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Detection of Gamma-Irradiated Mutant of Rodent Tuber (*Typhonium flagelliforme* Lodd.) In Vitro Culture by RAPD Molecular Marker

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Abstract

Rodent tuber (*Typhonium flagelliforme* Lodd.) is an Indonesian native plant which has a high potency to be used as anticancer medicine, but its genetic variation in Indonesia is low. Gamma irradiation can be used to increase genetic diversity. The objective of this research is to obtain in vitro culture of the first generation (MV₁) mutants of rodent tuber which had been irradiated by gamma rays and detect genetic changes by RAPD molecular markers. Irradiation was performed using ⁶⁰Co to obtain LD₅₀ value and induce mutation. Calli were then regenerated to obtain MV₁ plantlets and genetic changes were detected by RAPD molecular markers. Plantlets were successfully regenerated from 9 clumps of calli and produced 59 plantlets at 6 Gy. Genetic changes of 11 plantlets from calli were detected by 10 RAPD primers, which produced 69 fragments from 11 mutant plantlets. OPE-20 and OPC-5 primer showed the highest genetic changes with 11 and 8 polymorphic fragments respectively. Nine polymorphic primers showed genetic changes between 250 bp and 2 500 bp. This research had successfully induced mutation, regenerated, and detected genetic changes in 11 mutant plantlets from calli.

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Keywords: Gamma-irradiation; induced-mutation; in vitro regeneration; RAPD markers; *Typhonium flagelliforme* Lodd.

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Nomenclature

wk	week.
min	minute.
LD₅₀	lethal dose.
RAPD	randomly Amplified Polymorphic DNA.
Gy	gray.
bp	base pair.

1. Introduction

Rodent tuber (*Typhonium flagelliforme* Lodd.) is a herbal plant from the Araceae family which is widely cultivated in India, Australia, Sri Lanka, Indonesia, and the other a Asian countries with moderate temperature¹. Rodent tuber has a broad array of bioactive compounds with anticancer activity against lung, breast², liver³, blood⁴, intestine, prostate gland, and cervical cancer⁵ based on in vitro experiments. Anticancer compounds can be found in all parts of rodent tuber plant, including root, tuber, stem, and leaf⁶. The bioactive phytochemical constituents of rodent tuber are alkaloid, flavonoid⁷, saponin⁸, sterol², cerebroside, and glycoside⁹.

Rodent tuber has low genetic diversity⁸ while the potency of its bioactive compounds is very high. The diversity and quantity of bioactive compounds will be enhanced in line with genetic diversity. Mutation induction is one of the effective methods to enhance genetic diversity¹⁰. Gamma irradiation exerts a high proportion of kinetic energy which is able to induce mutation by changing chromosomal¹¹, organelle, and gene structure¹².

Gamma irradiation has been used to develop superior varieties¹³. Genetic diversity can be detected by molecular markers such as RAPD, ISSR, AFLP, and SSR¹⁴. RAPD marker is able to detect genetic diversity of plant whose genome sequence has not yet been known¹⁵ such as rodent tuber. Germplasm characterization is a very important aspect in order to utilize the potency of plant genetic diversity¹⁶. This research aimed to irradiate rodent tuber calli with gamma rays in order to induce mutation and utilize RAPD molecular marker methods to detect the genetic diversity of the results plantlets.

2. Material and methods**2.1. Plant material**

T. flagelliforme plants used in this research were obtained from Aromatic and Medicinal plant Research Agency (BALITTRO), Bogor, Indonesia. The plants were propagated in the Biology Laboratory, Pelita Harapan University, Indonesia, on optimized plant tissue culture medium containing Murashige and Skoog (MS) basal medium, 1 mg · L⁻¹ NAA, 0.5 mg · L⁻¹ BAP, 30 g · L⁻¹ sucrose, 10 % coconut water, and 8 g · L⁻¹ agar¹⁷. The calli produced were then used in analysis of radiosensitivity to Gamma irradiation.

2.2 . Analysis of *T. flagelliforme* rodent tuber radio sensitivity to gamma irradiation

Radiosensitivity analysis was performed to rodent tuber calli irradiated with (0, 10, 20, 30) Gy of Cobalt 60 (⁶⁰Co) exerted by irradiator gamma chamber 4 000 A at the Center for Application of Isotope and Radiation Technology, National Nuclear Energy Agency (BATAN), Jakarta. Each irradiation dose was repeated 12 times. Irradiation was done to 48 calli in 12 culture bottles which consist of four calli each. The percentage of death was observed in each irradiation dose.

