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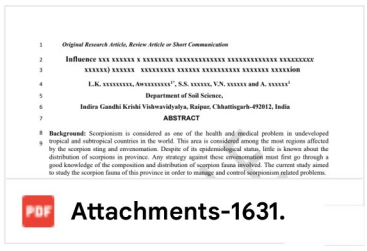
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5 **Department of Soil Science,**
6 **Indira Gandhi Krishi Vishwavidyalya, Raipur, Chhattisgarh-492012, India**
7 **ABSTRACT**
8 **Background:** Scorpionism is considered as one of the health and medical problem in undeveloped
9 tropical and subtropical countries in the world. This area is considered among the most regions affected
by the scorpion sting and envenomation. Despite of its epidemiological status, little is known about the
distribution of scorpions in province. Any strategy against these envenomation must first go through a
good knowledge of the composition and distribution of scorpion fauna involved. The current study aimed
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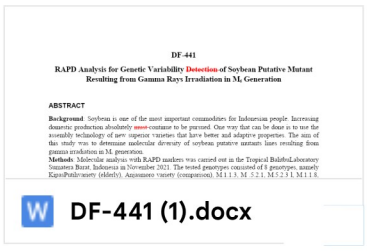
DF-441
RAPD Analysis for Genetic Variability Detection of Soybean Putative Mutant Resulting from Gamma Rays Irradiation in M6 Generation
ABSTRACT
Background: Soybean is one of the most important commodities for Indonesian people. Increasing domestic production absolutely must continue to be pursued. One way that can be done is to use the assembly technology of new superior varieties that have better and adaptive properties. The aim of this study was to determine molecular diversity of soybean putative mutants lines resulting from gamma irradiation in M6 generation.
Methods: Molecular analysis with RAPD markers was carried out in the Tropical BioInfoLaboratory Sumatera Barat, Indonesia in November 2021. The tested genotypes consisted of 8 genotypes, namely Kipas Putih=mutty (elderly), Anjasmoro variety (comparison), M 1.1.3, M 5.2.1, M 5.2.3 I, M 1.1.8,

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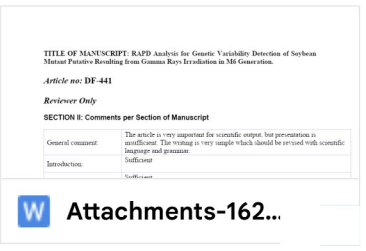
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General comment:	The article is very important for scientific output, but presentation is insufficient. The writing is very simple which should be revised with scientific language and grammar.
Introduction:	Sufficient

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Dr. Nilahayati, SP., M.Si <nilahayati@unimal.ac.id> Kam, 17 Mar 2022, 17.18
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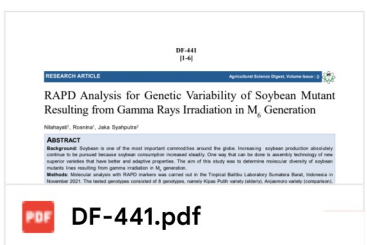
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Thank you for your information. There is no need corrections and changes in the manuscript.
Thank you so much.

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Nilahayati

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RESEARCH ARTICLE Agricultural Science Digest, Volume Issue : ()

RAPD Analysis for Genetic Variability of Soybean Mutant Resulting from Gamma Rays Irradiation in M_6 Generation

Nilahayati[1], Rosnina[1], Jaka Syahputra[2]

ABSTRACT

Background: Soybean is one of the most important commodities around the globe. Increasing soybean production absolutely continue to be pursued because soybean consumption increased steadily. One way that can be done is assembly technology of new superior varieties that have better and adaptive properties. The aim of this study was to determine molecular diversity of soybean mutants lines resulting from gamma irradiation in M_6 generation.

