UNIVERSITAS ISLAM RIAU

DIREKTORAT PENELITIAN DAN PENGABDIAN KEPADA MASYARAKAT

Alamat : Jl. Kaharuddin, Nasution No.13, Marpoyan, Pekanbaru, Riau, Indonesia - 28284

Telp: +62 761 72126 **Fax:** +62 761 72126 **Email:** dppm@uir.ac.id **Website:** dppm.uir.ac.id

Kontrak Penelitian
Tahun Anggaran 2023
Nomor : 439/KONTRAK/P-PT/DPPM-UIR/06-2023

Pada Hari Senin tanggal Empat September Dua Ribu Dua Puluh Tiga, Kami Yang Bertanda Tangan Di Bawah Ini :

1. Dr. ARBI HAZA NASUTION, B.IT, M.I.T	:	Ketua Direktorat Penelitian dan Pengabdian kepada Masyarakat, Universitas Islam Riau, dalam hal ini bertindak untuk dan atas nama Universitas Islam Riau, yang berkedudukan di Jl. Kaharuddin Nasution No. 113 P. Marpoyan, Pekanbaru, untuk selanjutnya disebut **PIHAK PERTAMA**
2. Dr. FATHURRAHMAN, S.P, M.Sc.	:	Dosen Fakultas Pascasarjana Prodi S2 Agronomi Universitas Islam Riau, dalam hal ini bertindak sebagai pengusul dan Ketua Pelaksana Penelitian Tahun Anggaran 2023 untuk selanjutnya disebut **PIHAK KEDUA**.

PIHAK PERTAMA dan PIHAK KEDUA, secara bersama-sama sepakat mengikatkan diri dalam suatu Kontrak Penelitian Tahun Anggaran 2023 dengan ketentuan dan syarat-syarat sebagai berikut:

Pasal 1
Ruang Lingkup Kontrak

PIHAK PERTAMA memberi pekerjaan kepada **PIHAK KEDUA** dan **PIHAK KEDUA** menerima pekerjaan tersebut dari **PIHAK PERTAMA**, untuk melaksanakan dan menyelesaikan Penelitian Tahun Anggaran 2023 dengan judul "Karakteristik Fenotip dan Genotip Kedelai Hitam (Glycine soja (L.) Merr) Varietas Detam 1 Hasil Poliploidisasi dengan Kolkisin".

Pasal 2
Dana Penelitian

1. Besarnya dana untuk melaksanakan penelitian dengan judul sebagaimana dimaksud pada Pasal 1 adalah sebesar Rp 17.000.000,00 (tujuh belas juta rupiah) sudah termasuk pajak.
2. Dana Penelitian sebagaimana dimaksud pada ayat (1) dibebankan pada Anggaran Pendapatan dan Belanja Universitas Islam Riau (APB UIR) tahun 2023.

Pasal 3
Tata Cara Pembayaran Dana Penelitian

1. **PIHAK PERTAMA** akan membayarkan Dana Penelitian kepada **PIHAK KEDUA** secara bertahap dengan ketentuan sebagai berikut:
   a. Pembayaran Tahap Pertama sebesar 70% dari total dana penelitian yaitu 70% x Rp. 17.000.000,00 = Rp. 11.900.000,00 (sebelas juta sembilan ratus ribu rupiah) yang akan dibayarkan oleh **PIHAK PERTAMA** kepada **PIHAK KEDUA** setelah **PARA PIHAK** membuat dan melengkapi usulan pelaksanaan penelitian yang mengikuti template yang dapat diunduh pada web: purse.uir.ac.id. Selanjutnya **PIHAK KEDUA** mengunggah usulan penelitian yang telah diseminarkan ke web: purse.uir.ac.id.
   b. Pembayaran Tahap Kedua sebesar 30% dari total dana penelitian yaitu 30% x Rp. 17.000.000,00 = Rp. 5.100.000,00 (lima juta seratus ribu rupiah) dibayarkan oleh **PIHAK PERTAMA** kepada **PIHAK KEDUA** setelah **PIHAK KEDUA** mengunggah Laporan Akhir Pelaksanaan Penelitian ke web: purse.uir.ac.id.
   c. Dana Penelitian sebagaimana dimaksud pada ayat (1) akan disalurkan oleh **PIHAK PERTAMA** kepada **PIHAK KEDUA**.
2. **PIHAK PERTAMA** tidak bertanggung jawab atas keterlambatan dan/atau tidak terbayarnya sejumlah dana sebagaimana dimaksud pada ayat (1) yang disebabkan karena kesalahan **PIHAK**

KEDUA dalam menyampaikan data peneliti, dan persyaratan lainnya yang tidak sesuai dengan ketentuan.

Pasal 4
Jangka Waktu

Jangka waktu pelaksanaan penelitian sebagaimana dimaksud dalam Pasal 1 sampai selesai 100%, adalah terhitung sejak Tanggal 21 Agustus 2023 dan berakhir pada Tanggal 30 Desember 2023.

