Live and Applied Science, Volume 1
Prosiding Seminar Bioteknologi Nasional (SimBioN) 2022
Diselenggarakan di Malang, 27 Agustus 2022
e-ISSN (2964-772X)

Development of Mutagenesis Method Using Ethyl Methane Sulfonate (EMS) as an Initial Stage of Breeding Red Ginger (Zingiber officinale Rosc.)

Khouw, B,[1] Yanti[1] Karmawan, L.U,[1]*
[1]Department of Biotechnology, Faculty of Technobiology, Atma Jaya Catholic University, Indonesia
*Corresponding email: listya.utami@atmajaya.ac.id

Abstract

Red ginger (*Zingiber officinale* Rosc.) is an herbal plant of the *Zingiberaceae* family that is commonly found and has the potential to be developed in Indonesia. Red ginger can be propagated vegetatively through the rhizome. This method of propagation can be said to be less effective. Efforts that can be made through the induction of mutagenesis with *ex vitro* and *in vitro* approach. Plants produced through the induction of mutagenesis have a high success rate of propagation with high quality plant yields, are more resistant to disease, have high similarity to the parent, the process is relatively fast, and has high potential for plant propagation. This study aims to examine the effect of EMS on the morphology and genetic material of red ginger as a potential mutagen for ginger breeding alternative. This study showed that the best surface sterilizing agent with the lowest level of contamination was 0.2% $HgCl_2$ with a contamination level of 0–33%. The *in vitro* effect of EMS immersion time on explant lethality and callus growth revealed that the longer the EMS immersion, the higher the explant lethality rate. Then, 45 minutes of EMS immersion was the best immersion time to support the growth of explant callus, despite the callus' poor quality. In *ex vitro* results, the effect of EMS immersion time on changes in plant morphological characteristics showed that the longer the EMS immersion, the lower the plant shoot height and the higher the mutation changes in the leaves compared to control plants. The molecular test results showed that the OPA-2 primer was the optimum primer to use because it produced the most bands of 4 compared to other primers. The results of the random amplified polymorphism DNA (RAPD) test and cluster analysis showed that the best results were shown by 12 hours of EMS-treated red ginger with a polymorphism level of 62.5%. Then the results of cluster analysis showed that ginger with 8 hours and 10 hours of EMS immersion red ginger had a close relationship, but the two immersions had a distant relationship with 12 hours EMS treated red ginger. The results of research studies show that EMS could induce mutations that results in the formation of a new mutant red ginger with a possible new characteristic. The EMS mutagen showed the potential to be used in ginger breeding for further research.*n.*

Keywords*: Ethyl Methane Sulfonate* (EMS)*, Ex vitro, In vitro, Random Amplified Polymorphism DNA* (RAPD), *Red ginger.*

1. INTRODUCTION

Red ginger (*Zingiber officinale* Rosc.) is one of the most popular types of herbal plants in the *Zingiberaceae* family and has the potential to be developed in Indonesia (Aidah 2020). In the red ginger plant, the important part is in the rhizome, which is often used as a complementary material in the food, pharmaceutical, and cosmetic industries because it has active compounds that are efficacious for the body's health and endurance (Silalahi 2019). Because of this, every year in Indonesia, the level of public consumption of ginger essential oil increases up to 10% (Saputri *et al*. 2018). This increase can occur due to public awareness of the importance of consuming red ginger for immune health and its use in the food and cosmetic industries, increasing the demand for essential oils in the market (Saputri *et al*. 2018). According to Aditama *et al.* (2015), in 2007, the supply of ginger in Indonesia to the world market experienced a drastic decline from 21.17% to 0.94%, while in 1999, Indonesia became the main country exporting ginger to the world mark. In the period from 2004 to 2011, there was a decline in red ginger productivity in Indonesia, reaching 5.88%/year (Rini 2013).

This decrease can occur due to several factors, namely there being errors in cultivation that have not been carried out intensively and the quality of results that change over time (Pribadi 2013; Saputri *et al.* 2018).

Red ginger can be propagated vegetatively through the rhizome (Silalahi 2019). This method of propagation can be said to be less effective for producing new and healthy ginger because the process takes a relatively long time (10–12 months) to reach the harvest period (Pribadi 2013). The vegetative propagation method has drawbacks, such as being susceptible to disease and contaminants such as plant pest organisms *Ralstonia solanacearum*, which cause the failure of ginger rhizomes, and the resulting rhizomes can sometimes not be sold (Zuraida *et al.* 2016; Fauzi & Nurcahyanti 2020). In the *Zingiberaceae* family, they generally experience a dormant phase (cells are not active), so this is also an obstacle in the process of propagation of new plants (Djadja 2004). Efforts to overcome existing problems with the addition of growth regulators can accelerate the dormancy period (Djadja 2004).

Another effort that can be made by producing new and superior varieties of red ginger plants can be done by developing ethyl methane sulfonate (EMS) chemical mutagenesis. EMS mutagens can induce mutagenesis in plant breeding through methylation processes in plant DNA strands (Kumari *et al.* 2020). Although mutagenesis using EMS is random, the plants produced using EMS have a high success rate of propagation with high-quality yields, are more resistant to disease, like their parents, and the process is relatively fast (Zuraida *et al.* 2016; Kumari *et al.* 2020). This study aims to examine the effect of EMS on the morphology and genetic material of red ginger as a potential mutagen for ginger breeding alternative.

