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The effect of colchicine treatment on phenotype and genotype characteristics of Detam-2 variety of soybean *Glycine max*

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Abstract. *Fathurrahman F, Ulpah S, Sodiq NAM, Mahadi I, 2024. The effect of Colchicine treatment on phenotype and genotype characteristics of Detam-2 variety of soybean* Glycine max. *Biodiversitas 25: 1230-1238.* The enhancement of soybean plant mutagenesis to obtain phenotypic and genotypic diversity is essential in agriculture. This study aimed to analyze the effect of colchicine concentration on phenotypic and genotypic characteristics based on RAPD markers of Detam-2 variety. The experiments were carried out from January to June 2023 in the experimental farm of the Agriculture Faculty, Universitas Islam Riau, Pekanbaru, Indonesia. Therefore, 600 plants were used, consisting of 200 controls and 400 samples treated with 3,500 ppm of colchicine. The growth parameter was analyzed using a DMRT statistical test at $p<0.05$, and stomata densities were compared using T-test. The mutant-1 produced the highest yield, a total of 235 pods (with 233 phity pods), seed weighed 93.20 g, which was significantly different from those of controls. Mutant-2 produced the highest dry weight of 100 seeds, which was 12.80 g, with a dry seed weight per plant of 63.30 g. Leaf types in mutant samples varied, including normal, purple leaf stems, and curly leaves. The genotypic study showed that primer OPAA-01 produced a different number of DNA bands. The highest genetic similarity was 0.961 among the controls, while the lowest was 0.451 between mutant-1 and the controls. Based on the results, the dendrogram produced two groups: control and mutant-1.

Keywords: Black soybean, growth, mutant, polymorphism, RAPD

INTRODUCTION

The Detam-2 variety of black soybean (*Glycine soya* L. Merr) is a commodity that is often processed into tempeh and tempeh milk to enhance protein digestibility. Apart from its use as a food ingredient, it also serves as a valuable resource in producing anti-aging products (Romulo and Surya 2021). The glutamate content has been reported to have a role in enhancing the taste of food, making black soybean a suitable main ingredient for soy sauce (Tang et al. 2023). In a previous study, the protein content of Detam-2 variety of black soybean was 45.36%, with fat levels of 33.06% (Fattah et al. 2022). This variety is highly resistant to insects, pod-shattering, and drought-sensitive. According to previous studies, it contains vitamin E, β-carotene, isoflavones, and anthocyanins, contributing to its antioxidant activity (Kim 2021). The total flavonoid content was 0.23 to 0.44 mg CE/g (Yusnawan 2018; Hasanah et al. 2019), and the findings showed that four soy products have a noticeably greater antioxidant content. Soy products have an antioxidant level ranging from 18.29 to 25.19 mg per g (Robbani et al. 2022).

The current limitations include the low improvement of soybean productivity and quality through time-consuming conventional breeding methods. For instance, the mutation of chromosomes and genes using mutagenic agents, such as colchicine, can tailor soybeans to agroclimatic conditions, fostering phenotypic and genotypic diversity. Colchicine-induced variations can cause polyploidy, such as triploid, tetraploid, and hexaploid. Tetraploid plants have larger size, increased vigor, and larger fruit and seed dimensions than diploid plants. Furthermore, their cells have larger nuclei, increased cell size, and larger vacuoles, leading to higher water content. Several studies have shown that larger stomata and vascular bundles enhance nutrient absorption, causing higher protein, vitamin, and metabolic yields. The phenotypic and genotypic trait changes induced by colchicine have been explored by Fathurrahman (2023).

Furthermore, it has the ability to inhibit the metaphase stage, preventing the polymerization of tubulin into microtubules and causing the inability of tubulin to form functional spindle fibers. Several studies showed that chromosome separation did not occur during the anaphase stage (Fathurrahman 2015; Fathurrahman et al. 2023a). Without a spindle, the division wall cannot form, leading to the retention of chromosomes and their copies in the same cell. Polyploid plants that develop from colchicine treatment have improved their adaptation to salt stress by efficiently regulating water usage by varying leaf latex osmotic potential (Mangena 2023). These plants also possess better adaptive abilities in regulating cation toxicity and ions that limit enzyme activity and nucleic acid metabolism than diploids (Manzoor et al. 2019).

