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In Vitro EMS Mutagenesis of Barangan Merah Banana: Assessing Phenotypic and Genetic Diversity for Crop Improvement

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ABSTRACT

Barangan Merah banana (*Musa acuminata* Colla) is a banana that can be developed as a table fruit because it is sweet, fragrant, high in vitamin A, and contains beta-carotene. However, this is constrained because this plant does not produce seeds and is susceptible to Fusarium wilt disease. This study aims to obtain diversity of Barangan Merah banana to provide genetic material for subsequent disease resistance screening through in vitro chemical mutation using ethyl methanesulfonate (EMS). This study used clumps of Barangan Merah banana shoots from in vitro propagation. The shoot eyes were soaked in 0.1% EMS solution for 1 hour, 2 hours, and 3 hours, then cultured in medium using Murashige and Skoog (MS) + benzylaminopurine (BAP) 3 mg L^{-1} for 4 weeks and continued subculture into medium MS without BAP for 16 weeks. The results showed that the duration of immersion in 0.1% EMS produced varying growth responses in Barangan Merah banana explants. A 1-hour immersion yielded the most optimal results for shoot initiation and growth. Longer immersion periods (2–3 hours) generated mixed effects, enhancing certain vegetative traits such as leaf and root size while reducing shoot height. The putative mutants exhibited darker leaf coloration and tetracytic stomata. The SSR marker STMS7 revealed clear genetic polymorphism, with putative mutant 1A displaying a distinct DNA banding pattern and forming a separate cluster in the phylogenetic analysis.

1. INTRODUCTION

Barangan Merah banana (*Musa acuminata* Colla) is a type of banana that is much sought after by people in the Sumatra region, especially Aceh, because of its sweet and more fragrant taste [1]. This banana contains beta-carotene, is high in vitamin A, and has a relatively high selling price [2]. The high demand for Barangan Merah bananas needs to be balanced with high and sustainable production. Based on data from the Central Statistics Agency in 2023, national banana production was 93,352 tons with a growth rate of 0.96%. Banana production in 2023 decreased by 25.86% in the Sumatra region. The decrease in banana production is often caused by disease attacks that can reduce the quantity and quality of banana plants.

The primary disease affecting many banana plants is Fusarium wilt, also known as Panama disease, which is caused by the fungus *Fusarium oxysporum* f. sp. *cubense* (Foc) [3]. Foc spores present in the soil or hosted by other plants, like weeds, can infect banana roots, penetrating through the root cortex and spreading into the epidermis and vascular system via fungal mycelium. Banana plantlets may become infected through vascular connections with their parent plants [4]. The spread of Fusarium wilt presents challenges in supplying banana seedlings, especially since they are typically propagated from tubers or shoots. Moreover, as banana plants do not produce seeds, traditional hybridization-based breeding methods are difficult to implement. As a result, efforts to produce disease-free seedlings and develop resistant banana varieties are being pursued through non-traditional methods, particularly tissue culture techniques.

Genetic variation generated through tissue culture, known as somaclonal variation, offers an alternative approach for developing new traits from existing banana germplasm. This type of variation can be induced through artificial mutation using the chemical mutagen ethyl methane sulfonate (EMS). EMS is commonly employed in plant breeding as a mutagenic agent to enhance genetic diversity at the gene level that plays an important role in agriculture. EMS contains an ethyl group (C_2H_5) bound to a methane sulfonate group (CH_3SO_3). The chemical formula of EMS is $C_3H_8O_3S$. This compound is mutagenic, teratogenic, and carcinogenic. The methane sulfonate group (CH_3SO_3) is part of the compound responsible for its reactive properties. The methane sulfonate group can react with nucleophilic groups, such as amino groups or sulfhydryl groups in proteins and DNA. The ethyl group (C_2H_5) is an alkyl group bound to the methane sulfonate group. This ethyl group gives EMS its volatile and soluble properties in

organic solvents. The mutagenic, teratogenic, and carcinogenic properties of EMS can cause damage to DNA and RNA, which can lead to mutations, congenital disabilities, and even cancer. EMS is a chemical compound with a relatively simple structure, but with hazardous properties. Its chemical formula consists of a methane sulfonate group and an ethyl group, which makes it an effective alkylating agent in triggering mutations and DNA damage [5, 6]. EMS functions as an alkylating agent due to its role in chemical reactions involving the ethylation of compounds. Although EMS is synthesized for research purposes, it is not produced on a large scale and is not known to exist naturally. It can be created by reacting ethyl alcohol with either methane sulfonic anhydride ($CH_3SO_2–O–SO_2CH_3$) or methane sulfonyl chloride ($CH_3SO_2–Cl$). The interaction between EMS and DNA has been thoroughly investigated, leading to a clear understanding of its reaction mechanism and the identification of specific DNA target sites affected by EMS [5]. Recent research has demonstrated that EMS is more efficient and effective at inducing mutations in agriculturally significant plants compared to other chemical or physical mutagens. Chemical mutagens like EMS primarily cause point mutations, offering an advantage over radiation, which tends to result in larger genetic disruptions such as deletions and chromosomal abnormalities [7]. MS is widely used in plant mutagenesis due to its simplicity and ability to reliably induce mutations. It works by alkylating DNA, specifically attaching an alkyl group to the oxygen atom in guanine involved in hydrogen bonding. This process produces O-6-alkylguanine, which mispairs with thymine instead of cytosine, ultimately leading to a base substitution from A/T to G/C, and causes single-nucleotide mutations [8]. EMS affects very short chromosome segments carrying one or a few genes, and can affect the cytological, genetic, physiological, and morphological properties of plant tissues and cells [9].

Several studies on the use of EMS on banana plants using tissue culture have been conducted on Ambon Kuning, Raja Sereh, Barangan Intan, and Barangan banana plants. Research result [10] showed that EMS treatment with a concentration of 0.1% and 30% fusaric acid produced 20 healthy plants from 88 Ambon Kuning banana plants (22.73%). The study [11] on banana cultivar Tella Chakkerakeli (AAA) showed that the EMS concentration and treatment duration significantly influenced explant survival, shoot initiation time, number of shoots, and root number. Lower EMS concentration (0.25% for 60 min) yielded the highest survival rate and early cluster-like bodies (CLBs) appearance, while higher concentrations reduced survival and growth parameters. Research [12] showed that changes in EMS concentration and duration at 13 weeks of age after EMS treatment of 150 mM (1.86%) for 30 minutes resulted in regrowth of Berangan Intan banana shoots of 78.00 ± 2.19%, Berangan of 75.67 ± 2.34%, and Rastali of 74.00 ± 1.79%. Treatment with the EMS solution of 250 mM (3.10%) for 60 minutes resulted in the regrowth of Berangan Intan banana shoots of 30.63 ± 1.05, Berangan of 33.33 ± 1.63, and Rasatali of 23.50 ± 1.76%, while the control treatment (buffer and water immersion) regrew its shoots by 90%.