Pasal 5
Target Luaran

1. **PIHAK KEDUA** berkewajiban untuk mencapai target luaran wajib penelitian berupa artikel minimal accepted (diterima) atau dimuat di SCOPUS Q3, selanjutnya **PIHAK KEDUA** mengunggah ke web: purse.uir.ac.id.
2. **PIHAK KEDUA** berkewajiban untuk melaporkan perkembangan pencapaian target luaran sebagaimana dimaksud pada ayat (1) kepada **PIHAK PERTAMA**.
3. Jika target luaran wajib tidak tercapai, maka sisa dana 30% tidak dibayarkan sampai luaran wajib diunggah ke web: purse.uir.ac.id hingga Tanggal 01 Desember 2024.

Pasal 6
Hak dan Kewajiban Para Pihak

1. Hak dan Kewajiban **PIHAK PERTAMA**:
    a. **PIHAK PERTAMA** berhak untuk mendapatkan dari **PIHAK KEDUA** luaran penelitian sebagaimana dimaksud dalam Pasal 7.
    b. **PIHAK PERTAMA** berkewajiban untuk memberikan dana penelitian kepada **PIHAK KEDUA** dengan jumlah sebagaimana dimaksud dalam Pasal 2 ayat (1) dan dengan tata cara pembayaran sebagaimana dimaksud dalam Pasal 3.
2. Hak dan Kewajiban **PIHAK KEDUA**:
    a. **PIHAK KEDUA** berhak menerima dana penelitian dari **PIHAK PERTAMA** dengan jumlah sebagaimana dimaksud dalam Pasal 2 ayat (1)
    b. **PIHAK KEDUA** berkewajiban menyerahkan kepada **PIHAK PERTAMA** luaran Penelitian dengan judul “Karakteristik Fenotip dan Genotip Kedelai Hitam (Glycine soja (L.) Merr) Varietas Detam 1 Hasil Poliploidisasi dengan Kolkisin”.
    c. **PIHAK KEDUA** berkewajiban untuk bertanggungjawab dalam penggunaan dana penelitian yang diterimanya sesuai dengan proposal kegiatan yang telah disetujui.

Pasal 7
Monitoring dan Evaluasi

PIHAK PERTAMA dalam rangka pengawasan akan melakukan Monitoring dan Evaluasi internal terhadap kemajuan pelaksanaan Penelitian Tahun Anggaran 2023 yang akan dilaksanakan mulai Tanggal 01 September 2023 hingga Tanggal 01 Oktober 2023.

Pasal 8
Laporan Pelaksanaan Penelitian

1. **PIHAK KEDUA** berkewajiban untuk menyampaikan kepada **PIHAK PERTAMA** berupa laporan akhir mengenai luaran penelitian dan rekapitulasi penggunaan anggaran sesuai dengan jumlah dana yang diberikan oleh **PIHAK PERTAMA** yang tersusun secara sistematis sesuai pedoman yang ditentukan oleh **PIHAK PERTAMA**.
2. **PIHAK KEDUA** berkewajiban mengunggah Laporan hasil penelitian yang telah dilaksanakan web: purse.uir.ac.id paling lambat Tanggal 01 Desember 2023.
3. **PIHAK KEDUA** berkewajiban mengunggah capaian luaran pada web: purse.uir.ac.id paling lambat Tanggal 01 Desember 2024.

Pasal 9
Sanksi

1. Apabila sampai dengan batas waktu yang telah ditetapkan untuk melaksanakan Penelitian ini telah berakhir, namun **PIHAK KEDUA** belum menyelesaikan tugasnya, terlambat mengirim laporan akhir, maka **PIHAK KEDUA** dikenakan sanksi administratif berupa penghentian pembayaran dan tidak dapat mengajukan proposal penelitian dalam kurun waktu satu tahun berturut-turut.
2. Apabila **PIHAK KEDUA** tidak dapat mencapai target luaran sebagaimana dimaksud dalam Pasal 5, maka kekurangan capaian target luaran tersebut akan dicatat sebagai hutang **PIHAK KEDUA** kepada **PIHAK PERTAMA** yang apabila tidak dapat dilunasi oleh **PIHAK KEDUA**, akan berdampak pada kesempatan **PIHAK KEDUA** untuk mendapatkan pendanaan penelitian atau hibah lainnya yang dikelola oleh **PIHAK PERTAMA**.

