Analysis of Genetic Variation Mutant Soybean (*Glycine max*. L.Merr) Gamma Rays and EMS *(Ethyl Methane Sulfonate)* Induction Through ISSR Molecular Markers

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Abtract

Mutation induction in soybeans was carried out to develop high genetic diversity as a basis for plant breeding to get superior varieties. Mutation induction can be done using physically mutagen gamma rays and chemically using EMS mutagens. This study aims to determine the effectiveness of chemical mutagen (EMS) and physical mutagen (gamma rays) in the induction of genetic diversity in soybeans. Mutation detection can be done with molecular markers to characterize plant genetic diversity. In this study, ISSR molecular markers were used consisting of 4 primers. The method of mutation induction used EMS and gamma rays treatment. The results of the primary PCR amplification of ISSR2, ISSR3 and UBC888 showed 100% polymorphism. The treatment of mutation induction using EMS chemical agents showed a higher degree of polymorphism compared to gamma rays treatment.

Keywords : induction mutation, gamma rays, EMS, ISSR

Abstrak

Induksi mutasi pada kedelai dilakukan untuk mendapatkan keragaman genetic yang tinggi sebagai dasar pemuliaan tanaman untuk mendapatkan varietas unggul. Induksi mutasi dilakukan secara fisik menggunakan gamma rays dan secara kimiawi menggunakan mutagen EMS. Penelitian ini bertujuan untuk mengetahui efektivitas mutagen kimiawi (EMS) dan mutagen fisik (gamma rays) dalam induksi keragaman genetic pada kedelai. Deteksi mutasi dapat dilakukan dengan penanda molekuler untuk mengkarakterisasi keragaman genetic tanaman. Dalam penelitian ini digunakan penanda molekuler ISSR yang terdiri dari 4 primer. Metode induksi mutasi yang digunakan dalam penelitian ini adalah perlakuan EMS dan perlakuan gamma rays. Hasil amplifikasi PCR primer ISSR2, ISSR3 dan UBC888 menunjukkan polimorfisme 100%. Perlakuan induksi mutasi menggunakan mutagen kimiawi EMS menunjukkan derajat polimorfisme yang lebih tinggi dibandingkan dengan perlakuan gamma rays.

Kata kunci : induksi mutasi, sinar gamma, EMS, ISSR

Introduction

The development of superior soybean varieties suitable for the land type / agroecosystem requires the availability of gene sources from the desired characteristics. The source of the gene from the desired trait is obtained from the availibility of germplasm. Development of superior soybean varieties related with the problems that are often obtained in the

field for drought tolerance, resistant to leaf rust disease, armyworm and pod sucking pests. Tolerance on acidic dry land types requires genotypes that have suitability (adaptation) in the environment of acidic soil, good agronomic growth (type of growth indeterminate / semideterminate, sturdy stem, not fall, medium / deep depth, leaf size classified medium), high yield potential and good seed quality.

Mutation breeding methods useful to improve the nature of the genetic resources that are not available and will allow for the development of new properties. Mutation breeding can be used to break gene linkage if the genes that control the properties are closely related to genes that control unfavorable properties. Mutation breeding is very effective to change certain traits without changing other properties that have been favored so that the time needed in the plant breeding program by mutation is relatively shorter. Mutation breeding is also effective for repairing perenial plants that take a very long time to be crossed because of waiting for the generative phase. Recently, mutation breeding has been used as a method of plant breeding in generating new variability and development of plant varieties. This method is proven by the fact that some induced mutant plants have been released as new varieties. (Khan and Tyagi., 2013).

There are so many molecular marker techniques are available, PCR-based approaches are desirable because of their simplicity and need only for small amounts of DNA samples. The sequence of sequences (ISSR) is a random multiloci marker produced by PCR amplification with microsatellite primers. They are beneficial because no previous genome information is needed for their use. ISSR markers are a good choice for DNA fingerprints (Bornet and Branchar., 2001). Previous researchers have shown that the ISSR analysis usually detects higher levels of polymorphism than those detected by restriction fragment length polymorphism (RFLP) or DNA amplified polymorphic analysis (RAPD) (Godwin et al., 1997)

The marker for inter-simple sequential sequences (ISSR) generated by microsatellite-primary repeats continuously to strengthen the area between the adjacent SSR locus. Inter-SSR provides a new fingerprinting approach that applies to taxonomic and phylogenetic comparisons and as a mapping tool in various organisms. (Zietkiewicz et al. 1994). These primers produce many polymorphic markers that are useful for mapping genomes (Nagaoka and Ogihara 1997). ISSR-