Methods: Molecular analysis with RAPD markers was carried out in the Tropical Balitbu Laboratory Sumatera Barat, Indonesia in November 2021. The tested genotypes consisted of 8 genotypes, namely Kipas Putih variety (elderly), Anjasmoro variety (comparison), M.1.1.3, M.5.2.1, M.5.2.3 I, M.1.1.8, M.1.1.9 and M.1.1.17 mutant lines. The primers used were RAPD 2 (5′-GTTTCGCTCC-3′), RAPD. 3 (5′-GTAGACCCGT-3′), RAPD 4 (5′-AAGAGCCCGT-3′) and RAPD 5 (5′-AACGCGCAAC-3′).

Result: The results of the RAPD analysis showed that there were differences in size of amplicon between the mutants and the parents, namely the RAPD 2 primer (1400 bp band) and the RAPD 5 primer (550 bp and 1000 bp band). The parents had a pattern of DNA bands but in the M.5.2.3 and M.1.1.9 mutants the DNA band was absent. The parent RAPD 5 primers (1000 bp, 550 bp) had a DNA banding pattern but in the M.1.1.3 and M.1.1.8 mutants there was no DNA banding pattern. This indicated that the M.1.1.3, M.5.2.3, M.1.1.8 and M.1.1.9 mutants had different genetic diversity from their parent (Kipas Putih variety).

Key words: Gamma Irradiation, Kipas putih soybean, Mutant lines, RAPD.

INTRODUCTION

Kipas Putih soybean is one of the local soybean varieties in Aceh Province which was released as a Indonesia national superior variety in 2008. This variety has the advantage of having a robust appearance and well adapted to the local environment. Recenly, this variety has become less attractive to farmers due to its long harvesting age and low production. The use of this variety has begun to be evicted with many other national superior varieties that have higher yield potential and shorter harvesting ages.

Nilahayati (2018) carried out a plant breeding program to improve the genetic characteristics of Kipas Putih soybean for early maturity and high yielding characters using gamma ray irradiation. The results of the study until the fifth generation (M_5) produced 33 mutant lines that were early maturity and had high yields. These mutants include 6 early-aged mutant lines (4-14 day faster harvesting with low seed weight, 3 mutant lines that harvest 8 days earlier, 19 mutant lines with high yields but not early maturity and 7 mutant lines with large seed weight (≥14 g/plant).

Furthermore, these mutant lines were purified to the M_6 generation and need to be verified to obtain information that these mutant lines are different from their parents. Mutation detection in plants can be done through phenotypic and genetic approaches. Matus and Hayes (2002) said that phenotypic detection using morphological traits are not considered as accurate markers due to environmental influences on morphological traits and insufficient polymorphism resulted among closely related cultivar. Therefore, the utilization of the genetic approach becomes a more reliable method and suitable for early mutation detection in the plant. Some genetic approaches which can be used for mutation detection are random amplification polymorphic DNA (RAPD). It has advantages such as it is applicable for anonymous genome, low DNA quantities and resulted in a high number of DNA fragments (Kumari and Thakur, 2014). However, it has some disadvantages regarding their low sensitivity and reproducible, which caused unstable results; the different study will result in a different outcome. In spite of the contrary argument regarding their usage for genetic diversity study, RAPD was still recommended and acceptable for detecting genetic variability than AFLP, ISSR and SSR (Sun and Wong, 2001).

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According to Sharma *et al*. (2008), RAPD is a technique for amplifying DNA with a PCR machine using a single primer measuring 10 nucleotides. The primer used is a random primer that amplifies the target genome at random. Random primers mixed with amplification reactions will bind to complement sequences along the target genome. Furthermore, the target sequence will be amplified, which can be visualized on the agarose gel. The previous study proved that RAPD had been successfully used for mutation detection in chili (Mullainathan *et al*. 2014), chickpea (Rajolia *et al*. 2020), pea genotype (Thakur *et al*. 2018), blackgram (Vyas *et al*. (2016) and soybean (Agam *et al*. 2020). Wahyudi *et al*. (2020) have also succeeded in detecting and evaluating genetic diversity induced using gamma ray irradiation in Grobogan soybeans with RAPD markers using 20 OPA 1-OPA 20 primers.