Pasal 10
Pembatalan Perjanjian

1. Apabila dikemudian hari terhadap judul Penelitian sebagaimana dimaksud dalam Pasal 1 ditemukan adanya duplikasi dengan Penelitian lain dan/atau ditemukan adanya ketidakjujuran, itikad tidak baik, dan/atau perbuatan yang tidak sesuai dengan kaidah ilmiah dari atau dilakukan oleh **PIHAK KEDUA**, maka perjanjian Penelitian ini dinyatakan batal dan **PIHAK KEDUA** wajib mengembalikan dana penelitian yang telah diterima kepada **PIHAK PERTAMA** yang selanjutnya akan disetor ke Kas Universitas Islam Riau.
2. Bukti setor sebagaimana dimaksud pada ayat (1) disimpan oleh **PIHAK PERTAMA**.

Pasal 11
Penyelesaian Sengketa

Apabila terjadi perselisihan antara **PIHAK PERTAMA** dan **PIHAK KEDUA** dalam pelaksanaan perjanjian ini akan dilakukan penyelesaian secara musyawarah dan mufakat, dan apabila tidak tercapai penyelesaian secara musyawarah dan mufakat maka penyelesaian dilakukan melalui proses hukum.

Pasal 12
Lain-Lain

1. **PIHAK KEDUA** menjamin bahwa penelitian dengan judul tersebut di atas belum pernah dibiayai dan/atau diikutsertakan pada Pendanaan Penelitian lainnya, baik yang diselenggarakan oleh instansi, lembaga, perusahaan atau yayasan, baik di dalam maupun di luar negeri.
2. Segala sesuatu yang belum diatur dalam Perjanjian ini dan dipandang perlu diatur lebih lanjut dan dilakukan perubahan oleh **PARA PIHAK**, maka perubahan-perubahannya akan diatur dalam perjanjian tambahan atau perubahan yang merupakan satu kesatuan dan bagian yang tidak terpisahkan dari Perjanjian ini.

Demikianlah surat perjanjian ini dibuat pada hari ini, tanggal, bulan dan tahun seperti tersebut diatas dan ditanda tangani oleh kedua **PIHAK** secara elektronik sebagai kekuatan dan untuk dipergunakan sebagaimana semestinya.

PIHAK PERTAMA	**PIHAK KEDUA**
Dr. ARBI HAZA NASUTION, B.IT, M.I.T	**Dr. FATHURRAHMAN, S.P, M.Sc.**
NIDN: 1023048901	**NIDN : 1018106903**

Effect of Colchicine Treatment on Phenotypic and Genotypic Characteristics of Black Soybean (Glycine max L. Merrill) from Detam-2 Variety

F. Fathurrahman[1], Saripah Ulpah[1], Noer Afny Muliyati Sodiq[2]
[1]Department of Agrotechnology, Faculty of Agriculture, Universitas Islam Riau, Pekanbaru 28284, Indonesia
[2]Department of Agronomy, Postgraduate Program, Universitas Islam Riau, Pekanbaru, 28284, Indonesia
Correspondent author : fathur@agr.uir.ac.id

ABSTRACT

The enhancement of soybean plant mutagenesis to obtain phenotypic and genotypic diversity is an essential process in agriculture. This process is often carried out to augment population traits using various methods and produce high-quality mutants. Therefore, this study aims to analyze effect of colchicine concentration on phenotypic (growth, stomata anatomy) and genotypic characteristics of black soybean from Detam-2 variety. The study procedures were carried out from January to June 2023 in the experimental field of the Faculty of Agriculture, Universitas Islam Riau, Pekanbaru, Indonesia. A total of 600 plants were studied, consisting of 200 controls and 400 samples treated with 3500 ppm of colchicine. Growth analysis was performed using the DMRT statistical test at $p<0.05$ and stomata analysis was carried out with T-test. Genotypic characteristics assessment used RAPD markers, and cluster observations were made using Jaccard's coefficient of similarity. The results showed that mutant-1 had the highest production, with a total of 235 pods, 233 fertile pods, 93.20 grams seed weight, and significant differences from the controls. Mutant-2 had the best 100 dry seed weight of 12.80 grams, and dry seed weight per plant of 63.30 grams, but not significantly different from the control. Stomata density characteristics were higher in the controls, while stomata length and width were higher in mutant samples. Furthermore, leaf types in mutant samples included normal, purple stems, and curly leaves. The results showed that primer OPAA-01 produced a different number of DNA bands. For mutant-3, lines 10 and 12 detected 9 DNA bands, while line 11 detected 4 bands, which was the lowest polymorphism among the treated samples. Primer OPAA-02 showed lower DNA bands in all mutant compared to the controls. In this study, primer OPAA-09 detected polymorphism in mutant-3, while others had fewer DNA bands. The highest genetic similarity analysis result 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, namely control and mutant-1.