PCR provides fast, reliable and very informative information systems for DNA fingerprinting that are suitable for applications (Prevost, and Wilkinson., 1999). This study aims to compare the effectiveness of chemical mutagen with physical mutagen for induction of genetic diversity using ISSR molecular markers. The results of the study can be used as treatment recommendations for induction of soybean genetic diversity as a basis for the development of germplasm

Material and Methods

Tool. The tools used are mortar and pestle, tip size of 1000 µl and 200 µl, micropipette, micro 22 R Hettich centrifuge, waterbath, refrigerator, autoclave, microwave, plate chamber electrophoresis, vortex, spindown, Genesys 10 UV Spectrophotometer, Macro Vue-20 Hoefer UV transiluminator (Gel Doc), PCR (Master Cycler Gradient Eppendorf). The material used for DNA sampling is the leaves of the Dering-1 soybean variety.

Material. Material used for DNA isolation: liquid nitrogen, CTAB extraction buffer, phenol: chloroform: Isoamilalkohol (PCI) = 25:24:21, chloroform: Isoamilalkohol (CI) = 24: 1, ammonium acetate, absolute ethanol, 70% ethanol, Tris-EDTA buffer (TE buffer), distilled water, ethidium bromide, loading dye, DNA marker, 10x buffer taq, dNTP, MgCl2, primer, taqDNA polymerase, ddH2O.

Mutation induction. The treatment of physically mutagent using gamma rays. Gamma Cell-220 irradiator with a dose: 100Gy; 200 Gy, 300 Gy. Chemically mutagent using EMS (Ethyl Methane Sulfonate) at a dose of 1% and soaking time in EMS respectively: 4.6 and 8 hours. A 1% EMS dose was obtained from a preliminary study that at these doses that showed the highest diversity compared to lower doses.

DNA isolation is obtained from samples of young leaves when the plant enters the R1 phase, when the plant begins to flower. The isolation method used is the method of Doyle and Doyle (1987). Measurement of purity and DNA concentration using a genesys 10 UV spectrophotometer

DNA amplification using PCR. The pure isolated DNA and has known concentration is used as a PCR template. DNA Amplification with Polymerase Chain Reaction (PCR). Amplification was carried out on a reaction mixture of 12.5 µl containing 7.5 µL of PCR mix, 2.5 µl of ddH2O, each of which was 1.5 µl and 1 µl (10-100 ng / µl) of soybean genomic DNA. PCR reaction using a programmed PCR machine was 30 cycles. The PCR program was set at 95 ° C for 3 minutes preheating, 30 cycles consisting of 1

minute denaturation at 95 ° C, 1 minute annealing at 50 ° C, and 2 minutes extension at 72 ° C. The last extension was done for 4 minutes at 72 ° C. The PCR amplification product was visualized on 1% agarose gel.

Data Analysis

Data from the amplicon band visualization were given a score with the Gel Analyzer software (Lazar, 2010). Each band seen in the gel is considered as an allele, DNA bands with the same rate of movement are considered as one allele. DNA bands with the same rate of movement, each band that looks to the size of the target is given a score of 1, while the invisible band is given a score of 0, so the results of scoring are binary data. Scoring data were then analyzed with UPGMA (Unweight-Pair Group Method with Arithmetic Means) software based on Jaccard genetic similarity index on PAST (Paleotological Statistics Software Package for Education and Data Analysis). The analysis results are presented in the form of dendogram and genetic similarity matrix. Scoring data were analyzed by PAST software to determine the main allele frequency values, genetic diversity, polymorphic information content (PIC) and the resulting heterozygosity

Results and Discussion

The quantitative data and the purity of DNA isolation measure using nano spectrophotometer in Table 1. Qualitative data on electrophoresis results in showing total genes that have been isolated in band sizes above 1000 bp. TheDNA concentration ranging from 2655.8 to 4261.9 ng / µL and the purity level ranged from 1.17 to 1.83. The lowest concentration is in sample code 8 (EMS 1% with 4 hours immersion) which is 2,655.8 (ng / µL), and the highest DNA concentration is in sample code 3 (300 Gy gamma rays). Measurement of DNA quantity was carried out by spectrophotometric method using spectrophotometer at wavelength (λ) 260 and 280 nm. (Darmono, 2011). DNA purity is determined by calculating the absorbance ratio in A260 with A280 (Ratio A260: A280). DNA molecules are pure if the absorbance ratio are between 1.8 - 2.0 (Novita, 2013)

