

## EFFECT OF GAMMA IRRADIATION ON PROTEIN PROFILE OF RODENT TUBER (*TYPHONIUM FLAGELLIFORME* LODD.) IN VITRO MUTANT BASED ON 1D AND 2D PAGE ANALYSES

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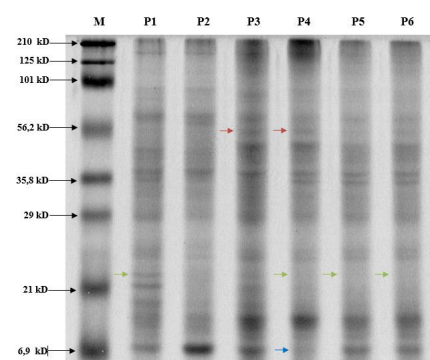
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### Graphical abstract



### Abstract

Rodent tuber is an Indonesian anticancer herbal plant whose genetic variation is low. Genetic variation is crucial to be increased to increase the content of anticancer bioactive compounds. In vitro calli of rodent tuber had been mutated by gamma irradiation. Mutant plants had their genome modified and therefore also affected protein expression. The purpose of this research is to identify protein expression differences between mutant and control rodent tuber plants by 1D and 2D PAGE. 1D PAGE had shown differences in the number of protein bands between control and mutant. M6/2 did not show any expression of 6,9 kD protein. 2D PAGE had identified 24 proteins which were expressed by control and M6/2 plants. Among them, 13 spots were up-regulated and 9 spots were down-regulated in M6/2 plants. In conclusion, 1D and 2D PAGE analyses had successfully proven the changes in protein expression between control and mutant rodent tuber plants.

**Keywords:** *Typhonium flagelliforme* Lodd., in vitro mutant, 1D PAGE, 2D PAGE, gamma irradiation

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## 1.0 INTRODUCTION

Rodent tuber (*Typhonium flagelliforme* Lodd.) is an Indonesian native herbal plant which is commonly found in Java Island [1]. Bioactive compounds of rodent tuber are alkaloid, saponin [2], terpenoid, steroid [3], flavonoid, and glycoside [4]. Rodent tuber has anticancer activities against cancer of liver [5], breast, colon, prostate gland, cervix [2], and leukemia [6]. Rodent tuber has been propagated conventionally by tuber separation method. Thus, the genetic diversity of rodent tuber is low [2], while the

potency of its bioactive compounds as anticancer medicine is high [7].

Rodent tuber has been propagated in vitro on Murashige and Skoog (MS) media supplemented with the optimum concentration of plant growth regulators, i.e. 1 mgL<sup>-1</sup> 1-Naphthaleneacetic acid (NAA) and 0.5 mgL<sup>-1</sup> 6-Benzyl Amino Purine (BAP) [8]. In vitro calli of rodent tuber had been irradiated with gamma rays and therefore produced in vitro mutant plants of rodent tuber. Rodent tuber in vitro mutants and control plants, which are not irradiated by gamma rays, had shown differences in plant heights

and mean number of shoots [9]. Gamma irradiation is one of the most common mutation methods [10] because it is effective to induce biochemical and physical changes of a plant [11]. Genetic mutation is able to induce changes in protein expressions [12] and secondary metabolite components [13] of a plant.

Proteomics is the systematic and quantitative analysis of the sequence, structure, function, modification, translocation, and biological expression control of cellular proteins [14]. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS PAGE) is one of proteomics methods [15, 16, 17]. SDS PAGE separates proteins based on their molecular weights. A protein with bigger molecular weight will move slower to the positive pole rather than a protein with smaller molecular weight [12].

Two Dimensional Polyacrylamide Gel Electrophoresis (2D PAGE) is one of PAGE methods which combines the principle of SDS PAGE and Isoelectric Focusing (IEF) [18]. 2D PAGE separates proteins based on their molecular weights and isoelectric points. 2D PAGE is commonly used to identify new proteins and compare the quantity of proteins in different samples [19]. 2D PAGE is able to identify more than 2000 proteins in one gel, so this method is very useful for proteomics analysis [18]. Protein expression of rodent tuber in vitro mutants had never been identified before. This research is aimed to analyze the effect of gamma irradiation on protein expression of rodent tuber in vitro mutants by identifying the 1D SDS and 2D PAGE protein profile differences between control and mutant plants.