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

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 the production of anti-aging products (Amalia et al., 2021). In addition, several studies have shown that it contains higher levels of glutamate, serine, and tyrosine compared to yellow soybean. 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 (Nurrahman 2015). In a previous study, the protein content of Detam-2 variety of black soybean was 45.36%, with fat levels of 33.06%. This variety is highly resistant to insects, pod shattering, and sensitive to drought (Bean and Root Research Institute, 2022). According to previous studies, it contains vitamin E, β-carotene, isoflavones, and anthocyanins, which contribute to its antioxidant activity (Nurrahman, 2015) . The total flavonoid content was 0.26 mg/grams (Hasanah et al., 2019) and its antioxidant activity was considered moderate, with an IC50 value of 220.42 ppm products (Amalia et al., 2021. Dried black

soybean has been reported to contain 140 calories per 35 grams, while the fresh variant contains 5-6 grams polyunsaturated fat, 2 grams soluble fiber, and 5 grams insoluble fiber in its nutritional composition.

In 2022, the soybean cultivation area in Indonesia covered 362,612 hectares, with a production volume of 613,318 tons and a productivity of 1.65 tons per hectare, which was considered relatively low. Despite the rising domestic consumption, the country imported 3,093,318 tons in 2021 (BPS 2022) to bridge the gap between demand and local production limitations. The current limitations include the low improvement of soybean productivity and quality through time-consuming conventional breeding methods. To address this issue, technological innovations, particularly genetic engineering is required. For instance, the mutation of chromosomes and genes using mutagenic agents, such as colchicine can tailor soybean to agroclimatic conditions, fostering phenotypic and genotypic diversity. Colchicine-induced variations can cause polyploidy, such as triploid, tetraploid, and hexaploid, leading to the possession of superior phenotypic characteristics. Tetraploid plants have larger size, increased vigor, and larger fruits and seed dimensions compared to 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 changes in phenotypic and genotypic traits induced by colchicine have been explored by Fathurrahman (2023).

Colchicine functions as a mitosis inhibitor, disrupting microtubule formation and doubling chromosome number. This compound is also used to induce polyploidy by inhibiting proper segregation of chromosomes during cell division, leading to the production of gametes with an uneven chromosomal number. 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, 2016). 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 often have improved adaptation to salt stress by efficiently regulating water usage through the variation of 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 compared to diploids (Manzoor et al., 2019).

The optimal concentration of colchicine that determines the selection of successfully polyploid soybean can be measured by seed germination, shoot length, and root length during the germination stage, as well as field germination during the M1 generation (Tabeli et al., 2012). This compound can also affect stomata, as shown in a study conducted by Aili et al. (2016) 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 Rochmah et al. (2017) on olive trees *(Olea europaea L.*). Detecting plant mutations using phenotypic method has been reported to face several limitations, and the traits obtained are often unstable (Garcia et al., 2016). Therefore, a genetic method must be used, as it is a more reliable method for early mutation detection in plants. Several genetic methods that can be used include Random Amplification Polymorphic DNA (RAPD) (Ashraf et al., 2007), Restriction Fragment Length Polymorphism (RFLP), Inter-Simple Sequence Repeat (ISSR) (Mishra et al., 2014), and DNA sequencing (Wahyudi et al., 2013; Nikmah et al., 2016) (Kumari and Thakur, 2014). 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 compared to other genetic markers, such as ISSR and RFLP and do not require 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 obtained can be used for genotypic clustering to determine the relatedness, which is often presented as a dendrogram. Therefore, this study aims to analyze effect of colchicine concentration on phenotypic (growth, stomata anatomy) and genotypic characteristics of black soybean from Detam-2 variety.

MATERIALS AND METHODS

The equipment and materials used in growth phase included a tractor, rotary cultivator, plow, label paper, measuring tape, sprayer, seed tray, glass beaker, pipette dropper, spatula, water bath, glass bottles/flasks, aluminum foil, digital scale, magnetic stirrer, hotplate, Bunsen burner, and spirit lamp. Furthermore, the materials used were soybean seeds of Detam-2 variety, urea fertilizer, TSP (Triple Super Phosphate), KCl (potassium chloride), compost, and distilled water (aquades). For stomata analysis, the equipment and materials included watch glasses, Petri dishes, a microscope, a camera, tweezers, brushes, glass microscope slides, transparent nail polish, and acetocarmine stain. For DNA analysis, loading dye, liquid nitrogen, Mix GP1 Buffer, GPX1 Buffer and RNase, Elution Buffer, filter columns, GP3, W1, Wash, Elution Buffer, electrophoresis gel, thermal cycler, NanoDrop spectrophotometer, and a digital camera (Olympus) with a UV filter were used. The process also required a centrifuge, distilled water (ddH2O), GD columns, water bath, mortar and pestle, test tubes, and mini test tubes. For PCR and RAPD analysis, DNA markers from Thermo Scientific, PCR buffer comprising dNTPs, forward primers, Taq DNA polymerase, DNA samples, and ddH2O were used. 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).

