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Analysis of Gamma Irradiated-Third Generation Mutants of Rodent Tuber (*Typhonium flagelliforme* Lodd.) Based on Morphology, RAPD, and GC-MS Markers

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ABSTRACT

Rodent tuber is an anticancer plant. The natural genetic diversity of rodent tuber is low due to vegetative propagation. It is important to increase the plant's genetic diversity in order to obtain plants with a high amount of anticancer compounds. *In vitro* calli were irradiated with gamma rays to increase its genetic diversity. Seventeen clones of the first generation of vegetative mutants in a green house (MV1) were propagated until MV3. This research aimed to analyse the stability of mutation in MV3 based on morphology, RAPD and GC-MS markers. Clone 6-1-2 had the highest increase of shoots and leaf number than the control and the other MV3 clones while clone 6-1-3-4 had the highest fresh and dry weight. RAPD analysis using 15 primers produced 67 polymorphic DNA bands and showed four main clusters at the similarity coefficient cut-off of 0.87. The GC-MS showed that MV3 contained at least eight types of anticancer compound in the leaves and six types in the tubers; these were higher than in the control. MV3 leaves and tubers contained at least eight new anticancer compounds that were not found in the control. This research proved that rodent tuber MV3 clones were solid mutants and had a high potential for being developed into anticancer drugs.

Keywords: anticancer compounds, gamma irradiation, GC-MS, morphology, RAPD, *Typhonium flagelliforme* Lodd

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INTRODUCTION

Rodent tuber (*Typhonium flagelliforme* Lodd.) is a herbal plant from the Araceae family (Essai, 1986) that contains detoxification and anticancer compounds. Rodent tuber is a plant native to Indonesia and has been used as traditional medicine for years. Bioactive compounds of rodent tuber are alkaloids, saponins, steroids and glycosides (Syahid, 2007). Anticancer compounds can be found in all parts of the rodent tuber plant, including the root, tuber, stem and leaf (Choo et al., 2001).

Rodent tuber has been reported to be cytotoxic against cancer of the lung, breast (Lai et al., 2010), liver (Lai et al., 2008), blood (leukemia) (Mohan et al., 2010), colon, prostate gland and cervix (Hoesen, 2007). Rodent tuber extract has also been reported to be able to prevent breast and cervical cancers (Syahid & Kristina, 2007). Rodent tuber hexane extract was toxic against Artemia salina (Sianipar et al., 2013a). Other biological activities of rodent tuber have been found to be antibacterial and antioxidant (Mohan et al., 2008) and able to induce apoptosis of cancer cells (Lai et al., 2008).

The development of Indonesian rodent tuber into anticancer drugs was inhibited by its low genetic diversity, which is caused by the conventional clonal vegetative propagation method. Low genetic diversity is followed by low bioactive compound diversity in rodent tuber (Syahid, 2008). Mutation induction of *in vitro* somatic cell population (calli) or shoot culture could increase its genetic diversity, which in turn might increase the probability of generating plant clones that contain a higher amount of anticancer compounds. Mutation can be induced by irradiation with physical mutagens such as gamma ray.

Genetic diversity can be analysed using molecular markers such as RAPD, RFLP, AFLP and SSR (Powell et al., 1996). The Randomly Amplified Polymorphic DNA (RAPD) marker is able to detect genetic diversity of a plant whose genome has not been sequenced yet (McClelland et al., 1994), such as rodent tuber. Genetic characterisation of a plant's germplasm is essential for harnessing the maximum potency of the plant's genetic diversity (Rout, 2006).

Embryogenic calli of rodent tuber plant have been induced, proliferated and regenerated using the single node culture method (Sianipar et al., 2011). Rodent tuber mutant clones have also been successfully generated by combining the effects of gamma irradiation with somaclonal *in vitro* culture variation. A somatic cell population (calli) of rodent tuber was irradiated with 6-Gy gamma rays. The irradiated *in vitro* plantlets exhibited various growth responses (Sianipar et al., 2013b). Those irradiated in vitro plantlets (mutant) and control plants were found to have genetic differences based on analysis with RAPD molecular markers (Sianipar et al., 2015a).

Thirty-seven clones of first generation vegetative mutant of rodent tuber induced in a green house (MV1) were analysed based on morphological and RAPD markers. MV1 clones had various morphological

characteristics (Sianipar et al., 2013c). Out of those 37 MV1 clones, there were 17 that had a diversified genetic profile and showed genetic differences from the control plants based on RAPD molecular analysis (Sianipar et al., 2015b).