The optimal concentration of colchicine that determines the selection of successful polyploid soybeans can be measured by seed germination, shoot length, root length

during the germination stage, and field germination during the M1 generation (Talebi et al. 2012). This compound can also affect stomata, as shown in a study conducted by Mulyono et al. (2022) on induced hybrid maize lines (*Zea mays* L.), where it caused lower stomatal density but larger stomatal size. Similar results were also obtained in a study by de Oliveira Neves et al. (2022) on olive trees (*Olea europaea* L.). Detecting plant mutations using the phenotypic method has been reported to face several limitations (Garcia et al. 2016). Several genetic methods that can be used include Random Amplification Polymorphic DNA (RAPD) (Backeljau et al. 1995), Restriction Fragment Length Polymorphism (RFLP), Inter-Simple Sequence Repeat (ISSR) (Mishra et al. 2014) and DNA sequencing (Kumari and Thakur 2014; Nikmah et al. 2016; Wahyudi et al. 2020). Several previous studies have shown that RAPD has been successfully used to detect mutations in rice (Ashraf et al. 2007), melon, and sugarcane (Kawar et al. 2009). RAPD markers are easier to reproduce than other genetic markers, such as ISSR and RFLP, and do not require initial knowledge of the genomic background (Kumari and Thakur 2014). Several studies have also shown the successful use of RAPD to detect mutations in melon (Daryono et al. 2011). The results can be used for genotypic clustering to determine the relatedness, often presented as a dendrogram. Therefore, this study aims to analyze the effect of colchicine concentration on phenotypic (growth, stomata anatomy) and genotypic characteristics of black soybeans from Detam-2 variety based on RAPD markers.

MATERIALS AND METHODS

The plant materials used were soybean seeds of Detam-2 variety; 600 plants were used, consisting of 200 controls and 400 samples treated. The equipment and materials for stomata analysis glass microscope slides, transparent nail polish, and acetocarmine stain. For DNA analysis, loading dye, Mix GP1 Buffer, GPX1 Buffer and RNase, Elution Buffer, filter columns, GP3, W1, Wash, Elution Buffer used Genomic DNA Mini Kit Plant (Geneaid), electrophoresis gel, thermal cycler, NanoDrop spectrophotometer, and a digital camera (Olympus) with a UV filter were used. For PCR and RAPD analysis provided by Thermo Scientific, DNA markers from Thermo Scientific, PCR buffer comprising dNTPs, forward primers, Taq DNA polymerase and DNA samples. The primers used included six types with sequences of AGACGGCTCC (OPAA-01), GAGACCAGAC (OPAA-02), TTAGCGCCCC (OPAA-03), AGATGGGCAG (OPAA-09), AACGGGCCAA (OPAA-14), and ACGGAAGCCC (OPAA-15) provided by Thermo Scientific.

The field experiment was conducted using 600 plants of Detam-2 variety of black soybean 400 plants (8 plots) were treated with colchicine at 3,500 ppm, and the remaining 200 plants (4 plots) as controls. Seeds were sowed in plots spacing 50 cm x 50 cm apart. Each plot measuring 1.2 m x 4.6 m x 0.2 m consisted of 50 plants planted at 25 cm x 40 cm space. Organic compost, 10 kg per plot, was applied one week before planting, and inorganic fertilizers, including 30 g urea, 75 g Triple Super Phosphate, and 50 g potassium chloride per plot, were applied during planting. As many as 32 plants were randomly selected as samples per plot. Phenotypic characteristics were observed, including number of pods (pcs), pithy pods (pcs), planting seed weight (g), seed moisture content on a wet weight (%), seed moisture content dry weight (%), weight of 100 dry seeds (g) and dry seed weight per plant (g). Growth analysis was done using statistical analysis, DMRT at $p<0.05$ significance level, and SAS 9.1 software.

Stomatal observations were conducted on the broadest leaves, collected at 9:00 am. The lower surface of the leaf (abaxial) was cleaned with a tissue, followed by applying a transparent clear nail polish over a width of one cm on the lower surface at the base, middle, and tip. After the nail polish had dried, transparent tape was placed over or covering the areas that had been painted. Subsequently, the tape was removed from the leaf, causing the nail polish to adhere. The tape was then affixed to a glass microscope slide and prepared for observation under 400x magnification microscope. Stomatal anatomy was analyzed using the T-test in this study. When there were differences in variance among treatments, the T-test was conducted using two samples assuming unequal variances. The T-test was performed using SPSS 23 to determine the influence of the various treatments.

Furthermore, for RAPD analysis, the DNA extraction was done using 50-100 mg of fresh leaves. The leaves were crushed into fine powder in a mortar with liquid nitrogen. The powder was then transferred 1.5 mL tube. Lysis was then conducted using GPX1 Buffer, RNase, and other necessary chemicals with skilled methods to obtain high-quality DNA for PCR analysis as manufactured instructions (Geneaid). The PCR analysis was carried out using pre-PCR at 95°C for 3 minutes, denaturation at 95°C for 30 seconds, annealing at 38.8°C for 30 seconds, elongation at 72°C for 1 minute, and post-PCR at 72°C for 10 minutes, with 35 cycles. Electrophoresis of PCR products was performed using 1.5% agarose gel and 1.5 µL of ethidium bromide solution (used as a DNA band stain) in 1xTBE buffer (pH 8). The PCR product solution of 3 µL and loading dye solution of 10 µL were loaded into the gel well. Electrophoresis was conducted at an electric voltage of 50 volts for 40 minutes. The results were then visualized using an Olympus camera with a UV filter to observe the formed DNA bands. RAPD analysis is conducted by observing the distance and number of DNA bands for each sample based on the DNA marker.