2.3. Determination of lethal dose 50 (LD₅₀)

Determination of LD₅₀ was performed by collecting *T. flagelliforme* survival percentage data on each irradiation treatment and analyzed using Data Fit V.8.2 (Oakdale Engineering) software. A regression equation was then used to determine the LD₅₀.

2.4. DNA isolation of plantlet mutant rodent tuber

DNA isolation was done by using modified CTAB procedure¹⁸. Plantlets of mutant rodent tuber leaves were homogenized in eppendorf tube in liquid nitrogen. One mL of CTAB buffer solution (2 % CTAB, 100 mM Tris-HCl pH 8.0, 20 mM Na₂EDTA pH 8.0, 1.4 M NaCl) and 0.2 % 2-Mercaptoethanol was added into the homogenized leaf samples. Samples were then incubated in water bath at 65 °C for 45 min. 500 µL of chloroform : isoamyl alcohol solution (24 : 1) were added into the samples. The samples were then reversed for 12 times. Samples were centrifuged for 1 min at 13 000 rpm (1 hertz is equal to 60 rpm). The supernatant obtained were separated into another tube. One tenth volume of natrium acetate solution (NaOAc) pH 5.2 and one volume of cold isopropanol were added into the supernatants. Samples were incubated at -20 °C for 30 min and centrifuged at 13 000 rpm for 10 min. Pelets obtained were washed with 200 µL of cold etanol 70 % and were room dried for 30 min. Pelets were resuspended in 200 µL of TE buffer pH 8.0. 3 µL of RNase A (10 mg · mL⁻¹) were added to the resuspended DNA samples and were incubated at 37 °C for 1 h. DNA samples were kept at -20 °C, and were ready to use.

2.5. PCR- RAPD

PCR RAPD reaction was done with the total volume of 25 µL which consisted of 5 µL of GoTaq Flexi Buffer (5X), 2.5 µL of MgCl₂ (25 mM), 0.5 µL of PCR Nucleotide Mix (10 mM), 1 µL of primer (10 µM), 0.2 µL of GoTaq DNA Polymerase (5U · µL⁻¹), 2 µL of DNA template (100 ng · µL⁻¹), and 13.8 µL of ddH₂O. PCR cycles consisted of a cycle of predenaturation (95 °C for 2 min), followed by 7 cycles of denaturation (95 °C for 1 min), annealing (37 °C to 34 °C for 1 min), and extension (72 °C for 3 min), and 38 cycles of denaturation (95 °C for 1 min), annealing (34 °C for 1 min), and extension (72 °C for 3 min), followed by a final extension cycle (72 °C for 7 min), and storage at 4 °C.

2.6. PCR-RAPD visualization

PCR RAPD products were visualized with electrophoresis using 1.2 % agarose gels with 110 volt for 90 min. Agarose gels were then soaked in ethidium bromide solution for 10 min and washed with water. Visualization and documentation of PCR RAPD were done with Chemidoc XRS System. DNA bands size were determined by comparing them with 1kb DNA ladder.

2.7. Data analysis

DNA bands obtained were scored into binary format of 0 and 1. Binary matrix obtained were analyzed by using Ntsys software to determine the similarity matrix with DICE coefficient¹⁹. Clustering analysis was done with UPGMA method.

3. Result and discussion

3.1. Analysis of rodent tuber radiosensitivity to gamma irradiation

Radiosensitivity analysis was done to rodent tuber calli. Mutation induction and plantlets regeneration will produce potential rodent tuber mutants. The success of mutation induction to obtain solid mutant depends on selection of plant material. Radiosensitivity curve of rodent tuber calli had equation $y = -0.0052x^3 + 0.3959x^2 + 10.105x + 100$ with LD₅₀ value 6.4 Gy. Radiosensitivity analysis of rodent tuber had not been done before. This research proved that rodent tuber has different radiosensitivity characteristics in comparison with the other plants.

