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Assessment of genetic diversity in EMS-induced porang (*Amorphophallus muelleri* Blume) revealed by RAPD markers

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Abstract

Increasing genetic diversity of porang through mutation is urgently needed since porang has low genetic diveristy. Therefore, this study aims to increase the genetic diversity of porang using ethyl methanesulfonate (EMS) and analyze the genetic diversity of porang using RAPD markers. Porang explants were subjected to *in vitro* EMS induction at various concentrations (control, 0.1%, 0.2%, 0.3%, 0.4%, and 0.5%) and evaluated after 35 days. DNA amplification was carried out using 20 RAPD primers. Genetic diversity within and between porang populations was analyzed using POPGENE software, while cluster analysis was performed using PAST software to identify genetic diversity patterns. The results showed that EMS mutation induction effectively increased the genetic diversity of porang. Primers OPA-2 and OPA-9 were identified as the most effective primers for detecting genetic diversity resulting from mutation induction. The 0.2% EMS treatment is recommended because it produced the most notable genetic changes compared to the control and shows potential for generating superior mutants to support porang breeding programs.

Introduction

Porang (*Amorphophallus muelleri* Blume) is a tuberous plant belonging to the Araceae family (Wahyudi et al., 2024). Porang tubers are known to contain high levels of glucomannan (Ekowati et al., 2015). The glucomannan content in porang tubers has been reported to be higher than several other *Amorphophallus* species, such as *Amorphophallus variabilis* with a content of 47.56% (Ibrahim et al., 2022), as well as *Amorphophallus paeoniifolius* (Dennst.) Nicolson, which contains only 2.52-3.2% glucomannan (Ekowati et al., 2015). The high glucomannan content of porang highlights its significant potential for further development in the food industry, particularly in the production of porang rice (Azhari et al., 2025), flour, noodles (Kamsiati et al., 2022), as well as syrup and jelly (Sharma & Wadhwa, 2022).

Currently, most porang farmers in Indonesia still rely on vegetative propagation methods using both bulbils and tubers (Yoseva et al., 2022). This propagation system produces offspring that are genetically similar to the parent plant, which may limit the new genetic diversity in porang (Marantika et al., 2025). Limited genetic diversity in porang is generally associated with increased susceptibility to both biotic and abiotic stresses (Salgotra & Chauhan, 2023). Therefore, increasing genetic diversity through mutation induction is an important strategy to broaden the genetic base of crop species, including porang.

Mutagenesis in porang has been successfully achieved through both physical and chemical approaches. Physical mutagenesis in porang using gamma rays, as reported by Wahyudi et al. (2024), affects

morphological traits (number of roots, number of leaves, plant height, and leaf color) as well as anatomical characteristics (stomatal traits and oxalate crystal density). Meanwhile, chemical mutation induction can be performed using oryzalin (Dwiati et al., 2025), colchicine (Suyono et al., 2023; Wahyudi et al., 2025), and ethyl methanesulfonate (EMS) (Poerba et al., 2009). Mutagenic compounds such as colchicine and oryzalin are widely recognized for their ability to induce polyploidy (Rachmatia, 2022; Suyono et al., 2023). However, these approaches primarily modify chromosome number rather than nucleotide sequences. In contrast, EMS induces genetic diversity through base substitutions at the nucleotide level. The use of EMS to generate genetic diversity at the nucleotide level in porang remains underexplored.

Ethyl methanesulfonate can induce point mutations through alterations in G/C (guanine-cytosine) base pairs to A/T (adenine-thymine) in the DNA sequence (Sikora et al., 2011; Dlamini, 2021). Consequently, EMS can damage DNA bases and trigger errors during gene code reading, thereby enabling the generation of broader genetic diversity in plant populations (Sonsan et al., 2023; Türkoğlu et al., 2023). EMS-induced mutagenesis in porang has been reported by Poerba et al. (2009), however the study was limited to observations of morphology and viability, and thus information on genetic diversity at the molecular level remains unavailable. Therefore, this study aims to assess the genetic diversity of porang after EMS mutation induction using RAPD molecular markers.