RESULTS AND DISCUSSION

All soybean plants untreated with colchicine (plants in control plots) showed normal growth. In comparison, those treated with colchicine at a concentration of 3,500 ppm (colchicine treatment plots) exhibited drawback growth (Figure 1). The plants that successfully grew in colchicine-treated plots the considered mutants.

Phenotype characteristics: The number of pods, pithy pods, planting seed weight, fresh weight of seeds, dry weight of seeds, weight of 100 dry seeds, and dry seed weight per plant

Observations on the generative growth, such as the number of pods in black soybean of Detam-2 variety, were conducted on control samples and colchicine-treated mutant with different growth types, namely normal growth (mutant-1), curled leaves (mutant-2) and purple leaves stem (mutant-3). After statistical analysis, significant differences were observed in the generative characteristics, including the number of pods, pithy pods, seed weight, and dry seed weight. The data from the follow-up DMRT at the $p<0.05$ level is presented in Table 1.

Table 1 shows the highest number of pods found in Detam-2 black soybeans, with mutant-1 producing 235 pods, followed by mutant-3 and mutant-2 with 220 and 188 pods, respectively. These numbers significantly differed from the control soybean sample, which only yielded 147 pods. The increase in the number of pods in mutant-1 was 88 of pods higher compared to the control. The results of this study were consistent with Fathurrahman (2023), where F1 mutant cucumbers produced more than 3.17 of fruits number. Furthermore, there was a significant increase in the number of fertile pods (Pithy Pods) in mutant-1 compared to other mutation samples and the control. After conducting statistical analysis and the follow-up DMRT test at $p<0.05$, it was evident that there was a significant difference between mutant-1, producing 233 pithy pods, and mutant purple stem, with 215 pithy pods, in comparison to the control possessing only 143 pithy pods.

Other phenotypic characteristics, such as the weight of seeds, increased after statistical analysis. The results showed a significant difference between the control, mutant-1, and mutant-2, which yielded 44.30 g, 93.20 g, and 70.84 g, respectively. The increase in seed weight in mutant-1 was 110.38%. This result was in line with Mangena and Mushadu (2023), where colchicine increased in leguminous crops enhances morpho-physiological.

Table 1. Growth and yield characteristics of the Black Soybean from Detam-2 variety in control and mutant samples

Character	Control	Samples		
		Mutant-1	Mutant-2	Mutant-3
Number of pods (pcs)	147.00±13.27 b	235.00±1.85 a	188.00±17.77 ab	220.00±31.88 ab
Pithy Pods (pcs)	143.00±13.22 b	233.00±4.93 a	150.00±18.07 b	215.00±1.76 a
Planting seed weight (g)	44.30±7.18 c	93.20±1.89 a	70.80±5.23 b	42.70±1.10 c
Fresh weight of seeds (%)	14.90±0.45	18.97±1.22	17.13±2.06	19.37±1.44
Dry weight of seeds (%)	9.73±0.06	9.87±0.06	12.53±1.50	10.03±0.17
Weight of 100 dry seeds (g)	13.50±0.38	14.10±0.86	12.80±1.11	13.10±0.18
Dry seed weight per plant (g)	38.70±6.63 b	63.00±5.56 a	63.30±7.05 a	48.30±5.36 b

Note: Mean±Standard Error (SE) followed by different letters of the same days of treatment is significantly tested using the Duncan multiple range test at $p<0.05$

Figure 1. The growth appearance of Detam-2 black soybean plants untreated and treated with colchicine at a concentration of 3,500 ppm. Note: ← are plots for mutant samples

Observations of the moisture content in wet weight showed no significant difference between the control sample and mutants. However, mutant-3 had a higher moisture content of 19.37% in numerical terms. The results also showed a 30% increase in moisture content in mutant-3 compared with control samples. There was no significant difference between the control and mutant for observing moisture content in dry weight. Mutant-2 had a 12.53% higher value than the control with 9.73%. The results of the observation of the weight of 100 dry seeds showed no significant difference. The lowest weight was 12.80 g in mutant-2, which was better than others. Colchicine was a mitosis inhibitor widely used to induce plant polyploidy during cell division by inhibiting chromosome segregation (Manzoor et al. 2019).