Genetic mutation might also affect the relative abundance of bioactive compounds. Gas Chromatography-Mass Spectrometry (GC-MS) is a method for analysing the metabolomic profile of an organism. GC utilises gas as the mobile phase to separate chemical compounds. GC is able to separate a lot of compounds in one run and can be combined with MS to identify the compounds based on the database (Kayser & Quax, 2007). Rodent tuber MV1 clones were propagated and regenerated into MV3 clones. This research aimed to analyse the third generation of vegetative mutant clones of rodent tuber (MV3) based on morphological analysis, molecular marker with RAPD profiling and GC-MS to identify the relative abundance of bioactive compounds.

MATERIALS AND METHODS

Plant Material

This research analysed the third generation of vegetative mutant clones of rodent tuber (MV3) (in the patenting process). Rodent tuber mother plants were obtained from Bogor (West Java, Indonesia).

Morphological Characterisation

The parameters of observation were the number of shoots, number of leaves, plant height and fresh and dry weight of the rodent tuber control and mutant plants. Morphological characteristics were analysed using the NTSYS DIST coefficient and UPGMA.

Molecular Analysis with RAPD Marker

DNA isolation was done based on Doyle and Doyle (1987). In this method, 2.5 g of leaf sample was homogenised with PVP 0.1% and liquified nitrogen. A volume of 2 mL of CTAB buffer (CTAB 10% b/v, EDTA 0.5 M pH 8.0, Tris-HCl 1M pH 8.0, NaCl 5M) and 10 µL of 1-merkaptoetanol 1% (b/v) was added to the sample powder. The sample was homogenised with vortex, incubated at 60°C for 20 min and cooled at room temperature. About 750 µl of chloroform:isoamyl alcohol (24:1) solution was added to the sample and then vortexed. The sample was centrifuged at 11.000 rpm for 10 min. A supernatant was added to 1 mL of chloroform:isoamyl alcohol (24:1) and centrifuged at 11.000 rpm for 10 min. About 750 µL of cold isopropanol, homogenised and stored at -20°C for one night was added to the supernatant. The sample was centrifuged at 11.000 rpm for 10 min. A DNA precipitate was dried in a vacuum for one h. The dried DNA sample was solubilised in 200 µL of buffer TE (Tris-HCl 1M pH 8.0; EDTA 0.5M pH 8.0). About 200 µL of DNA solution was added to 20 µL of RNase (10 mg/mL) and incubated at 37°C for one h. DNA was incubated at 4°C for one night. DNA solution was stored at -20°C. The DNA sample was amplified with 15 decamer primers using

the Thermal Cycler Gene PCR (ABI 9700). The composition of 1x PCR reaction was 5 µL of the DNA template (5 ng/µL), 0.2 µL of dNTP 0.2 mM, 2.5 µL of PCR buffer + $MgCl_2$ (1x), 1 µL of 10 pmol primer, 0.2 µL of 1U Taq polymerase and 16.1 µL of ddH_2O with a total volume of 25 µL. The PCR reaction thermal cycle was repeated 45 times in stages as follows: 94°C for one min, 36°C for one min, 72°C for 2 min and 72°C for 4 min. Extension time was conditioned at 72°C for 4 min. PCR product was fractionated by electrophoresis method in 1.4% agarose gel (w/v) submerged in 40 mL of 1x TAE. Electrophoresis was run at 75 volt for 1.5 h. Agarose gel was submerged in ethidium bromide solution for 10 min and washed with distilled water. Electrophorised gel was visualised under UV light and documented with Kodak gel logic. PCR-RAPD for each sample was replicated at least three times. The size of DNA bands was determined by comparison with 1 kb DNA ladder. Quantitative data were standardised based on Steel and Torrie's (1981) findings. The molecular data of the DNA bands were converted to binary numbers (0 and 1) and formulated in matrix. The relationship between mutant clones was analysed using SHAN clustering UPGMA. Genetic distance and clone grouping were determined using NTSYS ver. 1.70. The similarity index used in this research was Jaccard's (1901) Version 2.4. Chemical Compounds Analysis with GC-MS.

The leaves (including the stem) and tubers of rodent tuber were macerated in 96% ethanol. Extraction was done twice with a sonificator and incubated at room temperature for 48 h. The extracts were filtered with Whatman filter paper and analysed with a GC-MS detector. A volume of 5 µL of extract was injected into the column at a split ratio of 5:1 at 250°C. Helium was used as a carrier gas with a flow rate of 0.8 µL/minute. The initial column oven temperature was 70°C. It was then increased at a rate of 5°C/min until the temperature reached 200°C, after which it was stabilised for 1 min and then increased at the rate of 20°C/min until it reached 280°C for 28 min. The mass spectrometer was adjusted at electron impact ionisation mode at a voltage of 70 eV. Chemical compounds were identified by comparing the MS fragmentation pattern profile with the NIST database.