The plant population treatment was carried out using 400 black soybean seeds of Detam-2 variety treated with a concentration of 3500 ppm colchicine and 200 control seeds, totaling 600. These samples were planted in plots measuring 1.2 m x 4.6 m, each containing 50 seeds. The planting spacing was 25 x 40 cm, with a plot height of 20 cm and a spacing of 50 x 50 cm between plots. Furthermore, there were 4 plots for the control group and 8 plots for mutant, leading to a total of 12. Organic compost, 10 kg per plot, was applied one week before planting, and inorganic fertilizers, including 30 grams urea, 75 grams TSP (Triple Super Phosphate) and 50 grams KCl (potassium chloride) per plot, were applied during planting. Sampling was performed randomly for each treatment, with 32 plants selected for observation. Growth observations of mutation samples included three types, namely leaves with a normal color and shape, those with a purple stem, and curled leaves. Phenotypic characteristics were observed, including the number of pods, fertile pods, seed weight per plant, moisture content of fresh seeds, dry seeds (12%), dry seed weight per plant, and the weight of 100 seeds.

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 the application of 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 a 400x magnification microscope. Growth analysis was carried out using statistical analysis, using DMRT (Duncan's Multiple Range Test) at $p<0.05$ significance level and SAS 9.1 software. Stomatal anatomy was analyzed using the T-test in this study. When there were differences in variance among treatment, 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 treatment.

DNA extraction was performed by weighing 50-100 mg of fresh leaves, followed by the addition of liquid nitrogen to a mortar. The sample was placed in the mortal, crushed into fine powder, and transferred to a 1.5 ml tube. Lysis was then conducted using a mix of GP1 Buffer or GPX1 Buffer, RNase A, and other necessary chemicals with skilled methods to obtain high-quality DNA for use in the subsequent process, namely PCR analysis. Furthermore, 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 a 1% agarose gel and 1.5 µl of ethidium bromide solution (used as a DNA band stain) in a 1x TBE buffer (pH 8). A 1 kb DNA ladder solution of 2 µl and a loading dye solution of 2 µl were used as standards and loaded into the first well. The PCR product solution of 2 µl and a loading dye solution of 2 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 a UV transilluminator and an Olympus camera, with a UV filter to observe the formed DNA bands.

RESULTS AND DISCUSSION

Black soybean of Detam-2 variety grown in the control plots showed a full population of healthy plants. Meanwhile, in colchicine treatment plots (with a concentration of 3500 ppm), several plants did not grow. The condition of soybean in the study field is presented in Figure 1.

Figure 1. The plots of control and mutant samples of Detam-2 black soybean plants many don't grow. Note ← is plots for mutant samples

Phenotypic Characteristics: the number of pods, fertile pods, seed weight per plant, moisture content in wet weight, moisture content in dry weight, 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 stem leaves (mutant-3). After statistical analysis, significant differences were observed in the generative characteristics, including the number of pods, fertile 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. Generative characteristics of the Black Soybean from Detam-2 Variety in control and mutant samples

Generative Character	Samples			
	Control	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
Bernas 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
Seed moisture content wet weight (%)	14.90± 0.45	18.97± 1.22	17.13± 2.06	19.37± 1.44
Seed moisture content dry weight (%)	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 letter of the same days of treatment is significant tested using Duncan multiple range test at $p<0.05$

Based on Table 1, the highest number of pods were found in Detam-2 black soybean, with mutant-1 producing 235 pods, followed by mutant-3 and -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 59.86% higher compared to the control. The results of this study were consistent with Fathurrahman (2023), where F1 mutant cucumbers produced more fruits compared to the controls. Furthermore, there was a significant increase in the number of fertile 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 pods, and mutant purple stem, with 215 pods, in comparison to the control possessing only 143 pods. The increase in the number of pods in black soybean mutant samples was 62.93%.

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 sample-1 was 110.38%. This result was in line with Ajayi et al. (2014), where colchicine increased the weight of cowpea (*Vigna unguiculata L. Walp*) pods.

Observations of the moisture content in wet weight, after undergoing statistical analysis, showed no significant difference between the control sample and mutant. However, in numerical terms, mutant-3 had a higher moisture content of 19.37%. The results also showed a 30% increase in moisture content in mutant-2. For the observation of moisture content in dry weight, there was no significant difference between the control and mutant. Mutant-2 had a 12.53% higher value compared to 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 compared to 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 (Bharadwa 2015), and their expression affected 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.

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

Characteristics	Average		t count	t table	
	Control	Mutant		0.05	0.01
Stomatal Density (0.12 mm2)	143.52±2.31	114.2±1.33	2.5^{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 significan, Mean ± = standard deviation (SD)

Stomatal density in mutant plants was lower, measuring 114.2 per 0.12 mm², compared to that of the control, namely 143.52/0.12 mm². Furthermore, the standard deviation value 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 the presence of a low range of variation between the samples. The standard deviation for stomatal length and width was smaller in treatment group compared to the control.