This study was conducted to analysis genetic variability using RAPD markers on 8 soybean genotypes. The present study was aimed to evaluate and compare the genetic diversity in the M6 generation soybean mutants compared to their parents (Kipas Putih variety).

MATERIALS AND METHODS

This research was conducted at Laboratory of Balai Penelitian Tanaman Buah Tropika (Balitbu), Solok, Sumatera Barat from October to November 2021. The plant material used was soybean young leaves (2 weeks after planting soybeans). The genotype used in this study were M.1.1.3 , M.5.2.1, M.5.2.3, M.1.1.8, M.1.1.9 and M.1.1.17 mutants lines, Kipas Putih and Anjasmoro variety. The primers used were RAPD 2 (5′-d[GTTTCGCTCC]-3′), RAPD 3 (5′-d[GTAGACCCGT]-3′), RAPD 4 (5′-d[AAGAGCCCGT]-3′) and RAPD 5 (5′-d[AACGCGCAAC]-3′).

Extraction of leaf DNA using extraction buffer made from CTAB. A total of 100-120 mg of leaves are ground until smooth in liquid nitrogen. The refined samples were then put into a new 1.5 ml tube containing 1 ml of extraction buffer which was heated in a water bath of 65°C. The tube containing the sample was then incubated in a water bath at a temperature of 65°C. Added 500 µl of chloroform; isoamyl-alcohol (24:1) into a tube and shaken using a vortex until dissolved. Then the tube containing the sample was centrifuged at 12,000 rpm for 10 minutes. The top liquid was then transferred to a new tube and 600-800 µl of CIA (24:1) was added, then centrifuged at 12,000 rpm for 10 minutes. This step is repeated until the layer between the two liquid phases is not visible (5-7x). The clean supernatant was then transferred to a new tube and 500µl of cold iso-propanol was added, then dissolved by slowly inverting the tube. The tube containing the solution was then stored at -20°C for at least 30 minutes, then centrifuged at 12,000 rpm for 10 minutes. Then the liquid was removed by carefully spilling and placing the tube upside down on a tissue paper to dry the DNA pellet. 200 µl TE buffer, 20 µl 3 M sodium acetate pH and 500 µl absolute ethanol were added and dissolved successively to the tube containing the DNA pellet. The mixture was dissolved by thawing, then stored at -20°C for 30 minutes. Centrifuge tube filled with solution at 12,000 rpm for 10 minutes. The liquid portion was then discarded and the DNA pellet at the bottom of the tube was dried in a vacuum for 10 minutes or left in the open air until sufficiently dry. After the pellet is dry, add 70% ethanol as much as 500 µl to wash the DNA and the tube wall. The DNA pellets were dried again in the same manner as the previous step. 100 µl of TE buffer was added to the tube to dissolve the DNA pellet and it was ready to be tested for quality and quantity.

DNA quantification was carried out by electrophoresis using 0.8% agarose gel. A total of 2 µl of DNA stock was mixed with 8 µl of distilled water and 2 µl of loading buffer. The sample is mixed, then put into the gel wells in the electrophoresis chamber which has been filled with 0.5x TBE buffer. As a comparison, a DNA ladder was used and placed in the first and/or last well. Connect the electricity from the power supply with a voltage of 70V for 30 minutes, or until the DNA migrates / moves approximately 4-5 cm.

Staining and visualization of the results after the electrophoresis process was carried out by immersing the gel in a solution of Ethidium bromide (40 µl 1% et.br/1 liter of water) for 10 minutes. Soak in distilled water for 20 minutes. After that the gel is placed on top of the u.v. transilluminator and ready to be photographed. The quality of DNA is indicated by the level of clarity/cleanliness of the DNA band image. The quantity of DNA was determined by making a comparison between the thickness of the sample DNA band and the standard DNA ladder band.

