, Purwokerto, Central Java 53122, Indonesia

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ABSTRACT
Trigas orange is a nationally superior variety from Sambas, West Kalimantan, but its fruit quality remains less competitive than imported varieties, which are typically brightly colored and seedless. Genetic improvement through gamma-ray irradiation was applied to enhance its traits. This study aimed to evaluate the effects of 6 kRad gamma-ray irradiation on the morphological and fruit characteristics of Terigas orange mutants. Conducted from May to July 2023 at the Screenhouse, Faculty of Agriculture, Universitas Jenderal Soedirman. The experiment used a Randomized Block Design (RBD). The data were analyzed using LSD and the Least Significant Increase (LSI) Test at the 5% level is used to compare the diversity of mutants with normals. Results showed significant variations in plant height, stem diameter, leaf width, and petiole length. Qualitative changes were observed in leaf shape, margin, base, type, color (upper and lower surfaces), and length. Mutant fruits exhibited stronger aroma, higher brix levels, a sweeter yet slightly sour taste, and more seeds compared to the normal.

Keywords: Citrus improvement; Fruit quality; Gamma-ray irradiation; Morphological variation; Terigas orange

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INTRODUCTION
The Terigas citrus was a nationally superior citrus fruit from Tebas District, Sambas Regency, West Kalimantan. It was released as a superior variety in 2009. The fruit has a sweet, slightly sour, and refreshing taste. When ripe on the tree, the juice tastes like honey. Citrus fruits are in high demand in the market. They are high-quality, have a sweet taste or high organoleptic properties, are easy to peel, and have a good skin color. However, current consumer preferences also tend towards seedless or few seeds fruit. Seedlessness also was an essential criterion in the international citrus industry. Despite their high organoleptic characteristics, the presence of numerous seeds is a marketing obstacle for citrus fruits. Furthermore, various imported citrus fruits also exhibit seedless characteristics. (Kosmiatin and Husni 2018).

One way to improve the quality of local citrus fruits is through plant breeding. High juvenile seedling rates and heterozygosity with polygenic phenotypes often hamper conventional citrus breeding (Munankarmi et al. 2023). These obstacles can be overcome through mutation methods, namely mutation induction, using gamma-ray radiation. Gamma rays are a form of electromagnetic radiation with high energy and strong penetrating power, making them frequently used in mutation induction techniques in plants (Anggriansyah et al. 2024). Their ability to penetrate plant tissue deeply allows these rays to reach and affect the genetic material in meristematic cells, which are the centers of plant growth. Therefore, gamma rays are an effective mutagenic agent in generating random but potent genetic variation. This technique has been widely used in plant breeding programs, particularly to obtain genetic diversity in plants with a narrow genetic base that is difficult to cross conventionally (Hassanat et al. 2019).

Mutation induction by irradiation resulted in the highest number of mutants (approximately 75%) compared to other treatments, such as chemical mutagens (Hase et al. 2023). Changes caused by administering physical and chemical mutagens can occur at the genome, chromosome, and DNA levels. The gamma-ray irradiation mutation technique is more advantageous because of its rapid process and the ability to improve one or two plant traits (Nandariyah et al. 2020). Mariana et al. (2018) stated that gamma-ray mutation effectively produces seedless Pummelo, because gamma ray irradiation can disrupt meiosis, resulting in incompletely developed ovules or aborted embryos, resulting in seedless or very few seeded fruits. Furthermore, irradiation can also affect the activity of plant hormones such as auxin and gibberellin, which play

***Corresponding Author*:
E-Mail: noor.farid@unseod.ac.id

a role in seed formation and fruit development. With the disruption of hormonal balance due to mutations, seed formation can be inhibited, but the development of the fruit wall (pericarp) continues, resulting in parthenocarpic fruit.

In this study, citrus mutants were characterized to obtain distinctive traits. (Seminara et al. 2023) stated that characterization is crucial in identifying essential characteristics of economic value or defining the variety. This study aims to determine 1) the effect of gamma ray irradiation on the performance of Terigas orange mutants, 2) compare mutants with normal ones and select mutants that are superior to control plant.

MATERIALS AND METHODS

The mutation was carried out at the National Nuclear Energy Agency in June 2019. The research was conducted at the Screen House, Faculty of Agriculture, Universitas Jenderal Soedirman, Karangwangkal, North Purwokerto, from May to July 2023. The materials used in this study were Terigas orange mutants resulting from gamma-ray irradiation with a dose of 6 krad, Terigas orange normals, soil, water, pH meter paper, and distilled water. The tools used in this study included cloth, calliper, refractometer, dropper pipette, 250 ml Erlenmeyer, thermohygrometer, lux meter, knife, camera, stationery, hoe, sickle, analytical balance, RHS Color chart, and Munsell color chart.