2. METHODS

This research was conducted at the Laboratory of Tissue Culture and Greenhouse, Faculty of Biotechnology, Atma Jaya Catholic University of Indonesia. This research method consists of 2 major stages: *in vitro* and *ex vitro*. The initial stages of the *in vitro* research were the preparation of EMS mutagens and red ginger pre-treatment, initiation, and callus induction of red ginger plants. In the *ex vitro* stage, starting with the preparation of EMS mutagens (Merck, Cat.number M0880-5G) red ginger pre-treatment, red ginger acclimatization of treated-red ginger, DNA extraction and isolation, qualitative and quantitative testing of DNA isolation, *Random Amplified Polymorphism* DNA (RAPD)-PCR, cluster analysis of treated-red ginger and statistical data analysis.

2.1. EMS Mutagen Preparation and Red Ginger Pre-treatment (*In Vitro*)

The preparation of the EMS solution was carried out according to the method of Taolin *et al*. (2018), starting with the manufacture of 1 M phosphate buffer with a pH of 7. The preparation was carried out with as much as 70 mL of K_2HPO_4 (1M) mixed with 20 mL of KH_2PO4 (1M). After mixing, the pH of the solution was measured at pH 7. If the pH has not reached a value of 7, then KH_2PO4 is added. After the formation of 1 M phosphate buffer at pH 7, dilution was carried out to obtain a concentration of 0.1M because to dissolve EMS, a phosphate buffer concentration of 0.1M was required. The dilution was carried out by diluting 10 times the concentration of 1M phosphate buffer. Making treatment with 0.5% EMS concentration by taking 0.5 mL of EMS and calibrating it to 5 mL with the addition of pH 7 phosphate buffer. Pre-treatment was carried out according to the modification of Pawar *et al.* (2010) by dipping into 0.5% EMS mutagen which had been previously prepared with 45 minutes and 90 minutes of immersion time.

2.2. Initiation and Callus Induction of Red Ginger (*In Vitro*)

The initiation and induction of red ginger were carried out according to the method of Ibrahim *et al.* (2010). The meristem tissue derived from red ginger rhizome shoots was cleaned and sterilized with Masalgin 50 WP (fungicide) and Starner 20 WP (bactericide) as much as 0.2 g/100 mL for 120 minutes, then transferred to a 70% alcohol solution for 5 minutes and 0.2% $HgCl_2$ for 5 minutes. Then they were transferred to a commercial sodium hypochlorite (NaClO) solution (Bayclin) 30% for 8 minutes, after which the explants were rinsed with sterile distilled water for 3 minutes. After that, the explants (meristems) were cultured into culture bottles containing MS medium (Murashige & Skoog 1962), which had been sterilized by autoclaving (121^{o}C, 20 minutes), and the medium was measured with a pH meter ranging from 5.6 until 5.8. The explants at each concentration combination were cultured into 10 culture bottles and contained 3% sucrose, 1% agar, 100 mg/L L-glutamine, and growth regulators with various concentrations of treatment (Table 1) (Ibrahim *et al.* 2010).

Table 1 Growth regulators concentrations

Medium	Growth Regulators Concentrations
B0	-
B1	BAP 3.5 mg/L + 2.4-D 1.5 mg/L
B2	BAP 3 mg/L + 2.4-D 1 mg/L
B3	BAP 2.5 mg/L + 2.4-D 0.5 mg/L

B0: without growth regulators, B1: (BAP 3.5 mg/L and 2.4-D 1.5 mg/L), B2: (BAP 3 mg/L and 2.4-D 1 mg/L), and B3: (BAP 2.5 mg/L and 2.4-D 0.5 mg/L).

After completion, the explants were incubated at 25-27^{o}C in a dark environment for 8 weeks until callus grew and then checked at 1 until 2 weeks to check for contamination or infection in the culture media.

2.3. EMS Mutagen Preparation and Red Ginger Pre-treatment (*Ex Vitro)*

The preparation of the EMS solution was carried out according to the method of Taolin *et al*. (2018), starting with the manufacture of 1 M phosphate buffer with a pH of 7. The preparation was carried out with as much as 70 mL of K_2HPO_4 (1M) mixed with 20 mL of KH_2PO_4 (1M). After mixing, the pH of the solution was measured at pH 7. If the pH has not reached a value of 7, then KH_2PO_4 is added. After the formation of 1M phosphate buffer at pH 7, dilution was carried out to obtain a concentration of 0.1M because to dissolve EMS, a phosphate buffer concentration of 0.1M was required. The dilution was carried out by diluting 10 times the concentration of 1M phosphate buffer.

Preparation of a 0.15% EMS solution was carried out according to the modification of Pawar *et al*. (2010) by diluting 2 mL of pure EMS dissolved in 1.3 L of phosphate buffer pH 7. After completion, meristem tissue derived from red ginger rhizome shoots was cleaned and pre-treated. Pre-treatment was carried out with red ginger explants dipped in 0.15% EMS mutagen, which had been prepared previously with 0, 8, 10, and 12-hour immersion times.

