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Effects of Gamma Ray Irrad iation to Induce Genetic Variability of Teak Planlets (*Tectona grandis* Linn. F.)

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Abstract. Teak planlets (Tectona grandis) of Salomon clones were irradiated by gamma rays to induce genetic variability for growth improvement. The objectives of this research were to analyze the radiosensitivity of T. grandis Salomon clones by gamma ray irradiation and to analyze the genetic variations using Random Amplified Polymorphic Deoxyribonucleic Acid (RAPD). For those purposes, teak planlets were irradiated using gamma rays at 5 different dosages, those were 0 Gy, 10 Gy, 20 Gy, 30 Gy, and 40 Gy. Lethal doses50 (LD50) and reduced doses50 (RD50) were obtained using Curve-fit Analysis. Furthermore, the irradiated planlets were subcultured on medium MS + 0.1 kinetin (M1V0) the surviving M1V0 plantlets were multiplied to MS + 0.1 kinetin, so it is obtained the M1V1 generation. Genetic variation of the mutant was molecularly analyzed using RAPD methods and the variability was calculated using Analysis of Molecular Variance (AMOVA). Results of this research showed that lethal doses50 (LD50) and reduced doses50 (RD50) values were obtained at 24.5 Gy and 7.85 Gy, respectively. It includes genomes of small size and a small number of genomes. The genetic variation of mutant individuals in among and within the treatment of gamma irradiation was 16% and 84% according to AMOVA. It means that the dominant effect of genetic material caused genetic variation in mutant M1V1 generations. This dominance of genetic material could be caused by the response combination of tissue culture treatment and genetic factors that had the potency to be used as materials to select desired clones in the next stage.

Keyword: Genetic Variation, Mutant, Radiosensitivity, RAPD, Teak (*Tectona grandis*)

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1 Introduction

Teak (*Tectona grandis*) is one of the most valuable timber trees for the material of wood construction and carpentry. Naturally, inherited desirable teak timber characteristics have remarkable physical and mechanical properties, particularly strength II grades, durability I grades, workability, and aesthetic features. These characteristics have been led to the

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consequence, with a short shortage of supply (teak timber production), the demand for teak timber increases [1]. The total requirement for teak timber in Indonesian and international markets reaches ± 1.5-2.5 million m3 per year, of which at least 90% is harvested in India, Indonesia, and Myanmar [2]. However, this timber supplies currently available only ± 20% (4.11.893 m3) of the volume of teak timber used in Indonesia, resulting from a market gap in demand at ± 1.1 million m3 [3]. The decrease in site quality had a negative effect to determine the growth and development of plants; it is related to the quality and quantity of fertilization proportion blossoms, which is reflecting the decrease in productivity. Low genetic diversity of teak is caused by the quantitatively limited availability of natural germplasm among existing plants since it is an exotic plant [4] and has open pollination system (chasmogamy) [5]. Plant improvement requires high genetic diversity to obtain an extended opportunity of desired trait combinations according to the strategy of tree breeding.

Plant breeding technique is used to increase plant productivity through the utilization of genetic diversity and develop new varieties in certain specific traits [6]. Teak breeding has been established by cross-breeding (hybridization) [7], mutation breeding [8], and genetic transformation [9]. The mutation technique is commonly most popular on strategies for breeding to induce mutants and increase plant production, because it could induce recessive genes [10]-[11] in several new generated mutants.