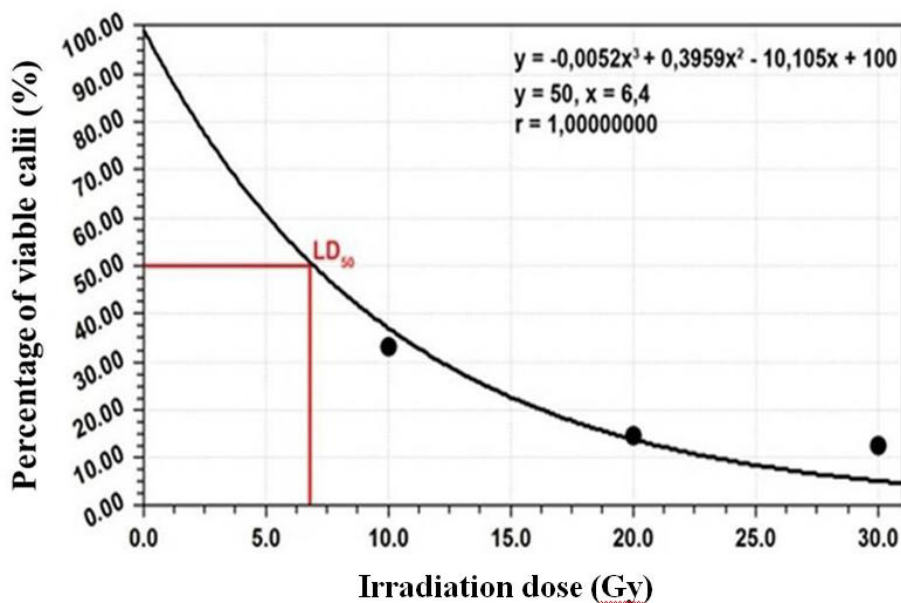


Fig. 1. LD₅₀ regression equation of gamma irradiation doses on Calli of *Typhonium flagelliforme*

3.2. Mutation induction and morphological characteristics of rodent tuber first generation mutant (M1) originated from Calli

Rodent tuber calli were irradiated by (0, 10, 20, and 30) Gy gamma irradiation to induce mutation. Rodent tuber mutation induction had been successfully done to 9 calli at the dose of 6 Gy, while 10 Gy gamma irradiation produced no viable calli. Viable irradiated calli also had been regenerated to first generation mutant plantlets (M1) (Fig. 2). This research had regenerated 8 viable calli which had not been irradiated (0 Gy) to produce 93 plantlets, nine viable calli which had been irradiated with 6 Gy gamma rays to yield 59 plantlets, while calli irradiated with 10 Gy gamma rays yield no viable plantlets. Lethality of 10 Gy gamma irradiation proved that irradiation could damage calli cells. Viable calli irradiated 6 Gy were therefore mutants because they could resist the negative effects exerted by gamma irradiation.

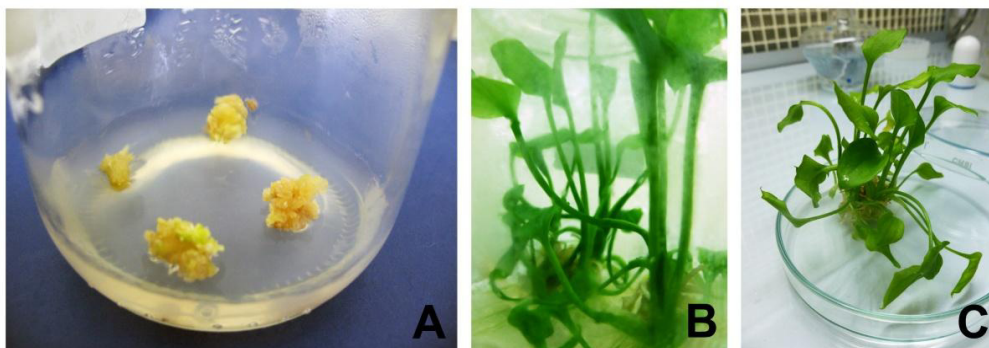


Fig. 2. Regeneration of calli which had been irradiated with 6 Gy of gamma irradiation; (A) rodent tuber calli in 1wk after irradiation; (B) rodent tuber M1 plantlets in 22 wk after irradiation; (C) rodent tuber M1 plantlets in 24 wk after irradiation.