The high and low levels of stomatal density were categorized based on Mulyono et al. (2022). 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 (Luttge and Buckeridge 2020). Therefore, in this study, it was only observed on the abaxial side. Ni & 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.

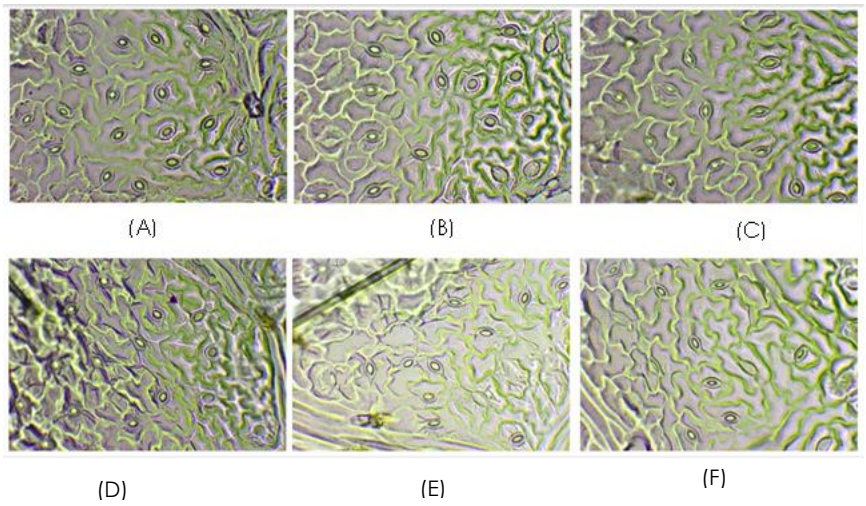

Figure 2 Stomatal density of black soybean plants var. detam-2 with control samples (A) leaf base, (B) leaf middle, and (C) leaf tip. Colchicine treatment on (D) leaf base, (E) middle of leaf, and (F) leaf tip (400x).

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, leading to some cells being affected. The leaves of plants treated with the compound were larger compared to 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. (2022) 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 showed by leaf shape and margins. The pointed and rounded shapes induced by treatment positively enhanced the production of the secondary metabolite stevioside.

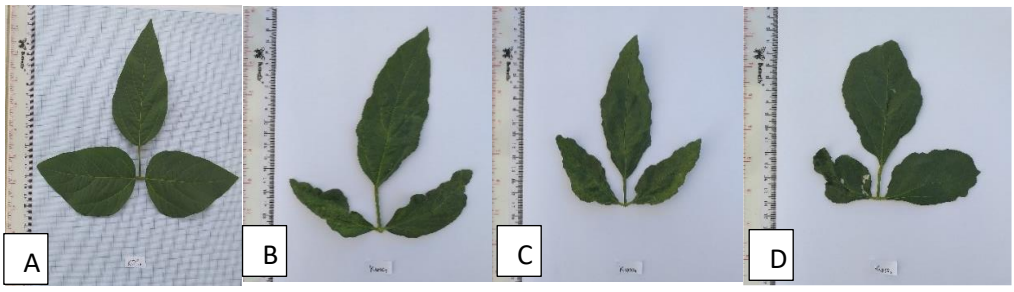

Figure 3. Effect of colchicine concentration on leaf morphology mutations of black soybean var. detam-2 (A) control sample leaves (B) truncate (truncated), (C) elliptical and (D) obtuse (blunt tip)

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.

Genotypic Characteristics

Selection of RAPD Markers

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 had also been used for genetic mapping, taxonomic studies, and detecting genetic mutations in treated plants (Dhakshanamoorthy et al., 2014). Traits caused by mutations could be detected using molecular markers. This method could be used for directly detecting genotypic differences at the DNA level (Dhillon et al., 2014). RAPD markers assessed genetic diversity among EMS-induced soybean mutant (Didik et al., 2020). In this study, they served as initial information for a mutation breeding program for soybean with genetic variations that could be used for heredity selection. Polymorphism occurred because the random sequence primer used 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. When the bands in treatment and the control plant DNA were the same, the condition was considered monomorphism.

The results of the use of 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 soybean is presented in Figure 4. 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.