Materials and methods

1. Sample preparation and *in vitro* propagation

Sample propagation was performed through subculture techniques. Explants were subcultured on Murashige and Skoog (MS) medium supplemented with 2 mg L^{-1} benzyladenine (BA). *In vitro* shoots were removed from the culture bottles and excised approximately 0.5 cm above a Petri dish containing distilled water supplemented with betadine. Subsequently, the explants were transferred onto culture media under sterile conditions. The explant initiation process was conducted in a Laminar Air Flow (LAF) cabinet. Following initiation, the culture bottles were covered with plastic wrap, secured with rubber bands, and incubated at 21 °C under a light intensity of approximately 1000 lux for four months.

2. Porang mutation induction by EMS

Ethyl methanesulfonate (EMS) induction on porang shoots was carried out using semi-solid MS medium sterilized at 121 °C for 30 minutes. Under sterile conditions in LAF cabinet, the medium was allowed to reach a temperature of approximately 50-60 °C, after which EMS was added at concentrations of 0% (control), 0.1%, 0.2%, 0.3%, 0.4%, and 0.5% through a 0.22 µm Millipore filter. The explants were transferred to media containing EMS and incubated at 21°C with a light intensity of ±1000 lux for 7 days. After 7 days of EMS treatment, the explants were transferred to MS media with 2 mg/L BA incubated at a temperature of 21°C with a light intensity of ±1000 lux for 35 days.

3. DNA extraction

About 100 mg of porang leaf tissue was used for DNA extraction. Genomic DNA was extracted using the Tiangen Genomic DNA kit following the manufacturer's instructions. The quality of the extracted DNA was qualitatively verified by electrophoresis using a blueGel™ electrophoresis system on a 1% agarose gel stained with 2 µg mL^{-1} DNA stain. NEXmark 100 bp Plus Blue DNA ladder was used as the standard.

4. DNA Amplification and Visualization

DNA amplification was performed using a Thermal Cycler ELVE-32G with 20 OPA primers (Operon Technologies) (Table 1). The PCR reaction was prepared in a total volume of 10 µL, consisting of 1 µL of DNA template (20 ng µL), 1 µL of forward primer (10 pmol), 1 µL of reverse primer (10 pmol), 3 µL of nuclease-free water, and 5 µL of GoTaq Master Mix. RAPD-PCR amplification was performed with an initial denaturation at 94 °C for 5 minutes, followed by 45 cycles of denaturation at 94 °C for 30 seconds, annealing temperatures were varied each primer for 60 sec (Table 1), the extension for 90 seconds at 72 °C and terminated by final extension for 7 minutes at 72 ℃ (Wahyudi et al., 2020). The amplification products were analyzed by electrophoresis on a 1.5% agarose gel in 20 mL 1× TBE buffer containing 2 µg/ml nucleic

acid stain (NEX View) and visualized using a blueGel™ electrophoresis system with a 100 bp DNA ladder (NEXmark Ladder).

Table 1. DNA sequences, GC content, annealing and melting temperature of OPA primers used in this study

Primer	Sequences ('5-'3)	GC Content (%)	TA (℃)
OPA-01	CAG GCC CTT C	70	34
OPA-02	TGC CGA GCT G	70	38
OPA-03	AGT CAG CCA C	60	33
OPA-04	AAT CGG GCT G	60	33
OPA-05	AGG GGT CTT G	60	34
OPA-06	GGT CCC TGA C	70	33
OPA-07	GAA ACG GGT G	60	31
OPA-08	GTG ACG TAG G	60	36
OPA-09	GGG TAA CGC C	70	35
OPA-10	GTG ATC GCA G	60	31
OPA-11	CAA TCG CCG T	60	34
OPA-12	TCG GCG ATA G	60	32
OPA-13	CAG CAC CCA C	70	35
OPA-14	TCT GTG CTG G	60	32
OPA-15	TTC CGA ACC C	60	32
OPA-16	AGC CAG CGA A	60	36
OPA-17	GAC CGC TTG T	60	33
OPA-18	AGG TGA CCG T	60	34
OPA-19	CAA ACG TCG G	60	32
OPA-20	GTT GCG ATC C	60	34

5. Data analysis

The effectiveness of EMS in inducing mutations in porang was evaluated based on polymorphism, genetic diversity, clustering, and genetic distance analyses. The presence or absence of amplified DNA fragments was assessed using a binary scoring system, where each DNA band detected on the agarose gel for each primer was assigned a score of "1", while undetected bands were assigned a score of "0".