The research [11] shows that EMS mutagen treatment can also cause morphological changes in the resulting mutants. Changes in morphological characteristics in Raja Sereh banana plantlet mutants are changes in leaf color to pale yellow and leaves to yellow, stem color to pale yellow, small, irregular leaf shape, and spiral-shaped leaves. Although the use of EMS has been carried out on several types of bananas, the use of EMS mutagens on Barangan Merah banana explants has never been reported.

Tissue culture techniques using banana shoots with EMS treatment can be an alternative strategy to induce phenotype variation. Research [13] showed that banana shoots produced by tissue culture and given EMS mutagen produced 2-14% phenotypic variations three months after culture. Selection of banana plant resistance can also be done at the nursery stage using DNA markers. This method is effective in determining its resistance to Fusarium [13, 14]. Phenotypic variation can be identified at the molecular level using simple sequence repeat (SSR) markers through Polymerase Chain Reaction (PCR) techniques. SSR markers are effective tools for assessing genetic diversity, as well as for detecting and selecting variants with desirable agronomic traits during in vitro selection processes. Variability of EMS mutagenesis results can be confirmed using simple sequence repeats (SSR) primers, namely Ma3/2, Ma15, Ma1/17, and STMS7 [15].

This study aims to evaluate the impact of EMS-induced mutants and soaking duration on the growth of putative mutants of Barangan Merah bananas derived from tissue culture, as well as their identification through DNA fingerprinting analysis.

2. MATERIALS AND METHODS

2.1 Materials

The planting material is the shoot resulting from multiple bud clump (MBC) induction. The explant is the result of in vitro culture from 2 stages of culture. The first stage is the induction of shoots of Barangan Merah banana stem explants using Murashige and Skoog (MS) media + 3 mg L^{-1} benzylaminopurine (BAP) for 4 weeks, then the second stage is the induction of multiple bud clumps using MS media + 5 mg L^{-1} BAP + 0.5 mg L^{-1} indole acetic acid (IAA) for 8 weeks.

The culture medium used was Murashige and Skoog (MS) from Sigma (catalog number M5524). Other ingredients were ascorbic acid, myoinositol, agar, sucrose, and benzylaminopurine (BAP). The EMS chemical mutagen used was from Sigma (catalog number M0880). The EMS mutagen solvent used was phosphate buffer solution, pH 7.

Leaves from putative mutant seedlings resulting from mutations were used for DNA fingerprint analysis, deoxyribonucleic acid (DNA) isolation, and the PCR process using SSR primers (Ma3/2, Ma15, Ma1/17, and STMA7). The primers can be seen in Table 1.

Table 1. Marker primer simple sequence repeats (SSR)

Primer	Forward	Reverse
Ma3/2	TGCGCGTCCACAC ACACACA	GGCGATACGCAACAA ATAGACTTAGG
Ma15	AGGCGGGGAATCG GTAGA	GGCGGGGAGACAGAT GGAGT
Ma1/17	TCGGCTGGCTAAT AGAAGGAA	TCTCGAGGGATGGTG AAAGA
STMS7	AAGAAGGCACGAG GGTAG	CGAACCAAGTGAAAT AGCG

Source: Hegde et al. [15].

2.2 Mutation induction using EMS with the in vitro technique

EMS treatment and in vitro mutation induction were conducted at the Plant Tissue Culture Laboratory, Faculty of Agriculture, Syiah Kuala University, Banda Aceh, Indonesia. The EMS solution was prepared by mixing EMS with phosphate buffer solution. Phosphate buffer solution was prepared by mixing solution A (prepared by dissolving 17.799 g of sodium hydrogen phosphate (Na_2HPO_4) in 1 L of water) and solution B (prepared by dissolving 15.601 g of sodium dihydrogen phosphate (NaH_2PO_4) in 1 L of water). Phosphate Buffer Solution pH seven was prepared by mixing 61.1 mL of solution A and 87.7 mL of solution B [16].

Immersion for 50 explants was done by immersing each explant in a small light-tight bottle containing a single-use EMS solution. The explants were immersed in 0.1% (v/v) EMS solution for 1 hour, 2 hours, and 3 hours according to the treatment carried out in dark conditions, rinsed with sterile distilled water, and cultured on Shoot induction media (MS + BAP 3 mg L^{-1}) for 4 weeks. Explants that remained sterile were subcultured into MS medium without adding growth regulators. The explants were incubated in an incubation room at a temperature of 25℃ and a light intensity of 1,000 lux for 16 hours per day. The explants were incubated until they grew into plantlets and were ready for hardening for 2 weeks, and acclimatized by individual confinement according to the method [17].

The parameters observed were the growth parameters of putative mutant explants, namely the number of shoots and shoot height at the ages of 7 and 10 weeks after induction (WAI) in 0.1% EMS solution according to the treatment of immersion time. Observations of growth (number of leaves, leaf length, leaf width, plantlet height, number of roots, and root length were carried out per individual putative mutant plantlet at the age of 20 WAI. The growth data was calculated as the average of three replicates and their standard deviation. Leaf color was observed in seedlings aged 6 weeks after acclimatization, while chlorophyll content and stomata shape were observed in seedlings aged 18 weeks after acclimatization.

Leaf color was measured using the Nihon Engei Shokubutsu Hyojun Iro Hyo CAT 154-D leaf color chart, Evelwell Corporation, Tokyo, Japan. Chlorophyll content was measured using the Soil Plant Analysis Development (SPAD) tool, expressed in units. Observation of the shape of the stomata was carried out using stomata preparations observed under an Olympus CX23 microscope with a magnification of 100x and an Am-Scope FMA050 Digital Camera microscope. Stomata preparations were made using the stomata print method. This method is carried out by smearing the leaf's abaxial part (lower surface) with clear nail polish containing acetone until it dries, then covering it using transparent tape on the object glass where the smear was applied.

2.3 DNA fingerprint analysis procedure

The isolated DNA was derived from putative mutant plants that were still alive 18 weeks after acclimatization. This analysis was conducted at the Center for Standard Testing of Biotechnology and Genetic Resources Instruments (BBPSI Biogen), Bogor, Indonesia.