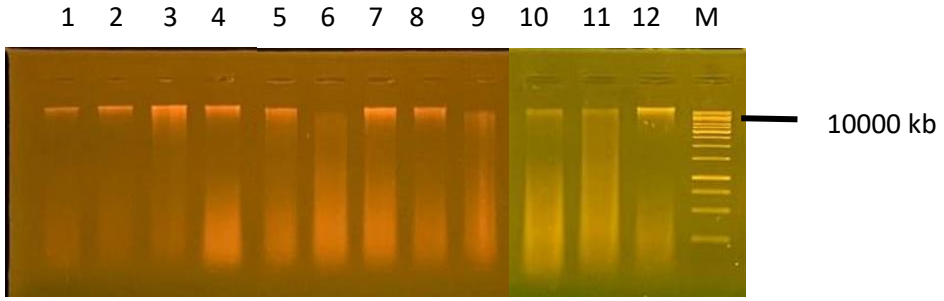

Figure 4. Profile of Total DNA band of Detam-2 black soybeans. Description: (1-3) control, (4-6) mutant-1, (7-10) mutant-2, (10-12) mutant-3, (M) 1 kb DNA ladder (Thermo Scientific)

From the six primers used for amplifying polymorphism and genetic diversity, three primers successfully produced DNA bands, namely AGACGGCTCC (OPAA-01), GAGACCAGAC (OPAA-02), and AGATGGGCAG (OPAA-09). These primers successfully amplified each sample, both in the control and mutant, as shown in Figure 5. Primer OPAA, 1 in lines 4-5 (mutant-1), produced 9 detected bands, which was the same as the control. In mutant-2 sample, line 7 detected 6 bands, while lines 8-9 detected 8. Therefore, in this sample, their gene expression was the same phenotypically, but there were different numbers of DNA bands and gene loci genotypically. For 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.

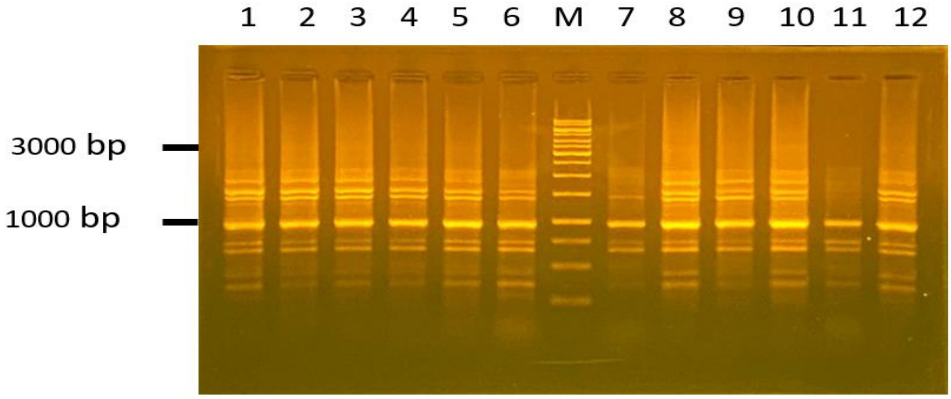

Figure 5. RAPD profile of OPAA 1 primer in black soybeans detam-2 variety, line 1-3 DNA band control, line 4-6 DNA band mutant-1, M is market marker 1 kb Thermo scientific, line 7-9 band DNA mutant-2 and line 10-12 of mutant-3.

The detection of DNA bands in these mutant samples showed the same phenotype, which was the purple color of the leaf stalk, while genotypically, there were differences in the number of DNA bands by 5. 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 (Simone et al., 2022).

The use of primer OPAA-2 (Figure 6) in the control samples showed 7 DNA bands detected on lines 1-3, although line 1 had 2 less distinct bands. In line 4 (mutant-1), it 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. The RAPD PCR results for mutant-2 showed fewer DNA bands amplified compared to 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. Loss of DNA bands occurred due to direct damage to the cells from soaking soybean seeds in colchicine solution. This led to the breaking of 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.

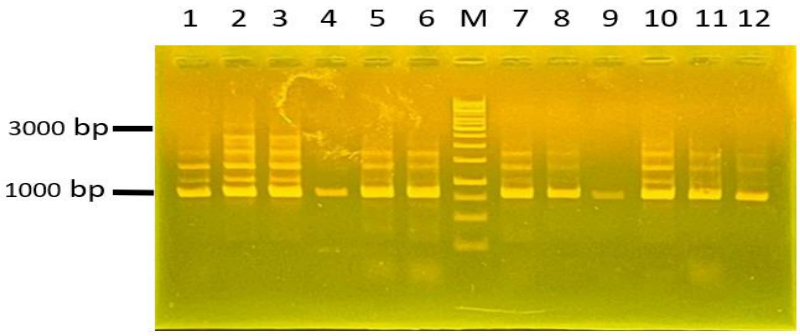

Figure 6. RAPD profile of OPAA-2 primers in black soybeans detam-2 variety, line 1-3 DNA band for control, line 4-6 DNA band for mutant sample-1, M is market marker 1 kb Thermo scintific, line 7-9 DNA band of mutant-2 and lines 10-12 of mutant-3.

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 7). 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 being produced on line 9.