The PCR procedure was carried out starting with the amplification of genomic DNA. A master mix of 25 µl consisted of 9 µl distilled water, 2.5 µl 10 pM primer, 1.0 µl 50 ng genomic DNA and 12.5 µl Green Go Tag Master Mix. Each PCR tube containing the sample (master mix) is placed in the PCR machine. The amplification process in the program is as follows; total number of cycles 45, preheating at 94°C for 2 minutes, heating for denaturation at 94°C for 1 minute, annealing process at 36°C for 1 minute, elongation process at 72°C for 2 minutes, in the last cycle added elongation time at 72°C for 10 minutes.

After the PCR process is complete, then do electrophoresis to see the results of amplification of genomic DNA. Each sample mixture was made up to 7 µl, consisting of; 5 µl amplicon and 2 µl buffer loading gel. The sample mixture was put into 2% gel wells in an electrophoresis apparatus filled with 0.5x TBE buffer. Fill one well with 1Kb DNA marker as a scale. Electrophoresis was run at 50 volts for 30 minutes.

The gel was immersed in a solution of Ethidium bromide (40 µl 1% et.br/1 liter of water) for 10 minutes for DNA band staining and visualization of the results. Then soaked in distilled water for 20 minutes. After that, the gel was exposed on the gel doc and was ready to be photographed and ready to be scored.

Interpretation of results

The genotypic data obtained from the RAPD gel photo shoot was in the form of a DNA band pattern of a certain size. The size of the genomic DNA pieces was carried out by comparing with the standard molecular weight of 1 kb DNA ladder. Differences between plants are indicated by the number of bands and the distance of migration. If there is no difference between the DNA band patterns of the plant, it means that there is no genetic variation. Assessment (scoring) is carried out on clear and sharp bands consistently. The bands that have bands that look firm are given a score of 1 (present) and if they are not, they are given a score of 0 (absent). Cluster analysis was performed using the NTSys (Numerical Taxonomy and Multivariate Analysis) program. This analysis uses the SAHN (Sequential, Agglomerative, Hierarchical and Nested Clustering) method. The genotypic similarity/disparity matrix was calculated based on the Jaccard coefficient using the Unweighted Pair Group Methode Arithmetic (UPGMA) Qualitative Similarity (SIMQUAL) method.

The grouping shows the similarity relationship between each individual soybean in the form of a genetic similarity dendogram. Genetic distance is the difference between the percentage of similarity values to the value of 100%. From the dendogram, it can be concluded how far the mutant lines have changed when compared to control plants.

RESULTS AND DISCUSSION

The results of DNA analysis using 4 primers, namely RAPD 2, RAPD 3, RAPD 4 and RAPD 5 can be seen in Fig 1, 2, 3 and 4. The results of the analysis of the appearance of the banding pattern indicate a polymorphic band pattern (RAPD 2, RAPD 3 and RAPD 5 primers) and monomorphic banding pattern (RAPD 4). Table 1 shows the bands resulting from DNA amplification of mutants with parents are polymorphic in RAPD 2 primer (1500 bp and 1400 bp bands), RAPD 3 primers (1000 bp bands) and primer RAPD 5 (1000 bp, 900 bp, 550 and 450 bp bands).

The difference between mutants and parents is that in the RAPD 2 primer (1400 bp band), the parents have these bands but in the G3 (M.5.2.3) and G5 (M.1.1.9) mutants these bands are absent. RAPD 5 primers (1000 bp, 550 bp band), the parent had these bands but in G1 (M.1.1.3) and G4 (M.1.1.8) mutants these bands were absent. This shows that the G1, G3, G4 and G5 mutants have different genetic diversity from their parents. Dhakshanamoorthy *et al*. (2014) said that the disappearance of regular bands in soybean mutants may cause by DNA damage, modified bases, base oxidation, point mutation and even chromosomal rearrangements induced by mutagen. The appearance of new bands in soybean mutants may be related to the changes in oligonucleotide priming site due to mutation, deletion and homolog recombination.

Table 1: Polymorphic amplification loci between parents, comparison and six soybean mutant genotypes.