## 2.0 METHODOLOGY

### 2.1 Preparation of Planting Materials

Rodent tuber in vitro mutants was propagated in vitro on Murashige and Skoog (MS) media supplemented with  $1 \text{ mgL}^{-1}$  NAA and  $0.5 \text{ mgL}^{-1}$  BAP and acclimatized in the green house of Indonesian Center for Agricultural Biotechnology & Genetic Resources (BB-Biogen). After 8 weeks being acclimatized, leaves of rodent tuber mutant and control plants were harvested and processed for 1D and 2D PAGE analyses.

### 2.2 Protein Isolation

1D and 2D PAGE utilize different methods of protein isolation. Protein samples for SDS PAGE were isolated by using 50 mM of sodium phosphate buffer and 10% *insoluble polyvinylpyrrolidone* (PVPP) [20]. Protein samples for 2D PAGE were isolated by using ReadyPrep Protein Extraction Kit Total Protein (BioRad) and then purified by *ReadyPrep 2-D clean up kit* (BioRad).

### 2.3 Protein Quantification

Proteins were quantified by Bradford method at wavelength of 595 nm. Standard of protein

quantification was 2 mg/ml Bovine Serum Albumin (BSA).

### 2.4 SDS PAGE Analysis

Protein samples were separated by electrophoresis with SDS PAGE according to [21]. SDS PAGE gel was composed of 6% stacking gel and 12% resolving gel. Protein sample concentration was  $20 \mu\text{g}/\mu\text{l}$ . Gel was immersed in staining solution and then washed with destaining solution. Protein marker used in this experiment was Broad Range (Biorad) marker which composed of proteins with molecular weight of 6900 – 210000 Da. SDS PAGE data was obtained in the form of protein bands.

### 2.5 Analysis of Protein Profile by 2D PAGE

2D PAGE analysis was done through 3 steps, i.e. rehydration of IPG Strips, protein separation in one dimension, and protein separation in two dimension. IPG strip (7 cm) with pH 3-10 (BioRad) was rehydrated in ReadyPrep 2D Starter kit Rehydration/Sample Buffer which had been added with  $150 \mu\text{g}/\mu\text{l}$  protein samples for 1 hours in room temperature. Protein separation in one dimension was done through the principle of isoelectric focusing. Briefly, IPG strips were rehydrated at  $10^\circ\text{C}$  for 12 hours and then separated on graduated voltages (250 V for 20 minutes, 4000 V for 120 minutes, and 4000 V for 10000 seconds). IPG strips were then immersed in equilibrium buffer 1 and 2 (BioRad). IPG strips were separated by inserting IPG strips in 12% resolving gel and closed with agarose overlay. 5  $\mu\text{l}$  of marker protein was pipetted on filter paper. Gel was electrophorized at 100 V for 120 minutes. Gel was immersed in staining solution for 15 minutes and washed with destaining solution. The resulted protein spots were then analyzed by PDQuest 2D Analysis Software (Biorad).

## 3.0 RESULTS AND DISCUSSION

### 3.1 SDS PAGE Analysis

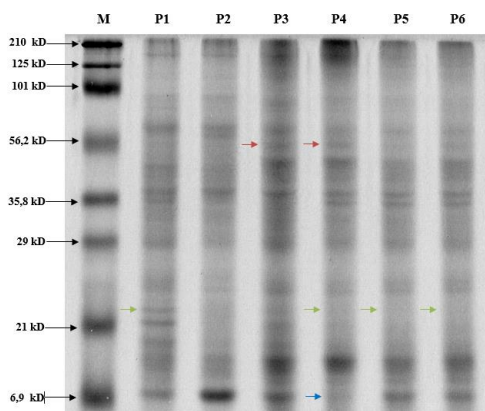
The availability of rodent tuber in nature is limited because rodent tuber only propagate on damp, shady areas [22]. Plant tissue culture is therefore crucial to produce many rodent tuber plants in a short amount of time [23]. In vitro culture is able to increase genetic variation by inducing somaclonal variation. Somaclonal variation is caused by mutation, changes in chromosome structure, gene amplification, gene methylation, or transposon activation during the in vitro culture of a plant [24].