Isolation of Genomic DNA - Banana genomic DNA was isolated using the modified method [18]. A total of 0.5 g of banana leaf pieces was ground in a sterile mortar with 500 µL of extraction buffer, containing 100 mM Tris-HCl (pH 8.0), 1.4 M NaCl, 20 mM EDTA (pH 8.0), 2% (w/v) CTAB (cetyltrimethylammonium bromide), 2% (w/v) PVP (polyvinylpyrrolidone), and 0.38% (w/v) sodium bisulfite, until fully homogenized. The grinding results were then put into a 2 mL microtube, and the extraction buffer was added until the volume reached 800 µL. The sample was then incubated at 65℃ for 15 minutes and turned every 5 minutes until homogeneous. The sample was then extracted using 800 µL of chloroform: isoamyl alcohol (24:1) solution, followed by centrifugation at 12,000 rpm for 10 minutes at 20℃. The supernatant formed was then carefully transferred into a 1.5 mL microtube. Next, 3M sodium acetate, pH 5.2, was added to each sample as much as 1/10 times the volume of the supernatant, and cold isopropanol as much as one time the volume of the supernatant, then the sample was slowly inverted and incubated at -20℃ for one hour. After that, the sample was centrifuged at 12,000 rpm for 10 minutes at 20℃. The supernatant was discarded, and the DNA pellet was washed using 500 µL of cold 70% (v/v) ethanol solution. The sample was centrifuged again at 12,000 rpm for 5 minutes at 20℃. The supernatant was discarded again, and the clean DNA pellet was then air-dried overnight at room temperature to remove residual ethanol. The DNA pellet was then dissolved using 100 µL of TE solution (10 mM Tris, pH 8.0, and 1 mM EDTA) to which the RNAse enzyme (10 mg/mL) had been added. The samples were then incubated at 37℃ for 30 minutes. The stock DNA solution was then stored at -20℃ until ready for use.

DNA Amplification - Each sample was amplified in a 10 µL total reaction containing 10 ng/µL of 2 µL template DNA; 2x My-Taq HS (Bio-line, UK), 5 µL; forward and reverse primers at a concentration of 10 µM each in 0.5 µL, and sterile ddH_2O. Amplification was performed using four pairs of banana SSR primers. PCR reactions were performed in a T100 Thermal Cycler PCR machine (Bio-Rad, USA) with the following PCR profile: initial denaturation at 95℃ for 5 minutes, followed by 35 cycles of denaturation at 94℃ for 30 seconds, annealing at 55℃ for 1 minute, initial extension at 72℃ for 1 minute, and ending with a final extension at 72℃ for 10 minutes [19].

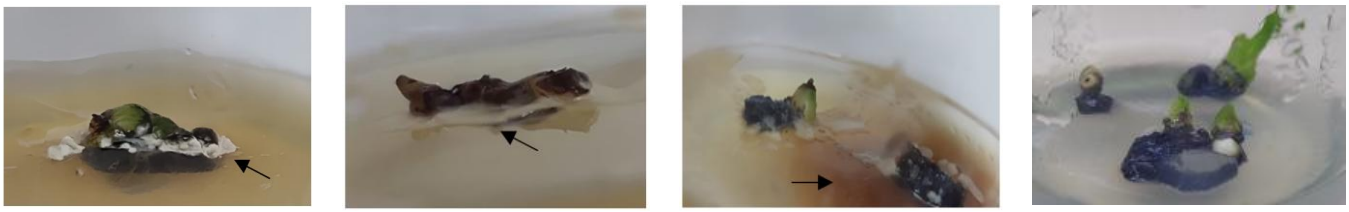
Polyacrylamide Gel Electrophoresis - The PCR results were then electrophoresed according to the method [20] on a 6% polyacrylamide gel in a tank containing 1x Tris Borate EDTA (TBE) buffer at a voltage of 80 V for 115 minutes. The electrophoresis results were then stained with ethidium bromide solution (10 mg/mL) and visualized under UV light using a UV Transilluminator (Bio-Rad, USA). The results of polyacrylamide gel visualization in molecular tests were scored as binary data. Each visible band scored 1, and the invisible band scored 0. The scoring data were then analyzed using the Sequential Agglomerative Hierarchical and Nested-Unweighted Pair Group Method with Arithmetic (SAHN-UPGMA) program on NTSYS software version 2.1 to obtain a phylogenetic tree and genetic similarity matrix.

3. RESULTS AND DISCUSSION

3.1 Mutant putative growth spectra features

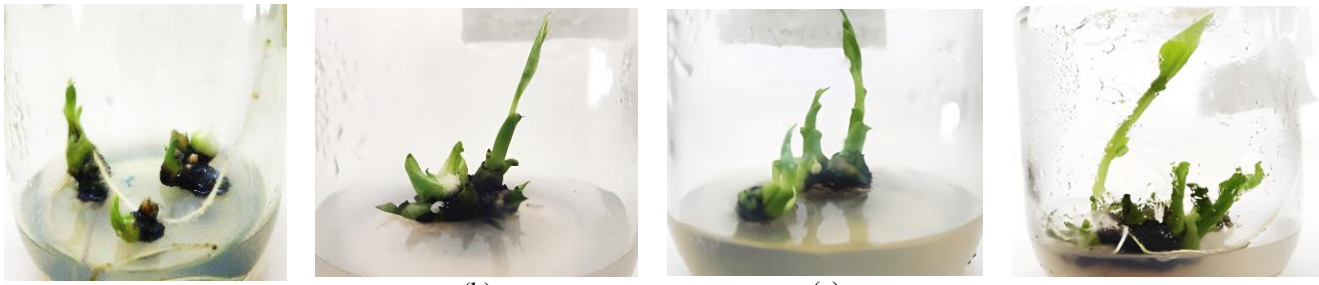
The results showed that EMS treatment on 30 shoots of Barangan Merah banana resulted in 26 (86.7%) putative mutant shoots that survived at 7 weeks after induction (WAI) and

decreased to 16 (53.3%) shoots at 10 WAI. The number of surviving explants decreased due to contamination caused by fungi and bacteria and browning (Figure 1). As many as 9 (30%) of the surviving putative mutant shoots became putative mutant plantlets. EMS induction to the acclimatization process was 20 weeks. The acclimatization process was carried out for 4 weeks and continued with transplanting to the field. The surviving plantlets grew into putative mutant seedlings after the acclimatization and transplanting process (6 weeks after acclimatization (WAA)), as many as 8 (88.9%) putative mutant seedlings.



