

Diversity analysis in soybean (*Glycine max* [L.] Merrill) mutant lines grown in saline soil using agronomic traits and RAPD markers

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Abstract. Soybean is one of the strategic food crops in Indonesia, but its production is far below the demand due to inadequate area for soybean cultivation. Shifting soybean cultivation to marginal land such as saline soil has been suggested as a realistic solution to increase soybean production. Development of soybean variety tolerant to salinity is the key step to support the cultivation of soybean in saline soil. The objective of this research was to evaluate the genetic diversity in mutant soybean lines generated using gamma rays based on agronomic traits and RAPD markers. A total of 200 irradiated seeds of cultivar Detam-3 were planted in saline soil with electrical conductivity of 1.2–4.3 dS/m. Agronomic traits were evaluated on plants until harvesting time. Genetic analysis using two RAPD markers (OPAA-02 and OPAA-14) was done on 11 plants of each radiation treatment. The results showed that 54 plants survived in saline soil. High level of variation based on agronomic traits was observed in these plants. RAPD analysis revealed 60% and 83.3% polymorphism among 11 plants for OPAA-02 and OPAA-14 markers, respectively.

Keywords: Detam-3, gamma rays, OPAA-02, OPAA-14, saline soil, soybean.

1. Introduction

Soybean (*Glycine max* L. Merr.) is one of the main crops widely planted in many countries. Soybean is used as food, feed and raw materials of industrial product [1]. It has a relatively high protein content (35–46%) [2]. However, national soybean production is far below the demand. Domestic production was 963.18 thousand tons in 2015, while domestic consumption reached 1.56 million tons [3]. The domestic consumption increased by 1.73% each year [4]. On the other hand, the area for soybean cultivation has decreased. The harvested area in 2009 was 722,291 ha, but decreased to 550,793 ha in 2013, and increased to 614,095 ha in 2015 [5]. However, domestic soybean production is unable to meet the soybean demand. Harsono [6] estimated that 2 million ha of cultivation area is needed to fulfil the demand in 2020.

Salinity becomes a global challenge in agricultural production [7]. Up to 80% of plant yield can be lost because of drought and salinity [8]. Development of salinity tolerant variety is the key step to support the cultivation of soybean in saline soil. The first step in a breeding program is creating genetic variation through hybridization [9], exploration [10] and mutation [11]. Mutation breeding has been widely used for inducing variation. Two types of mutation are known, i.e. chemical mutation



using mutagen EMS [12] and physical mutation using gamma rays [13]. Mutation breeding is considered effective to improve traits and efficient to screen new traits [14]. RAPD technique is one alternative for identification plant genetic diversity [15].

This study aimed to evaluate the diversity of gamma rays-mutated soybeans lines based on agronomic traits and RAPD markers. This research is a part of a breeding program to obtain adaptive black soybean mutant plants with better productivity compared to its parents in saline soil.

2. Materials and methods

This research was conducted at saline soil in Dresi Wetan Village, Kaliori Sub-district, Rembang District, Central Java Province and Central Laboratory of Diponegoro University, Semarang City, Central Java Province, from December 2017 to July 2018. Soybean cultivar Detam-3 was irradiated with gamma rays at 0 (control), 160, 208, 258, 304, 352, 400, 448, 496, 544 and 592 Gy at the Center for Isotopes and Radiation Application, National Nuclear Energy Agency (CIRA-NNEA). Two-hundred seeds were planted in saline soil with electrical conductivity (EC) of 1.2–4.3 dS/m. The mutant population was named BSMG (for Black Soybean Mutant Gamma).

Agronomic traits were assessed based on the number of leaves, plant height, number of pods, average seed per pod, seed weight per plant, weight of 1 seed and 100 seeds. Analysis of agronomic traits was done by analysis of variance (ANOVA) at 5% significance level, followed by Dunnett's test to compare each treatment to control plant (Detam-3).