RESULTS AND DISCUSSION

Morphological Characterisation

Morphological characteristics, specifically the number of shoots, number of leaves, plant height and the fresh and dry weight of 17 MV3 clones were different from those of the control plants (Figure 1 and Table 1). After eight weeks, clone 6-1-2 was found to have the highest increase in shoot (4.5) and leaf number (15) compared to the control and the other MV3 clones. Clone 6-3-2-5

Figure 1. MV3 clones after eight weeks of growing in a green house. a: 6-3-2-5; b: 6-1-1-2; c: 6-9-1

Table 1
Morphological Characteristics of MV3 Clones and Control Plants

Num	Clone	Increase from 1st to 8th week			Total plant weight (g)*	
		Shoot	Leaf	Plant height (cm)	Fresh weight	Dry weight
1	Control	0.0	1.0	3.5	24.33	4.13
2	6-3-3-6	1.0	6.0	4.0	7.25	1.59
3	6-9-3	2.5	3.5	4.0	12.36	1.54
4	6-9-4	0.4	4.0	12.5	14.43	1.9
5	6-2-5-3	0.5	7.0	12.0	7.32	1.77
6	6-3-2-5	1.5	8.0	13.5	27.96	4.10
7	6-1-1-2	3.5	2.0	6.0	24.59	3.67
8	6-9-1	2.5	11.0	4.5	15.82	3.36
9	6-2-4-1	0.0	2.0	3.0	12.6	2.7
10	6-6-3-7	0.5	6.0	7.5	9.03	2.05
11	6-6-3-6	1.0	6.0	12.5	16.03	5.99
12	6-2-7	0.0	5.5	12.0	4.13	0.82
13	6-2-6-3	0.0	5.0	5.5	10.98	2.24
14	6-1-2	4.5	15.0	8.3	22.17	3.96
15	6-1-1-6	1.0	2.0	5.0	16.17	3.62
16	6-2-8-2	2.5	11.5	6.5	21.17	4.67
17	6-9-5	0.0	12.5	10.3	5.32	1.29
18	6-3-3-10	0.0	1.5	7.5	5.54	0.99

* Fresh and dry weight were cumulative of root, leaf, stem, tuber and flower weight

had the highest increase in plant height (13.5 cm) compared to the control and the other MV3 clones. Clone 6-3-2-5 had the highest fresh weight (27.96 gr), while clone 6-6-3-6 had the highest dry weight (5.99 gr) compared to the control and the other MV3 clones. Thus, based on morphological characterisation, MV3 clones were able to produce the higher biomass (number of shoots, number of leaves and plant height) compared to the control (Table 2).

The morphological characteristics of the rodent tuber control and MV3 clones were analysed using the NTSYS programme to generate relative similarity between the clusters. The dendrogram showed six clusters at the similarity coefficient cut-off of 0.73 (Figure 2). Clones in one cluster had a short genetic distance between one another based on morphological observation.

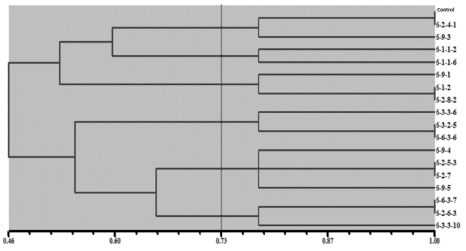

Figure 2. A dendrogram of MV3 clones based on morphological characteristics. The dendrogram was made from binary scores of the number of shoots, number of leaves and plant height data using the NTSYS software at the similarity coefficient cut-off of 0.73

Table 2
Grouping of MV3 Clones Based on Morphological Characteristics

Morphology	Interval	Clone
Increase in the number of shoots from 1st to 8th week	<1	Control, 6-9-4, 6-2-5-3, 6-2-4-1, 6-6-3-7, 6-2-7,6-2-6-3, 6-9-5, 6-3-3-10
	1-2	6-3-3-6, 6-3-2-5, 6-6-3-6, 6-1-1-6
	>2	6-9-3, 6-1-1-2, 6-9-1, 6-1-2, 6-2-8-2
Increase of the number of leaves from 1st to 8th week	<5	Control, 6-9-3, 6-9-4, 6-1-1-2, 6-2-4-1, 6-1-1-6, 6-3-3-10
	5-10	6-3-3-6, 6-2-5-3, 6-3-2-5, 6-2-6-3, 6-6-3-7, 6-6-3-6, 6-2-7
	>10	6-1-2, 6-2-8-2, 6-9-5, 6-9-1
Increase of plant height from 1st to 8th week	<5	Control, 6-3-3-6, 6-9-3, 6-9-1, 6-2-4-1
	5-10	6-1-1-2, 6-6-3-7, 6-2-6-3, 6-1-2, 6-1-1-6, 6-2-8-2, 6-3-3-10
	>10	6-9-4, 6-2-5-3, 6-3-2-5, 6-6-3-6, 6-2-7, 6-9-5