2.4. Acclimatization of Pre-treated Red Ginger Plants

Acclimatization of pre-treated red ginger plants is carried out in the Faculty of Biotechnology's greenhouse. The first stage involves preparing planting media by combining burnt husks with fertile soil (1:1). Polybags are filled with mixed planting media. Treated red ginger shoots are planted in 10 polybags for each immersion treatment that already contains

the previously prepared planting media. Post completion, shoot height and leaf mutations were observed.

2.5. DNA Extraction and Isolation

The stages of DNA extraction and isolation were carried out using the plant genome Tiangen Plant Genomic DNA kit (Cat. number DP305-3). First, samples of red ginger leaves that had been acclimatized 30 days after each treatment were taken to yield 100 mg of plant leaf samples, then ground in a mortar and pestle with liquid nitrogen added. After the plant samples were ground, 700 µl of 65°C pre-heated GP1 (β-Mercaptoethanol (β-ME) was added to Buffer GP1 before use. The final concentration of β-ME was 0.1%) to the powdered plant tissue, vortexed for 10–20 seconds to mix, then incubated for 20 minutes at 65°C, mixed by inverting the tube several times.

In the next step, 700 µl of chloroform was added, then mixed by inverting the tube several times and centrifuged for 5 minutes at 13.400×g. The supernatant aqueous phase was transferred to a new tube, 700 µl of buffer GP2 was added, then mixed by inverting the tube several times. Furthermore, all the mixtures from the previous stages are pipetted into the Spin Column CB3 (the Spin Column CB3 is placed in the Collection Tube first). The CB3 cover was closed and centrifuged for 30 s at 13.400×g. The filtrate was discarded, and the spin column was placed by CB3 into the collection tube.

In the next step, 500 µl of buffer GD was added and centrifuged at 13.400×g for 30 seconds, then the filtrate was discarded and the Spin Column CB3 was put back into the collection tube. A total of 600 µl of PW buffer was added to Spin Column CB3 and centrifuged for 30 seconds at 13.400×g. Then flow-through was removed, Spin Column CB3 was placed back into the collection tube, and the cycle was repeated once. The Spin Column CB3 was placed back into the Collection Tube and centrifuged for 2 min at 13.400×g, discarding the flow-through. The CB3 cover is opened and left at room temperature for a while to dry the membrane. The CB3 membrane was then loaded with 100 µl of buffer TE, incubated for 2-5 minutes at (15 until 25°C), and centrifuged for 2 minutes at 13.400×g to be eluted.

2.6. Quantitative and Qualitative Testing of DNA Isolation

Visualization and quantitative testing of DNA isolation results were carried out according to modifications Istiqomah *et al.* (2016). Visualization was carried out by means of electrophoresis (BioRad) and 2% agarose gel. In each well, the agarose gel that has been made is injected with sample and loading dye in a ratio of 5:1 (µl). Then 1 Kb ladder marker (Geneaid, Cat. number DL006) was injected as much as 5 µl. After completion, 80-volt electrophoresis was performed for 60 minutes. Then the reading of the results is done using UV transilluminator. Quantitative DNA testing was carried out using NanoDrop™2000 UV Spectrophotometers to see the DNA concentration.

2.7. Random Amplified Polymorphism DNA (RAPD)-Polymerase Chain Reaction (PCR)

A mutation detection test with RAPD was carried out according to the modification Istiqomah *et al.* (2016). DNA amplification was carried out using the OPA-2 (TGCCGAGCTG) primer with a PCR reaction composition of 10 µl with a 40× cycle, consisting of GoTaq Green Master Mix (GMM) (Cat. number M7121) 5 µl, DNA template 3 µl, Nuclease Free Water (NFW) 1 µl, and primer 1 µl. PCR was performed with pre-denaturation of 94°C for 2 minutes, denaturation of 94°C for 1 minute, primer attachment of 36°C for 1 minute, the extension of 72°C for 5 minutes, and post-extension of 72°C for 5 minutes. Visualization was carried out by

means of electrophoresis (BioRad) and 2% agarose gel. In each well, the agarose gel that has been made is injected with sample and loading dye in a ratio of 5:1 (µl). Then 1 Kb ladder marker (Geneaid, Cat. number DL006) was injected with as much as 5 µl. After completion, 80-volt electrophoresis was performed for 90 minutes. Then the reading of the results is done using a UV transilluminator.

2.8. Cluster Analysis of Treated Red Ginger

Analysis of genetic relationships in a plant can be determined based on the genetic similarity between individuals was carried out according to the modification Istiqomah *et al.* (2016). The genetic relationship between the mutant red ginger plant treatments was carried out by cluster analysis in the form of a dendrogram using the Unweighted Pair Grouping Method with Arithmetic Averaging (UPGMA) software with FreeTree and TreeView X software. The electrophoresis results were converted into binary data with a scoring system with a value of 1 for existing bands and a value of 0 for those that do not produce bands. Then this binary data will be processed in FreeTree to obtain a dendrogram, which is visualized in TreeView X software.