Gamma rays as a physic mutation type technique are widely used to increase genetic variation. This mutation technique in vitro culture, can be able to induce in a short time and more easily created a stable mutant, which is a superior genetic and a desirable phenotypic appears to obtain individual lines with a specific characteristic to improve productivity [12] compared to ex Vitro and field experiment mutation breedings. Mutants result from interactions between free radicals generated by gamma rays with water substances in the vacuole, causing morphological, anatomical, biochemical, and physiological changes [13]. The frequency of mutation also serves as an index of radiosensitivity, which is influenced by mutagenic effectiveness (dose of gamma rays irradiation) and efficiency explants (phases, size, and thickness) [14]. Gamma rays at low doses (0-10 KGy) and the use of plant parts that are actively dividing; such as meristem cells are important factors for the successful induction of genetic variation [15]. The appropriate utilization of irradiation dose may be determined by genome size, the smaller genome size the lower dose needed for irradiation, as well as for the larger genome size, the use of higher doses of irradiation. The teak genome has 36 chromosomes with the size of 0.94 Pg (868.5 Mbp) [16], including small size chromosomes. In Vitis vinifera (n = 19; V genome = 475 Mbp), with explant dormant buds have used a dose of gamma rays at 25-35 Gy; *Persea americana* (2n = 24; V genome = 907 Mbp), with explant zygotic embryo, is irradiated using a dose of gamma rays at 25 Gy [17]. Teak plantlets have potentially been used as explants (plant materials) for irradiation because the multiplication of teaks has been carried through micropropagation

techniques [18]. Planlet requires a relatively shorter time for producing mutant compared to other vegetative organs due to meristematic cell development.

Application of gamma ray irradiation on meristematic cells may develop a new genetic variation, the variation in which needs to detect the stability in any event expression of genetic variations arising among mutant generations [19]. Genetic variation is determined by radiosensitivity [20]. The plant radiosensitivity does not always affect the cells and DNA material [21]. Therefore, it is necessary to study the use of gamma ray irradiation on teak plantlets with various levels of dosages. Information on irradiation doses is very useful in studying teak growth in vitro and ex vitro conditions. Planlet of Post-irradiated generation or mother material1 vegetative1 generation (M1V1) may also be seen through morphological and genetic analysis using RAPD (Random Amplified Polymorphic DNA) to detect the genetic variation that has occurred as a result of gamma ray irradiation. The purpose of this study was to analyze the value radiosensitivity and genetic variation using the RAPD method on M1V1 mutants.

2 Methods

This research was carried out at the Tissue Culture Laboratory Forest Resource Conservation and Ecotourism (KSHE), Faculty of Forestry, Bogor Agricultural University. Gamma ray irradiation treatment was conducted at the Central Laboratory of Isotope and Irradiation Technology Applications (PATIR), National Nuclear Energy Agency (BATAN), and Laboratory of Molecular Genetics of Forest and Forestry for Analysis of genetic variation.

2.1 Material Preparation Plant

The teak planlets were cut into pieces using scissors with a single nodal segment before multiplication (M0V0). These were done in a petri dish, containing sterile distilled water to maintain osmotic balances and transpiration of plants, so that plants were not withered and stressed. M0V0 plantlets were further placed on medium MS containing 0.1 ppm BAP + 0.1 ppm kinetin [22] and incubated at room temperature at 24 oC for 3 months. The light duration was 16/8 hours (MIVI).

2.2 Mutations Induced by Gamma Rays

Mutation induction was mediated by gamma ray using irradiators Chamber Cobalt60 (60Co) in BATAN. Gamma ray irradiation was applied to planlets at 5 different dosages, which were 0, 10, 20, 30, and 40 Gy. Irradiated plantlets were transferred into new media MS0 (MS without hormone). The survived plantlets after irradiation were called M1V0 generations, whereas before irradiated planlets were called M0V0 generations. Each plantlets were cultured and observed in each culture bottle. The observed parameters were LD50, RD50, and the number of leaves. The data analysis was done using Curveexpert Software 1.3. The regression calculation

model of LD50 and RD50 was Gaussian models, is a normal distribution curve with equation [23]:

$$y = a^{\frac{-(x-b)}{2c}} \quad (1)$$

LD_{50} calculation was obtained from survived planlets after irradiation, incubated in media MS0 during 3 months and 16/8 hours light duration.

This study was arranged in a completely randomized design (CRD) in four replication. Each experimental unit consisted of 5 plantlets, so the total of planlets for irradiation treatment was 100 plantlets in M1V0 generations. Each bottle was planted by a single planlet and all irradiated planlet were observed. The data were analyzed and documented by photography for visualizing the development stages of plantlets.