Primer	Locus size	G1	G2	G3	G4	G5	G6	G7	G8
RAPD-2	1500	-	-	-	-	-	-	-	+
	1400	+	+	-	+	-	+	+	+
RAPD-3	1000	-	-	-	-	-	-	-	+
RAPD-5	1000	-	+	+	+	+	+	+	-
	900	-	-	-	-	-	-	-	+
	550	+	+	+	-	+	+	+	+
	450	+	+	+	+	+	+	+	-

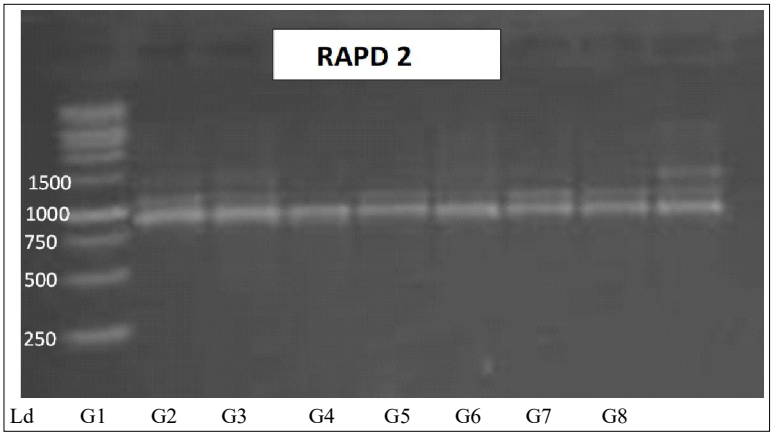

Fig 1: Pattern of DNA bands from RAPD analysis using RAPD Primer 2. Ld: DNA ladder, G1: M.1.1.3 mutant, G2: M.5.2.1 Mutant, G3: M.5.2.3 mutant, G4: M.1.1.8 mutant, G5: M.1.1.9 mutant, G6: M.7.1.17 mutant, G7: Kipas putih variety (parent), G8: anjasmoro variety.

Van Harten (1998) and Mudibu *et al*. (2012) stated that gamma rays produce free electrons that are radical, causing cell damage that can change the morphology of plants to be different from their parents. Mullainathan *et al*. (2014) reported that RAPD analysis of the M3 generation plants exposed to mutagen treatments produce clear difference from the mutant and untreated control, thus indicating that mutagenic treatments produce polymorphic regions in the chilli mutant.

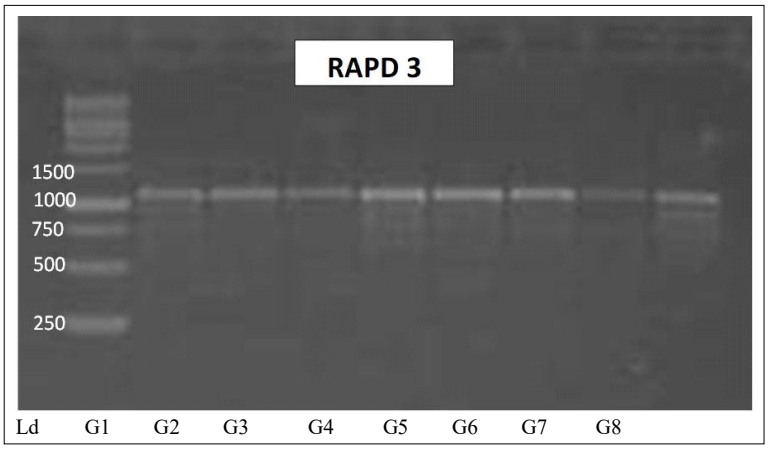

Fig 2: Pattern of DNA bands from RAPD analysis using RAPD primer 2. Ld: DNA ladder, G1: M.1.1.3 mutant, G2: M.5.2.1 mutant, G3: M.5.2.3 mutant, G4: M.1.1.8 mutant, G5: M.1.1.9 mutant, G6: M.7.1.17 mutant, G7: Kipas putih variety (parent), G8: anjasmoro variety.