Molecular variations were revealed using Random Amplified Polymorphic DNA (RAPD) markers with two primers (OPAA-02 and OPAA-14) which were previously used as DNA markers for soybean genotypes grown under salt stress [16,17]. Leaf samples were collected from 3-week-old plants of each gamma-rays dose. DNA extraction was carried out using Plant Genomic DNA Kit (Tiangen). DNA amplification was performed by PCR following a modified method from Khan et al. (2013). PCR-mix was composed of 22 μ l Master Mix (12.5 μ l AmpliTaq Gold 360, 1 μ l 360 GC Enhancer, 8.5 μ l ddH_2O), 1 μ l primer (working solution 25 μ M) and 2 μ l DNA template to make a total volume of 25 μ l per sample. The amplification reaction was carried out in a Thermal Cycler (Labnet, MultiGene OptiMAX). The first cycle consisted of denaturation of template DNA at 95°C for 10 min, followed by primer annealing at 37°C for 30 sec and primer extension at 72°C for 2 min. For the next 43 cycles, the period of denaturation was reduced to 30 sec while annealing and extension time remained the same as in the first cycle. The last stage of PCR amplification was final extension at 72°C for 8 min. PCR products were separated on a 1.5% agarose gel and DNA fragments were visualized using GelDoc. Bands were scored as present (1) or absent (0) for each primer. The data was arranged into a binary data matrix as discrete variables. This matrix was subjected to Unweighted Pair-Group Method for Arithmetic Average Analysis (UPGMA) to generate a dendrogram using average linkage procedure. All these computations were carried out using NTSYSpc software.

3. Results and discussion

3.1. Agro-morphological characteristics

The leaf number of BSMG-304, BSMG-400 and BSMG-592 was significantly different from Detam-3. The plant height of BSMG-160, BSMG-208, BSMG-304 and BSMG-544 was significantly different from Detam-3 (Table 1). Under optimum condition the plant height of Detam-3 was approximately 56.9 cm [18]. In our study, the growth of this variety at saline soil was strongly inhibited (14.33 cm), which may be caused by the effect of salinity stress. The decrease in plant height of mutant lines may also be caused by both salinity stress and gamma radiation that can create a mutation in plant, such as dwarfism. Other studies reported that the decrease in plant height was due to the effect of salinity stresses on plant growth [19] or the effect of gamma radiation [8].

Table 1. Morphological characteristics of soybean mutant lines and its wild type (Detam-3) grown in saline soil.

Genotype	Number of crops	Number of leaves	Plant height (cm)
BSMG-160	7	5.33±1.16	8.67±0.76*
BSMG-208	6	13.33±1.53	8.50±3.12*
BSMG-256	3	8.67±0.58	14.00±2.00
BSMG-304	8	24.00±18.25*	7.33±1.89*
BSMG-352	3	10.67±1.15	9.33±2.31
BSMG-400	5	28.00±10.15*	9.83±2.75
BSMG-448	3	13.00±3.46	11.50±2.78
BSMG-496	3	9.33±3.51	12.17±4.75
BSMG-544	0	0.00±0.00	0.00±0.00*
BSMG-592	4	19.00±6.08*	18.33±7.50
Detam-3	12	6.00±5.20	14.33±2.08
CV (%)		46.74	28.88

*Significantly different from the control (Detam-3) based on Dunnett's test at $P \leq 0.05$.

CV = coefficient of variation.

Plants affected by salinity stress show delayed growth responses such as decrease of plant height, leaf area, dried apical buds and even plant death [20]. In other crops such as bean, salinity stress in high concentration of NaCl decreases plant height, number of leaves and leaf area [21]. These decreasing parameters are due to the negative effects of ions Na^+ and Cl^- on the rate of photosynthesis, changes in enzyme activity, and also decreased levels of carbohydrates and growth hormones which can cause growth inhibition [21]. Gamma radiation in soybeans can affect the length, width and density of stomata that influence the process of respiration and photosynthesis [13].

3.2. Yield components

The number of seeds per pod for BSMG-256, BSMG-352 and BSMG-544 and seed weight per plant for BSMG-208 were significantly different from control ($P > 0.05$) (Table 2). Under optimum condition, variety Detam-3 produces approximately 51 pods per plant [18]. The same variety produced on average of 4 pods per plant or 96% less (range from 2.00 to 6.67) under salinity stress condition in our study. In the previous study, treatment of variety Dering-1 to three levels of salinity stress, i.e. 3, 6 and 9 dS/m, led to the decreasing number of pods per plant by 32.7, 68.03 and 98.10%, respectively [20].

The weight of 1 seed and 100 seeds of BSMG-544 and BSMG-592 were significantly different from the control (Table 2). Under optimum condition, the weight of 100 seeds of Detam-3 is approximately 11.8 g [18], but under salinity stress condition in our study, its weight decreased to 7.95 g. On the contrary, the weight of 100 seeds of BSMG-592 in saline soil increased to 12.67 g, which is categorized as medium-size seed according to Adie and Krisnawati [22] and Putra et al. [23]. Our result showed that gamma radiation with the dose of 592 Gy gave a positive response on the weight of 100 seeds.