2.3 Multiplication of Teak Planlets Post-Irradiation

After irradiation, planlets were multiplicated in MS medium + 0.1 ppm kinetin that aimed to avoid chimera and diplontic selection phenomenon. It was called M1V1 generation. Multiplicated planlets were originated from teak plantlets of Solomon clones (M1V0) with a single nodal segment and the leaves cut in half to reduce transpiration. The M1V0 planlet length was around a single nodule segment and the leaves were cut in half to reduce transpiration. These were carried out in a Laminar Air Flow Cabinet (LAFC), sterilized using ultraviolet light for 1 hour, and with 70% ethanol.

2.4 Genetic Analysis using RAPD

DNA extraction was done using about 5 gram calli of planlets M1V1, selected randomly from 3 mutant planlets in each treatment. The protocol DNA of extraction was essentially a method of CTAB (Cetyl Trimethyl Ammonium Bromide) (modified by [24]) and amplified by RAPD (Table 1). The DNA banding pattern of electrophoresis results was interpreted in the form of a scoring system [25]. Scoring results were processed using Microsoft Excel software that comes with GenAlex 6.5, Version Popgen 1:32, and NTSYSpc Version 20.1d.

Table 1 RAPD primers used for amplification of Tectona grandis

Primers	*Sequence* (5'-3')	*Annealing* (^{0}C)
OPC-1	TTCGAGCCAG	37
OPC-2	GTGAGGCGTC	37
OPC-8	TGGACCGGTG	37
OPC-9	CTCACCGTCC	37
OPC- 10	TGTCTGGGTG	37

3 Results and Discussion

3.1 Radiosensitivity

Radiosensitivity is used as an indicator of the quantitative effect of gamma rays [26], an indicator to determine the antioxidant activity of plant systems [27], and as a defense system of plants (defensive mechanism system) to gamma rays [28]. Radiosensitivity is also used as a parameter for assessing the effectiveness of mutations through physiological changes [29], as well as the growth and development of plants [30]. The variable sensitivity level is calculated based on the survival rate of planlets and height growth. The survival rate of planlet and planlet height growth are calculated based on the LD50 (Lethal doses50) and RD50 (Reduced doses50).

The survival rate of planlets depends on given irradiation doses, at a higher dose of irradiation caused the decrease of the plant viability [31]. The LD50 value of T. *grandis* planlets of salomon clone was 24.5 Gy (Figure 1A). In control, all planlets survived; however, there were 5 planlets contaminated by the fungus (Table 2). Gamma ray irradiation dose at 30 and 40 Gy reduced the survival rate of planlets by 80%. These doses (30 and 40 Gy) are categorized as high dose irradiation that affected the survival rate of planlets. High and low irradiation doses depended on biological responses both negative and positive correlations on plant material used. The increasing frequency of chromosomal damages was proportional to the irradiation doses, the increasing irradiation dose corresponded to increasing levels of chromosome damage; so it inhibited the plant survival [29], plant growth, and mitotic delays [32].