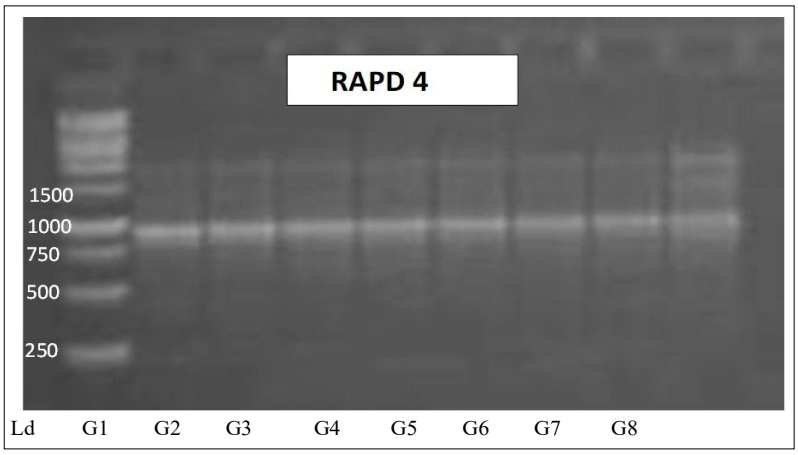

Fig 3: Pattern of DNA bands from RAPD analysis using RAPD primer 2. Ld: DNA ladder, G1: M.1.1.3 mutant, G2: M.5.2.1 mutant, G3: M.5.2.3 mutant, G4: M.1.1.8 mutant, G5: M.1.1.9 mutant, G6: M.7.1.17 mutant, G7: kipas putih variety (parent), G8: anjasmoro variety.

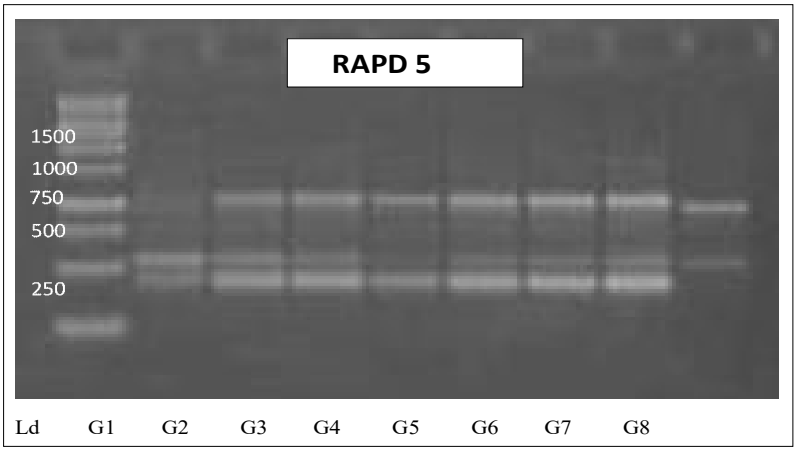

Fig 4: Pattern of DNA bands from RAPD analysis using RAPD Primer 2. Ld: DNA ladder, G1: M.1.1.3 mutant, G2: M.5.2.1 mutant, G3: M.5.2.3 mutant, G4: M.1.1.8 mutant, G5: M.1.1.9 mutant, G6: M.7.1.17 mutant, G7: Kipas putih variety (parent), G8: anjasmoro variety.

