The Effects of Subculture on The Mutant Plant Regeneration of Rodent Tuber (*Typhonium flagelliforme*) *In Vitro* Mutagenesis Using Gamma-Ray Irradiation

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**Abstract.** The combination of gamma-ray irradiation treatment and somaclonal variation may cause morphological changes in vitro culture of rodent tuber. *In vitro* shoots were irradiated with several gamma-ray doses. Rodent tuber plantlets were irradiated with the doses of 20 Gy. The plantlets were subcultured up to 5 times then produced MV1 to MV5. Plantlets MV4 and MV5 were acclimatized and transplanted at the greenhouse. The purpose of this study was to analyze the morphological characteristics in the fourth mutant vegetative propagation (MV4) and the fifth mutant vegetative propagation (MV5) of rodent tuber from the combination of gamma-ray irradiation and somaclonal variation. Subcultures were performed to regenerate from MV1 to MV5 with optimal MS (Murashige & Skoog) medium. Subcultures were done at eight weeks. The morphological measurement in MV4 with the average number of shoots, number of leaves and plant height, i.e. 5.21, 14.41 and 5.52 cm, respectively. The highest average number of shoots, number of leaves and plant height was generated in MV5, i.e. 5.23, 16.54 and 5.18 cm, respectively. Pre-acclimatization were successfully produced in the greenhouse of about 30% (MV4) and 39.5% (MV5). Post acclimatization was obtained 100% in control, MV3, and MV5 at the greenhouse. MV5 had better morphological characteristics. The morphological changes were occurring in MV4 and MV5 compared with controls due to the combination of gamma-ray irradiation and somaclonal variation.

**Keywords:** *Typhonium flagelliforme* Lodd., morphology, gamma-ray irradiation, somaclonal variation.

1. **Introduction**

Rodent tuber is an Indonesian plant commonly found in Java island and grows well at 1-300 m altitude above sea level [6]. Rodent tuber has slow propagation rate, and it is distributed only in damp and shady place in its natural habitat. Rodent tuber is a medicinal plant which is useful for treat cancer such as breast cancer, intestine, prostate gland, liver, leukemia, and cervix [6, 10]. Rodent tuber contains a bioactive compound that can inhibit pathogenic microorganism such as bacteria, fungi, and virus [15], and has a toxic activity from hexane extract in *Artemia salina* [29]. Choon et al. [3] said that rodent tuber has activity as an anticancer and induces apoptosis.

The problems faced in the development of rodent tuber for anticancer drug materials are the bioactive compounds from the existing rodent tuber plant which still very low, while the rodent tuber...
in Indonesia has a low genetic variation. One of the technologies that have the potential to increase genetic variation is through \textit{in vitro} mutagenesis and combined with physical mutagens (gamma irradiation). Gamma irradiation is the most commonly used mutation breeding methods [25] because it can change the chemical and chromosomal aberration plants [28, 20]. The technology has been applied on rodent tuber, and LD$_{50}$ was produced at a dose of 20 Gy. The adventitious shoots that have been obtained, subcultures were performed on the same media formulation with vegetative mutants 5 (MV5). Rajeswari & Paliwal [24] reported that the subcultures could increase the regeneration plants ability which the shoots are becoming juvenile. The high frequency of regeneration ability will decrease physiological performance and regeneration potential. The decreasing of regeneration potential can be seen from morphological performances both on shoot height, number of shoots, number of leaves and acclimatization ability.

The morphological variation in rodent tuber (\textit{T. flagelliforme} Lodd.) needs to be improved. The higher genetic variation also influences the variety and the number of bioactive compounds. Mutant induction is one of the effective ways to increase genetic variation [8]. Mutation induction can be done by physical mutation by using gamma-ray irradiation. Gamma irradiation ionizes the cellular atom so that it can damage the DNA base and make it mismatched. Irradiation can also alter the structure of chromosomes through deletion, inversion, duplication, and translocation. DNA changes may eventually increase phenotypic variation, one of which is plant morphology [35]. The results of gamma-ray irradiation in putative buds of rodent tuber showed a vary growth response [30].

Rodent tuber had been successfully propagated through \textit{in vitro} techniques used Murashige and Skoog (MS) medium with an optimal concentration of NAA 0.5 mg L$^{-1}$ and BAP 0.5 mg L$^{-1}$ [29]. Plant growth regulator (PGR) has a function as a chemical messenger, also known as phytohormones to promote and stimulate plants growth and development. Auxin and cytokinin play important roles as interaction regulates meristem development, and \textit{in vitro} organogenesis [26]. The application of using PGR in the right concentrations can optimize plant multiplication [26]. Somaclonal variation is the genetic variation of plants produced through tissue cultures [17]. The genetic variation that occurs within tissue culture is caused by the doubling of chromosome number (fusion, endomitosis), changes in chromosomal structure, gene and cytoplasmic changes [7, 14].