The experimental design used in this study was a Randomized Block Design (RBD). The factors tested in this study were the Terigas orange variety normals and the Terigas mutant resulting from 6 kRad gamma-ray irradiation. The plant material used was Terigas orange scions, with three replications, each replication consisting of 10 scions, for a total of 30 scions. Observations were conducted based on the guidelines for compiling descriptions and testing the correctness of plant varieties by the Decree of the Minister of Agriculture of the Republic of Indonesia concerning the Technical Compilation of Descriptions and the Correctness of Horticultural Plant Varieties 2019. Quantitative characters include plant height (cm), stem diameter (mm), leaf length (cm), leaf width (cm), leaf stalk length (cm). Qualitative characters include crown shape, crown condition, stem cross-section shape, branching, bark texture, bark color, leaf shape, leaf surface, leaf edge, leaf type, leaf tip, leaf base, leaf surface, leaf direction facing, upper leaf color, lower leaf color, leaf stalk color, thorns, fruit character.

Quantitative data obtained from the research results were tested using ANOVA. If the calculated F value > F table indicates an influence between the independent and dependent variables, further testing was conducted using the Least Significant Difference (LSD) test at the 5% level to determine the best treatment. Furthermore, the Least Significant Increase (LSI) test at the 5% level was used to compare the diversity of mutants with their normals. Qualitative data are presented in tables and descriptions with images.

RESULT AND DISCUSSION

The Terigas citrus mutant exhibited several significant phenotypic differences compared to its normal. Quantitative traits showing differences included stem diameter, leaf width, and petiole length. Table 1 the results of the study showed that all Terigas mutants had lower plant height, stem diameter, and leaf length compared to normal plants. In the research of Çimen et al. (2020) on Ortanique citrus mutant population that shoots irradiated with 50 Gy, some clones showed stunted growth and smaller fruit size compared to the control. The research from Hartati et al. (2021), also reveals that gamma ray irradiation was reduce plant growth due to its effects on cell structure and function. Gamma rays are high-energy ionizing radiation capable of penetrating plant tissue and causing DNA damage, including double-strand breaks, mutations in growth-regulating genes, and changes in the expression of genes that control cell division and differentiation.

The mutant plant high was lower than of the Terigas normal plant. Goldenberg et al. (2014) research results also stated that gamma mutagenesis sometimes reduces the size or growth of citrus mutant, but can also reduce the number of seeds, change the ripening time and size of the fruit. The research of (Supanjani et al. 2024) on mungbean also result that gamma-ray irradiation significantly affects plant height. According to Fadli et al. (2018), the decrease in plant height due to gamma-ray irradiation occurs because this radiation can damage the structure of plant chromosomes. Gamma rays are a form of ionizing radiation with high energy so that they can cause genetic damage. This chromosome damage will impact the expression of essential genes that regulate plant growth and development, including genes involved in synthesizing growth hormones such as auxin and gibberellin. Disruption in the synthesis or distribution of these hormones will cause a slowdown in cell division and elongation, directly affecting plant height.

Table 1 reveals that the length of normal plant leaves was 6.09 cm, the size of mutant leaves is all shorter than normal. The research research of (Faiz et al. 2023)a also showed that the high dose of gamma ray irradiation effect on leaf length leaf length reduction. The results of Saini and Gill (2009) research on Rough Lemon, also stated that the dose of gamma irradiation on rough lemon seeds caused a decrease in seedling height and leaf size as the dose increased. A decrease in plant growth was also observed in the research of (Choi et al. 2023) reveals that gamma irradiation in citrus caused significant changes in plant physiological processes, decreasing the rate of photosynthesis, respiration, and vegetative growth, especially at high doses. Irradiation causes oxidative stress due to the formation of free radicals (ROS), which damage cell membranes, chloroplasts, and important enzymes, thus disrupting photosynthetic efficiency and energy metabolism. Desouky et al. (2015) also added that exposure to ionizing radiation in biological systems, such as plant cells, triggers a series of chemical and physical reactions that begin with the initial energy absorption by molecules within the cell. When radiant energy is absorbed, excitation and ionization occur, namely the transfer or release of electrons from atoms or molecules. One of the main molecules affected is water (H_2O), as water is a significant component of cells. As a result, water ionizes, producing H_2O^+ ions and free electrons. This process forms various free radicals, such as hydroxyl radicals, hydrogen radicals, and superoxide anions (O_2^-), through a chain reaction. These highly reactive free radicals can interact with various cellular components, such as lipids, proteins, nucleic acids (DNA/RNA), and carbohydrates (Dwinanda et al. 2020).