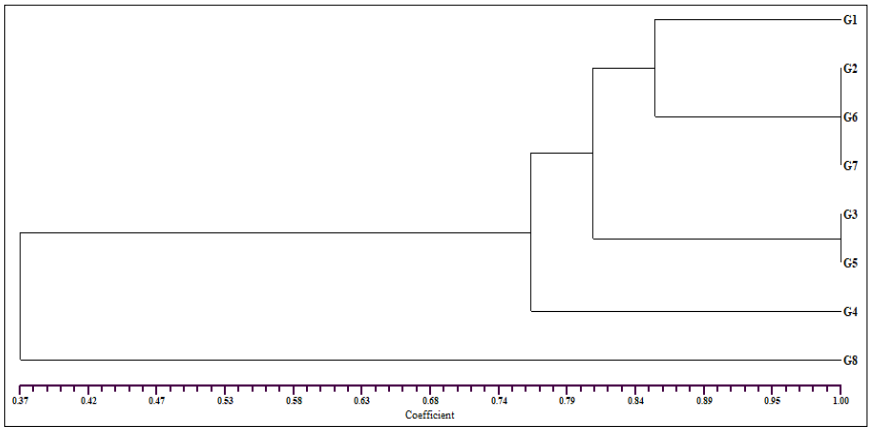

Fig 5: The results of the analysis of the relationship between 8 soybean genotypes using the NTSys program.

DNA amplification depends on the match of the primer with the DNA sequence of the mutant soybean. Primers that did not match the soybean DNA sequence did not produce amplification products. This is because there are no complementary sites in soybean DNA with these primer sequences. Primer attachment and amplification of a DNA locus is caused by the presence of complementary primary nucleotide base pairs used in the DNA strand. The presence or absence of a locus in plant samples can be caused by differences in the nucleotide arrangement in the DNA sample, so that the same primer cannot attach and therefore cannot amplify the locus. This difference is called polymorphism. According to Yuwono (2006), primers that are not specific can cause amplification of other regions in the genome that are not targeted or there are no amplified genomic regions.

The results of cluster analysis of several soybean genotypes tested showed that the soybean genotypes could be divided into two main groups, namely group 1 (Anjasmoro variety) and group 2 (Kipas Putih variety and 6 genotypes of putative mutant results from gamma ray irradiation) (Fig 5). Anjasmoro and Kipas Putih varieties as well as mutant genotypes have a very far genetic distance with a similarity value of 37%. Anjasmoro variety (G8) is the genotype that is farthest from other genotypes which shows that this genotype is the most different among other genotypes. In group 2, the percentage of similarity between Kipas putih and their mutants was seen. The farthest similarity percentages were M.1.1.8 mutants with 76% similarity, M.5.2.3 and M.1.1.9 mutants with 80% similarity and M.1.1.3 mutants with 85%. Meanwhile, the M.5.2.1 and M.7.1.17 mutants had 100% similarity, which means that there were no genetic differences with their parents. Agam *et al.* (2020) in the previous studies have also used RAPD analysis to detect genetic diversity in Detam 3 soybean mutants. RAPD analysis using OPAA-02 and OPAA-14 primers showed 60% and 83.3% polymorphism, respectively, among the mutant lines. The highest genetic distance was observed between BSMG-256 and the wild type 46% similarity.

CONCLUSION

In conclusion, there was a diversity of DNA banding patterns between the parents and the four mutant lines tested, namely M.1.1.3, M.5.2.3, M. 1.1.8 and M.1.1.9 mutant lines. The results of cluster analysis showed that Anjasmoro and Kipas Putih varieties and the mutant genotypes had very far genetic distances with a similarity value of 37%. The percentage of similarity between Kipas Putih and M.1.1.8 mutant was 76%, the M.5.2.3 and M.1.1.9 mutants were 80% and the M.1.1.3 mutant had 85% similarity. While the M.5.2.1 and M.7.1.17 mutants have 100% similarity, which means that there are no genetic differences with their parents.

ACKNOWLEDGEMENT

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Conflict of intrest: None.

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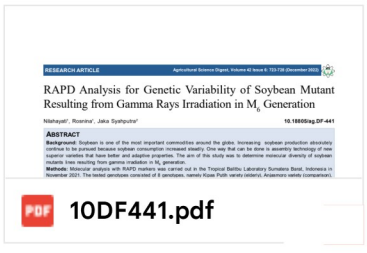
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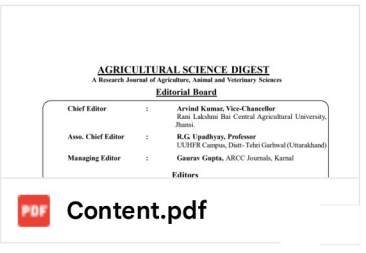
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