Observations of dry seed weight showed a significant difference between the control and mutant-1 and -2 samples. The weight of dry seeds per plant in mutant-1, mutant-1-2, and the control was 63.00 g, 63.30 g, and 38.70 g, respectively. Furthermore, there was a 63.56% increase in the weight of dry seeds in mutant-2. Mutations caused by the mutagen treatment could lead to various abnormalities in the processes of mitosis and meiosis in cells (Bharadwaj 2015), and their expression affects both vegetative and generative growth.

Stomatal characteristics

The results of colchicine effect observation on stomatal characteristics in black soybean plants of Detam-2 variety (Table 2) showed that stomatal density, length, and width in both the control and mutant treatment had t-values lower than the t-table. Therefore, there was no significant difference in stomatal density, length, and width between control and mutant plants based on the t-test, as shown in Table 2. Colchicine treatment affected stomatal characteristics, and mutations occurred due to the toxic nature of colchicine, which induced random mutations.

Stomatal density in mutant-1 plants was lower, measuring 114.2 per 0.12 mm², compared to the control of 143.52/0.12 mm². Furthermore, the standard deviation for stomatal density in control and mutant plants was approximately ±2.31 and ±1.33, respectively. This shows that the mean value was higher than the standard deviation, showing a low range of variation between the samples. The standard deviation for stomatal length and width was smaller in the treatment groups compared to the control.

Table 2. Characteristics of density, length, and width of stomata between samples of black soybean var. Detam-2 control and mutant samples

Characteristics	Control	Mutant	T count	T-Table	
				0.05	0.01
Stomatal Density (0.12 mm2)	143.52±2.31	114.2±1.33	2.50^{ns}	2.57	4.03
Stomatal length (µm)	16.43±1.10	17.90±0.20	2.27^{ns}	2.57	4.03
Stomatal Width (µm)	11.01±0.56	11.17±0.51	0.41^{ns}	2.57	4.03

Note: ns: non significant, Mean ±: Standard Deviation (SD)

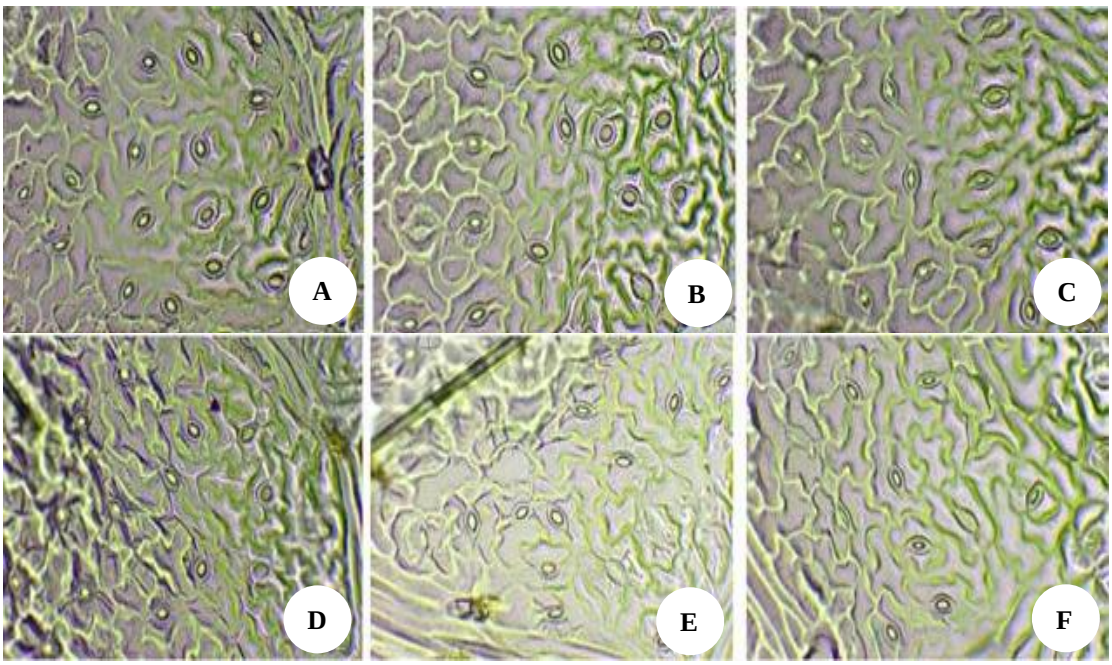

Figure 2. The stomatal density of black soybean plants var. Detam-2 with control samples: A. Leaf base, B. Leaf middle, and C. Leaf tip. Colchicine treatment on: D. Leaf base, E. Middle of leaf, F. Leaf tip (400x)

The high and low levels of stomatal density were categorized based on Mulyono et al. (2022), which included low (<300 per mm²), moderate (300-500/mm²), and high density (500 per mm²). This parameter was related to the size of the stomata, where the highest density was associated with small organs and vice versa, as shown in Figure 2. Stomata could be present on both the abaxial and adaxial surfaces, but sometimes only on the abaxial aspect (Lüttge and Buckeridge 2020). Therefore, in this study, it was only observed on the abaxial side. Wiendra and Pharmawati (2019) also reported a reduction in the number of stomata but with larger sizes in plants derived from seedlings of *Impatiens balsamina* L. treated with colchicine. This was supported by Moghbel et al. (2015) on *Glycyrrhiza glabra* plants, which also showed larger stomatal sizes with the same treatment.