2.9. Statistical Analysis

The data were statistically analyzed with the IBM Statistical Package for the Social Sciences (SPSS) to determine the effect of duration on callus growth and lethality. The analysis was carried out using the Kruskal-Wallis test for *in vitro* data. If there was a significant effect ($P<0.05$), then it was continued with the *Stepwise step-down* test at a 95% significance level. ANOVA test for *ex vitro* if there is a significant effect ($P<0.05$), then continued with *Duncan's* post hoc test at a 95% significance level.

3. RESULTS AND DISCUSSION

3.1. Picture and Table

Table 2 Comparison of sterilization agents on the level of contamination

Sterilization agents	Contamination rate
NaClO	100 ± 0^{a}
$HgCl_2$	16.67 ± 19.24^{b}

The post hoc test was conducted using the *Mann-Whitney* method. Different letters showed significant difference between samples.

Table 3 Lethal dose of red ginger callus results

Parameter	Immersion Time		
	0 (Control)	45 Minutes	90 Minutes
Lethality rate	0 ± 0^{a}	16.67 ± 19.24^{a}	66.67 ± 23.57^{b}

The post hoc test was conducted using the *Stepwise step-down* method. Different letters showed significant difference between samples.

Table 4 Red ginger callus growth rate

Parameter	Immersion Time		
	0 (Control)	45 Minutes	90 Minutes
Callus growth rate	0 ± 0 [a]	25± 31.91 [a]	0 ± 0 [b]

The post hoc test was conducted using the *Stepwise step-down* method. Different letters showed significant difference between samples.

Table 5 Shoot growth length of EMS- treated red ginger results

Parameter	Immersion Time			
	0 (Control)	8 hours	10 hours	12 hours
Shoot growth lengths	33.18 ± 1.24 [b]	24.65 ± 1.74 [a]	23 ± 2.01 [a]	19.17± 0.74 [a]

The post hoc test was conducted using Duncan's method. Letters differences represent a significant difference between samples.

Tabel 6 Concentration and purity of the isolated genomic DNA results

EMS Immersion Treatment (Hours)	Concentration (ng/µl.)	$A_{260/280}$	$A_{260/230}$
0 (Control)	161	1.41	0.72
8	23.3	2.03	0.49
10	25.2	2.04	0.53
12	18.7	2.19	0.38

Table 7 RAPD primer optimization for red ginger

Primer code	Total number of bands
OPA-13	0
OPA-11	0
OPA-2	4
OPA-1	2
OPA-9	3
OPA-7	1
OPA-16	2
OPN-19	0
OPE-19	1

Table 8 RAPD Analysis of pre-treated red ginger

Primer code	Samples (Hours)	Number of bands			Polymorphism rate (%)
		Total	Monomorphic	Polymorphic	
OPA-2	8	5	3	2	40
	10	6	3	3	50
	12	8	3	5	62.5

Table 9 Similarity matrix of EMS treated red ginger.

	8 hours	10 hours	12 hours
8 hours	1.0		
10 hours	0.57	1.0	
12 hours	0.30	0.27	1.0

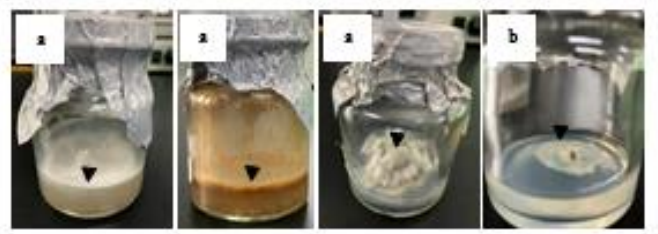

Figure 1 Contamination of media. (a) multilevel NaClO sterilization and (b) $HgCl_2$ 0.2% sterilization. ▲ indicates the contamination.

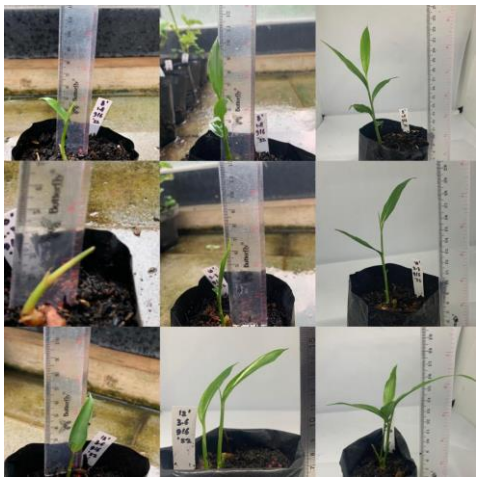

Figure 2 Shoot Stages of growth EMS treated red ginger (a) 8 Hours, (b) 10 Hours, and (c) 12 Hours.

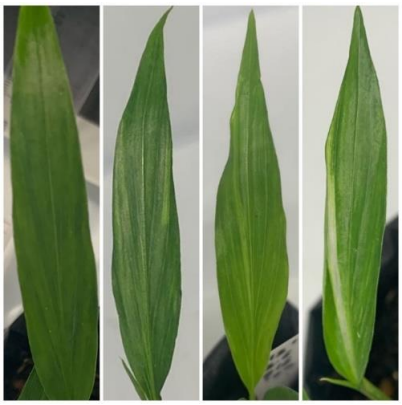

Figure 3 Leaves mutation stages of EMS-treated red ginger plants. (a) 0 Hours (Control), (b) 8 Hours, (c) 10 Hours, and (d) 12 Hours. ▲ indicates leaf mutations (LM).