The mean value of plantlets regenerated from calli irradiated by 0 Gy is 11.63 plantlets, plantlets regenerated from calli irradiated by 6 Gy is 6.56 plantlets, but no plantlet regenerated from calli irradiated with 10 Gy (Fig. 3).

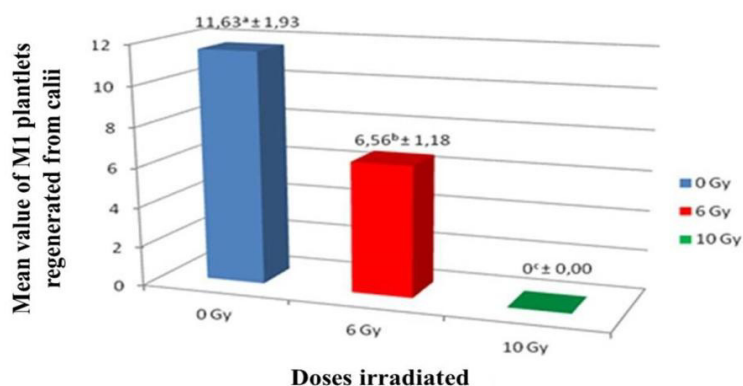


Fig. 3. Mean value of rodent tuber M1 plantlets regenerated from calli irradiated by (0, 6, and 10) Gy gamma rays.

Irradiation can inhibit calli regeneration to plantlets²⁰. The number of viable mutants depends on the effects of irradiation against somatic cells, which could induce abnormality and reduce viability²¹. Reduced regeneration capacity at higher dose of gamma rays was due to toxicity of irradiation towards cells and also caused by low cell competency²². Plantlets regenerated from irradiated calli are possibly solid mutants because it originated from one cell whose genome has been destructed while they were undergoing cell division²³.

3.3. Genetic changes detection of rodent tuber mutants generated from calli

Genetic changes detection was done on 11 M1 rodent tuber mutants originated from calli to analyze genetic changes between irradiated rodent tuber plants. This research had been successfully detected genetic changes of 11 M1 rodent tuber mutants originated from calli. The DNA bands generated had been categorized into two categories, i.e. monomorphic and polymorphic bands. Polymorphic bands gave important information in detecting genetic changes because it showed genetic changes of DNA which served as primer annealing sites. Nine out of 10 primers showed polymorphic changes. Specifically, 10 primers used in this research yield 69 bands in total which 38 of them are polymorphic (Table 1).

Table 1. Quantity of total bands and polymorphic bands yielded from 12 rodent tuber plants (normal mother plantlet and 11 M1 rodent tuber mutants originated from calli) detected using 10 RAPD primers.

Primers	Sequence	Size (bp)	Number of DNA Bands	Number of Polymorphic Bands
OPA-2	TGCCGAGCTG	400 to 1 500	6	0
OPA-9	GGGTAACGCC	950	1	1
OPA-13	CAGCACCCAC	600 to 2 000	8	5
OPB-18	CCACAGCAGT	850 to 2 500	6	1
OPC-5	GATGACCGCC	500 to 2 500	10	8
OPC-8	TGGACCGGTG	750 to 4 000	7	2
OPD-8	GTGTGCCCCA	600 to 2 400	7	4
OPD-20	ACCCGGTCAC	600 to 2 600	8	5
OPE-3	CCAGATGCAC	300 to 1 800	5	1
OPE-20	AACGGTGACC	300 to 2 700	11	11
TOTAL			69	38

OPE-20 primer generated most of the polymorphic bands, i.e. 11 bands (Fig. 4), followed by OPC-5 primer which generated eight polymorphic bands (Fig. 5), and OPA-13 along with OPD-20 primer which generated five polymorphic bands. Primer that did not produce any polymorphic bands was OPA-2, while OPA-9, OPB-18, and OPE-3 primer only produced 1 polymorphic band.