(a) (b) (c) (d)

Figure 1. Explant contamination (indicated by arrows) caused by (a) fungi, (b) bacteria, (c) browning, and (d) sterile explant



(a) (b) (c) (d)

Figure 2. Explants after soaking in 0.1% EMS solution for (a) control, (b) 1 hour, (c) 2 hours, and (d) 3 hours, at 10 WAI

Table 2. Average number of shoots and shoot height of putative mutants of Barangan Merah bananas after being soaked in EMS solution with time soaking treatment

Soaking Time (hour)	WAI (weeks)	Average Shoots Number	Average Shoots Height (cm)
0	7	1 ± 1	1.13 ± 1.13
1	7	1 ± 0	0.55 ± 0.55
2	7	2 ± 1	0.35 ± 0.35
3	7	4 ± 1	0.78 ± 0.78
0	10	1 ± 0	1.64 ± 1.64
1	10	7 ± 1	1.87 ± 1.87
2	10	4 ± 1	1.81 ± 1.81
3	10	7 ± 0	1.71 ± 1.71

The study results in Table 2 show that immersion in 0.1% EMS for 3 hours at 7 WAI tends to produce a greater number of shoots (4 shoots) than other treatments. Immersion in 0.1% EMS for 1 and 3 hours at 10 WAI tends to produce more shoots (7 shoots) than other studies. The treatment of immersion in 0.1% EMS for 1, 2, and 3 hours tends to produce lower shoot heights than the control at 7 WAI, and the shoot height is higher than the control at 10 WAI. Figure 2 shows an example of an explant treated with mutase using 0.1% EMS at 10 WAI.

The growth of putative mutant plantlets of Barangan Merah banana 20 weeks after induction (WAI) tended to vary (Table 3). In general, the control showed the leaves number (3.8 ± 0.5 leaves), leaf length (8.2 ± 0.8 cm), leaf width (2.6 ± 0.1 cm), plantlet height (19.2 ± 0.6 cm), roots number (7 ± 1 roots), and root length (10.4 ± 0.9 cm) tended to be better compared to the average growth of putative mutant plantlets from immersion in 0.1% EMS, namely the leaves number (3.3 ± 0.7 leaves), leaf length (5.3 ± 0.6 cm), leaf width (1.7 ± 0.2 cm), plantlet height (11.7 ± 0.3 cm), roots number (6.4 ± 1.4 roots), and root length (7.7 ± 0.4 cm). Soaking for 1 hour (sample 1A) and 3 hours (sample 1C) in 0.1% EMS solution tended to produce a better number of leaves (5 ± 1 leaves) and plantlet height (15.7 ± 0.2 cm) compared to the average number (3.3 ± 0.7 leaves) and plantlet height (11.7 ± 0.3 cm) in other 0.1% EMS soaking treatments. The best leaf length (7.2 ± 0.2 cm) and root length (16.0 ± 0.4 cm) were found in putative mutant plantlets soaked for 2 hours (sample 1B) in 0.1% EMS solution compared to the average leaf length (5.3 ± 0.6 cm) and root length (7.7 ± 0.4 cm) from other 0.1% EMS soaking treatments. The best leaf width (2.9 ± 0.1 cm) and number of roots (9 ± 2 roots) were found in the 2-hour soaking treatment (sample 2B) in 0.1% EMS compared to the average leaf width (1.7 ± 0.2 cm) and number of roots (6.4 ± 1.4 roots) from other 0.1% EMS soaking treatments. The phenotypic variations, as indicated by the growth patterns of Barangan Merah banana plantlets, are shown in Table 3.

Table 3. Leaf number, leaf length, leaf width, plantlets height, roots number, and root length of putative mutant plantlets Barangan Merah bananas at 20 WAI

Sample Code	EMS Concentration (%)	Soaking Time (hour)	Leaves Number (leaves)	Leaves Length (cm)	Leaves Width (cm)	Plantlet Height (cm)	Roots Number (roots)	Roots Length (cm)
1A	0.1	1	5 ± 1	6.2 ± 1.2	1.6 ± 0.2	15.7 ± 0.3	7 ± 2	12.0 ± 0.2
2A	0.1	1	1 ± 0	4.7 ± 0.3	1.5 ± 0.1	9.7 ± 0.4	6 ± 1	10.5 ± 0.7
3A	0.1	1	2 ± 0	3.8 ± 0.2	1.3 ± 0.2	6.3 ± 0.3	4 ± 1	3.0 ± 0.4
4A	0.1	1	3 ± 1	4.0 ± 1.0	1.2 ± 0.2	6.5 ± 0.1	5 ± 1	3.5 ± 0.3
5A	0.1	1	4 ± 1	3.5 ± 0.6	1.0 ± 0.1	8.0 ± 0.5	6 ± 2	4.2 ± 0.2
1B	0.1	2	4 ± 1	7.2 ± 0.2	2.2 ± 0.2	14.2 ± 0.2	8 ± 2	16.0 ± 0.4
2B	0.1	2	3 ± 1	5.5 ± 0.5	2.9 ± 0.1	14.5 ± 0.2	9 ± 2	6.0 ± 1
1C	0.1	3	5 ± 1	6.2 ± 0.5	2.1 ± 0.1	15.7 ± 0.2	6 ± 1	9.5 ± 0.4
2C	0.1	3	3 ± 0	6.5 ± 0.5	1.9 ± 0.2	15.0 ± 0.7	7 ± 1	5.0 ± 0.4
Average			3.3 ± 0.7	5.3 ± 0.6	1.7 ± 0.2	11.7 ± 0.3	6.4 ± 1.4	7.7 ± 0.4
Control 1.1	0	0	5 ± 1	12.0 ± 1.0	3.5 ± 0.1	25.0 ± 0.3	10 ± 2	11.0 ± 1.1
Control 1.2	0	0	4 ± 0	8.5 ± 0.5	2.7 ± 0.1	20.0 ± 0.8	6 ± 0	7.0 ± 0.7
Average			3.8 ± 0.5	8.2 ± 0.8	2.6 ± 0.1	19.2 ± 0.6	7 ± 1	10.4 ± 0.9

Table 4. Leaf color variations in putative mutants of Barangan Merah banana seedlings at 6 weeks after acclimatization

Sample Code	Treatment		Leaf Color	Figure	
	EMS Concentration (%)	Soaking Time (hour)		a	b
1A	0.1	1	deep yellow green 3507		
3A	0.1	1	bright yellow green 3504		
4A	0.1	1	deep yellow green 3507		
5A	0.1	1	deep yellow green 3507		
1B	0.1	2	vivid yellow green 3506		
2B	0.1	2	deep yellow green 3507		
1C	0.1	3	deep yellow green 3507		
2C	0.1	3	deep yellow green 3507		
Control 1.1	0	0	vivid yellow green 3306		
Control 1.2	0	0	vivid yellow green 3306		

Description: a = Leaf color chart, b = Leaf blade color.