Phenotypic mutations

The morphological changes in black soybean plants due to colchicine induction led to mutations in leaf shape. Leaf shape mutations occurred due to gene expression in response to treatment. Colchicine, as a mutagen, was toxic and caused random changes, affecting some cells. The leaves of plants treated with the compound were larger than those of the control plants. Furthermore, there was heterogeneity in phenotypes due to colchicine-induced mutations, particularly in the varying leaf shapes, as seen in Figure 1. Azizan et al. (2021) showed that colchicine induction in stevia plants led to physical changes in leaf margins, alternating between serrated and non-serrated edges. Changes in plant genes caused mixed phenotypes, as shown by leaf shape and margins. The pointed and rounded shapes induced by treatment positively enhanced the production of the secondary metabolite stevioside.

The leaves of black soybean plants, such as Detam-2 variety, had an elongated leaf shape under normal conditions, as described for variety. Colchicine induced changes in this variable, transforming them from serrated to non-serrated, from ellipses to more ellipses, and from pointed to blunt-ended. Furthermore, the margins exhibited various patterns, ranging from wavy to serrated, as presented in Figure 3.

Phenotypic characteristics of black soybean var. Detam-2 appeared to contrast into 4 groups (Figure 4), and all 200 seeds of control plants developed and created typical generations (Figure 4A). Plants treated with colchicine experienced changes in phenotype development into 3 groups. The foremost prevailing ones are those with typical development (Figure 4B). Therefore, compared to the control, the ordinary change plants are taller and deliver more pods. The characteristic of the Detam-2 soybean sort, which has purple leaf petioles (Figure 4C), physically looks alluring. Within the control and mutant-1, purple does not appear on the leaf petioles. The comes about of the study also appeared that there was an alteration within the shape of curly leaves in mutant-3 (Figure 4D) due to the treated of 3,500 ppm colchicine. Plants with this change can also deliver cases, but not as numerous as other mutant plants.

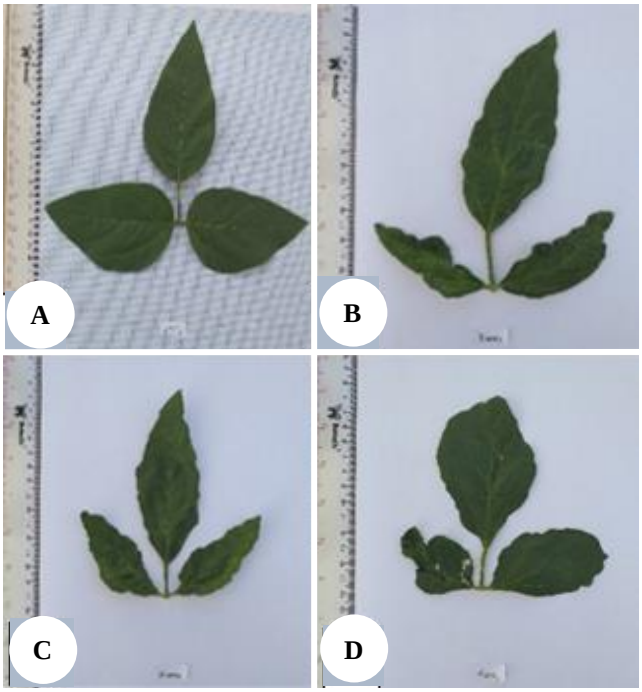

Figure 3. Effect of colchicine concentration on leaf morphology mutations of black soybean var. Detam-2: A. Control sample leaves, B. Truncate (truncated), C. Elliptical, D. Obtuse (blunt tip)