Table 3 shows the similarity coefficient matrix, which represents the genetic distance between the mutant clones based on morphological characteristics. A similarity coefficient of 1.00 (100%) indicates that there is no genetic difference, while one lower than 0.95 (95%) indicates that there is genetic difference between two mutant clones. Based on the morphological analysis, MV3 clones 6-3-2-5, 6-6-3-6, 6-1-2 and 6-2-8-2 were shown to have the lowest genetic similarity (33%) to the control, while MV3 clone 6-2-4-1 showed 100% similarity to the control. The other MV3 clones had 56% (nine clones) and 78% (three clones) genetic similarities to the control. According to this research, gamma irradiation at a dose of 6 Gy was able to increase the number of shoots, number of leaves and plant height of rodent tuber MV3 clones compared to the control plants (Table 1). The observed increase in the MV3 plant's biomass compared to that of the control is a crucial beneficial factor for commercial production of medicinal plants.

Gamma irradiation could induce the release of free radicals in plant cells; this induces somatic genetic cross-over, DNA sequence modification and chromosomal aberration (changes in the number and structure of chromosome). These genetic modifications alter the structure and metabolism of a plant as well as its morphological characteristics (Kovacs & Keresztes, 2002). A low dose of gamma irradiation has also been able to induce morphological alteration of potato plants (Afrasiab & Iqbal, 2010). The diversity of morphological characteristics observed between MV3 clones (Figure 2 and Table 3) was due to random mutation induced by gamma irradiation, which could change a gene's structure and function in a different way in different plants (Surya & Soeranto, 2006; Pillay & Tenkouano, 2011).

Table 3
Genetic Similarity Matrix of MV3 Clones Based on Morphological Characterictics

	Kontrol	6-3-3-6	6-9-3	6-9-4	6-2-5-3	6-3-2-5	6-1-1-2	6-9-1	6-2-4-1	6-6-3-7	6-6-3-6	6-2-7	6-2-6-3	6-1-2	6-1-1-6	6-2-8-2	6-9-5	6-3-3-10
Kontrol	1.00																	
6-3-3-6	0.56	1.00																
6-9-3	0.78	0.56	1.00															
6-9-4	0.78	0.33	0.56	1.00														
6-2-5-3	0.56	0.56	0.33	0.78	1.00													
6-3-2-5	0.33	0.78	0.33	0.56	0.78	1.00												
6-1-1-2	0.56	0.33	0.78	0.56	0.33	1.00												
6-9-1	0.56	0.56	0.78	0.33	0.33	0.33	0.56	1.00										
6-2-4-1	1.00	0.56	0.78	0.78	0.56	0.33	0.56	0.56	1.00									
6-6-3-7	0.56	0.56	0.33	0.56	0.78	0.56	0.56	0.33	0.56	1.00								
6-6-3-6	0.33	0.78	0.33	0.56	0.78	1.00	0.33	0.33	0.33	0.56	1.00							
6-2-7	0.56	0.56	0.33	0.78	1.00	0.78	0,33	0.33	0.56	0.78	0.78	1.00						
6-2-6-3	0.56	0.56	0.33	0.56	0.78	0.56	0.56	0.33	0.56	1.00	0.56	0.78	1.00					
6-1-2	0.33	0.33	0.56	0.33	0.33	0.33	0.78	0.78	0.33	0.56	0.33	0.33	0.56	1.00				
6-1-1-6	0.56	0.56	0.56	0.56	0.33	0.56	0.78	0.33	0.56	0.56	0.56	0.33	0.56	0.56	1.00			
6-2-8-2	0.33	0.33	0.56	0.33	0.33	0.33	0.78	0.78	0.33	0.56	0,33	0.33	0.56	1.00	0.56	1.00		
6-9-5	0.56	0.33	0.33	0.78	0.78	0.56	0.33	0.56	0.56	0.56	0.56	0.78	0.56	0.56	0.33	0.56	1.00	
6-3-3-10	0.78	0.33	0.56	0.78	0.56	0.33	0.78	0.33	0.78	0.78	0.33	0.56	0.78	0.56	0.78	0.56	0.56	1.00

According to Van Harten (1998), the normal irradiation doses that are commonly applied to plant cells were at the range of 5-100 Gy. Morphological diversity, which is induced by gamma irradiation, has also been observed in mutant plants of yardlong bean [*Vigna unguiculata* (L.) Walp.], whose number of shoots, number of leaves and plant height were higher compared to those of the control plants (Gnanamurthy et al., 2012). Potato var. Silana (Hamideldin & Hussin, 2013) and soybean (Mudibu et al., 2012), which had been induced by gamma irradiation also had higher plant height compared to controls. However, gamma irradiation at doses of 5-24 Gy on ginger vegetative plants (*Zingiber officinale*) (Rashid et al., 2013) and at doses of 10 and 20 Gy on *Curcuma alismatifolia* (Taheri et al., 2014) resulted in a lower number of leaves and shorter plants compared to the controls. Like MV1 clones (Sianipar et al., 2013c), MV3 clones also had morphological variations due to gamma irradiation (Table 3). Besides genetic mutation, environmental factors also influence morphological characteristics. Therefore, morphological characterisation should be accompanied with molecular marker analysis in order to obtain a more accurate estimation of genetic diversity.