Rodent tuber from Bogor had been propagated on MS media supplemented with  $1 \text{ mgL}^{-1}$  NAA and  $0.5 \text{ mgL}^{-1}$  BAP [8]. Rodent tuber was also irradiated with gamma rays to increase its genetic diversity. Gamma irradiation resulted in differences in shoot lengths and mean number of shoots between control and mutant plants [8]. Genetic variation, which is

induced by mutation, could affect the plant's morphological features [13]. Gamma irradiation caused genetic changes through induction of site mutation on a gene (single nucleotide changes) and chromosomal aberration (deletion, translocation, insertion) [25]. RAPD analysis of rodent tuber in vitro mutants had proven the genetic changes of 11 mutant plantlets from control. This genetic variation was detected at molecular weight of 250-2500 bp by 9 polymorphic primers [26].

Mutation of gene could affect the expression and function of proteins [27, 28]. Protein profile was analyzed by 1D SDS PAGE and 2D PAGE methods. The first step in proteomics analysis was protein isolation [29]. Protein extraction from plant tissue samples is commonly hard to be achieved due to the low content of protein and the presence of a large amount of polysaccharide, lipids, and phenolic compounds [30]. In this research, total protein extraction from rodent tuber leaves was done by using sodium phosphate buffer and 10% insoluble PVPP. PVPP isolates protein through phenolic precipitation. PVPP is able to inhibit protease activity and remove contaminants such as phenolic and nucleic acid so the isolated protein has a higher quantity and purity [31].

Standard curve equation was obtained from Rf value and log of marker protein's molecular weight. This equation was used to determine the molecular weights of each protein band on 1D SDS PAGE gel (Figure 1). Rf values of each band on gel were counted and substituted in standard curve equation. Molecular weights of proteins on each band were determined by  $10^y$  (y is a log of protein's molecular weight).



**Figure 1** SDS PAGE Protein Analysis of Rodent Tuber Control and Mutant Plants

M (Marker Prestain 6.9-210 kD), P1 (control plants grown in green house), P2 (control in vitro plant), P3 (M6/1), P4 (M6/2), P5 (M6/3), P6 (M6/6). Samples were electrophorized with 6% stacking gel and 12% running gel at 35 mA for 120 minutes. Protein concentration of each sample was 20  $\mu\text{g}/\mu\text{l}$ . Blue arrows indicate 6.9 kD protein which was expressed by control but not in M6/2. Red arrows indicate 56.2 kD protein which was not expressed by control but expressed by M6/1. Green arrows indicate 24.3 kD protein which was expressed by control but not in M6/2, M6/3, M6/6.

SDS PAGE analysis showed the difference in protein bands pattern between control and mutant plants. Rodent tuber in vitro control plant which was cultured on MS medium showed the fewest number of protein bands, i.e. 10 bands (Table 1). Rodent tuber in vitro control, M6/1, M6/2, M6/3, dan M6/6 have the same protein bands at molecular weights of 77,5 kD, 53,6 kD, 50,70 kD, 29 kD, 24,9 kD dan 10,4 kD. Several mutant plants did not express proteins which were expressed by control plant grown in green house.