Somaclonal variation has a potential source of new plant genotypes. The occurrence of variation may be due to genetic changes at the level of DNA, genes, or chromosomes that occur during cultures [21]. The combination of gamma radiation and somaclonal variation can cause morphological changes in the plant. Repeated subcultures may cause morphological and genetic changes. This genetic modification leads to changes in plant physiology and biochemistry that affect morphology. The purpose of this study was to examine the regeneration potential which expressed in the differences morphological on regenerated plants from \textit{in vitro} mutagenesis combined by gamma irradiation.

2. Materials and Methods
2.1 Regeneration and initiation of explant
The plant material used was mutant shoots generated from combined gamma-ray irradiation with a somaclonal variation. Putative mutant shoots were obtained at the dose of 20 Gy. The first mutant vegetative propagation (MV1) was obtained and subcultured in the same medium until the fifth mutant vegetative propagation (MV5). MV4 and MV5 were acclimatized at the greenhouse.

2.2 Shoot multiplication
Shoots of gamma-ray irradiation were cultured in Murashige and Skoog (MS) media. The multiplication shoots was using Murashige & Skoog medium with BAP treatment with 0.5 mg L$^{-1}$ and NAA 0.5 mg L$^{-1}$ [29], with pH 5.7-5.8 treated with KOH or HCL 0.1 N. The irradiated shoots were cultured and incubated in the medium for 8 weeks at 22°C with 1,000 lux with 16 hr/day fluorescent lighting in the culture room. At this stage, plants that can survive and regenerate seedling produce were called first-generation shoots which irradiated gamma rays \textit{in vitro} (MV1). The MV1 shoots were subcultured into the new medium through the separation of the shoots which produced MV5.
The plantlet was cleaned from the agar. The average number of hoots vegetative propagation obtained the highest number of shoots in the 4th generation influenced by gamma irradiation of 20 Gy in buds and somaclonal variations during the mutation process. The putative mutant plants were identified with1:1:1 ratio for post-acclimatization at the Center for Research and Development of Biotechnology and Agricultural Genetic Resources (BB-BIOGEN), Bogor.

2.4 Statistical analysis
Morphological data (number of shoots, number of leaves, and plant height) were analyzed using the Kruskal-Wallis test. The homogeneity of variances was tested with the Levene test and normality of residuals with the Shapiro-Wilks-W-Test. Mann-Whitney U test was performed to run over post hoc test.

3. Results and Discussion
The shoots were produced by the combination treatment of gamma-ray irradiation with somaclonal variation generated putative mutants. The putative mutant is obtained at a dose of 20 Gy. Gamma-irradiation shoots were successfully subcultured many times until the putative mutant of the fifth vegetative propagation (MV5) was obtained. The percentage of shoots produced from the first to the fifth vegetative propagation obtained the highest number of shoots in the 4th generation (MV4) (Figure 1). This condition indicates that cell division by mitosis for budding organ formation is highest in the 4th subculture. Growth and development are physiologically influenced by genes expressed in the morphology in vitro cultures. Plants that can be subcultured because of the irradiation of gamma-rays in such high intensity can drastically alter the genomes of plants, thus disrupting cell structure and function [36].

3.1 In vitro regeneration of rodent tuber
The MV1 clone (20-1-1, 20-1-2, 20-1-3) has been successfully in vitro propagated and regenerated to produce the fifth vegetative propagation (MV5). This study was determined the morphological changes in the number of shoots, number of leaves, and plant height at the control plants and irradiated plants at MV4 and MV5 which is consisting of clones 20-1-1, 20-1-2 and 20-1-3. Irradiated plants have a different number of shoots and number of leaves in the control plants. The average number of shoots and height of MV4, and MV5 plants at the first until the eight weeks can be seen in Figure 1. The differences between MV4, MV5, and the control plant showed a change in genetic traits caused by in vitro mutagenesis combination with gamma-ray irradiation.

Mutants were identified with significant morphological changes from the morphological characteristics (Table 1). The average number of shoots in MV4 and MV5 at eight weeks old is higher than control plants (Table 1). The average number of shoots in MV4 and MV5 were 5.21 and 5.23 respectively, while control was only 4.24. The average number of leaves in MV4 and MV5 were 14.41 and 16.54, respectively. MV5 is the best regeneration in the morphological characteristic.