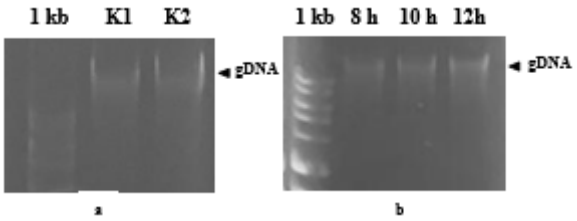

Figure 4 Isolated red ginger DNA. (a) 0 (control) and (b) 8, 10, and 12 hours EMS immersion. ▲ indicates genom DNA.

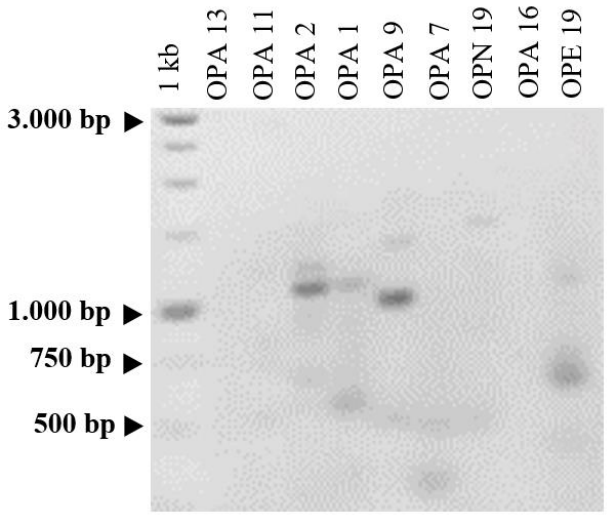

Figure 5 RAPD-PCR visualization of primer optimization for red ginger.

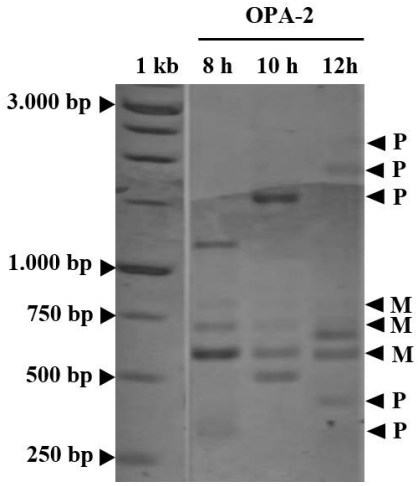

Figure 6 RAPD-PCR visualization of treated red ginger. ▲ indicates polymorphic bands (P), monomorphic bands (M)

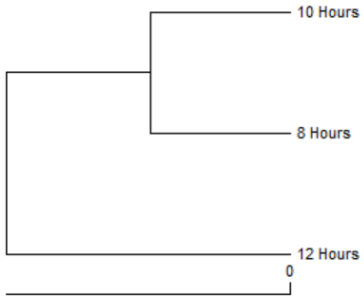

Figure 7 Dendrogram of cluster analysis among treated EMS red ginger.

3.2 RESULT

3.2.1 Comparison of Surface Sterilization Agents on The Level of Contamination

A comparison of surface sterilization with $HgCl_2$ and NaClO on the level of contamination is shown in Figure 1. A comparison of the results of media contamination levels is shown in Table 2. The result showed that the level of contamination had a significant effect on the media (P:0.00, Mann-Whitney test; Table 2).

3.2.2 Lethality Rate of Red Ginger Callus (*In Vitro*)

The lethality rate of red ginger callus obtained revealed that the level of lethality had a significant effect on the immersion time of EMS. (P:0.015, Kruskall-Wallis test followed post hoc test *Stepwise step-down*; Table 3). The mutation rate increased linearly with the lethality rate in explants as the EMS immersion time increased. The results of the lowest lethality rate were then obtained after 45 minutes of immersion.

3.2.3 Morphological Characteristics of EMS-treated Red Ginger (*Ex Vitro).*

The morphological characteristics of EMS-treated red ginger revealed that the growth rate of shoots had a significant effect on the immersion time in the EMS solution (P:0.050) (Table 5). The longer the immersion time in the EMS solution, according to observations made 30 days after planting, resulted in morphological structural changes in shoot lengths and increased leaf mutations (Figures 2 and 3).

3.2.4 Isolated DNA genomic of red ginger and EMS-treated red ginger

The results of DNA isolation from control and EMS-treated red ginger are shown in Figure 4. The gel electrophoresis visualization revealed that the control and treatment samples contained isolated DNA with the production of an unfragmented band. The quantitative DNA test analysis showed that all treated ginger samples had an $A_{260/280}$ ratio number higher than 2. In all samples, the $A_{260/230}$ ratio was less than 2.0 to 2.2. The DNA concentration ranged from 18.7 to 167 ng/µl in all ginger samples (Table 6).

3.2.5 RAPD Analysis of Pre-treated Red Ginger

The optimization results of nine RAPD-PCR primers are shown in Figure 5. The best primer optimization results with the production of 4 bands obtained more OPA-2 primers than other primers (Table 7).