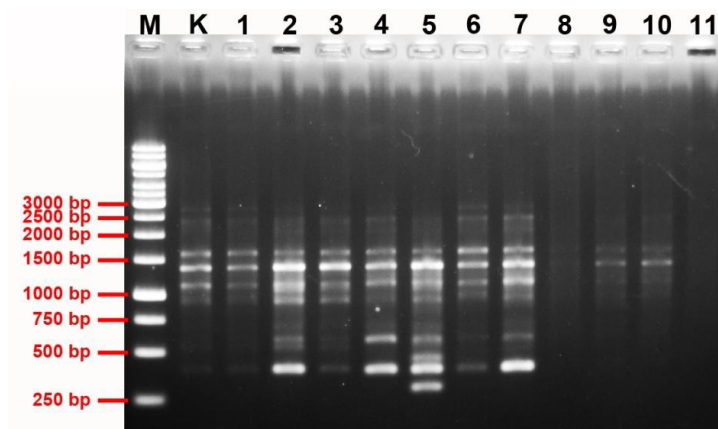


Fig. 4. DNA bands profile of M1 rodent tuber mutants analyzed by OPE-20 primer. (M) marker; (K) control; (1) M-6/1-1, (2) M-6/1-3; (3) M-6/2-1; (4) M-6/2-3; (5) M-6/2-7; (6) M-6/3-1; (7) M-6/3-3; (8) M-6/4-1; (9) M-6/5-1; (10) M-6/5-2; (11) M-6/6-1. Red arrows indicate specific DNA bands of M-6/2-7

The effect of irradiation towards DNA can be analyzed by detecting changes of DNA bands profile between normal mother plantlet and mutant plants by using OPE-20 and OPC-5 RAPD molecular markers. Mutation can induce changes in genome sequence which effect primer annealing sites and therefore also change DNA bands in RAPD profile. One base difference in genome sequence may inhibit the annealing of primers²⁴. Changes in OPE-20 DNA bands profile occurred in size ranging from 250 bp to 1 500 bp, while changes in OPC-5 occurred in the size ranging from 500 bp to 1 500 bp. One of the specific changes detected in OPE-20 RAPD profile can be seen in 300 bp and 450 bp DNA bands of M-6/2-7. Specific changes in DNA profile of one mutant indicated that this mutant is unique among others in term of genetic profile.

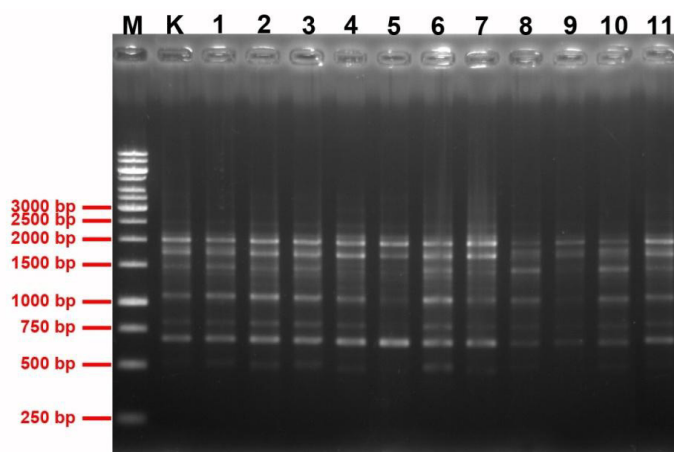


Fig. 5. DNA bands profile of M1 rodent tuber mutant originated from calli by using OPC-5 primer. (M) marker; (K) control; (1) M-6/1-1; (2) M-6/1-3; (3) M-6/2-1; (4) M-6/2-3; (5) M-6/2-7; (6) M-6/3-1; (7) M-6/3-3; (8) M-6/4-1; (9) M-6/5-1; (10) M-6/5-2; (11) M-6/6-1

DNA bands profile of normal mother plantlet and 11 M1 rodent tuber mutants originated from calli which had been analyzed by using 10 RAPD primers shown a high degree of genetic variation, where some mutants produced bands that were different from control. Even mutants irradiated by the same dose of gamma rays could yield a different DNA bands profile. Mutant plants in this research were generated from different cells of irradiated calli which also had different responses to irradiation. Therefore, DNA bands profile depends on the extent by which irradiation affecting cells. The same size of DNA bands between mutants proved amplified DNA sequence homology, while specific bands in one mutant proved the unique genetic variety²⁴.