Table 1. Quantitative whole genome using nano spectrofotometer

Sample	260/230	Abs 230	Abs 260	Abs 280	260/280	Con (ng/µL)
Kontrol	1,61	32,95	53,12	45,31	1,17	2655,8
8	1,92	39,98	76,69	43,32	1,76	3834,3
9	1,92	41,50	79,58	44,73	1,78	3978,9
10	1,56	54,71	85,24	54,70	1,56	4261,9
1	1,91	37,00	70,61	38,59	1,83	3530,6
2	1,94	40,49	78,52	44,07	1,78	3925,8
3	1,81	44,64	80,79	46,08	1,75	4039,4

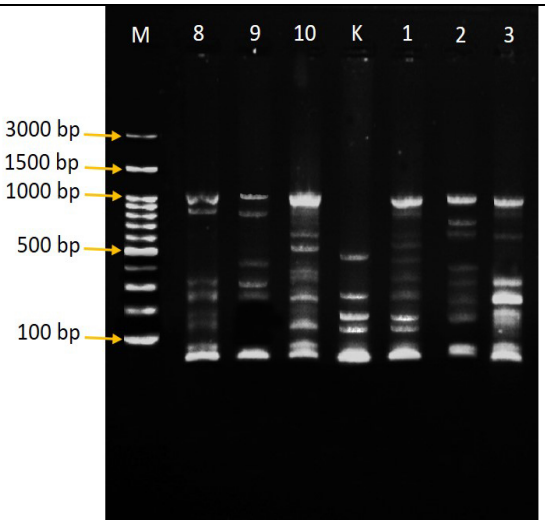

Figure 1. Amplification pattern of ISSR primers AG8CT/ISSR1 for soybean. 8: EMS 1% with 4 hours immersion; 9: EMS 1% with 6 hours immersion; 10: EMS 1% with 8 hours immersion 1 : 100Gy gamma rays; 2: 200 Gy gamma rays; 3: 300 Gy gamma rays

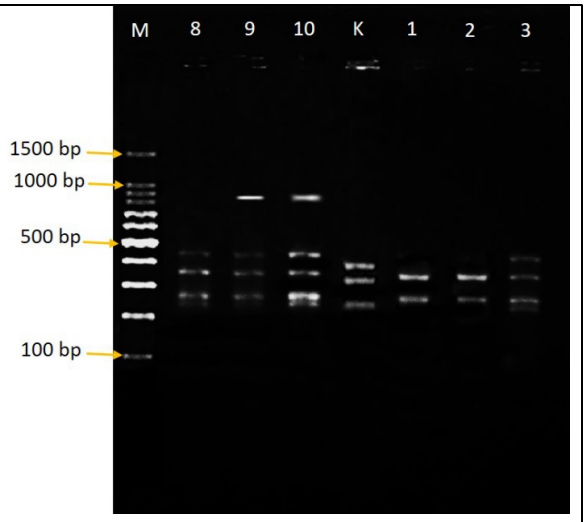

Figure 2. Amplification pattern of ISSR primers CA8G/ISSR2 for soybean. 8: EMS 1% with 4 hours immersion; 9: EMS 1% with 6 hours immersion; 10: EMS 1% with 8 hours immersion 1 : 100Gy gamma rays; 2: 200 Gy gamma rays; 3: 300 Gy gamma rays

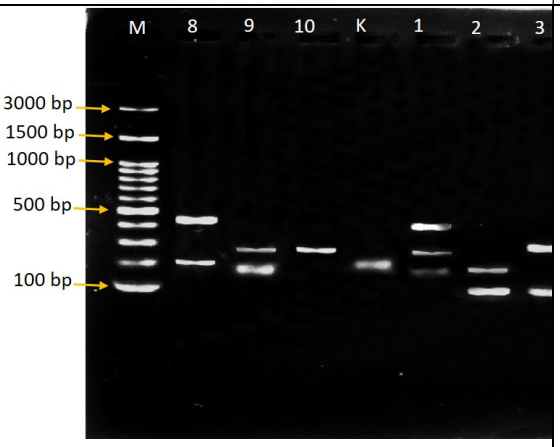

Figure 3. Amplification pattern of ISSR primers GT_7AC /ISSR3 for soybean. 8: EMS 1% with 4 hours immersion; 9: EMS 1% with 6 hours immersion; 10: EMS 1% with 8 hours immersion 1 : 100Gy gamma rays; 2: 200 Gy gamma rays; 3: 300 Gy gamma rays