Almost all mutants have reduced leaf width and leaf stalk, but there was the TR 19 2102 mutant has wider leaf than the Terigas normal. The TR 19 3203 mutant also has longer leaf stalk values than the Terigas normal. This is in line with what Ibáñez et al. (2024) stated that the results of mutations due to gamma-ray irradiation are unpredictable because radiation works randomly at the molecular level. When gamma rays hit plant tissue, they can penetrate cells and cause DNA damage by breaking the double chain of DNA or by forming free radicals that react with DNA molecules. This process does not occur at a specific location or a specific gene but randomly throughout the genome, so the type and location of mutations vary significantly from one cell to another, even within the same individual. (Karyanti et al. 2015) added that mutations can occur in point mutations, insertions, deletions, or even significant changes such as chromosomal translocations. Purba et al. (2021) stated that the effects of these mutations also vary significantly due to their random nature. Some mutations may have no impact, while others can cause significant phenotypic changes.

The qualitative characteristics of the Terigas orange mutant show significant differences compared to its normal, particularly in leaf morphology. Characteristics of leaf shape, leaf margins, leaf bases, leaf type, and the color of the upper and lower surfaces of the leaves undergo significant changes. For example, leaf shape and margin changes may indicate modifications to the genes encoding these qualitative traits. In contrast, differences in leaf color may indicate changes in chlorophyll or other pigment content, potentially affecting photosynthetic efficiency. The research results (Figure 1) exhibited that TR 19 2001 has oval leaves, serrated edges, a rounded leaf base, a convex leaf type, upper leaf color RHS 137A, and lower leaf color RHS 138B. The Terigas elder has serrated leaves, a split leaf base, a flat leaf type, upper leaf color RHS 143A, and lower leaf color RHS 141A. The extent of genetic changes caused by gamma-ray irradiation in plant tissue is determined by the type of tissue irradiated. This was also demonstrated by Pérez-Jiménez et al. (2020), phenotypic changes in citrus mutants are determined by the origin of the mutated plant material, such as seeds and shoots that are more tolerant to radiation exposure. Therefore, this study showed a narrow variation, with only one plant exhibiting changes in leaf characteristics, because the scion's tolerance to gamma-ray irradiation is higher than that of other planting materials. Based on the research of Eun and Kim (2022), explained that gamma ray irradiation on Satsuma mandarins citrus (*Citrus unshiu* Marc.) can produce phenotypic diversity. These changes occur randomly across various genes, resulting in altered gene expression that results in differences in the morphological appearance of the plants.

The characteristics of the mutant leaves (Figure 2) can be seen on the surface of the TR 19 4002 mutant leaves which are duller than the normal leaves which are shiny (Figure 2). The color of the upper leaves of the TR 19 4002 mutant is RHS 138A, the TR 19 3202, TR 19 2002, and TR 19 4102 mutants have a color of RHS 139A, indicating a difference in color with the normal leaf color, namely RHS 143A. The presence of changes in leaf color also occurred in Manjusha et al. (2024) gamma ray irradiation caused changes in leaf color in acid lime (*Citrus aurantifolia*) cv. Agamalai, because it can damage the genes that involved in chlorophyll biosynthesis and chloroplast formation. This high-energy radiation can cause genetic mutations in nuclear DNA and chloroplast DNA, including genes that regulate important enzymes such as protochlorophyllide reductase and chlorophyll synthase, which play a direct role in the formation of green leaf pigments. As a result, chlorophyll production decreases or is disrupted, so that the leaves show different colors depending on the level of damage or altered gene expression. Hong et al. (2022) also stated that genetic changes due to gamma-ray irradiation occur due to the interaction between ionizing radiation and DNA molecules, which can cause direct or indirect damage to the genetic structure. One form of this damage is genetic mutation, which is characterized by the addition or loss of one or more nitrogen bases in the DNA sequence. Alanazi et al. (2024) also added that gamma radiation has high energy that can break DNA chemical bonds, especially between nucleotides. In addition, this radiation can produce free radicals from water molecules in cells, damaging nitrogen bases, breaking DNA chains, or causing the formation of abnormal cross-links. As a result of these changes, the genetic information encoded by the base sequence can change, causing changes in gene expression.