The results of the RAPD-PCR visualization of red ginger treated with EMS with OPA-2 primer were quite diverse, as there were many polymorphic fragments (>40%) (Table 8). The results of DNA amplification with OPA-2 primers on all samples can produce polymorphic and monomorphic fragments and bands. The red ginger sample with EMS immersion for 12 hours had the highest polymorphic percentage (62.5%).

3.2.6 Cluster Analysis of Treated Red Ginger

Based on the dendrogram constructed in treated red ginger (Figure 7), two clusters were indicated; the first cluster was subdivided into two clusters, which had 8-hour and 10-hour treated red ginger. The second cluster, which had a 12-hour treated red ginger, has the lowest similarity to other treated red ginger.

Based on the similarity matrix value of red ginger treated with EMS (Table 9), the highest similarity value is 0.57, which is between 8 hours and 10 hours for mutant ginger samples. While the lowest similarity value is 0.27, which is between 10 hours and 12 hours of mutant ginger samples.

3.3 DISCUSSION

Based on the results obtained, there is a difference in the percentage level of contamination that occurs, in graded NaClO sterilization the results are 100% contaminated on all treatment media (Figure 1), whereas in $HgCl_2$ (0.2%) sterilization the results are 0-33% contaminated media and contamination-free culture 67-100%. According to Khatun *et al.* 2015, showed that $HgCl_2$ was the most effective sterilization treatment out of all the treatments tested with the highest rate of contamination-free culture rate of 86.66%. Sterilization with H_2O_2, NaClO, and Bavistin, on the other hand, was ineffective. (Khatun *et al*. 2015) Although increasing the exposure time and sterilant concentration reduced contamination, it resulted in many damaged explants (Govindaraj *et al*. 2008).

The existence of this contamination (Figure 1) is because in the red ginger rhizome there are endophytic fungi that live in it that are in symbiotic mutualism or neutral with their hosts, so the high percentage of contamination is because the sterilization of the explant surface is not maximal; because the endophytic fungi that live in it are not sterilized (Sari *et al*. 2020). Endophytic fungi on *Zingiber officinale* sp have been isolated and identified with DNA sequencing, and several endophytic fungi on rhizomes have been identified, including *Acremonium sp. 3 JMr5*, *Acremonium sp. 4 JMr7*, *Beltraniella sp. JMr1*, *Colletotrichum sp. 1 JMr2*, *Cylindrocarpon sp. 2 JMr3*, and *Mycelia sterilia 2 JMr4* (Ginting *et al*. 2013)

The percentage of graded NaClO sterilization and $HgCl_2$ sterilization results in a significant difference in contamination (Table 2), the decrease in the percentage of contamination was due to NaClO being able and highly reactive to oxidize protein compounds (Ex: amino acids, amines, and amides) and nucleic acids contained in the molecular structure of microorganisms because it has one chlorine atom (Cl), whereas in $HgCl_2$ it has the same working principle an oxidizing agent but has two Cl atomic ions which have a stronger sterilizing ability when bound to proteins (Yildiz *et al*. 2012; Padhi & Singh 2017).

In this research the effect of EMS immersion on the lethal dose of explants (Table 3) is the best result showing that EMS immersion for 45 minutes in explants can still grow, while at 90 minutes of immersion most of the explants died with a blackish-brown physical appearance, this is because the increase in immersion time can cause explants lethality. (Qosim *et al*. 2015) From the results of the influence of EMS immersion time on the lethal dose of explants related to the growth of callus explants where from the data obtained (Table 4) the best results of callus growth were obtained at 45 minutes of EMS immersion whereas at 90 minutes of immersion callus did not grow because most of the explants were lethal, so they were not able to grow to form callus. According to this result, *in vitro* studies by Junaid *et al.* (2008) showed that low concentrations of EMS significantly increased callus induction and biomass production; therefore, it could be concluded that EMS exposure could affect plant morphological.

The longer the immersion time in the EMS solution, according to observations made 30 days after planting, resulted in lower morphological structural changes in short lengths and increased leaf mutations (Table 5, Figures 2 and 3). Related to these findings, Pawar *et al.* (2010) investigated the effect of physical and chemical double mutagens (γ rays and EMS) on *Zingiber officinale* Rosc. chlorophyll mutations. Their research a revealed that EMS immersion produced more chlorophyll mutants than γ ray exposure. In comparison, Abdullah *et al.* (2018) conducted another study that supports explaining changes in height and growth of *Zingiber officinale* Rosc. due to mutagen exposure. Mutations caused by exposure include stunted plant growth, shorter plant stature, leaf deformities, and chlorophyll mutations.

Based on the results of the quantitative test of red ginger plant DNA isolation (Table 6). The $A_{260/280}$ ratio value was greater than 2.0 in all mutant ginger samples (8, 10, and 12 hours), but less than 2.0 in control red ginger samples. The results of the $A_{260/230}$ ratio value measurement in all samples of red ginger have a ratio value of less than 2.0 to 2.2. The DNA concentration results in all samples were good, with a concentration range of 18.7 to 167 ng/µl. According to Istiqomah *et al.* (2016), the comparison value of $A_{260/280}$ indicates the quality of the DNA obtained because nucleic acids and proteins have maximum absorbance at wavelengths of 260 and 280 nm. A good $A_{260/280}$ ratio value is between 1.8 and 2.0, while a good $A_{260/230}$ ratio value is between 2.0 and 2.2. According to William *et al.* (1997), the presence of phenolic residues during the DNA extraction process can cause a low $A_{260/230}$ value.