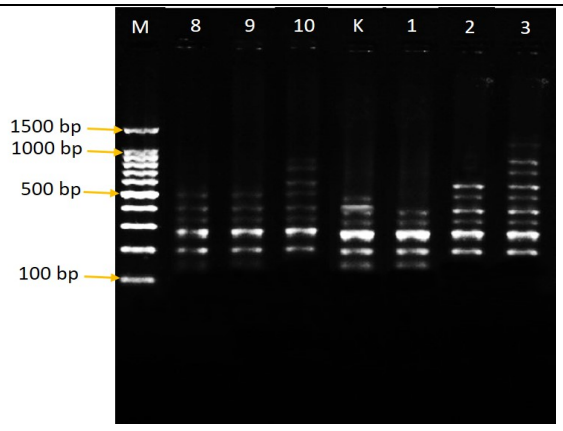

Figure 4. Amplification pattern of ISSR primers BDB$(CA)_7$ /UBC888 for soybean. 8: EMS 1% with 4 hours immersion; 9: EMS 1% with 6 hours immersion; 10: EMS 1% with 8 hours immersion 1 : 100Gy gamma rays; 2: 200 Gy gamma rays; 3: 300 Gy gamma rays

Table 2. Polymorphism band produced by ISSR primers in genotype soybean mutation induction gamma rays and EMS (Ethyl methane sulfonate)

Primer Name	Sequence (5'-3')	Annealing Temperature (C°)	Size (bp)	Amplified Bands	Polmorphic Bands	P%
Gamma rays						
ISSR1	$(AG)_8CT$	53	80-1000	16	15	93.75
ISSR2	$(CA)_8G$	50	220-430	7	7	100
ISSR3	$(GT)_7AC$	50	80-480	6	6	100
UBC888	$BDB(CA)_7$	47	150-1100	13	10	76.92
EMS						
ISSR1	$(AG)_8CT$	53	80-1000	16	15	93.75
ISSR2	$(CA)_8G$	50	230-850	8	8	100
ISSR3	$(GT)_7AC$	50	170-450	4	4	100
UBC888	$BDB(CA)_7$	47	150-850	14	13	92.86
Combination of polymorphisme						
ISSR1	$(AG)_8CT$	53	80-1000	19	18	94.74
ISSR2	$(CA)_8G$	50	220-850	12	12	100
ISSR3	$(GT)_7AC$	50	80-480	8	8	100
UBC888	$BDB(CA)_7$	47	150-1100	19	19	100

The results of ISSR primary amplification work out in amplifying DNA in DNA products ranging from 80 to 1100 bp. The total amplification was 4-19 bands with polymorphisms between 4-19 polymorphisms, and the percentage of diversity ranged from 76.92-100% (Figure 1-4). Table 2 showed that gamma rays treatment of ISSR1 mutation induction showed the highest band amplification, namely 16 bands, 15 band polymorphic bands and

93.75% amplification percentage. In the mutagen EMS treatment using the ISSR 1 primary, the highest amplification band was 16 bands, 15 polymorphism bands and 93.75% amplification percentage. Whereas the combination treatment showed the UBC 888 primer had the highest amplification band, namely 19 bands, 19 polymorphism bands and 100% amplification percentage, respectively.

Table 3. ISSR primer amplification, % polymorphism of mutant soybean EMS induction

Primer Name	Sequence (5'-3')	Annealing Temperature (C°)	Size (bp)	PIC	EMR	MI	RP
ISSR1	$(AG)_8CT$	53	80-1000	0.359	**240**	**86.25**	**16**
ISSR2	$(CA)_8G$	50	220-430	0.375	49	18.37	5.5
ISSR3	$(GT)_7AC$	50	80-480	0.417	36	15	3.5
UBC888	$BDB(CA)_7$	47	150-1100	0.337	130	43.75	13

Table 4. ISSR primer amplification, % polymorphism of mutant soybean gamma rays induction

Primer Name	Sequence (5'-3')	Annealing Temperature (C°)	Size (bp)	PIC	EMR	MI	RP
ISSR1	$(AG)_8CT$	53	80-1000	0.359	**240**	**86.25**	**14**
ISSR2	$(CA)_8G$	50	230-850	0.391	64	25	8.5
ISSR3	$(GT)_7AC$	50	170-450	0.437	16	7	3
UBC888	$BDB(CA)_7$	47	150-850	0.348	182	63.375	13.5