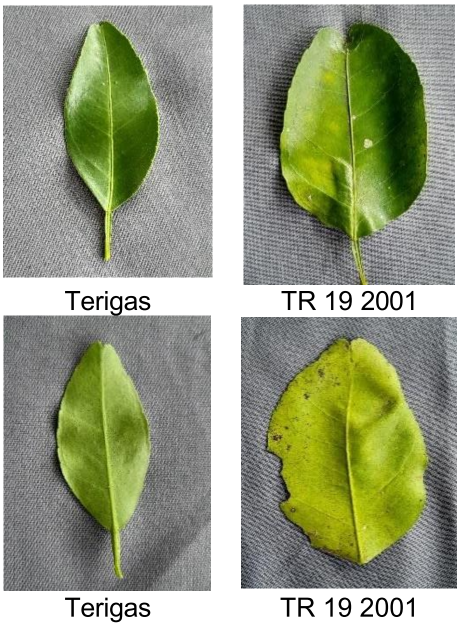

Figure 1. Differences between normal and mutant leaves include leaf shape, leaf edges, leaf base, leaf type, upper and lower leaf color

Table 1. Comparison of normal and mutant characters of Terigas variety

Mutant	Plant height (cm)	Stem diameter (cm)	Leaf length (cm)	Leaf width (cm)	Leaf stalk length (cm)
TR 19 2202	85,45	0,37	0,53	0,32	0,11
TR 19 2001	56,95	6,67	3,13	1,32	0,51
TR 19 2002	49,45	1,83	2,73	0,62	0,41
TR 19 1101	49,45	1,33	0,83	1,22	0,39
TR 19 1102	49,45	8,97	3,23	1,32	1,11
TR 19 2101	122,45	0,13	4,43	0,32	0,41
TR 19 2102	122,45	0,53	3,73	4,32 a	1,01
TR 19 2001	36,45	4,27	4,23	2,32	0,61
TR 19 3202	56,45	15,87	3,63	1,72	0,11
TR 19 3203	126,45	2,03	5,13	2,52	1,81 a
TR 19 4002	55,45	2,77	2,43	1,02	0,09
TR 19 4201	66,45	2,07	3,73	1,82	0,11
TR 19 4202	66,45	10,37	2,03	1,12	0,21
TR 19 4102	42,45	3,87	3,13	0,82	0,31
Terigas (a)	156,57	18,07	6,09	2,60	1,23
LSI	41,55	6,43	3,57	1,78	1,09

Remarks: The numbers in the variables are observation data minus the LSI value at the 5% level.

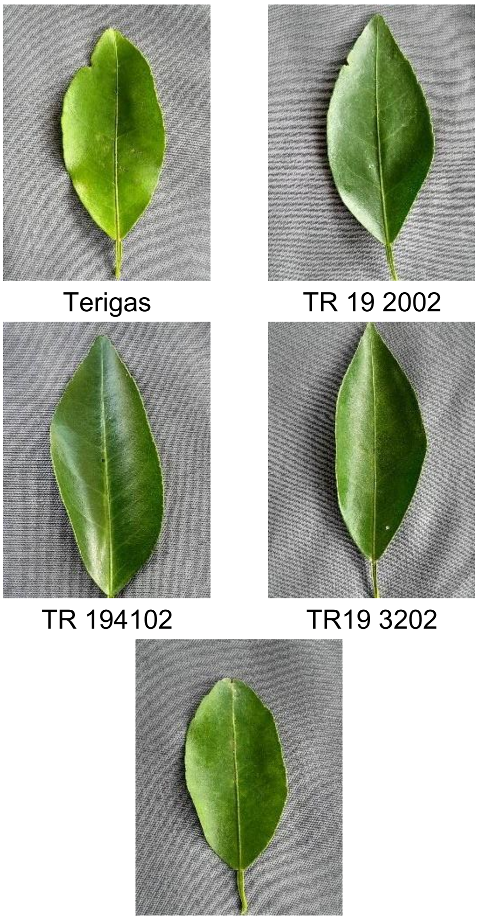

Figure 2. Differences between the normals and the Terigas orange mutant in the color characteristics of the upper leaf surface

The TR 19 2202 mutant has more seeds, more acidic flesh, and a more pungent aroma than its normal. The mutant orange's sugar content is higher, at 16 brix, compared to its normal's, at 15.1 brix (Table 2). According to Bermejo et al. (2015) research, a mutant of Murcott oranges also showed a significant increase in Brix. This is because gamma irradiation affects the activity of enzymes such as invertase and sucrose synthase, which play a role in the breakdown and accumulation of sugars. Therefore, an increased conversion of starch to simple sugars in the fruit resulting in an increase in the Brix value. Mutations also can increase fruit sugar content because they affect genes involved in carbohydrate metabolism, such as those that regulate sugar synthesis, transport, and storage. These changes can increase the activity of enzymes like sucrose phosphate synthase or invertase, which play a role in sugar formation and accumulation (Wang et al. 2021). Certain mutations can even increase photosynthetic efficiency or alter assimilates distribution, directing more sugar to the fruit (Lukaszuk et al. 2017). Gamma irradiation also acts as a mild abiotic stress, triggering the plant's natural defense mechanisms, including the accumulation of osmoprotectants like sugars to stabilize cell function (Khan et al. 2015). This combination of changes makes mutant fruit potentially have higher Brix levels than the normal variety.