RAPD molecular markers had successfully detected genetic changes between 11 M1 rodent tuber mutants originated from calli. The main changes in DNA bands profile of M1 rodent tuber mutants in comparison with normal mother plant were the presence of new DNA bands, the loss of DNA bands, and also the variation of DNA bands intensity. Genetic differences between mutants were resulted from DNA damage due to irradiation which caused changes in DNA structure²⁵. Irradiation can induce genome, chromosome, gene, and organelle mutation¹². RAPD molecular markers had been used for detecting mutant resulted from gamma ray irradiation, such as mutants of *Rhododendron*²⁶, *Ipomoea batatas*²⁷, *Saccharum officinarum*²⁸, and *Chrysanthemum mori folium*²⁹.

DNA bands of M1 rodent tuber mutants had been converted to binary number (Supplementary 1), where “1” indicates the presence of band and “0” indicates the absence of band. Binary data of RAPD profile can be used to produce genetic similarity matrix (Table 2) using NTSys software and Dice similarity coefficient¹⁹. This research proved the genetic similarity between M1 rodent tuber mutants with coefficient ranging from 0.75 to 0.96 (75 % to 96 %).

Table 2. Genetic similarity matrix between rodent tuber control plant and 11 M1 rodent tuber mutants originated from calli.

	Control	M-6/1-1	M-6/1-3	M-6/2-1	M-6/2-3	M-6/2-7	M-6/3-1	M-6/3-3	M-6/4-1	M-6/5-1	M-6/5-2	M-6/6-1
Control	1.00											
M-6/1-1	0.96	1.00										
M-6/1-3	0.92	0.95	1.00									
M-6/2-1	0.96	0.95	0.93	1.00								
M-6/2-3	0.93	0.92	0.90	0.95	1.00							
M-6/2-7	0.88	0.85	0.85	0.91	0.92	1.00						
M-6/3-1	0.92	0.91	0.90	0.95	0.95	0.91	1.00					
M-6/3-3	0.90	0.91	0.87	0.92	0.94	0.90	0.94	1.00				
M-6/4-1	0.83	0.83	0.81	0.84	0.80	0.75	0.82	0.75	1.00			
M-6/5-1	0.88	0.87	0.85	0.89	0.87	0.84	0.85	0.81	0.88	1.00		
M-6/5-2	0.91	0.90	0.88	0.92	0.89	0.86	0.90	0.86	0.90	0.93	1.00	
M-6/6-1	0.86	0.85	0.83	0.87	0.84	0.80	0.81	0.78	0.95	0.92	0.89	1.00

There was no M1 rodent tuber mutant with 100 % genetic similarity which indicated that all mutants had undergone different mutation. M-6/2-7 and M-6/4-1 alongside with M-6/2-7 and M-6/3-3 showed 75 % genetic similarities which were the lowest similarity among others. Normal mother plantlet and M-6/1-1 alongside with control plant and M-6/2-1 mutant showed 96 % genetic similarity which is the highest among others. This result indicated that M-6/1-1 and M-6/2-1 mutants were not so different with control, while M-6/4-1 mutant that has 83 % genetic similarity with normal mother plantlet was the most different mutant among others.

Variations of polymorphic bands quantity and genetic similarity matrix produced in this research indicate that 10 primers used were not so effective to detect genetic changes between M1 rodent tuber mutants. This was due to the

lack of information regarding the suitable primers to detect genetic changes of rodent tuber. The primers OPA-2, OPA-9, OPB-18, OPC-5, and OPC-8 had been proven to be effective in detecting genetic changes between *Typhonium trilobatum*, *Typhonium roxburgii*, and *Typhonium flagelliforme*¹⁶, but this research shown that only OPC-05 generated the most polymorphic bands, while the others only greeted a few polymorphic bands. The differences between literature and this research results may be due to the difference of plant variety, although they were all *Typhonium flagelliforme*. This research also showed that RAPD primers that can be used to detect genetic changes between species do not always suitable to be used as markers to detect genetic changes due to mutation induction.

Cluster analysis also performed to know the grouping of mutants based on genetic similarity between individual plants. Fig. 6 shows dendrogram and clustering of control and 11 M1 rodent tuber mutants.