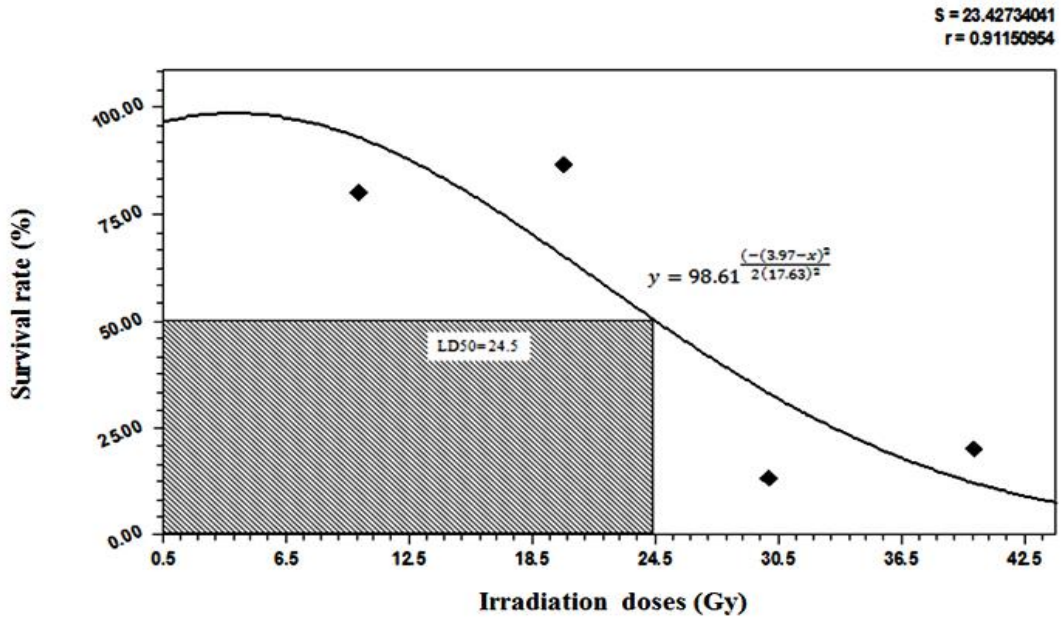

Figure 1 LD_{50} of teak planlets in vitro conditions

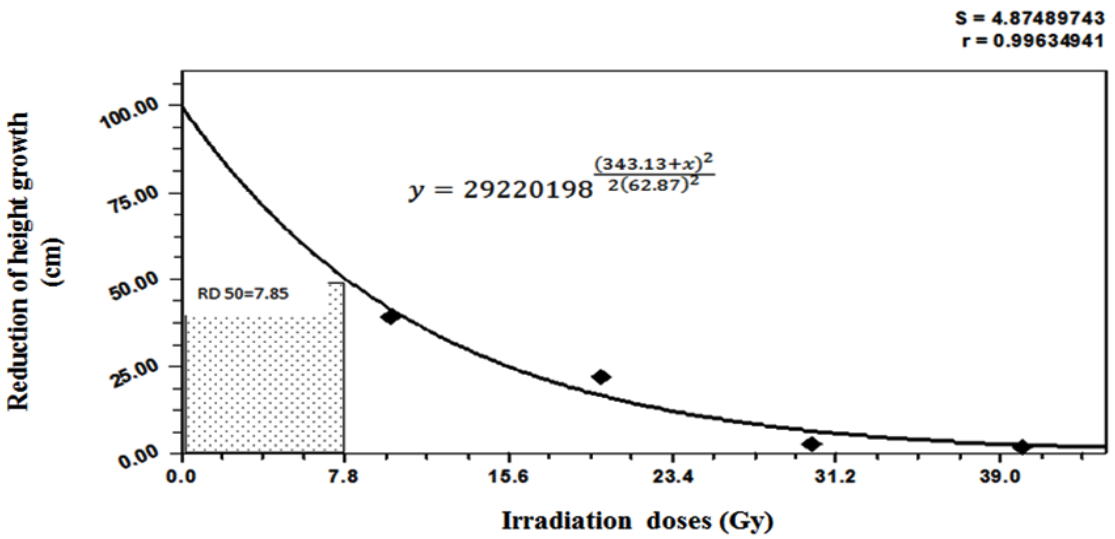

Figure 2 RD_{50} of teak planlets of clones in vitro conditions

Table 2 Effect of irradiation on height growth and leaves number of teak plantlets in *in vitro*

Doses (Gy)	$\Sigma_{survival}$ planlets	$\Sigma_{Contaminated}$ planlets	T0* (cm)	T1* (cm)	ΔT = T1-T0	N0* *Mean ± SD*	N1* *Mean ± SD*	ΔN = N1*-N0* *Mean ± SD*
0	15	5	6.4	10.0	3.6	5.45 ± 1.8	7.6 0± 1.7	2.2 ± 2.4
10	12	5	5.1	6.6	1.4	4.15 ± 1.4	6.15 ± 1.3	2.0 ± 1.4
20	13	1	4.5	5.3	0.8	4.65 ± 1.5	5.60 ± 1.9	0.9 ± 1.7
30	2	1	5.2	5.1	0.1	4.75 ± 1.8	3.80 ± 1.2	-0.9 ± 2.0
40	3	0	5.2	5.3	0.07	4.65 ± 0.9	3.80 ± 1.1	-0.8 ± 1.3