3.2 Leaf color, chlorophyll content, and stomatal shape

Phenotypic variations caused by EMS-induced mutations include changes in leaf color, chlorophyll content, and stomatal characteristics. Leaf color in putative mutant seedlings of Barangan Merah banana (Table 4) showed three leaf color variations, namely bright yellow green (leaf color code number 3504), vivid yellow green (leaf color code numbers 3506 and 3306), and deep yellow green (leaf color code number 3507) at 6 weeks after acclimatization (WAA). Putative mutant seedlings had dark yellow-green leaves (78%) compared to the control, except for putative mutant seedling samples 3A and 1B, which were bright yellow-green (22%), the same as the control. The difference in leaf color in the putative mutant seedlings sample 3A was different from that of other putative mutant seedlings soaked for 1 hour. Likewise, putative mutant seedlings 1B were different from other putative mutant seedlings soaked for 2 hours.

Table 5. Chlorophyll content in leaves of putative mutant and control seedlings of *Musa* cv. Merah at 18 weeks post-acclimatization

Sample Code	EMS Concentration (%)	Soaking Time (hour)	Chlorophyll Content (unit)
1A	0.1	1	31.1
4A	0.1	1	39.6
1B	0.1	2	38.0
2B	0.1	2	39.9
1C	0.1	3	37.1
2C	0.1	3	28.7
		average	35.7
Control 1.1	0	0	41.4
Control 1.2	0	0	37.6
		average	39.5

This study shows that the color of the leaves of the putative mutant seedlings aged 6 WAA (Table 4) which were initially darker green than the control tended to change after the putative mutant seedlings aged 18 WAA became lighter green, seen from the chlorophyll content (Table 5) where the average chlorophyll content of the putative mutant seedlings (35.7 chlorophyll units) was lower than the control (39.5 chlorophyll units).

Table 5 shows that 33.3% of putative mutant seedlings (sample codes 1A and 2C) have lower chlorophyll content (average 29.9 chlorophyll units) and 66.7% of putative mutant seedlings have higher chlorophyll content (average 38.65 chlorophyll units) compared to the average chlorophyll content of all putative mutant seedlings (35.7 chlorophyll units).

Figure 3 shows that the type and shape of stomata do not differ in the control and putative mutants of Barangan Merah banana seedlings, namely the tetracytic stomata type, which shows four guard cells arranged in series and parallel to the guard cells, and is round like a kidney [21, 22].

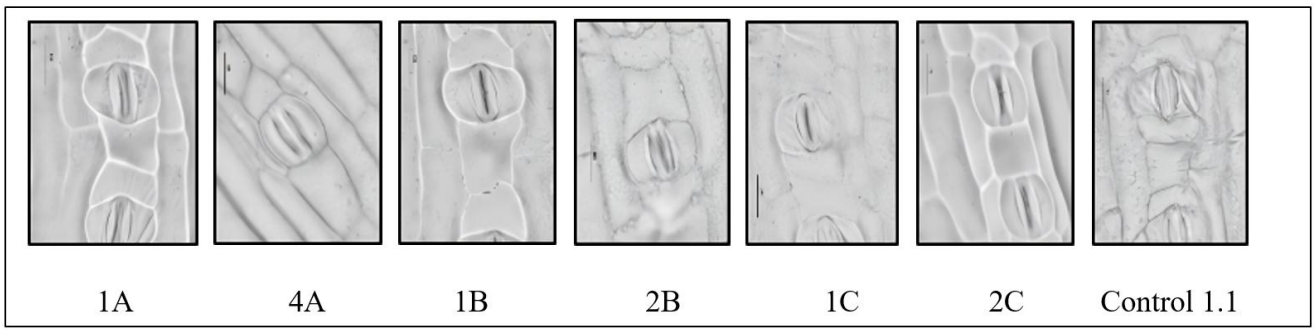


Figure 3. The shape of lower leaf stomata in the leaves of banana seedlings and putative mutant seedlings (Barangan Merah banana) at 18 weeks after acclimatization

3.3 DNA fingerprint analysis

The study results in Table 6 show DNA polymorphism of four markers used for DNA analysis of putative mutants of Barangan Merah banana seedlings at 6 WAA. The range of allele sizes listed on each marker (Table 6) corresponds to the location of the gene band (Figures 4-7). The total number of alleles detected was 18 for all markers tested and differed for each marker, according to the DNA band in Figures 4-7.

The four markers' major allele frequency (MAF) values tend to be low at the STMS7 marker, which is 0.38 compared to other MAF values in Table 6. MAF is related to expected heterozygosity (He). The lowest MAF value in this study (0.38) was found in marker STMS7, but it showed the highest He value (0.80). The low average MAF value (0.47) on the four markers used in this study (Table 6) turned out to indicate a high observed heterozygosity (Ho) value, which was close to 1 (0.97). This result indicated that the level of heterozygosity obtained from the putative mutant of the Barangan Merah banana in this study was high.

The results showed that the Polymorphic Information Content (PIC) for the Barangan Merah banana mutant tended to be higher in the STMS7 marker, which was 0.78 PIC. The relatively high PIC value for the STMS7 marker (Table 6) indicates the potential of the marker to provide diverse genetic information.

The results of molecular analysis of eight banana DNAs consisting of six test samples (samples 1A, 1B, 1C, 2B, 2C, and 4A) and two DNAs (samples 1.1 and 1.2) are presented in Figures 4-7. In this study, the putative mutations of Barangan Merah bananas showed different major band locations (300 bp and 100 bp) when amplified using the four primers. This result indicated that mutations occur randomly in the genome. Based on the analysis results, two of the four SSR primers used, namely primers Ma3/2 and Sa15, are monomorphic, where the DNA band locations of all samples analyzed are the same (Figures 5 and 7), which means that there is no allelic variation in the eight banana DNAs analyzed.

Based on the results of the primer analysis, Ma1/17 and STMS7 are polymorphic, where both primers can distinguish the two DNAs used (Figures 5 and 7). Primer Ma15 (Figure 6) shows allele variations between the samples tested with the control (K1.1 and K1.2). The DNA band arrangement of sample 2C differs from the other samples and K1.1, but is the

same as that of sample K1.2. Primer Ma1/17 (Figure 5) can show a more varied allele arrangement, indicated by sample 2C, which has a different DNA band arrangement from the other five test samples and K1.1, however, sample 2C is not different from K1.2.