Specifically, M6/3 and M6/6 did not show any protein expression at molecular weight of 132,7 kD. M6/6 did not show protein expression at 94,4 kD. M6/2, M6/3, and M6/6 did not show protein expression at 24.3 and 11 Kd. M6/2 and M6/3 did not show any protein expression at 21 kD. M6/1 did not show any protein expression at 35,6 kD. M6/2 did not show protein expression at 6.9 kD, which was expressed by the other five samples including control plants. On the other side, several mutant plants produced proteins which were not produced by control plant grown in green house. For example, M6/1 expressed protein at 56,2 Kd; M6/2 and M6/3 expressed protein at 34.1 kD which were not expressed by control plants grown in green house.

Rodent tuber in vitro control plant expressed protein at 27.2 kD, which was not expressed by the other five samples including control plant grown in greenhouse (Table 1). Cells of gamma-irradiated plant had undergone a series of DNA repair mechanism. This repair mechanism has induced changes in gene sequence, and therefore also affected protein expression [32]. Thus, gamma irradiation is able to modify the physical and biological system properties of a plant. Gamma irradiation initiate the release of  $\text{OH}^-$  and  $\text{O}_2^-$  which destruct cell membrane, modify plastid structure, and induce the fragmentation endoplasmic reticulum, golgi apparatus, DNA, and protein [33].

The amount of total protein of rodent tuber in vitro mutant was also affected by gamma irradiation. Control plant grown in green house produced the same amount of protein bands with M6/1, i.e. 13 bands, which is the highest amount of protein bands compared to in vitro control, M6/2, M6/3, and M6/6 (Table 1). Gamma irradiation is able to increase the protein content of a plant cell [34]. There were also several proteins which were expressed by control plant grown in green house but not expressed by mutant plants. Gamma irradiation could inhibit the growth of plant in vitro culture [32] so there were some proteins that were not expressed by mutant plants. However, there were also several proteins which were not expressed by control but expressed by mutant plants. Gamma irradiation is able to induce or inhibit the expression of proteins [35]. Protein profile of M6/2 was different from the other mutants because M6/2 expressed protein with molecular weight of 6.9 kD which was not expressed by the other mutant plants (Figure 1).

**Table 1** Rf Value and Molecular Weight of Protein of Rodent Tuber

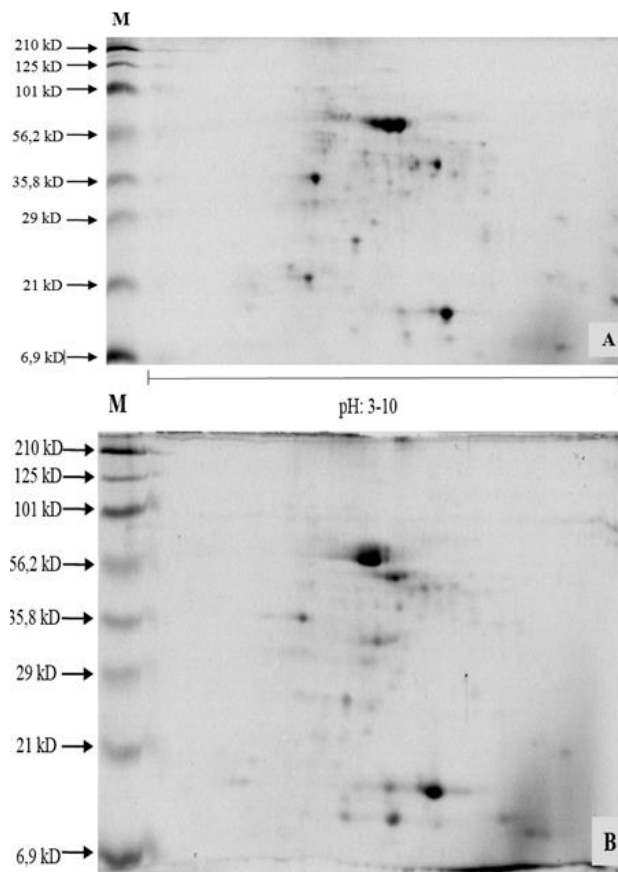
M (Marker Prestain 6.9-210 kD), P1 (control plants grown in green house), P2 (control in vitro plant), P3 (M6/1), P4 (M6/2), P5 (M6/3), P6 (M6/6). Rf value was obtained by comparing the relative migration of sample and solvent (cm). Standard curve equation is  $y = -1.4222x + 2.2164$  with x: Rf value (cm) and y: log of protein's molecular weight (kD). - indicated protein which was not expressed by rodent tuber in vitro mutant but was expressed by control plant.