Notes: *T0: Height M0V0; T1: height M1V1; N0: number of leaves M0V0; N1: number of leaves M1V0

Value RD50 of teak planlet of Solomon clones in vitro was at 7.8 Gy. (Figure 2). Reduction of height growth (enthalpy) on M1V0 plantlets decreased at all irradiation treatments (Table 2). The dose of gamma ray irradiation at 10 Gy may reduce the activity of endogenous cytokinin hormone, it caused the growth of planlet goes slow down. Decreasing the amount of endogenous cytokines synthesis caused by protein synthesis either decreased or damage and physiological damage after irradiation [31].

Based on Table 2, enthalpy of leaves number M1V0 occurred after gamma ray irradiation with doses at 30 and 40 Gy. The low enthalpy of leaves number may be caused by the dried and falt down leaves (senescences). It is possible to the doses of 30 and 40 Gy were able to subquentially increase glutathione compound [33], inhibit the synthesis of auxin hormone, and increase the hormone ethylene on leave plantlets.

3.2 Genetic variation

All M1V0's planlet were multiplicated in MS medium + 0.1 ppm kinetin, had a different response. It depended on the dose of irradiation (Figure 3). In control, planlets grew normally,

while irradiated planlets stimulated the occurrence of callus formation (callogenesis). At 10 Gy, planlets formed calli and had fragile structure, small size, and brownish, while the irradiation doses of 20, 30, and 40 Gy produced somatic embryogenic structure.

Morphological changes could not always be explained by genetic material changes [34]. PCR-based DNA marker techniques are used in determining the genetic relatedness and diversity [35]. RAPD is a dominant marker that is widely used for genetic mapping, analysis of mutants, and somaclonal variation during the culture period [36]. These markers were amplified genomic widely and randomly so that the RAPD primer used is not specifically required. There are many scientific papers about analysis for genetic relatedness and diversity using RAPD markers on *Solanum tuberosum* L. [37], *Tectona grandis* [38], *Pinus kwangtungensis* Chun ex Tsiang [39] *Dendranthema grandiflora* Tzvelev [40], and bamboo [41]-[42]. RAPD analysis using callus mutant M1V1 produced dendrogram shown in Figure 3.

The value of the genetic distance between the individual mutants and controls is shown in the dendrogram in Figure 4. The dendrogram is divided into three major clusters, reflecting the genetic variation among all mutant M1V1 individuals. Those are the first cluster consisted of individual control I and 10 Gray III. The second cluster is divided into two subclusters, namely subcluster 1 (control II, Control III, 20 Gy III, 20 Gy II, 30 Gy I, 30 Gy II, III 30 Gy, 40 Gy I, and 40 Gy III) and subcluster 2 (10 Gray I, 10 Gray II, and 20 Gy I), the genetic distance between subcluster 1 and subcluster 2 was 0.21. The third cluster was in an individual of 40 Gy II and formed their group. The genetic distance between individual mutants and controls based on dendrogram ranged from 0-0.85.