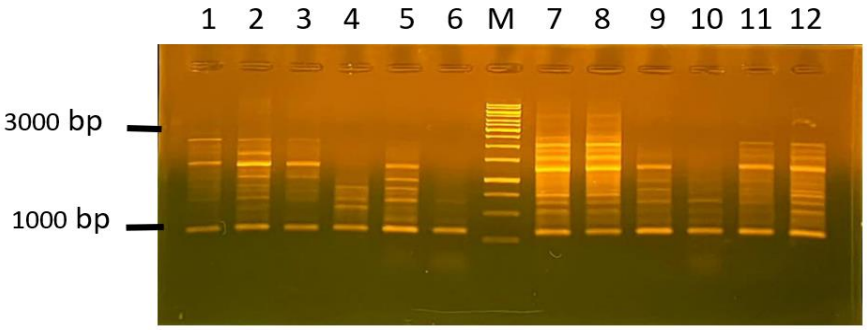

Figure 7. RAPD profile of OPAA 9 primer in black soybeans detam-2 variety, line 1-3 DNA band for control, line 4-6 DNA band for mutant-1, M is the 1 kb Thermo scientific marker, line 7-9 band DNA of mutant-2 and line 10-12 of mutant-3.

The RAPD results for mutant-2 sample showed fewer DNA bands compared to 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., 2023).

Cluster Analysis Result

Cluster analysis was performed on 3 groups of black soybean mutant of Detam-2 variety using the Jaccard algorithm. The genetic similarity between the control and mutant based on RAPD markers ranged from 0.41 to 0.961, as shown in Table 3. In the three groups of mutant samples observed, there was low similarity with the control sample. Among mutant samples, the average similarity was higher compared to the control. The lowest genetic similarity was found in three control samples, along with

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

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 a reduction in appearance and physiological damage, including inhibited primary stem growth and failure to grow (Leung et al. 2015).

The dendrogram of genetic relationships gave two major groups, as shown in Figure 8. The control soybean sample formed the first group, along with mutant-2 sample, which acted as an outgroup. Furthermore, it exhibited phenotypic characteristics of purple leaf stems. The second group consisted of the other two soybean mutant, namely Mutant-3, with phenotypic characteristics of curly leaves, and Mutant-1, which did not show any phenotypic changes. This showed that mutant-2 and -3 had a considerable genetic distance from the control. Colchicine treatment produced soybean mutant that were quite similar with low genetic distances. This study showed that colchicine treatment generated potential genetic variations in soybean mutant compared to the control. This was supported by the dendrogram, which showed differences in grouping between the groups.

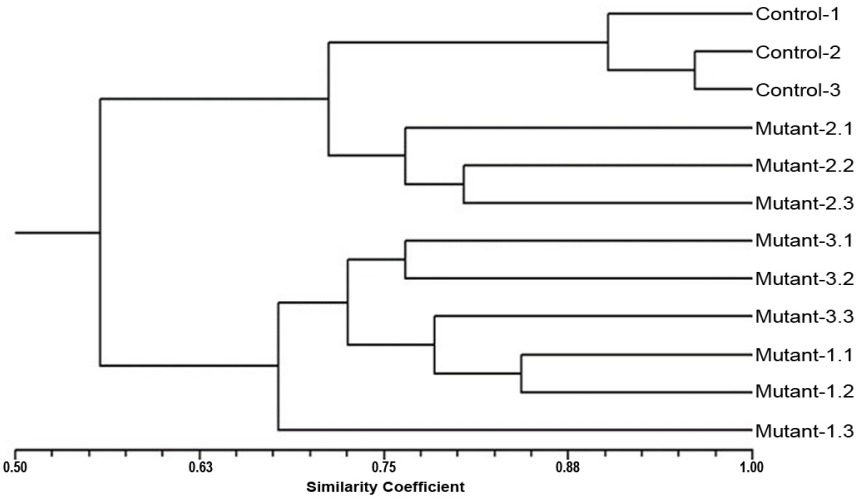

Figure 8. Dendrogram generated from Jaccard's coefficient similarity showing the genetic relationship of 9 colchicine induced individual soybean mutants and control.

Colchicine was 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 be used to enhance the genetic variability of soybean was somatic embryogenesis (somatic clone) using polyethylene glycol (PEG) as an osmotic solution. Somatic clone methods generated new soybean variety that enhanced production (Ghulam et al. 2017).

RAPD markers effectively detected variability and had been adopted in population studies, plant systematics (Dwivedi et al. 2018), and plant breeding (Fei et al. 2014). These molecules also had other advantages, such as applicability to anonymous genomes, requiring a low amount of DNA, and generating a high number of DNA fragments (Kumari and Thakur, 2014). 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 (Hromadova et al., 2023).

CONCLUSIONS

In conclusion, the results analyzing mutations caused by colchicine treatment provided evidence of mutations in 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 mutant. The production of pods, seed weight, and full seeds showed that mutant soybean yielded higher. RAPD marker analysis also confirmed the occurrence of polymorphism and loss of DNA bands in mutant samples. Mutations induced by colchicine treatment could be a source of new genetic diversity, with potential for selection in subsequent generations and development.