Thinning of the fruit's skin also occurred in this research (Table 2), that in line with Rattanpal et al. (2019) research on the Kinnow mandarin variety, which used shoots for irradiation. High-energy gamma rays cause DNA damage and gene mutations that affect the rate of cell division and enlargement in the skin tissue, thus the number of cell layers and the size of the cells making up the fruit's skin become fewer or smaller.

A stronger fruit aroma in TR 19 2202 mutant indicates increased volatile compounds that influence the aroma profile, which can be a sensory advantage for consumers. This research results was in line with He et al. (2024) research on the 'Ehime 38' (Citrus reticulata) mutant reveals a stronger aroma, this was indicated by the number and total concentration of volatile compounds increasing in the mutant, especially during the fruit ripening phase. Monoterpenes (such as limonene, γ-terpinene, and α-pinene) and esters that contribute to the fresh and sweet aroma showed higher accumulation in the mutant.

The taste of TR 19 2202 mutant fruit was sourer, according to Strazzer et al. (2019), mutations in the acid-regulating genes (CitPH1, CitPH5) affect how many protons (H^+) are pumped into the juice cell vacuoles, which affects acidity (sour taste). Sour varieties have high CitPH1/CitPH5 expression, while sweeter varieties tend to have mutations or decreased expression in these genes, that mutations can lead to increased acidity depending on the genes affected.

This research has not yet succeeded in producing seedless mutants, as explained by Huang et al. (2017) that mutations in the Meiguicheng orange (Citrus sinensis) can result in abnormal megagametogenesis, resulting in incomplete development of the embryo sac (female gametophyte) within the ovule. This can also be due to the mutant ovules that occur premature degeneration, preventing the megaspore mother cell (MMC) from completing meiosis properly. Consequently, the embryo sac does not form or is non-functional, resulting in failure of fertilization and the development of seedless fruit.

Table 2. Characteristics of the Terigas orange normal and mutant fruit

Fruit Character	Normal	Mutant
Fruit height	48,8 mm	41,2 mm
Fruit diameter	56,8 mm	48,7 mm
Ripe fruit skin color	7,5 GY 5/4	7,5 GY 4/6
Fruit weight	1,4 mm	1,3 mm
Fruit flesh flavor	91,91 gram	58,15 gram
Sugar content (brix)	Sour +	Sour ++
Fruit aroma	15,1	16
Number of seeds	Medium	Soft

Figure 3 shows that the size and weight of the mutant fruit are smaller than the size of the normal fruit, the color of the fruit skin on the mutant is slightly greener than the normal fruit, namely on the normal 7.5 GY 5/4 while on the mutant fruit 7.5 GY 4/6. This is in line with Çimen et al. (2020) research on the Ortanique tangor citrus mutant population (MV3 generation) from shoots irradiated with 50 Gy showed that most of the population had fruit size (diameter, height) similar to the control. However, there was variation on some mutant fruits which were smaller than the control fruit. The research of Kundu and Dubey (2017) on citus pollen also found that the use of gamma-irradiated pollen resulted in reduced fruit growth in size and weight compared to fruit treated with normal pollen. The effect of gamma radiation that has high ionization properties can penetrate plant tissue and cause DNA damage and cell division disorders, especially in meristem tissue that forms generative organs. In addition, radiation exposure also causes oxidative stress that reduces the efficiency of photosynthesis and the flow of nutrients to the fruit, further slowing its enlargement.

Figure 3. Normal fruit and TR 19 2201 (a); intact fruit of normal and mutant (b); intact split fruit and mutant

CONCLUSIONS AND SUGGESTIONS

Gamma ray irradiation mutations in citrus cv. Terigas generally produce diversity that increases leaf width in TR 19 2102, and increases leaf stalk length in TR 19 3203. Diversity in leaf shape, leaf edges, leaf base, leaf type, upper and lower leaf color in TR 19 2001, diversity in color characteristics of the upper leaf surface in TR 19 2002, TR 19 4102, TR 19 3202, TR 19 4002, decreases fruit skin thickness and increases fruit brix in TR 19 2202.

Suggestions for further research include trying various doses of gamma ray irradiation to increase the possibility of forming seedless mutants.

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