RAPD Molecular Marker Analysis

The concentration of DNA extracts was between 641.70 and 4246.8 ng/µL. The OD_{260}/OD_{280} ratio was between 1.8 and 2.0, which indicated that the extracts were relatively pure (without RNA or protein) (Sinden, 1994). PCR-RAPD of rodent tuber's DNA extracts was done using 15 primers (Table 4), which had also been used for research on the other plants from *Typhonium* genus (Acharya et al., 2005). Primers used in this research were reproducible according to previous RAPD profile analyses of rodent tuber MV1 clones (Sianipar et al., 2015b). There were 67 polymorphic bands (190-3000 bp) out of the total 132 bands produced by 15 primers. The OPB-18 primer produced the highest number of polymophic bands as well as total bands compared to the other primers. Specifically, the OPB-18 primer produced 14 polymorphic bands out of the total 15 DNA bands. RAPD amplification of MV3 with OPB-18 primer produced polymorphic bands at 390 bp, 750 bp, 1350 bp and 3000 bp (Figure 3). The RAPD profile of OPB-18 primer revealed that MV3 clones had new DNA bands but also underwent the loss of DNA bands compared to the RAPD profile of control plants.

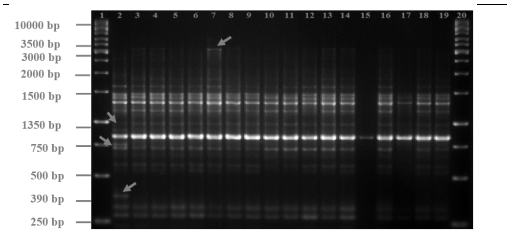

Figure 3. RAPD profile of MV3 clones amplified with OPB-18 primer. 1: Marker 1 kb; 2: Control; 3: 6-3-3-6; 4: 6-9-3; 5: 6-9-4; 6: 6-2-5-3; 7: 6-3-2-5; 8: 6-1-1-2; 9: 6-9-1; 10: 6-2-4-1; 11: 6-6-3-7; 12: 6-6-3-6; 13: 6-2-7; 14: 6-2-6-3; 15: 6-1-2; 16: 6-1-1-6; 17: 6-2-8-2; 18: 6-9-5; 19: 6-3-3-10; 20: Marker 1 kb

Table 4
The Number of DNA Bands Produced by RAPD Amplification of MV3 DNA with 15 Primers

Num	Primer	Sequence	Total bands	Polymorphic bands	Size (bp)
1	OPA-02	5'-TGCCGAGCTG-3'	5	1	450-1250
2	OPA-03	5'-AGTCAGCCAC-3'	10	6	530-2830
3	OPA-09	5'-GGGTAACGCC-3'	10	3	425-1900
4	OPA-14	5'-TCTGTGCTGG-3'	10	8	500-2500
5	OPB-18	5'-CCACAGCAGT-3'	15	14	310-3000
6	OPC-05	5'-GATGACCGCC-3'	13	9	250-1900
7	OPC-08	5'-TGGACCGGTG-3'	10	4	540-2170
8	OPC-11	5'-AAAGCTGCGG-3'	4	3	650-2200
9	OPC-14	5'-TGCGTGCTTG-3'	8	3	600-3000
10	OPD-08	5'-GTGTGCCCCA-3'	6	1	570-2830
11	OPD-10	5'-GGTCTACACC-3'	11	6	190-2000
12	OPD-18	5'-GAGAGCCAAC-3'	7	3	400-1350
13	OPD-20	5'-ACCCGGTCAC-3'	10	5	575-2400
14	OPE-03	5'-CCAGATGCAC-3'	5	0	350-1400
15	OPE-07	5'-AGATGCAGCC-3'	8	1	350-1500
Total			132	67	

RAPD profiles were scored using the NTSYS software to produce a dendrogram for determining relative genetic similarity between clones. According to Rout (2006), a dendrogram represents the genetic relationship between different plant varieties of the same species. A dendrogram of MV3 clones based on the RAPD analysis with a similarity coefficient cut-off of 0.87 showed four main clusters (Figure 4). The genetic similarity matrix showed the genetic distance between MV3 clones (Table 5). The matrix showed that there was no 100% genetic similarity between 17 MV3 clones and the control. Mutant clones 6-9-3, 6-2-5-3, 6-1-1-2, 6-2-6-3, 6-1-2 and 6-3-3-10 each had 71% genetic similarity to the control, which was the lowest among all the clones. Clone 6-6-3-6 had 80% genetic similarity to the control, which was the highest among all the clones. Table 5 showed that there were three clones with 73%, one with 74%, one with 80%, one with 75%, four with 76%, two with 77% and one with 78% genetic similarity to the control.