The differences between the number of shoots and the number of leaves between the control plants and the mutant plants indicate that the plant has undergone DNA mutations caused by gamma-ray irradiation of 20 Gy in buds and somaclonal variations during in vitro culture. According to Khan et al. [13] examined changes in plant morphological characteristics in the field due to gamma irradiation in sugar cane, Artemisia [23], and Phalaenopsis amabilis (L.) Bl. [32].
### Table 1. The morphological characteristics differences between rodent tuber control and mutant plant

| Rodent tuber plant | Number of shoots (Mean ± SD) | Number of leaves (Mean ± SD) | Plant height (Mean ± SD) |
|-------------------|------------------------------|-----------------------------|------------------------|
| Control           | 4.24 ± 2.49                  | 11.37 ± 6.58                | 5.71 ± 1.22            |
| MV4               | 5.21 ± 2.17**                | 14.41 ± 6.09**              | 5.52 ± 1.43**          |
| MV5               | 5.23 ± 1.58**                | 16.54 ± 6.16**              | 5.18 ± 1.70**          |

*Significantly different from control (P < 0.05); **(P<0.01) (Mann Whitney-U test)

### Figure 1. The number of plants after irradiated with gamma-ray from each generation from MV1 to MV5.

#### 3.2 Growth and development of rodent tuber mutant plants

The average number of shoots and plant height in MV4 and MV5 generation until eight weeks are shown in Figures 2 & 4. Statistical data showed that the growing number of shoots per week increased by eight weeks. Shoots that have been irradiated by gamma rays can be regenerated to the fifth generation (MV5). Subculture cultures up to 5 times in vitro cultures showed a declining number of shoots. This is due to the cell's ability to decrease proliferation the number of shoots produced in the optimal medium given the combination treatment between BAP and NAA. The number of shoots was decreased due to the increase of ethylene biosynthesis and abscisic acid (ABA). PGRs can reduce the ability of the differentiation process to both forms the number of shoots and plant height.

Rodent rodents can be rapidly in vitro propagated because they are grown on MS medium containing complete macro and micronutrients and supplemented with PGR in the form of NAA belonging to auxin and BAP belonging to cytokinin [29]. The combination of BAP and NAA has also been used for the propagation of various in vitro plants, including Aconitum balfourii [27], bitter apple [26], and Albizia odoratissima L.f. (Benth.) [24].

At the control, plants were followed by increasing the number of leaves in the 1st week into the 6th week but in the 8th week its decreasing. While in MV4 and MV5, the development of the number of leaves was increased from the first week until the eighth week (Figure 3). Data was shown the number of leaves was decreased due to the cell death in the leaves at the control plant. The growth plant was longer in putative mutant plants than in control. The number of leaves was declined in the control plant because of no genetic traits changed. In the mutant can occur specific genes that control the process of differentiation to form organs [17]. This specific gene can inhibit damages to the enzyme that plays a role in the formation of chlorophyll. The parameters of morphological changes in the number of leaves, it is due to the combination of gamma irradiation treatment with somaclonal variation.
The average number of shoots in MV4, MV5, and control at 8 weeks supplemented with MS medium 0.5 mg L$^{-1}$ NAA and 0.5 mg L$^{-1}$ BAP.

Figure 2.

The average number of leaves in control, MV4 and MV5 at 8 weeks old supplemented with MS medium 0.5 mg L$^{-1}$ NAA and 0.5 mg L$^{-1}$ BAP.

Figure 3.

The average plant height in control, MV4, and MV5 at 8 weeks old supplemented with MS medium 0.5 mg L$^{-1}$ NAA and 0.5 mg L$^{-1}$ BAP.

Figure 4.

The differences of the plant height between MV4 and MV5 can be inherited to the next generation. This is indicated by the different number of shoots and the number of leaves at the 4th and 5th generations. The consistency of MV4 and MV5 morphological also indicates that the morphological
differences between clones are not caused by environmental factors alone, but mainly because of genetic factors, because environmental factors may change while genetic factors can be passed into the next subculture [5, 20].

The morphological changes were also observed in experiments of mutation of gamma rays in Thai Tulip (*Curcuma alismatifolia*) which obtained LD$_{50}$ at a dose of about 25 Gy. At that dose, there is a change in the development of flowers, mutations of chlorophyll and alteration of plant morphology to produce some mutants [1]. Banerji & Datta [2] found that the optimum dose of gamma rays to shoot cuttings was 25 Gy. On the other hand, Lamseejan et al. [16] obtained LD$_{50}$ at 14 Gy for induction of *Chrysanthemum* of the purple clone.

In addition to gamma irradiation, somaclonal variation may also cause genetic differences or differences between plants as reflected by the morphological differentiation between the clones of the plant. According to van Harten [35], somaclonal variation is the result of the irregularity of cell division by mitosis that occurs during the process of *in vitro* plant regeneration. Genetic variation due to somaclonal variation can be caused by doubling the number of chromosomes (fusion, endomitosis), changes in chromosome structure, gene changes, and cytoplasmic changes [7, 14].