Genetic diversity analysis was conducted using POPGENE. Genetic diversity within the population was assessed using the following parameters: number of alleles (Na), number of effective alleles per locus (Ne), allele frequency (P_i), polymorphic loci (PPL), expected heterozygosity (He), and the Shannon index (I). The Ne value was calculated using the following formula (Crow & Kimura, 1970; Allendorf et al., 2024):

$$Ne = \frac{1}{(1-h)} = \frac{1}{\sum pi^2}$$

where P_i denotes the frequency of allele i at a locus, and $h = 1 - \Sigma p_i^2$ represents heterozygosity at that locus.

The allele frequency (P_i) was calculated using the following formula (Kanaka et al., 2023):

$$pi = \frac{ni}{N}$$

where n_i represents the number of individuals carrying allele i, and N denotes the total number of individuals.

The percentage of polymorphic loci was calculated using the following formula (Kanaka et al., 2023):

$$PPL = \frac{number\ of\ polymorphic\ loci}{total\ locus} \times 100\%$$

Expected heterozygosity (He) refers to the level of heterozygosity predicted based on allele frequency. The expected heterozygosity (He) value was calculated using the following formula (Crow & Kimura, 1970; Allendorf et al., 2024):

$$He = 1 - \sum pi^2$$

The Shannon index is used to measure genetic diversity by considering both the number and relative proportion of alleles. The Shannon index (I) was calculated using the following formula (Kanaka et al., 2023):

$$I = -\sum (piInpi)$$

Genetic diversity among populations was analyzed using POPGENE software version 1.32 based on the parameters of total genetic diversity (HT), genetic differentiation among populations (GST), gene flow (Nm), and genetic distance.

Cluster analysis was conducted using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm and the Jaccard similarity index in PAST software version 4.03. Marker efficiency was evaluated using the parameters Polymorphism Information Content (PIC), Effective Multiplex Ratio (EMR), and Marker Index (MI).

Each primer was analyzed to determine the PIC value, which was calculated using the following formula (Wu et al., 2021):

$$PIC = 2(f)(1 - f)$$

where PIC represents polymorphism information content, f is the frequency of observed band fragments, and $(1 - f)$ is the frequency of band fragments that are not observed.

EMR (Effective Multiplex Ratio) is used to determine the effective ratio between the total number of amplified bands and the number of polymorphic bands. The EMR value was calculated using the following formula (Sharma et al., 2022):

$$EMR = \beta$$

where β represents the total number of bands per primer, and the β value corresponds to the number of polymorphic bands.

Marker Index (MI) was calculated using the following formula (Sharma et al., 2022):

$$MI = PIC \times EMR$$

Marker Index (MI) is a parameter used to evaluate the effectiveness of a primer in detecting genetic polymorphism.

Results and discussion

1. Genetic Diversity of Porang After Mutation Induction with EMS

Induction of mutations using EMS has been proven to increase the genetic diversity of porang. The increased genetic diversity is indicated by the rise in the number of alleles (Na) and the number of effective alleles (Ne) in the treatment group compared to the control group (Table 2). The value of Na increased from 1.2381 to 1.8571. Similarly, the number of effective alleles (Ne) also increased from 1.2019 to 1.5893.

Table 2. Genetic diversity in porang populations after EMS induction

	Control	Treatment
P (%)	23.81	85.71
Na	1.2381 ± 0.4364	1.8571± 0.3586
Ne	1.2019 ± 0.3848	1.5893 ± 0.4025
H	0.1072 ± 0.2001	0.3242± 0.1951
I	0.1524 ± 0.2824	0.4739 ± 0.2603

Note: P (%) = percentage of polymorphic bands; N = number of samples; Na = number of alleles; Ne = effective number of alleles; H = genetic diversity index; I = Shannon's index.