M	P1		P2		P3		P4		P5		P6	
	Rf	BM	Rf	BM	Rf	BM	Rf	BM	Rf	BM	Rf	BM
210	-	-	-	-	-	-	-	-	-	-	-	-
	0.05	132.7	0.05	132.7	0.05	132.7	0.05	132.7	-	-	-	-
125	-	-	-	-	-	-	-	-	-	-	-	-
101	-	-	-	-	-	-	-	-	-	-	-	-
	0.17	94.4	0.17	94.4	0.17	94.4	0.17	94.4	0.17	94.4	-	-
	0.24	77.5	0.24	77.5	0.24	77.5	0.24	77.5	0.24	77.5	0.24	77.5
56.2	-	-	-	-	0.3	56.2	0.3	56.2	-	-	0.3	56.2
	0.37	53.6	0.37	53.6	0.37	53.6	0.37	53.6	0.37	53.6	0.37	53.6
	0.39	50.7	0.39	50.7	0.39	50.7	0.39	50.7	0.39	50.7	0.39	50.7
35.8	0.40	35.8	-	-	-	-	0.40	35.8	0.40	35.8	0.40	35.8
	-	-	-	-	-	-	0.53	34.1	0.53	34.1	-	-
29	0.55	29	0.55	29	0.55	29	0.55	29	0.55	29	0.55	29
	-	-	0.61	27.2	-	-	-	-	-	-	-	-
	0.64	24.9	0.64	24.9	0.64	24.9	0.64	24.9	0.64	24.9	0.64	24.9
	0.65	24.3	-	-	0.65	24.3	-	-	-	-	-	-
21	0.92	21	-	-	0.92	21	-	-	-	-	0.92	21
	0.93	11	-	-	0.93	11	-	-	-	-	-	-
	0.95	10.4	0.95	10.4	0.95	10.4	0.95	10.4	0.95	10.4	0.95	10.4
	-	-	-	-	-	-	-	-	0.96	10.1	0.96	10.1
6.9	0.97	6.9	0.97	6.9	0.97	6.9	-	-	0.97	6.9	0.97	6.9

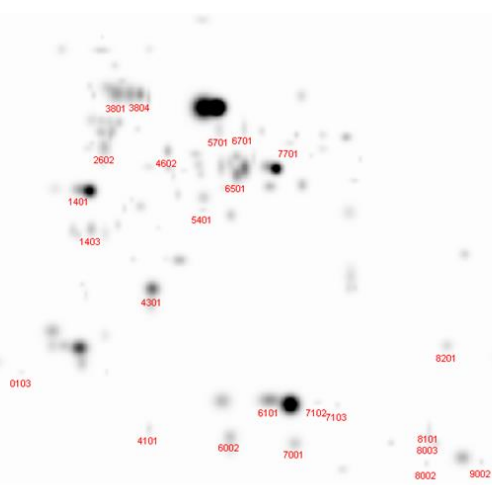
- indicated protein which was not expressed by P1