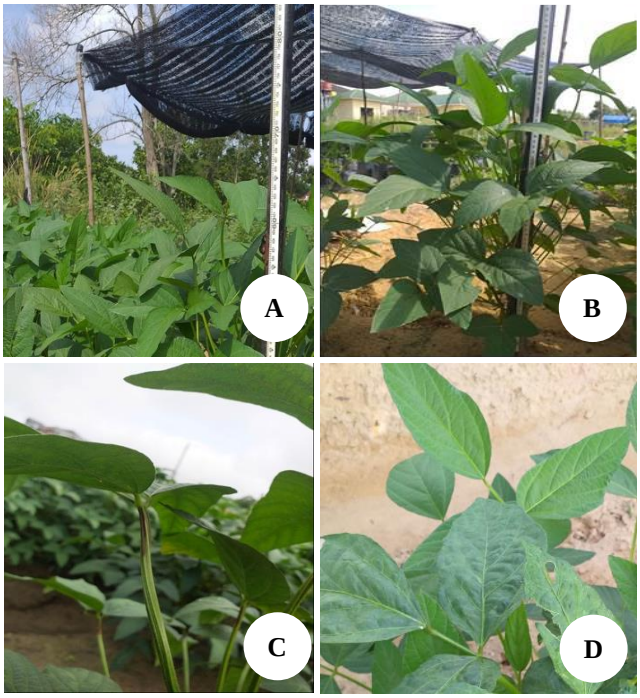

Figure 4. Effect of colchicine concentration of 3500 ppm on the growth of black soybean var. Detam-2: A. Control, B. Mutant-1, normal, C. Mutant-2 leaf stalks are purple, D. Mutant-3 curly leaves

Genotypic characteristics

The DNA band profiles

RAPD was a PCR-based method for identifying genetic variations, which had been used for intraspecific variation assessment (Kumari and Thakur 2014). Moreover, in recent years, it has also been used for genetic mapping, taxonomic studies, and detecting genetic mutations in treated plants (Dhakshanamoorthy et al. 2014). The traits caused by mutations could be detected using molecular markers; this method could directly detect genotypic differences at the DNA level (Dhillon et al. 2014). RAPD markers assessed genetic diversity among EMS-induced soybean mutants (Didik et al. 2020). In this study, they served as initial information for a mutation breeding program for soybeans with genetic variations that could be used for heredity selection. Polymorphism occurred because the random sequence primer was not specific to a particular gene; hence, the DNA bands were presumed to represent new traits. The number of DNA bands that appeared in mutant plants but not in the control was considered polymorphism. The condition was considered monomorphism when the bands in the treatment and the control plant DNA were the same.

The results of using 6 primers produced DNA bands, which were used to amplify 12 DNA samples from black soybean variety Detam-2. The total band profile of Detam-2 black soybeans is presented in Figure 5. Faint or unclear DNA bands were due to the very low total concentration, and almost all the samples were bright and thick, showing a high total concentration. The total DNA bands produced were greater than 10,000 bp, and thickening affected the duplication process.

These primers successfully amplified each sample, both in the control and mutant, as shown in Figure 6. Primer OPAA, 1 in lines 4-5 (mutant-1), produced 9 detected bands, the same as the control. In the mutant-2 sample, line 7 detected 6 bands, while lines 8-9 detected 8. Therefore, this sample had different numbers of DNA bands and gene loci genotypically. For the mutant-3 sample, lines 10 and 12 detected 9 bands, while line 11 detected 4, which was the lowest level of polymorphism among mutant samples.

The detection of DNA bands in these mutant samples showed the same phenotype, which was the purple color of the leaf stalk (Mutant-2) in the Figure 4C, while genotypically, there were differences in the number of DNA 5 bands. Polymorphism in the amplified fragments occurred due to mutations from colchicine treatment. These random mutations could be substitutions, deletions, or insertions in the DNA genome, altering the size of the DNA fragments in the samples (Savino et al. 2022).

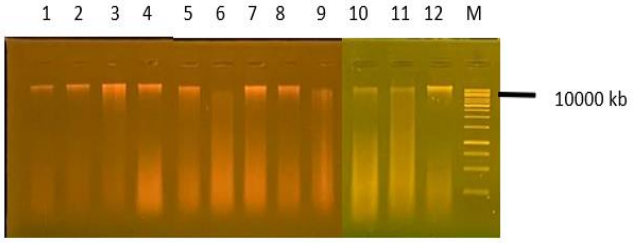

Figure 5. Profile of Total DNA band of Detam-2 black soybeans. Description: 1-3: Control, 4-6: Mutant-1, 7-10: Mutant-2, 10-12: Mutant-3, M: 1 kb DNA ladder (Thermo Scientific)

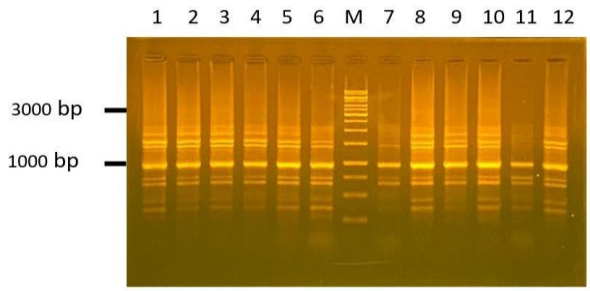

Figure 6. RAPD profile of OPAA 1 primer in black soybeans Detam-2 variety. Lines 1-3: Control, Lines 4-6: Mutant-1, Lines 7-9: Mutant-2, and Lines 10-12: Mutant-3, M: 1 kb DNA ladder (Thermo Scientific)