The STMS7 primer (Figure 7) was able to detect more allelic variations in the eight banana DNAs analyzed, especially variations between samples 1A, 1B, 2B, and 4A, which had different allele arrangements with K1.1 and K1.2 (control). The DNA band arrangement in sample 1A showed that alleles 2,3, and 4 differed from the control. Sample 1B differed from the control in alleles 4 and 5. Samples 2B and 4A showed a combination of allele arrangements between K1.1 and K1.2.

Table 6. Results of SSR marker polymorphism DNA: Ma3/2, Ma1/17, Ma15, and STMS7

Marker Name	Allele Size Range (bp)	Allele Number	Major Allele Frequency	Expected Heterozygosity (He)	Observed Heterozygosity (Ho)	Polymorphic Information Content (PIC)
Ma3/2	83-95	2	0.50	0.50	1.00	0.38
Ma1/17	203-268	4	0.50	0.59	1.00	0.51
Ma15	108-166	6	0.50	0.50	1.00	0.38
STMS7	153-319	6	0.38	0.80	0.88	0.78
Total		18				
Average		4.5	0.47	0.60	0.97	0.51

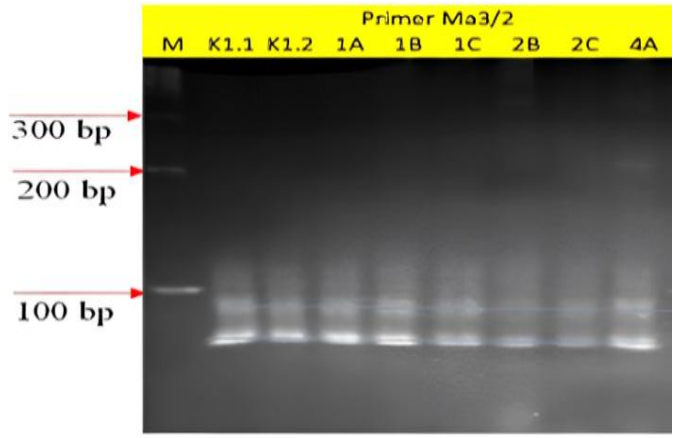


Figure 4. DNA bands from eight banana genotypes amplified using the SSR primer Ma3/2

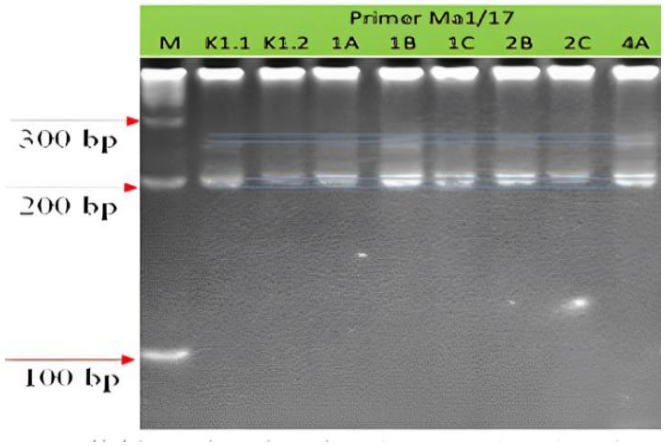


Figure 5. DNA bands from eight banana genotypes amplified using the SSR primer Ma1/17

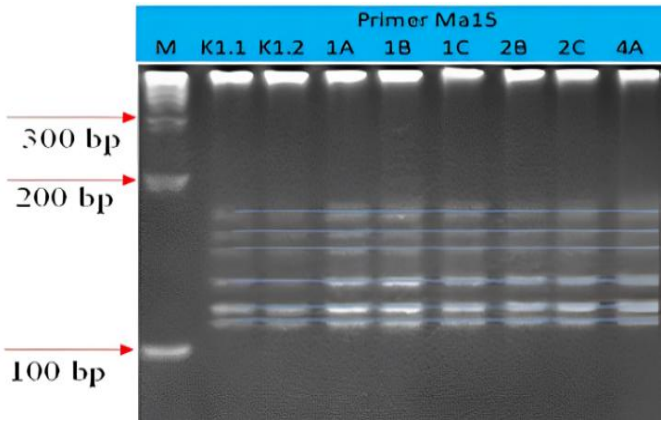


Figure 6. DNA bands from eight banana genotypes amplified using the SSR primer Ma15

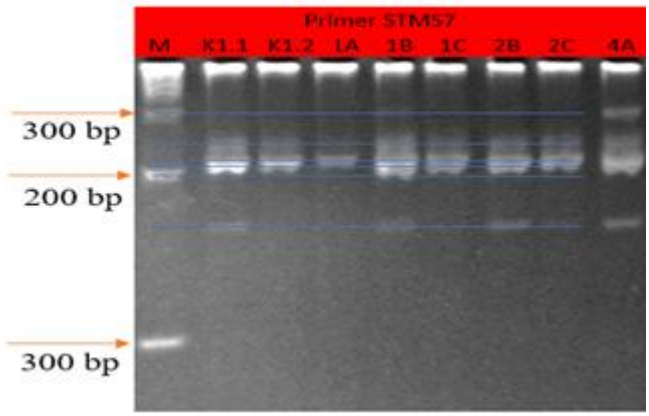


Figure 7. DNA bands from eight banana genotypes amplified using the SSR primer STMS7

3.4 Filogenetik

The results of phylogenetic analysis showed that the eight banana genotypes analyzed were divided into two primary groups with a genetic similarity coefficient of 0.75 (Figure 8). The first cluster (I) consisted of seven banana genotypes, except genotype 1A, which was divided into the second cluster. Meanwhile, the first cluster was divided into three subgroups: subgroup IA consisting of Control genotypes 1.1, 2B, 1C, and 4A; subgroup IB consisting of Control genotypes 1.2 and 2C; and subgroup IC consisting of genotype 1B.

Based on the genetic similarity matrix values as presented in Table 7, it can be seen that genotype 2B shows 100% similarity to Control 1.1. In contrast, genotype 1A shows the lowest similarity to Control 1.1, which is 77.8%. Meanwhile, genotype 2C shows the highest similarity to Control 1.2, 94.4%, while genotype 1B shows the lowest similarity to Control 1.2, 66.7%. Between genotypes Control 1.1 and Control 1.2, a genetic variation of 16.7% was found, with a similarity level between the two of 83.3%.