According to the quantitative DNA test results, the control and treatment samples contained isolated DNA with a thin band but were not fragmented. According to Harahap (2017), the thickness of the resulting bands can be caused by many factors, such as the amount of DNA isolated completely, the higher the concentration and the thicker the bands produced during the visualization process, and vice versa. Another factor that can affect the amount of movement is the amount of movement activity when injecting the sample into the well that is not evenly distributed.

The results of the RAPD-PCR visualization of red ginger treated with EMS with OPA-2 primer were quite diverse (Figure 6), as there were many polymorphic fragments (>40%)

(Table 8). The results of DNA amplification with OPA-2 primers on all samples can produce polymorphic and monomorphic fragments and bands. The red ginger sample with EMS immersion for 12 hours had the highest polymorphic percentage (62.5%) with 5 polymorphic bands compared to other treatment samples. Many bands and high polymorphism can be suspected as an influence of the given EMS mutagen. According to Primrose & Twyman (2006), the more polymorphic bands are produced, the more genetically diverse. Many factors can contribute to genetic diversity, one of which is the influence of mutations (Istiqomah *et al.* 2016).

The results obtained are in accordance with the research of Istiqomah *et al*. (2016). The percentage of polymorphic in three varieties of ginger using RAPD was 86.9%. According to Palai *et al*. (2007), the genetic relationships provided by RAPD markers provide an effective method for genetic diversity studies. According to Ashraf *et al.* (2014), RAPD analysis has been proven to be useful in distinguishing between closely related species.

Based on the dendrogram constructed in red ginger mutants (Figure 7), two clusters were indicated; the first cluster was subdivided into two clusters, which had 8-hour and 10-hour mutants. The second cluster, which had a 12- hour mutant, has the lowest similarity to other mutants. According to Nei (1987), the value of diversity genetics ranged from 0.1 to 0.4, which is classified as low; 0.5-0.7, which is classified as medium; and 0.8-1.0, which is classified as high. The similarity matrix value of red ginger treated with EMS (Table 9), the highest similarity value is 0.57, classified as medium, which is between 8 hours and 10 hours for mutant ginger samples. While the lowest similarity value is 0.27, classified as low, which is between 10 hours and 12 hours of mutant ginger samples. The low similarity and distant relationship between EMS-treated red ginger can be suspected as an influence of the given EMS mutagen. According to Sulistyawati & Widyatmoko (2017), Several factors influence a population's genetic structure, including environment, natural conditions, population size, and natural selection. Furthermore, mutations, gene flow, and genetic drift have all an impact on population genetic diversity.

4. CONCLUSIONS

This study showed that the *in vitro* and *ex vitro* results of the effect of EMS immersion time on changes in plant morphological characteristics showed that the longer the EMS immersion, the lower the plant shoot height, and the higher the mutation changes in the leaves compared to control plants, and the lethality rate increased. Then the results of the molecular analysis showed that 12 hours of EMS-treated red ginger had a distant relationship with low similarity to another red ginger mutant, which means that many induction mutations occur. The results of research studies show that EMS could induce mutations that result in the formation of a new mutant red ginger with new characteristics. The EMS mutagen showed the potential to be used in ginger breeding for further research.

ACKNOWLEDGEMENT

The author's gratitude goes to PT. Indofood Sukses Makmur Tbk. as the party who provided financial assistance for the continuation of this research through the Indofood Research Nugraha 2021 program.

References

Aditama LG, Yulianto E, Wilopo. 2015. Pengaruh produksi dan nilai tukar terhadap volume ekspor (Studi pada Volume Ekspor Jahe Indonesia ke Jepang Periode 1994-2013). JAB. 25(1): 1-9.

Aidah SN. 2020. Ensiklopedi jahe: deskripsi, filosofi, manfaat, budidaya, dan peluang bisnisnya. Yogyakarta (ID): KBM Indonesia.

Ashraf K, Ahmad A, Chaudhary A, Mujeeb M, Ahmad S, Amir M, Mallick N, 2014. Genetic diversity analysis of *Zingiber officinale* Rosc. by RAPD collected from subcontinent of India. J Biological Science. (2014)21, 159-165.

Djadja SHH. 2004. Kultur *in vitro* eksplan rimpang *Zingiber zerumbet* van *aromaticum* Val. J Biologi. 7(3): 1-9.

Fauzi YF, Nurcahyanti SD. 2020. Ketahanan tiga klon jahe (*Zingiber officinale* Rosc.) terhadap penyakit layu bakteri (*Ralstonia solanacearum*). J Proteksi Tanaman Tropis. 1(2): 62-69.

Ginting, R., Sukarno, N., Widyastuti, U., Darusman, L. And Kanaya, S. 2013. Diversity of Endophytic Fungi from Red Ginger (*Zingiber officinale* Rosc.) Plant and Their Inhibitory Effect to *Fusarium oxysporum* Plant Pathogenic Fungi. *HAYATI*, [online] 20(3), pp.127-137.