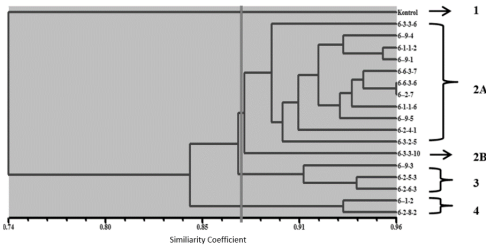

Figure 4. Dendrogram of MV3 clones based on RAPD profile. The dendrogram was generated by scoring of DNA bands in RAPD profiles of 15 primers using NTSYS UPGMA and SHAN clustering at the similarity coefficient cut-off of 0.87

The RAPD molecular marker is able to detect DNA polymorphism without taking environmental factors into account (Guimaraes et al., 2007). The RAPD molecular marker, which utilises random primers, was able to show genetic diversity between the control and the MV3 clones. Single random primers have been known to be able to amplify DNA and show the polymorphism of mutant DNA (Williams et al., 1990). DNA mutation could change primer annealing sites in a plant's genome, which is followed by change in the RAPD profile of DNA bands (Tindall et al., 1988). The number of DNA bands produced by a primer is determined by the number of complementary sequences in the genome (Singh & Singh, 1995).

DNA polymorphism of MV3 clones is due to gamma irradiation on somatic cell population. Gamma irradiation is able to induce the production of reactive free radicals, which can change the DNA sequence and break DNA double bonds. Gamma irradiation also causes chromosomal aberration such as deletion, invertion, translocation and duplication (Gorbunova & Levy, 1997). The loss of DNA bands observed in the RAPD profile of MV3 clones (Figure 3) was probably caused by destruction of DNA, rearrangement of chromosomes and deletion or insertion of DNA nucleotides. The existence of new DNA bands observed in the RAPD profile of MV3 but not in the control was probably caused by deletion or invertion of DNA nucleotides (Yunus et al., 2013).

RAPD analysis of *Rhododendron* also showed genetic differences between the control and MV3 clones, which were irradiated with gamma ray at doses of 5 and 10 Gy (Atak et al., 2011). Yunus et al. (2013) also proved that RAPD analysis was effective in deleting DNA polymorphism of ginger plants (*Etlingera elatior*) irradiated with 10 Gy gamma ray. Polymorphism analysis of gamma-irradiated DNA has also been done on potato plants (Yaycili & Alikamanoglu, 2012), Hibiscus *Sabdariffa* L. (Sherif et al., 2011) and anthurium plants (Puchooa, 2005). A genetic similarity matrix (Table 5) of the results shows the diversity of genetic modification between the MV3 clones. The observed genetic similarity variation between the MV3 clones was due to random mutation caused by gamma irradiation (Pillay & Tenkouano, 2011). According to Pillay and Tenkouano (2011), the DNA repair mechanism of potato plants was different from one cell to another; therefore, they showed different genetic profiles. Based on morphological markers, MV3 clone 6-2-4-1 had 100% similarity to the control, while, based on the molecular marker, clone 6-2-4-1 had 73% genetic similarity to the control. The difference in genetic similarity between morphological and molecular markers was due to the influence of the environment on morphological analysis.

The genetic difference between different rodent tuber mutant clones (MV0 and MV1) has also been assessed by applying the PCR-RAPD technique (Sianipar et al., 2015a; Sianipar et al., 2015b).