Table 2. Yield components of soybean mutant lines and control grown in saline soil.

Genotype	Number of pods	Average of seeds/pod	Seeds weight/plant	Weight of 1 seed	Weight of 100 seeds
BSMG-160	2.00±1.73	1.75±0.43	0.17±1.07	0.06±0.01	5.77±1.53
BSMG-208	6.67±2.89	1.67±0.61	1.15±0.54*	0.11±0.03	10.88±3.49
BSMG-256	6.00±3.46	1.88±0.13*	0.67±0.36	0.06±0.03	6.30±3.32
BSMG-304	4.33±1.15	1.53±0.46	0.58±0.26	0.10±0.05	9.61±4.60
BSMG-352	2.00±0.00	1.83±0.29*	0.30±0.02	0.08±0.01	8.27±1.11
BSMG-400	5.00±1.00	1.39±0.18	0.73±0.10	0.11±0.03	10.88±2.83
BSMG-448	4.67±3.79	1.59±0.36	0.79±0.74	0.10±0.02	10.29±2.09
BSMG-496	4.00±2.00	1.50±0.50	0.40±0.18	0.07±0.02	7.38±2.17
BSMG-544	0.00±0.00	0.00±0.00*	0.00±0.00	0.00±0.00*	0.00±0.00*
BSMG-592	3.33±3.21	1.16±0.29	0.43±0.31	0.13±0.02*	12.67±1.77*
Detam-3	4.00±1.00	1.30±0.26	0.43±0.19	0.08±0.01	7.95±1.42
CV (%)	47.96	20.79	53.28	25.21	25.21

*Significantly different from the control (Detam-3) based on Dunnett's test at $P \leq 0.05$.

CV = coefficient of variation.

3.3. Molecular analysis

Based on RAPD analysis, 60% polymorphism level (3 of 5 fragments) and 83.3% polymorphism level (5 of 6 fragments) were found for OPAA-02 and OPAA-14, respectively (Figure 1 and Table 3).

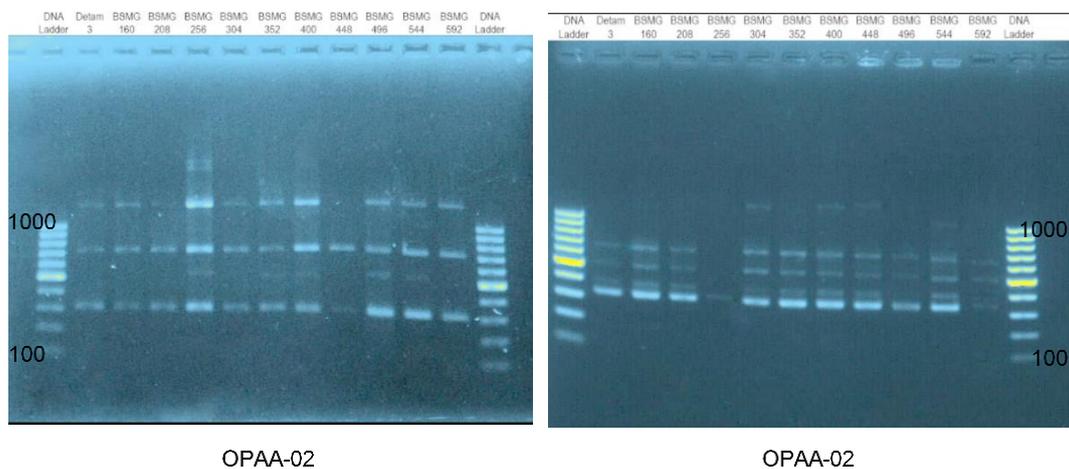


Figure 1. RAPD analysis of several soybean mutant samples derived from gamma-irradiated Detam-3 variety using OPAA-02 and OPAA-14 primers.

Table 3. Overview of DNA fragments amplified in soybean mutant lines derived from gamma-irradiated Detam-3 variety using OPAA-02 and OPAA-14 primers.