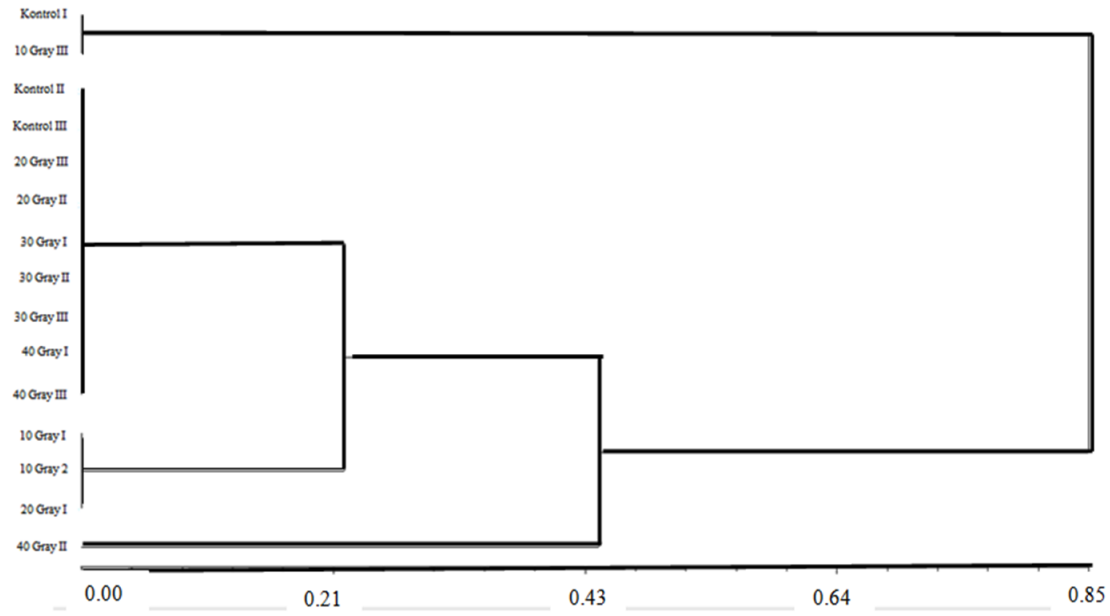

Figure 3 Dendrogram of genotypic characters of teak planlets of M1V1 Solomon clones. Individual treatment consists of Kontrol I: control replicated I; Kontrol II: control replicated II; Kontrol III: control replicated III; 10 gray I; 10 Gy replicated I; 10 Gy II: 10 Gy replicated II; 10 Gray III: 10 Gy replicated III; 20 gray I: 20 Gy replicated I; 20 gray II: 20 Gy replicated II; 20 gray III: 20 Gy replicated III; 30 gray I: 30 Gy replicated I; 30

gray II: 30 Gy replicated II; 30 gray III: 30 Gy replicated III; 40 Gray I: 40 Gy replicated I; 40 Gray II: 40 Gy replicated II; 40 Gray III: 40 Gy replicated III;