The genetic differences between irradiated plant clones are due to the clones coming from different cells or single nodes in a single plant irradiated with gamma rays of 20 Gy. Mutations caused by gamma-ray irradiation and somaclonal variation are random so that each cell in the irradiated plant is likely to have a genetic mutation with a different pattern than the others [33, 34, 22]. Random changes to the genome due to gamma irradiation also occur in coconut mutants [25]. Therefore, each cell will produce a clone of plants that have a unique DNA sequence. Differences in gene sequencing DNA sequences will produce proteins and enzymes with different structures, functions, and expression patterns [12], so that plant morphology will be diverse.

This study showed that rodent tuber plants MV4 and MV5 have a higher biomass number of shoots and number of leaves than control. The crucial factor for commercial production of plants because through *in vitro* culture techniques, it is expected that rodent tuber plants can be mass-produced in a short time to meet the needs of the pharmaceutical industry of anticancer drugs.

### 3.3 Acclimatization of rodent tuber at the greenhouse

Acclimatization was done after plants rooted at 8$^{th}$ weeks. Acclimatization was done at two stages which are pre-acclimatization and post-acclimatization. During pre-acclimatization, the lowest percentages live of regeneration mutant plants was MV4 by 30% (Figure 5). Mutant plants were successfully lived 100% at the greenhouse during the post–acclimatization, as shown in Figure 5. Rodent tuber mutant plants at 5$^{th}$ generation (MV5) were recovered before post acclimatization and had normal growth after acclimatization. It was probably an expression of mutagen that affected the temporary steady state physiology of the plantlet [5, 11].

The success of post-acclimatization in control plants reached 100% but in MV4 and MV5 only 30% and 39.53%, respectively. This condition is caused by control plants that do not change genetic traits in the acclimatization process of stomata conductivity. MV4 and MV5 mutants genetic changes occur that have not been able to adapt and control stomata conductivity *in vitro* conditions. This evidence during post-acclimatization after adaptation has been reached 100% success *in vitro* conditions. In micropropagation, the limiting factors that determine the success of shoots production and acclimatization in large numbers are the techniques of acclimatization. The roots of plantlets have not yet been able to play a role in absorbing water and nutrients. The stomata conductivity is still low in the process of adaptation is needed by gradually decreasing environmental humidity to transpiration which causes the plantlet may quickly wilt and die. The success of plant *in vitro* propagation can be seen from the survival rate during acclimatization [18]. Control plants, MV4, and MV5 clones have right *in vitro* propagation and regeneration capacity and can produce enough roots to succeed in pre-acclimatization. Plants that have good viability at the pre-acclimatization stage (laboratory) are then transferred to the greenhouse (post-acclimatization).
Rodent tuber plants that have been irradiated with gamma rays and successfully grown in the greenhouse are also called putative mutant plants. Conventional propagation of rodent tuber in the field was vegetatively through the production from the tubers. The rodent tuber mutant plant was successfully reproduced and regenerated until generation MV4, and MV5 can live in the greenhouse. Plant acclimatization is influenced by several factors, such as media [19], light intensity, and moisture [9].

The morphological differences between control and mutant plants are due to mutation induction by gamma rays and somaclonal variations that cause genetic alteration. Gamma irradiation contains enormous kinetic energy that can alter the DNA sequence and plant chromosome structure [4]. This genetic modification leads to changes in physiology and biochemistry that affect morphology. Other researchers also observed changes in morphological characteristics in the field due to gamma irradiation, for example in sugarcane [13], Artemisia [23], Sorghum bicolor [33], and Phalaenopsis amabilis (L.) [32].

Besides, it is generally seen that both in vitro culture of irradiated plants have a higher number of shoots and number of leaves than controls. The morphological consistency between in vitro plants and greenhouse plants is also likely due to genetic differences among stable inherited clones of in vitro generation (MV4 and MV5) in the greenhouses. Moreover, the morphological changes and mutation induction of rodent tuber from Pekalongan are also expected to increase the content of anticancer bioactive compounds.

**Conclusions**

In vitro mutagenesis using gamma-ray irradiation was employed to obtain rodent tuber mutant plants. Dosages of 20 Gy were determined morphological changes at all putative mutant plants. The shoots were irradiated with gamma rays can survive until the fourth mutant vegetative propagation (MV4) and the fifth mutant vegetative propagation (MV5). The highest number of shoots, number of leaves and plant height are in MV5, i.e., 5.23, 16.54 and 5.78, respectively. The regeneration percentage plants are produced in control, MV4, and MV5 about 100%, 30% and 39.53% at pre-acclimatization. The post acclimatization at the greenhouse is obtained 100% of the regeneration percentage plants. These morphological changes can be caused by the combination of gamma-ray irradiation and somaclonal variation.
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