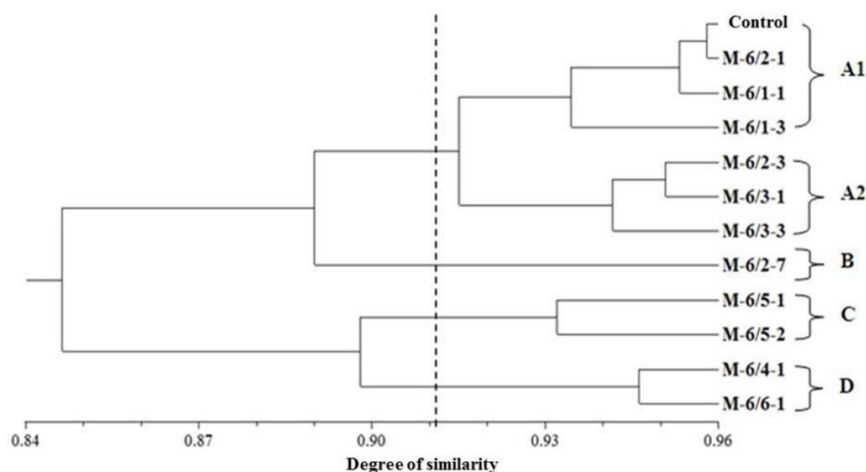


Fig. 6. Dendrogram of M1 rodent tuber mutants based on RAPD molecular markers. Vertical line indicates cut-off point at 0.90 similarity coefficient.

Cut-off at 0.90 similarity coefficient had made dendrogram clustered into 4 main groups (Table 3). Group A consisted of seven rodent tuber mutants. Group A therefore further clustered to 2 groups, i.e. A1 group at 0.93 coefficients which consisted of control plant, M-6/2-1, M-6/1-1, and M-6/1-3 mutants, alongside with A2 group which consisted of M-6/2-3, M-6/3-1, and M-6/3-3 mutants.

Group B consisted of 1 rodent tuber mutant, i.e. M-6/2-7, group C consisted of 2 rodent tuber mutants, i.e. M-6/5-1 and M-6/5-2 with 0.93 similarity coefficients. Group D consisted of 2 rodent tuber mutants, i.e. M-6/4-1 and M-6/6-1 with 0.95 similarity coefficients. Clustering of rodent tuber had shown that M-6/2-7 mutant was a unique mutant because it was the most different mutant among others.

Table 3. Clustering of 12 rodent tubers (control and 11 M1 rodent tuber mutants originated from calli) based on dendrogram

Main groups	Sub groups	Similarity coefficients	Plants
A	A1	0.93	kontrol, M-6/2-1, M-6/1-1, M-6/1-3
	A2	0.94	M-6/2-3, M-6/3-1, M-6/3-3
B		0.89	M-6/2-7
C		0.93	M-6/5-1, M-6/5-2
D		0.95	M-6/4-1, M-6/6-1

Detection of genetic diversity between normal mother plantlet and mutant of other plants had also been done using RAPD molecular marker. As for rice cultivars, irradiation was done to produce mutant plants which were

resistant to glufosinate herbicide and to obtain marker of the resistant gene by using RAPD detection method³⁰. RAPD molecular markers had also been used to detect genetic variation between tomato plants regenerated from calli with 90 to 99 % genetic similarity³¹. Other research had shown variation between Chrysanthemum mutants by using RAPD molecular markers. Clustering analysis had made the mutants grouped into five groups with high genetic similarity and two mutants with low genetic similarity³². Genetic variation between sugarcane irradiated with gamma rays and detected with RAPD molecular markers had shown that the genetic similarity between normal mother plantlet and mutants will be decreased in line with increasing irradiation dose which can produce mutant with unique DNA bands profile.

4. Conclusion

This research had successfully obtained first generation mutants (M1) of rodent tuber through gamma rays irradiation and detected genetic changes of mutants by using RAPD molecular markers. 59 plantlets of M1 rodent tuber mutants had been regenerated from nine calli irradiated by 6 Gy gamma rays with the mean value of 6.56 plantlets. Nine out of 10 RAPD primers yield polymorphic DNA bands when detecting genetic profile of 11 M1 rodent tuber mutants. OPE-20 primer showed 11 polymorphic DNA bands in size ranging from 250 bp to 2 500 bp which is the highest degree of genetic changes among others. Genetic similarity coefficient between M1 rodent tuber mutants originated from calli ranged from 0.75 to 0.96 (75 % to 96 %). Cluster analysis showed four main groups of M1 rodent tuber mutants at the 0.90 cut-off point.

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