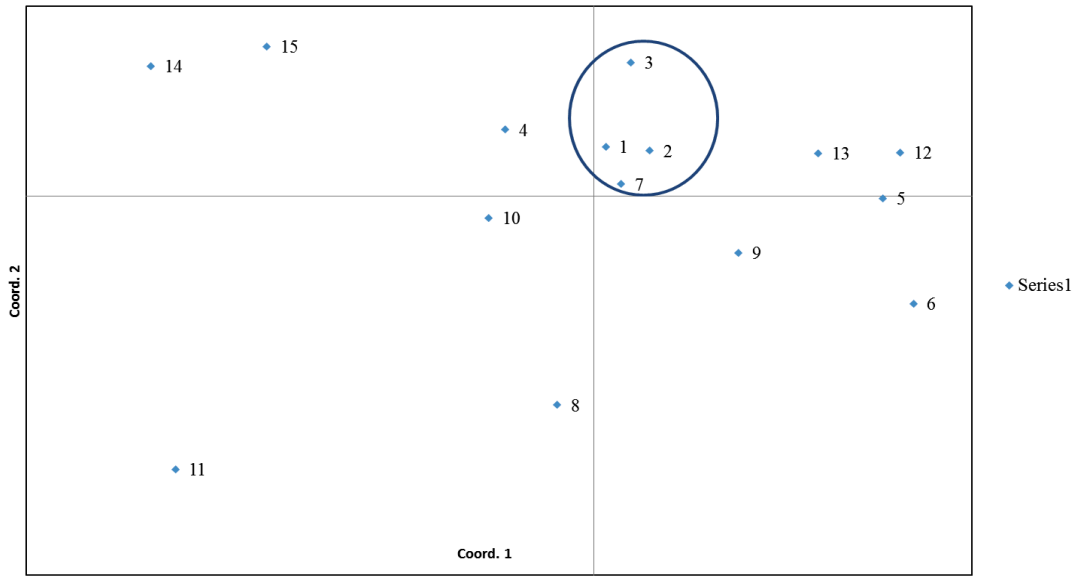

Figure 4 Analysis of principal coordinates analysis (PcoA) from 5 population (15 individual) of Salomon teak calli. Individual treatment including in 1 = control replicated I; 2= Control replicated II; 3 = control replicated III; 4 = 10 Gy replicated I; 5 = 10 Gy replicated II; 6= 10 Gy replicated II; 7 = 20 Gy replicated I; 8 = 20 Gy replicated II; 9= 20 Gy replicated III; 10= 30 Gy replicated I; 11= 30 Gy replicated II; 12 = 30 Gy replicated III; 13 = 40 Gy replicated I; 14 = 40 Gy replicated II; 15 = 40 Gy replicated III.

Based on the pattern, all individual control (I, II, and III) are clustered in quadrant 1 and formed their group, indicating a higher relationship. However, all mutant M1V1 scattered randomly throughout all quadrant sides (Figure 4); it has a great value of genetic distance, and the high level mutation relates to an extended heterogeneity. Mutants of irradiation dose at 10 Gy (I, II) and 20 (I, III) were a spacing adjacent to the control group (I, II, and III). It corresponded to the performance of irradiated planlets at 10 and 20 Gy, it was a few growths normally. The similarity rate of mutants and control group showed the genetic relationship, the greater values distance of mutants to control have a closer the kinship relationship is. A variety of factors, including gene flow, inbreeding, and mutation, influence the level of genetic relationship [43].

Higher values of genetic similarity and lower values of genetic distance between individual treatments indicate higher mutation rates and lower heterogeneity. In both analyzes, dendrogram and PoCA, not all mutant M1V1 is clustered together. It may be caused by the differences of chromosome numbers in primers used and plant individual mutants [41]. The differences of clustering in both these analyses occur in bamboo [41] and teak [38].

In this study, mutant M1V1 teak has a higher genetic diversity average (H) (0.1391) compared to teak diversity in India (0.075) [25]. It means that irradiated on teak planlet in vitro mutagenesis has higher genetic variation than the population in India. So gamma ray is better to induce genetic variation than natural population. In plant cells, gamma rays have LET ((linear

energy transfer) that can change the arrangement of nucleotide bases through ionization, dissociation, and excitation; which can methylate DNA from specific DNA damage (DDSs) and 64 pyrimidone photoproducts (64PPs) [35], [44].

Genetic diversity of mutant M1V1 maybe has potential lines and clones to select an effective parenting material for providing valuable information further genetic management and breeding programs aimed at improving the productivity and quality of the teak in the wood.

Table 3 Results of Analysis of Molecular Variance (AMOVA)

Source	DF*	SS*	MS*	DE*	%
Among treatments	4	55.87	13.967	1.656	16%
Within treatments	10	90.00	9.000	9.000	84%
Total	14	145.67		10.66	100%

Notes: DF (*Degrees of freedom*); SS (*Sum of squares*); MS (*Mean of squares*); DE (*Diversity Estimation*)

AMOVA (Table 3) showed that genetic variation is within the treatment of gamma ray irradiation was 84% and 16% for among treatments. It means that genetic variation in mutant M1V1 generations was caused by the dominant effect of genetic material. This genetic material dominance may be caused by a combination of responses to tissue culture treatment and its genetic. Tissue culture is caused by putative mutations in plant cells due to the increased transposable element [45] and DNA methylation [46]. The transposable element and DNA methylation occurs in tissue culture, is caused by high concentrations of inorganic nutrients in the medium [47], repeated subcultures, mutagen agents (adenine sulfate) [48].

Mutant M1V1 must be had stable genetics, so it should be multiplicated and propagated vegetative treatment simultaneously both in vitro and in the field to eliminate chimera cells. The genetic distance between treatments was quite large, both among treatments and within treatments. Thus the gamma ray irradiation techniques on the teak planlet of Salomon clones could produce a high genetic diversity compared to the natural population.

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