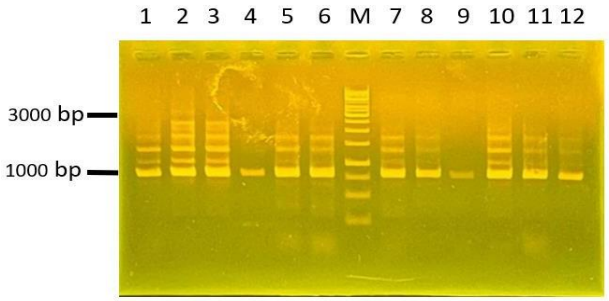

Figure 7. RAPD profile of OPAA-2 primers in black soybeans Detam-2 variety. Lines 1-3: Control, Lines 4-6: Mutant-1, Lines 7-9: Mutant-2, and Lines 10-12: Mutant-3, M: 1 kb DNA ladder (Thermo Scientific)

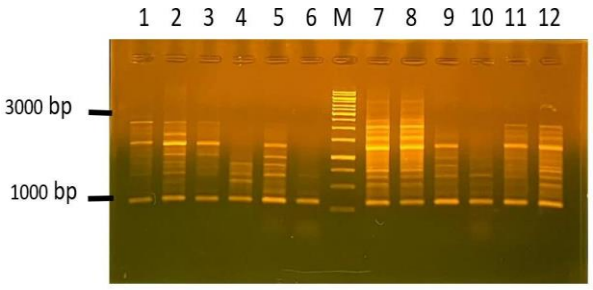

Figure 8. RAPD profile of OPAA 9 primer in black soybeans Detam-2 variety. Lines 1-3: Control, Lines 4-6: Mutant-1, Lines 7-9: Mutant-2, and Lines 10-12: Mutant-3, M: 1 kb DNA ladder (Thermo Scientific)

Primer OPAA-2 (Figure 7) used in the control samples showed 7 DNA bands detected on lines 1-3, although line 1 had 2 less distinct bands. Line 4 (mutant-1) produced 1 band, while lines 5-6 gave 4. The number of DNA bands in this mutant sample was due to mutations in the DNA sequence and changes in the position of the loci, leading to the primer amplifying fewer DNA bands. Mutant-2 showed fewer DNA bands amplified than the control, and there was a similar pattern with mutant-1. The patterns in mutant-3 (lines 10-12) were less than 3000 bp in size and resembled the patterns of the other mutant, but their numbers were lower compared to the control; the DNA band losses occurred due to direct damage to the cells from soaking soybean seeds in colchicine solution. This led to the breaking the bonds between the DNA's constituent compounds (Zhivagui et al. 2023). DNA consisted of phosphate, sugar, and nitrogenous bases, and when their covalent bonds were broken, the nucleotides became inactive. Furthermore, inactive nucleotides were degraded by the cell, leading to the loss of some DNA bands during the processing of the seeds soaked in colchicine solution.

Primer OPAA-09 in the control sample detected 9 DNA bands (Lines 1-3), but the thickness of the amplification results in the control was not the same (Figure 8). The loss of several bands in mutant-1 was visible in lines 4-6, with 1-5 bands missing. Meanwhile, polymorphism occurred in mutant-2, where the number of DNA bands detected was 13 on lines 7-8, with only 8 produced on line 9.

The RAPD results for mutant-2 sample showed fewer DNA bands than the control sample, where line 10 produced 6 bands and lines 11-12 experienced polymorphism, with 12 DNA bands detected. The addition of DNA bands was assumed to occur due to colchicine, leading to genetic changes (Manzoor et al. 2019). Furthermore, four types of genetic changes could occur due to gamma-ray exposure, including changes in the number of genomes, number of chromosomes, chromosome structure, and gene mutations (Fathurrahman et al. 2023b).

Cluster analysis result

Cluster analysis was performed using the Jaccard algorithm on 4 groups of black soybeans of Detam-2 variety. The genetic similarity between the control and mutant based on RAPD markers ranged from 0.451 to 0.961, as shown in Table 3. The three groups of mutant samples observed showed low similarity with the control sample; among mutant samples, the average similarity was higher compared to the control. The highest genetic similarity was found in three control samples, along with one sample from mutant-1 and one from mutant-2, while the highest similarity was between control-3 and control-2. High concentrations of colchicine showed reduced appearance and physiological damage, including inhibited primary stem growth and failure to grow (Leung et al. 2015).