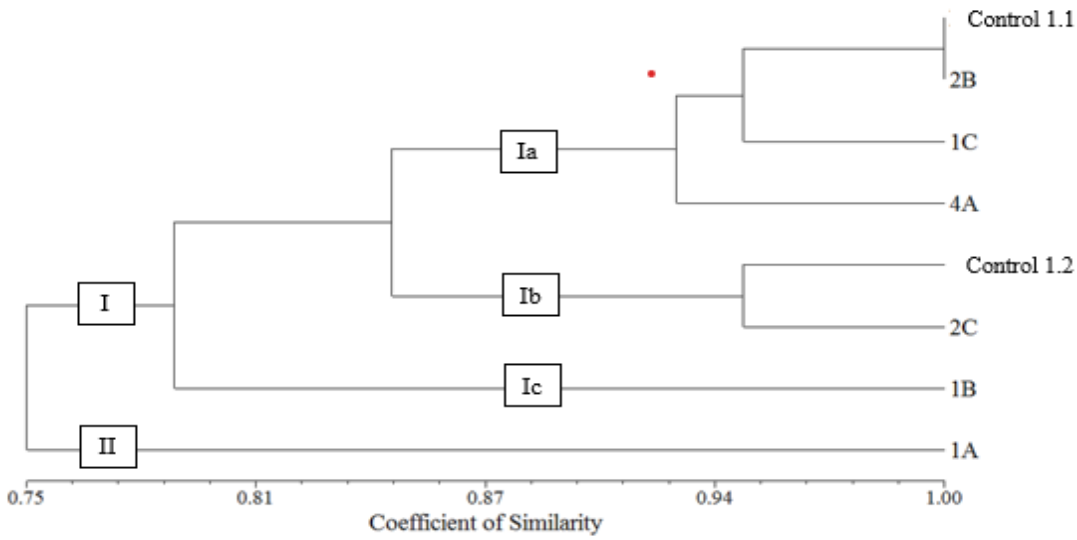


Figure 8. Phylogenetic tree showing the grouping of the eight banana genotypes analyzed in this test based on four SSR markers

Table 7. The genetic similarity matrix of the eight banana genotypes analyzed in this test is based on four SSR markers

Sample	Control_1.1	Control_1.2	1A	1B	1C	2B	2C	4A
Control_1.1	1.000							
Control_1.2	0.833	1.000						
1A	0.778	0.722	1.000					
1B	0.833	0.667	0.722	1.000				
1C	0.944	0.889	0.833	0.778	1.000			
2B	1.000	0.833	0.778	0.833	0.944	1.000		
2C	0.889	0.944	0.667	0.722	0.833	0.889	1.000	
4A	0.944	0.778	0.722	0.889	0.889	0.944	0.833	1.000

4. DISCUSSION

The lower EMS concentrations encourage increased shoot production, growth, and meristem growth in this study. As stated by researchers [23, 24], EMS treatment resulted in an increase in the number of tuna in orchid explants. The results of research [25] on the same plants also showed that EMS treatment could increase the number and height of tuna. This indicates that EMS mutagens can induce genetic variation.

The treatment of EMS immersion in this study tends to produce lower shoot heights than the control at 7 WAI, and the shoot height is higher than the control at 10 WAI. This is possibly due to the 0.1% EMS concentration affecting in vitro shoot growth. The immersion of explants in 0.1% EMS solution can inhibit or decrease plantlet growth at the age of 20 WAI. This is in line with research [24] that EMS can have the effect of reducing shoot growth. EMS also causes significant morphological and physiological changes. EMS-induced mutations caused phenotypic variations as indicated by the growth patterns of Barangan Merah banana plantlets, also mentioned by Kamila et al. [26]. Saraswathi et al. [27] also stated that EMS was effective in producing genetic variation in Rastali bananas.

This research showed that chemical mutation using EMS produces random variations in leaf color parameters at 6 WAA. Leaf color in this study did not find any variegated leaves, namely the appearance of a mixture of other colors, such as white or yellow, in the green color of the leaves of putative mutant seedlings. Widiarsih and Dwimahyani [28] stated that the emergence of variegated leaves displaying a mix of the typical green coloration with white (albino) or yellow (viridis) shades suggests a mutation in chlorophyll production. Such chlorophyll mutations occur within the chloroplasts and result from defects in mutant genes, which can interfere with the leaf's photosynthetic function. The presence of striped or uneven coloration on leaves is a hallmark of mutations in chloroplast genes. Meanwhile, mutations occurring outside the cell nucleus, particularly in mitochondrial genes, can lead to inhibited growth, altered flower structures, and other morphological abnormalities [29]. Restanto et al. [30] stated that color indicators are related to the age and quality of plants because the color can determine the condition of cells actively dividing or dying. The molecular mechanism for leaf color mutations shows that mutated genes disrupt the stability and synthesis of pigments in leaves directly or indirectly [31]. Leaf color in plants that experience mutations affects the efficiency of plant photosynthesis, which can result in stunted plant growth [32]. Environmental factors also affect plant physiology, including changes in leaf color and chlorophyll content [33, 34]. Huo et al. [35] stated that green-leafed mutants showed low chlorophyll levels and high carotenoid levels. Changes in temperature and light intensity, as well as nutrient content such as magnesium, iron, and nitrogen during growth in the field, affect photosynthesis in seedlings and can cause changes in leaf color and chlorophyll content [36].

The green leaf in the leaf tissue of the putative mutant plantlets and the control indicates chlorophyll. Martha et al. [23] stated that the deeper the green color of a leaf, the higher its chlorophyll content, whereas lighter or younger leaf colors suggest lower levels of chlorophyll. This study showed that the soaking treatment in 0.1% EMS affects the chlorophyll content of putative mutant seedlings of Barangan Merah banana in

various ways at 18 WAA. Variations in chlorophyll content due to EMS were also found in studies [37, 38]. Chemical mutation using EMS in this study tended to reduce the number of chlorophyll units in putative mutant seedlings aged 18 WAA. EMS can affect chlorophyll content in plants by inducing mutations that disrupt chlorophyll synthesis or stability. Several studies show that EMS treatment can cause a decrease in chlorophyll content are found in studies [39-41].

Stomata play an important role for plants in transpiration, respiration, and photosynthesis. Stomata on Barangan Merah banana leaves are found on the upper and lower sides of the leaves. The same thing is generally found in most plants [42]. Ploidy level is related to stomatal shape [43] and stomatal size [44]. Barangan Merah banana is a type of banana with an AAA (triploid) chromosome arrangement [45, 46]. Stomata are influenced by plant genetics and the environment. Stomata are derivatives of leaf epidermis cells [47]. They are one of the leaf anatomical structures directly influenced by the environment and cause variations in stomata's shape, structure, and arrangement [48, 49].