### 3.2 2D PAGE Analysis

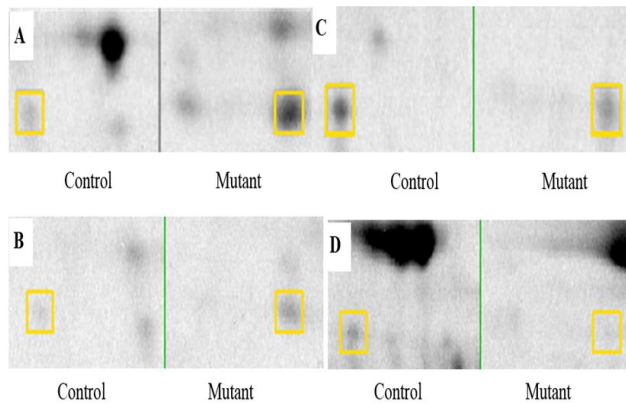
The objective of 2D PAGE analysis was to compare the protein profile of M6/2 and control plant (Figure 2). According to 2D PAGE, there were nine bands of protein marker at molecular weights of 6.9 kD, 21 kD, 29 kD, 35.8 kD, 56.2 kD, 101 kD, 125 kD and 210 kD. 2D PAGE showed 116 protein spots of control plant and 86 protein spots of M6/2 mutant plant which were expressed at pH 3-10 and molecular weight 6.9-210 kD. Most proteins obtained at the molecular weight range of 6.9-101 kD. PDQuest analysis had identified 24 protein spots which were expressed by both control and M6/2 plants. These protein spots had the same locations on both samples, but with different degree of expressions (Figure 3). The presence of protein spots was indicated by SSP (Standard Spot) value. SSP is a set of specific numbers which indicates the location of proteins. The first two digits in SSP indicates the location of protein spots at X and Y coordinates. M6/2 expressed 13 proteins which were up-regulated from control and 11 proteins which were down-regulated from control (Figure 4).



**Figure 2** 2D PAGE profile of rodent tuber protein samples M (Marker Prestain 6.9-210 kD), A (control plants grown in green house), B (M6/2). Protein were separated by IPGs pH 3-10.



**Figure 3** The location of 24 protein spots which were expressed by control and M6/2. The four digits number of each protein spot indicate the location of identified protein spots.



**Figure 4** Protein expression of M6/2 compared to control A(SSP 6002), B(SSP 4101), C(SSP 4301), D (SSP 4602) Control (control plant grown in green house), Mutant (M6/2)

Protein profile of M6/2 was further analyzed by 2D PAGE in order to compare it with control plant which was grown in green house. 2D PAGE gives a complete quantitative and qualitative protein data and it also indicate the modification of protein translation [36]. 2D PAGE analysis had shown the down-regulation of protein expression by mutants compared to control plant. Control plants showed the expression of 116 proteins spots, while M6/2 mutant only showed 86 spots (Figure 2). This result was in line with 1D SDS PAGE data, which also showed the decrease in the amount of protein bands expressed by M6/2 if compared to control plant (Figure 2). Gamma irradiation had induced mutation in rodent tuber which was followed by the up-regulation and down-regulation of protein expression. Mutant plants had 13 protein spots which were up-regulated (Fig. 4a and 4b) and 11 protein spots which was down-regulated (Figure 4c and 4d).

The other mutant plants also showed up-regulation and down-regulation of protein expression, such as rice [37], pear fruit [38], and *Aspergillus fumigatus* [39]. 2D PAGE had identified 50 proteins which were expressed by mutant rice plants (*Oryza sativa* L. variety Zhenhui 249 Y). Among those 50 proteins, three proteins were up-regulated and two proteins were down-regulated [37]. 2D PAGE also had identified 15 protein spots of red pear fruit which were up-regulated and six proteins which were down-regulated [38]. 2D-PAGE analysis of *Aspergillus fumigatus* showed 700 protein spots in the pH range of 3-10 and molecular weight of 14-100 kD. *Aspergillus fumigatus* mutant also showed 42 protein spots which were up-regulated [39].

#### 4.0 CONCLUSION

This research has proven the differences in protein profile between rodent tuber control and mutant plants by 1D SDS PAGE and 2D PAGE analyses. SDS PAGE showed the differences in the amount of

protein bands between control and mutant plants. M6/2 did not express protein with molecular weight of 6.9 kD. 2D PAGE had identified 116 protein spots of control and 86 spots of M6/2 in the pH range of 3-10 and molecular weight of 6.9-210 kD. Control and M6/2 expressed 24 protein spots at the same locations. 2D PAGE had identified 13 proteins of M6/2 which were up-regulated and 13 proteins which were down-regulated.

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