The dendrogram of genetic relationships has two major groups, as shown in Figure 9. The control soybean samples formed the first group, along with mutant-2 (3 samples). Phenotypic characteristics of mutant-2 plants, the leaf stems are purple, while the leaf stems are green in the control samples. The second group consisted of the other two soybean mutants, mutant-3, with phenotypic characteristics of curly leaves, and mutant-1, which showed no phenotypic changes shown in the mutant 2.1-2.3. The character of mutant-1 was higher growth and pod production than the control, even though it does not show changes in phenotype like other mutants. The genotypic DNA band of mutant-1 was also different from the control.

This showed mutant-2 and -3 had a considerable genetic distance from the control. Colchicine treatment produced soybean mutants that were quite similar with low genetic distances. This study showed that colchicine treatment generated potential genetic variations in soybean mutants compared to the control; and was supported by the dendrogram, which showed group grouping differences.

Colchicine was an effective mutagen that caused chromosome duplication and transitioned the DNA base G/C to A/T (Samadi et al. 2022). Therefore, it could lead to mismatched base pairs and induce polyploidy. Another method that could enhance soybean's genetic variability was somatic embryogenesis (somatic clone) using Polyethylene Glycol (PEG) as an osmotic solution. Somatic clone methods generated new soybean varieties that enhanced production (Raza et al. 2017).

Table 3. Jaccard's coefficient similarity among colchicine-induced soybean control and mutants

	Control-1	**Control-2**	**Control-3**	**Mutant-2.1**	**Mutant-2.1**	**Mutant-2.1**	**Mutant-3.1**	**Mutant-3.2**	**Mutant-3.3**	**Mutant-1.1**	**Mutant-1.2**	**Mutant-1.3**
Control-1	1											
Control-2	0.921	1										
Control-3	0.882	0.961	1									
Mutant-2.1	0.725	0.764	0.765	1								
Mutant-2.2	0.666	0.667	0.667	0.784	1							
Mutant-2.3	0.745	0.706	0.706	0.745	0.804	1						
Mutant-3.1	0.549	0.510	0.510	0.549	0.569	0.569	1					
Mutant-3.2	0.509	0.471	0.471	0.549	0.686	0.686	0.765	1				
Mutant-3.3	0.529	0.529	0.529	0.686	0.706	0.667	0.745	0.784	1			
Mutant-1.1	0.568	0.529	0.529	0.569	0.706	0.667	0.667	0.745	0.804	1		
Mutant-1.2	0.451	0.451	0.451	0.451	0.549	0.588	0.745	0.667	0.765	0.843	1	
Mutant-1.3	0.568	0.490	0.490	0.608	0.588	0.549	0.706	0.667	0.647	0.725	0.647	1

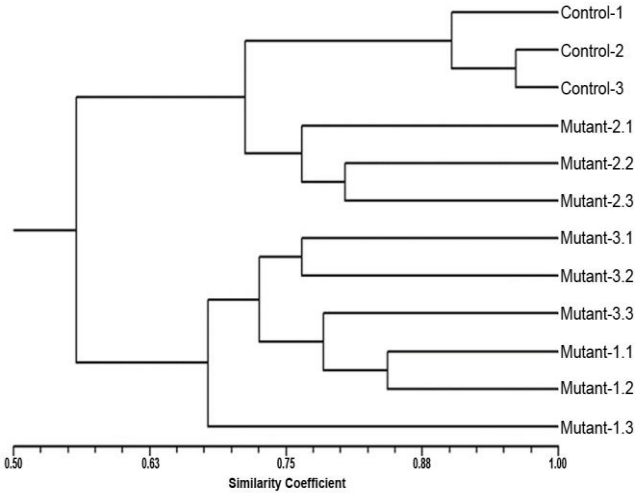

Figure 9. The dendrogram generated from Jaccard's coefficient similarity shows the genetic relationship between mutants and control

RAPD markers effectively detected variability and have been adopted in population studies, plant systematics (Dwivedi et al. 2018), and plant breeding (Fei et al. 2014). These markers also had other advantages, such as applicability to anonymous genomes, requiring a low amount of DNA, and generating many DNA fragments (Kumari and Thakur 2014). On the contrary, RAPD markers had disadvantages related to their low sensitivity and reproducibility, leading to unstable results. Despite conflicting arguments about their use in genetic diversity studies, RAPD markers were still recommended and accepted for detecting genetic variability compared to AFLP, ISSR, and SSR (Hromadová et al. 2023).

In conclusion, the results analyzing mutations caused by colchicine treatment provided evidence of mutations in the black soybean variety Detam-2. Furthermore, there were significant changes in phenotypic growth characteristics, such as the appearance of purple in the leaf petioles and curling. Stomata density in mutant samples was lower compared to the control, but stomata size was larger in the mutant. The pods production, seed weight, and pithy pods of mutant-1 soybeans had higher yields. RAPD marker analysis also confirmed the occurrence of DNA band polymorphisms in the mutant samples. Mutations induced by colchicine treatment could be a source of new genetic diversity, with the potential for selection in subsequent generations and development.

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