The shape and type of stomata affect the ability of leaves to exchange gases (carbon dioxide, oxygen, and water vapor) with the surrounding environment. Wider or more stomata will increase the surface area for gas exchange, thereby increasing conductance [50]. Besides chlorophyll content (particularly chlorophyll b), stomatal conductance plays a crucial role in influencing the net photosynthesis rate. It also serves as an indicator of the plant's water status and is used as a selection criterion in breeding superior varieties [51, 52].

DNA fingerprint analysis showed that the highest number of alleles was found in markers M15 and STMS7, each with six alleles, compared to other markers. This result showed that genetic variation in putative mutants is more common in these two markers. The number of alleles from SSR results indicates genetic diversity or variation at a particular locus. Alleles are different versions of the DNA sequence at a locus, and the number of distinct alleles detected by SSR provides an idea of how diverse the population or individuals being analyzed are genetically [53].

The result of major allele frequency (MAF) values tends to be low at the STMS7 marker indicates more genetic variation in the putative mutant of Barangan Merah banana at this marker. MAF is the frequency of occurrence of the most common allele in a population and the proportion of the most common allele found at a particular locus. MAF values range from 0 to 1 or 0% to 100%, where 1 or 100% indicates that only one allele is present in the population, while 0 or 0% indicates no allele. If the MAF is low, many sample individuals may carry different alleles. The allele frequency value is the basis for calculating the expected heterozygosity (He) value and provides an estimate of genetic variation [54]. MAF in genome-wide association studies (GWAS) to find relationships between genetic variation and disease [55] is also used in the analysis of natural selection and genome evolution [56]. High genetic diversity also shows that the sample individuals in this study have different alleles at specific gene loci, related to individual adaptation to environmental changes [57].

The highest He value on marker STMS7 showed that the expected genetic diversity for the putative mutant of Barangan Merah banana at marker STMS7 is greater than the other three markers. If the frequency of the major allele is very high, He will tend to be low, because most individuals will have homozygous genotypes for that allele. Conversely, if the frequency of the major allele is low, He will be higher, because more individuals are expected to have heterozygous genotypes [58].

The relationship between MAF and Ho was described by research [59], namely, if the major allele frequency is low, Ho will be higher, because more individuals will have heterozygous genotypes. The high Ho value on the four markers in this study indicates that the putative mutant sample of the Barangan Merah banana is heterozygous with a Ho value of 0.88 - 1.00. The high Ho in this study may have resulted from the mutation factor due to the EMS treatment. New mutations can create additional alleles and contribute to higher heterozygosity [59].

The Polymorphic Information Content (PIC) for the Barangan Merah banana mutant tended to be higher in the STMS7 marker. PIC is a measure used to assess the diversity of alleles in a genetic marker. PIC reflects the ability of the marker to detect polymorphism in a population. A high PIC value indicates that the marker is very informative and practical for genetic analysis [60].

This study used the simple sequence repeats (SSR) technique on the putative mutant of Barangan Merah banana seedlings with four pairs of markers, namely Ma3/2, Ma1/17, Ma15, and STMS7 (based on research [15]), because all four primers showed specific resistance bands, used to test the variation among mutants that confirmed their reproducibility, showed precise amplification, easy to read and showed polymorphic bands. EMS can increase the polymorphism of SSR markers in plant genetics. As a chemical mutagen, EMS causes point mutations in DNA, including substitutions and insertions/deletions, which can change the repeat sequence in SSR markers. These changes, if significant enough, will result in variations in the size of the DNA fragments amplified by the SSR primers, resulting in polymorphisms that can be identified through genetic analysis [61]. EMS causes point mutations, which means changes in individual DNA bases. These can be substitutions (one base replacing another) or insertions/deletions (adding or removing a base) [62]. SSRs are short, repetitive DNA sequences that are used as genetic markers. When EMS causes mutations in or near an SSR sequence, it can change the number of repeats, which causes changes in the length of the amplified fragment. These length differences are called polymorphisms, which are variations in DNA sequences that can be used to distinguish individuals or populations. By using SSR primers that amplify altered SSR sequences, geneticists can detect these polymorphisms and use them for genetic mapping, diversity analysis, or other genetic studies [63].

The result of the DNA band arrangement in this study showed that the STMS7 primer could distinguish the four DNAs used in this analysis. Therefore, the STMS7 primer can be recommended for DNA analysis because it showed more alleles and polymorphisms. The differences of each allele in samples 1A and 1B with the control may indicate disease resistance. This result showed that mutations can cause individuals to have different allele patterns than controls, so they can be used to see their disease resistance. The association of polymorphism in putative mutants with disease resistance must be confirmed in further research. Variations in DNA banding patterns are then used to determine the number of alleles when binary data assessment activities are carried out.

Identical alleles can differ in band size; this may not be detected because there is no genomic change since the plant has not been cultured for a long time, or the DNA changes that

occur do not change the length of the allele. The difference in alleles produced from putative mutants compared to alleles in control plants lies in the size of the bands. In this case, changes in size can be caused by deletions or insertions of nucleotides. Size differences that may occur in flanking areas or repeat sequences can be identified by sequencing [64].

EMS is a chemical mutagen used to create genetic variation in banana plants, resulting in mutants with new traits such as virus resistance. Phylogenetic studies of these mutants can reveal how EMS-induced mutations affect the evolutionary relationships between banana varieties. Phylogenetic studies analyze the evolutionary relationships between organisms based on their genetic differences. When studying EMS-induced mutants, phylogenetic analysis can help determine how a particular mutation affects the relationships between banana varieties. By comparing the DNA sequences of wild-type and EMS-mutated bananas, researchers can gain insight into how EMS-induced genetic changes have affected the evolution of these plants. This information can be used to understand the impact of EMS on banana diversity and how EMS can be used to develop new banana varieties [65].

5. CONCLUSION

The conclusion of this study is that the treatment of immersion in 0.1% EMS solution for 1 hour shows a tendency for a better number of shoots and shoot height. Increasing the immersion time to 3 hours in 0.1% EMS solution concentration decreases shoot height. The treatment of immersion for 1 hour and 3 hours in 0.1% EMS solution tends to increase the number of leaves and plant height. The treatment of immersion for 2 hours in 0.1% EMS solution tends to increase leaf size, number, and length of roots. Several samples of mutant seedlings aged 2 weeks after acclimatization showed darker leaf color than the control, but the chlorophyll content was not greater than the control at week 18 after planting. Mutant seedlings have a tetracytic stomata type and a rounded kidney shape, the same as the control stomata. The STMS7 marker used in this study can be used to distinguish mutants because there is polymorphism. The putative mutant sample 1A has a different DNA banding pattern, suspected to have a different DNA structure from the control. The eight banana genotypes analyzed were separated into two main groups with a genetic similarity coefficient of 0.75.

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