Table 5
Genetic Similarity Matrix of MV3 Clones Based on RAPD Molecular Marker

	Kontrol	6-3-3-6	6-9-3	6-9-4	6-2-5-3	6-3-2-5	6-1-1-2	6-9-1	6-2-4-1	6-6-3-7	6-6-3-6	6-2-7	6-2-6-3	6-1-2	6-1-1-6	6-2-8-2	6-9-5	6-3-3-10
Kontrol	1.00																	
6-3-3-6	0.73	1.00																
6-9-3	0.71	0.90	1.00															
6-9-4	0.74	0.90	0.88	1.00														
6-2-5-3	0.71	0.86	0.91	0.91	1.00													
6-3-2-5	0.75	0.89	0.90	0.90	0.89	1.00												
6-1-1-2	0.71	0.92	0.86	0.94	0.91	0.92	1.00											
6-9-1	0.76	0.89	0.83	0.92	0.89	0.87	0.95	1.00										
6-2-4-1	0.73	0.83	0.83	0.92	0.85	0.86	0.89	0.89	1.00									
6-6-3-7	0.78	0.92	0.86	0.93	0.87	0.89	0.93	0.93	0.90	1.00								
6-6-3-6	0.80	0.88	0.86	0.92	0.87	0.92	0.90	0.92	0.90	0.94	1.00							
6-2-7	0.77	0.90	0.89	0.92	0.88	0.92	0.91	0.92	0.92	0.95	0.96	1.00						
6-2-6-3	0.71	0.87	0.91	0.86	0.94	0.87	0.88	0.88	0.83	0.89	0.86	0.88	1.00					
6-1-2	0.71	0.80	0.79	0.85	0.82	0.83	0.85	0.85	0.86	0.86	0.87	0.86	0.80	1.00				
6-1-1-6	0.77	0.89	0.90	0.90	0.87	0.89	0.90	0.90	0.90	0.92	0.94	0.95	0.89	0.87	1.00			
6-2-8-2	0.73	0.82	0.80	0.87	0.80	0.85	0.84	0.86	0.87	0.88	0.91	0.89	0.80	0.93	0.89	1.00		
6-9-5	0.76	0.89	0.86	0.92	0.86	0.90	0.92	0.92	0.91	0.93	0.93	0.92	0.88	0.85	0.93	0.89	1.00	
6-3-3-10	0.71	0.87	0.85	0.85	0.85	0.86	0.89	0.88	0.85	0.87	0.87	0.88	0.86	0.82	0.90	0.81	0.91	1.00

Analysis of Chemical Compounds with GC-MS

GC-MS analysis revealed that the leaves of MV3 clones contained at least eight anticancer compounds i.e. more than in the control plants (Table 6). The quantity of anticancer compound stigmasta-5,22-dien-3-ol (3-beta) in leaves of clone 6-1-1-6 was 10.96% higher than in the controls and was also the highest compared to the other MV3 clones. The leaves of MV3 clones also contained new anticancer compounds that were not found in the control plants, such as coumaran, gamma tocopherol, 1-phenanthrenecarboxylic acid and oleic acid (Table 6).

Table 6
The Number of DNA Bands Produced by RAPD Amplification of MV3 DNA with 15 Primers

Compounds	% Relative abundance in control	% Relative abundance in mutant clones							
		6-9-1	6-3-3-6	6-1-1-6	6-3-2-5	6-6-3-6	6-1-1-2	6-1-2	6-2-8-2
Hexadecanoic acid	11,64	10,5	13,09	2,19	16,85	22,48	7,51	19,21	14,92
Alpha-tocopherol	3,36	0	0	0	2,84	0	3,5	4,11	0
Coumaran	0	0,7	0	0	0	0	0	0	0
Gamma-tocopherol	0	1,45	0	0	1,38	0	0	0	0
Ergost-5-en-3-ol (3 beta)	6	7,42	0	13,58	3,3	0	2,69	0	0
9-Octadecenamide	1,11	1,75	2,63	4,2	1,06	1,72	2,83	1,83	1,71
Squalene	3,8	2,65	0	0	3,46	0	4,6	0	0
Stigmasta-5,22-dien-3-ol (3 beta)	12,84	13,68	0	23,8	13,41	0	13,37	9,89	9,02
Stigmast-5-en-3-ol (3.beta.,24s)	2,42	4,02	0	7,28	0	0	0,52	0	0
1-Phenanthrenecarboxylic acid	0	0	0	0	0	0	0,38	0	0
Oleic acid	0	0	0	0	0	0	0,62	0	0
4-vinyl-2-methoxy-phenol	0,28	0	0	0	0	0	0	0,43	0

The highlights indicate the higher quantities of anticancer bioactive compounds in MV3 clones compared with the control

The GC-MS analysis of tubers of MV3 clones revealed that they contained six anticancer compounds, more than in the control plants (Table 7). The quantity of hexadecanoic acid methyl ester of tubers of clone 6-6-3-6 was 15.37% higher than in the control and was also the highest compared to the other MV3 clones. Tubers of MV3 clones also contained new anticancer compounds that were not found in the control plants i.e. alpha-tocopherol, stigmasta-5,22-dien-3-ol (3-beta), beta elemene and dodecanoic acid.

Table 7
The Number of DNA Bands Produced by RAPD Amplification of MV3 DNA with 15 Primers

Compounds	% Relative abundance in control	% Relative abundance in mutant							
		6-9-1	6-3-3-6	6-1-1-6	6-3-2-5	6-6-3-6	6-1-1-2	6-1-2	6-2-8-2
Hexadecanoic acid	8,91	11,81	5,2	10,91	13,57	13,2	0,06	0,1	14,29
Alpha-tocopherol	0	0,3	0,16	0	0	0	0	0	0
9-octadecenamide	0,76	0,35	1,13	0	0,5	0,64	0	0	0
Stigmasta-5,22-dien-3-ol (3 beta)	0	0	0	0	0	4,78	3,8	0	0
Stigmast-5-en-3-ol (3.beta.,24s)	2,44	2	0	0	2,21	2,83	0	0	0
Tanshinone II-b	0,17	0,59	0	0	0	0	1,64	0	0
Hexadecanoic acid ethyl ester	20,98	13,61	20,58	15,08	18,07	0	24,26	35,9	7,57
Hexadecanoic acid methyl ester	0,20	0,30	0,28	0	0,25	15,57	0	0	0
Beta elemene	0	0	0	0	0	0	0,12	0	0
Dodecanoic acid	0	0	0	0	0	0	0	0,7	0
Oleic acid	0	0	0	0	0	0	0,62	0	0
4-vinyl-2-methoxy-phenol	0,28	0	0	0	0	0	0	0,43	0