Primer	Sequence (5' to 3')	Fragment size (bp)	Total no. of fragment	No. of polymorphic fragment	Polymorphism (%)
OPAA-02	GAGACCAGAC	300–2220	5	3	60
OPAA-14	AACGGGCCAA	300–1210	6	5	83.3

OPAA-02 primer produced 2,220 bp fragment (in BSMG-256) and 520 bp fragment (in BSMG-256, BSMG-352, BSMG-400, BSMG-496 and BSMG-544), which did not appear in control (Detam-3). OPAA-14 primer generated 1,210 bp fragment (in BSMG-304, BSMG-400 and BSMG-448), and 850 bp fragment (in BSMG-544). A band with a size of 1,340 bp present in the control plant was missing in BSMG-448 for OPAA-02 primer. Missing bands with the size of 700, 520 and 370 bp were also observed in BSMG-256 for OPAA-14 primer. The presence and absence of different fragments may indicate the occurrence of genetic mutations in the irradiated seeds (Table 4).

Juwarno and Samiyarsih [24] reported the genetic differences between control plot and 80 mM NaCl plot for three soybean cultivars (Mahameru, Slamet and Dam) subjected to salt stress condition. Genetic instability on soybean subjected to salinity stress was manifested in RAPD profiles as the decrease or the increase in band intensity, disappearance of bands and appearance of new bands as compared to the controls [17].

Table 4. Presence of RAPD bands in black soybean mutant gamma (BSMG) lines derived from gamma-irradiated Detam-3 variety.

Primer name	Fragment size (bp)	Detam-3	BSMG lines									
			160	208	256	304	352	400	448	496	544	592
OPAA-02	2,220				+							
	1,340	+	+	+	+	+	+	+	+	+	+	+
	740	+	+	+	+	+	+	+	+	+	+	+
	520					+	+	+	+	+	+	
	300	+	+	+	+	+	+	+	+	+	+	+
OPAA-14	1,210					+		+	+			
	850										+	
	700	+	+	+		+	+	+	+	+	+	+
	520	+	+	+		+	+	+	+		+	+
	370			+	+		+	+	+	+	+	
	300	+	+	+	+	+	+	+	+	+	+	+

RAPD analysis of the mutant lines revealed four groups of soybean genotypes (Figure 2). The first cluster comprised of the wild type and mutant line BSMG-592, which showed 100% similarity. The second cluster included BSMG-160, BSMG-208, BSMG-304, BSMG-400 and BSMG-448 at 74% similarity. Two of the mutants (BSMG-304 and BSMG-400) occupied the same branch node. The third cluster included three mutant lines (BSMG-352, BSMG-496 and BSMG-544) with 70% similarity. The highest genetic distance was observed between BSMG-256 and control (Detam-3), which showed 46% similarity.

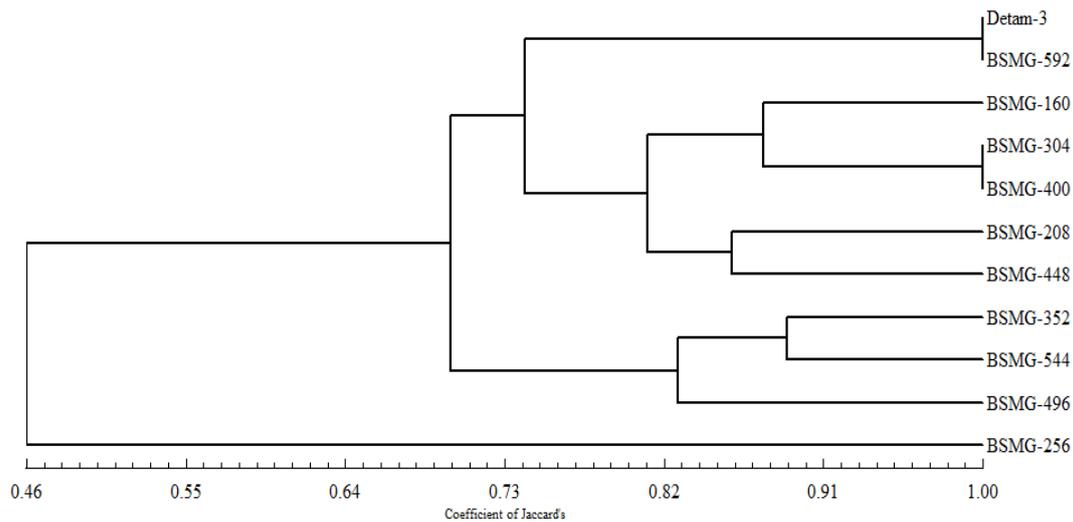


Figure 2. Dendrogram derived from UPGMA clustering analysis based on genetic difference in soybean mutant lines generated from gamma-irradiated of Detam-3 variety.

4. Conclusions

Mutant BSMG-256 had significantly higher weight of 100 seeds than the wild type Detam-3 under salinity stress condition. 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).

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