Govindaraj, S., Kumari, B., Cioni, P. and Flamini, G., 2008. Mass propagation and essential oil analysis of *Artemisia vulgaris*. J *Bioscience and Bioengineering*, [online] 105(3), pp.176-183.

Harahap AS.2017. Uji kulitas dan kuantitas DNA beberapa pohon kapur Sumatra. J Anima Science and Agronomy. 2(2): 1-6.

Ibrahim MSD, Rostiana O, Khumaida N. 2010. Pengaruh umur eksplan terhadap keberhasilan pembentukan kalus embriogenik pada kultur meristem jahe (*Zingiber officinale* Rosc). J Littri, 16(1):37-42.

Istiqomah CRP, Pancasakti H, Kusdiyantini E. 2016. Keragaman genetic jahe (*Zingiber officinale* Roscoe) menggunakan teknik penanda molekuler *Random Amplified Polymorphic* DNA (RAPD). J Biologi. 5(2): 87-97.

Junaid, A., Mujib, A. and Sharma, M., 2008. Effect of growth regulators and ethylmethane sulphonate on growth, and chlorophyll, sugar and proline contents in *Dracaena sanderiana* cultured in vitro. J *Biologia plantarum*, [online] 52(3), pp.569-572.

Khatun, M. M., Tanny, T., Razzak, A. M., Alam, M. F., Uddin, M. E., Amin, R. and Yesmin. S. 2015. Standardization Of In Vitro Sterilization Procedures For Micropropagation Of Ginger (*Zingiber Officinale* Rosc.). *International Journal of Applied Biology and Pharmaceutical Technology*, [online] 7(1), pp.131-36

Kumari M, Kumar M, Solankey SS. 2020. *Zingiber officinale* roscoe: ginger. Di dalam: Novak J, Blüthner, WD, editor. Medicinal, aromatic, and stimulant plants. Switzerland (EU): Springer. Hlm 605- 622.

Nei, M. (1987). Molecular Evolutionary Genetics. New York: Columbia University Press.

Palai, S. K., & Rout, G. R. (2007). *Identification and genetic variation among eight varieties of ginger by using random amplified polymorphic DNA markers. Plant Biotechnology, 24(4), 417–420.*

Pawar NV, Nimbalkar M, Pai SR, Kolar F. 2010. RP-HPLC analysis of 6-gingerol and assessment of antioxidant activitivies in EMS treated ginger. J Phytology. 2(3): 13-23.

Pawar NV, Nimbalkar M, Pai SR, Kolar F, Dixit G. 2010. Induction of chlorophyll mutants in *Zingiber officinale* Roscoe by Gamma Rays and EMS. J Food Agric. 22(5): 406-411

Pribadi ER. 2013. Status prospek peningkatan produksi dan ekspor jahe Indonesia. J Perspektif. 12(2): 79-90.

Primrose SB, Twyman RM. 2006. Principles of gene manipulation and Genomics 7th Ed. USA: BlackwelL.

Qosim WA, Yuwariah Y, Hamdani JS, Rachmadi M, Perdani SM. 2015. Effect of ethyl methane sulphonate mutagent to shoot regeneration on two genotypes derived from Purwakarta and Pandeglang. J.Hort. 25(1): 9-14.

Rini EP. 2013. Status dan prospek peningkatan produksi dan ekspor jahe Indonesia. Bogor (ID): Balai Penelitian Tanaman Rempah dan Obat.

Saputri L, Hastuti ED, Budihastuti R. 2018. Respon pemberian pupuk urea dan pupuk kandang sapi terhadap pertumbuhan dan kandungan minyak atsiri tanaman jahe merah [*Zingiber officinale* (L.) Rosc var. *Rubrum*]. J Biologi. 7(1): 1-7.

Sari M, Sukarno N, Batubara I, Ginting R. 2020. Potency of endophytic fungi isolated from *Zingiber officinale* Roscoe to control pathogenic fungus *Candida albicans* In Vitro. J Sumberdaya HAYATI. 6(1): 26-32.

Silalahi M. 2019. Keanekaragaman tumbuhan bermanfaat di pekarangan oleh etnis sunda di desa Sindang Jaya kabupaten Cianjur Jawa Barat. J Pendidikan Matematika dan IPA. 10(1): 88-104.

Taolin F, Refli, Mauboy R. 2018. Pengaruh konsentrasi dan lama perendaman biji dalam *ethyl methane sulfonate* (EMS) terhadap variabilitas morfologi padi (*Oryza Sativa L*.) varietas lokal Ende. J Biotropikal Sains. 15(3): 57-72.

William W, Karol M, Piotr C, 1997. Effect of pH and Ionic Strength on the Spectrophotometric Assessment of Nucleic Acid Purity. J BioTechniques 22:474-481.

Yildiz M, Fatih OST, Kahramanogullari C, Tuna E. 2012. The Effect of Sodium Hypochlorite Solutions on the Viability and In Vitro Regeneration Capacity of the Tissue. *J Natural Products,* [online] 2(4), pp.328-331

Zuraida AR, Mohd SMA, Erny SMN, Ayu NO, Che RCZ, Pavallekoodi G, Sreeramanan S. 2016. Micropropagation of ginger (*Zingiber officinale* var. rubrum) using buds from microshoots. J Botanica. 48(3): 1153-1158.