The highlights indicate the higher quantities of anticancer bioactive compounds in MV3 clones compared with the control

The GC-MS analysis showed that the leaves of MV3 clones contained at least eight anticancer compounds, more than in the control plants. The quantity of anticancer compound stigmasta-5,22-dien-3-ol (3-beta) of clone 6-1-1-6 was the highest compared to the control and the other MV3 clones (Table 6). Stigmasta-5,22-dien-3-ol (3 beta) (stigmasterol) is a phytosterol that could reduce the number of *Ehrich Ascites Carcinoma* (EAC). Stigmasterol is able to activate the protein phosphatase 2A by ceramide to promote the apoptosis of cancer cells. Stigmasterol also exerts an antioxidant effect because it is able to reduce lipid peroxidation and increase gluthathionin, superoxide dismutase and catalase activities in EAC mice liver (Ghosh et al., 2011).

The leaves of the MV3 clones also contained new anticancer compounds that were not found in the control plants, such as coumaran, gamma-tocopherol, 1-phenanthrenecarboxylic acid and oleic acid. Coumaran (2,3-dihydrobenzofuran) and its derivatives are able to inhibit the polymerisation of tubulin, thus inhibiting mitosis (Pieters et al., 1999). Vitamin E (α dan γ-tocopherol) has been proven to be able to reduce the risk of developing several kinds of cancer (Jiang et al., 2001). The compound, 1-phenanthrenecarboxylic acid, shows anticancer activity against KB nasopharyngeal cancer, Hop62 lung cancer, ME180 cervical cancer and K562 leukemia (Tatiya et al., 2014). Oleic acid is a monounsaturated fatty acid n-9 with anticancer activity against breast cancer (Escrich et al., 2008) and colon adenocarcinoma (Carrillo et al., 2011).

The GC-MS analysis of tubers of MV3 clones revealed that they had six anticancer compounds; this was more than in the control plants. The quantity of hexadecanoic acid methyl ester of tubers of clone 6-6-3-6 was the highest compared to the control and the other MV3 clones. Hexadecanoic acid methyl ester has been able to induce the apoptosis of human gastric cancer cells (Yu et al., 2005).

The tubers of the MV3 clones also contained the new anticancer compounds that were not found in the control plants i.e. alpha-tocopherol, stigmasta-5,22-dien-3-ol (3-beta), beta elemene and dodecanoic acid. Beta-elemene has anti-proliferative activity against prostate, lung, colon, cervical, breast and brain cancers (Li et al., 2010). Dodecanoic acid (lauric acid) has been proven to be able to induce apoptosis of colon cancer cells (Fauser et al., 2013). This finding was consistent with previous analysis of rodent tuber MV1 phytochemicals, whose shoots and tubers contained at least eight anticancer compounds, which is a higher number of anticancer compounds than in the control. The shoots and tubers of MV1 also contained new anticancer compounds that were not found in the control plants (Sianipar et al., 2015c). Because MV3 clones have a higher plant biomass than the control and contain a higher amount of anticancer compounds than the control, they have a high potential for becoming a new source of anticancer bioactive compounds for the formulation of commercial anticancer drugs.

CONCLUSION

MV3 clones underwent genetic changes not seen in the control according to morphological, RAPD profile and bioactive compound analyses. Based on the RAPD molecular marker, four genetic diversity clusters were shown, indicating that MV3 clones were different from the control. The GC-MS analysis showed that the leaves of the MV3 contained at least eight kinds of anticancer compound, more than in the control. MV3 leaves also contained coumaran, gamma-tocopherol, 1-phenanthrenecarboxylic acid and oleic acid, which were new anticancer compounds not found in the control. The GC-MS analysis also showed that tubers of MV3 contained at least six types of anticancer compound, more than in the control. MV3 tubers also contained new anticancer compounds that were not found in the control i.e. alpha-tocopherol, stigmasta-5,22-dien-3-ol (3-beta), beta elemene and dodecanoic acid. Clone 6-1-1-2 had the lowest genetic similarity to the control, contained a higher amount of anticancer compounds compared to the control and contained new anticancer compounds that were not found in the control. Therefore, rodent tuber MV3 clones are solid mutant clones that have a high potential for being developed into anticancer drugs.

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