Tables 3 and 4 show the potentiality of 4 primers to amplify DNA as shown in the table of primary identification which is most informative, known from the value of PIC (Polymorphic Information Content) ranging from 0-0.5. The high PIC value shows that primers are very good for genetic diversity (Roldan-Ruiz et al., 2000; Soengas et.al., 2006). EMR (Effective Multiple Ratio) is used to determine the number of polymorphic fragments in the observed sample. The higher the EMR value the more effective the primer is in producing polymorphic fragments. The MI (Marker Index) value is used to determine the primary index in producing polymorphic fragments (Varshney et.al., 2007). RP (Resolution Power) values are used to determine the strength of a primer in producing clear fragments. The higher the RP value, the better a primer in producing clear frgamen (Prevost and Wilkenson, 1999). Of the four primers for primary ISSR 1 is the best primary to get polymorphism in mutant individuals.

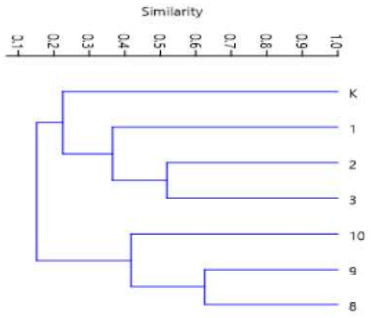

Figure 5. Dendogram of Mutant EMS and Gamma Radiation

The level of similarity in some mutants shows the lowest value of 0.083 and the highest of 0.519, this means that the diversity is very high in mutant individuals. There were 2 mutant clusters from the results of the dendogram, namely group 1 consisting of mutants resulting from the induction of gamma-ray mutations grouped with control with a genetic distance of 0.24 ie sample codes 1, 2 and 3. Cluster 2 is consisted of mutants resulting from mutation induction by EMS with a genetic distance of 0.407 in the sample code 8.9 and 10 (Figure 5).

The results of the dendogram showed that treatment with EMS mutagen showed greater genetic differences than control when compared with treatment with gamma ray mutagen. This shows that treatment with EMS mutagent is enough to produce genetic diversity in soybean plants. Mangaiyarkarasi, et.al. (2014) study results on mutagenic effectiveness and gamma ray efficiency, EMS and combined treatments were studied in the genotype of cowpea varieties. Mutagenic seeds are tested for LD50 percent for all mutagens, separately and a 50% dose of seed germination is considered as LD (lethal dose) value. EMS becomes more effective and efficient in causing mutations compared to gamma rays and combined treatments

Two mutagens, physical (gamma ray) and chemical (EMS) were tested on Uma beans (Phaseolus lunatus). Two mutagens, physical (gamma ray) and chemical (EMS) were tested on Uma beans (Phaseolus lunatus). Dosage / concentration is determined with an LD50 value of 16 KR for gamma rays and a concentration of 40 mM for EMS tested on germination and percentage of survival. EMS treatment is more effective in producing chlorophyll mutants. Physical mutagen gamma rays produce more suitable mutants including mature plants, dwarfness and higher yields. Low dose gamma rays, 8 to 20 KR, can be used to obtain beneficial mutants in Uma bean plants. (Kumar *et.al.,* 2003).

A study of the effectiveness and efficiency of gamma rays, ethylmethane sulphonate (EMS) and their combinations (gamma + EMS light) was carried out in two varieties of urdbean

(*Vigna mungo* (L.) Hepper) namely, T-9 and Pant U-30. Based on effectiveness, the mutagen sequence is EMS> gamma rays + EMS> gamma rays in both varieties (Goyal *et.al.,* 2012). The highest frequency of morphological mutations was observed at 300 Gy gamma irradiation (4.11%) from CH40 / 91 followed by 0.4% EMS (2.08%) from *Cicer arietinum* genotype C44 (Shah *et.,al,* 2011). The results of this study are different from those of Pavadai et al. (2010) reported variability, heritability and genetic progress for plants treated with mutagen rather than plants not treated for all generations. 50 KR gamma ray treatment is effective compared to other mutagenic treatments and controls.

Based on this research, EMS mutation induction treatment can be used to produce plant diversity in soybeans. The results of this study provide a very important basis for further research in assembling new soybean superior varieties

Conclusion

The research shows that treatment with EMS chemical mutagen shows a higher degree of polymorphism compared to gamma rays treatment.

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