The heterozygosity value (h) also increased from 0.1072 to 0.3242. According to Nei's classification (1973), the heterozygosity value in the control sample was categorized very low (h < 0.30), reflecting a relatively homozygous population condition. The heterozygosity value after mutation induction using EMS remains in the low category (0.3242), however it indicates an increase in genetic diversity. The Shannon index (I) value also increased from 0.1524 to 0.4739. According to the Shannon and Weaver (1997) classification, the Shannon index value in the control sample falls into the low category (< 1.00), indicating the dominance of certain alleles and a very limited level of genetic diversity. The Shannon index value after EMS-induced mutation increased, although it is still classified as low (0.4739), demonstrating an increase in allelic diversity.

Overall, genetic diversity among populations also increased. he analysis of genetic diversity showed that the total genetic diversity (Ht) was 0.2589 ± 0.0267, while the average heterozygosity within populations (Hs) reached 0.2157 ± 0.0217 (Table 3). The relatively small difference between Ht and Hs indicates that most of the genetic diversity is maintained within populations. Approximately 83.03% of the total genetic diversity is distributed within populations, whereas only 16.97% occurs among populations.

Table 3. The Effect of EMS Induction on Genetic Diversity between Porang Populations

Parameters	Value
Ht	0.2598 ± 0.0267
Hs	0.2157 ± 0.0217
Gst	0.1669
Nm	2.4964

Note: Ht = total genetic diversity; Hs = within-population heterozygosity; Gst = coefficient of genetic differentiation among populations; Nm = gene flow.

An increase in genetic diversity was also reflected by the coefficient of genetic differentiation among populations (Gst), which was 0.1669, indicating that 16.69% of the variation occurs among populations, while the remaining 83.31% is found within populations. The relatively high Gst value indicates substantial genetic differentiation among populations. The gene flow value (Nm) was 2.4964, indicating a relatively high level of gene flow among populations.

Ethyl methanesulfonate (EMS) is an effective mutagen that induces point mutations by altering DNA bases from G/C to A/T (Sikora et al., 2011; Savitri & Fauziah, 2018). Therefore, it can cause complementary base mispairing and increase the frequency of gene mutations, thereby enhancing genetic diversity (Sonsan et al., 2023). A similar phenomenon was reported by Wahyudi (2020), where EMS was able to increase the genetic diversity of *Glycine max* (L.) Merr., as detected using RAPD molecular markers. In addition, EMS has been widely used as an agent for inducing genetic variability in several other plant species, such as *Capsicum frutescens* L. (Dwinianti et al., 2019), *Neolamarckia cadamba* (Roxb.) Bosser, and *Leucaena leucocephala* (Lam.) de Wit (ZakyZayed et al., 2014). Overall, these findings highlight the potential of EMS as an effective mutagenic agent for enhancing genetic diversity.

2. Clustering analysis of porang after EMS mutation induction

Cluster analysis of porang after EMS mutation induction indicates that EMS treatment can increase the genetic diversity of porang. The clustering analysis generated six clusters (Figure 1), with treatments E1 (0.4%) as well as B2 and B1 (0.1%) forming clusters I, II, and III, which were separated from other concentration groups with a similarity value of approximately 0.56, indicating that EMS induction effectively increased the genetic diversity of porang. A similar pattern was observed in the 0.2% EMS treatment (C1 and C2), which was separated from the other clusters into clusters IV and VI with a similarity value of approximately 0.60–0.67, indicating a high level of genetic diversity. However, several treatments at 0.3% (D1, D2, and D3), 0.4% (E2), and 0.5% (F1 and F2) were grouped in the same cluster as the control with a similarity value of approximately 0.77, indicating a high level of genetic similarity.

The similarity values between the control group (A1–A3) and the 0.1% EMS treatment group (B1–B2) ranged from 0.31 to 0.63, indicating very high genetic diversity in porang. Meanwhile, the EMS 0.2% treatment group (C1–C2) showed higher similarity values, ranging from 0.50 to 0.69. At an EMS concentration of 0.3% (D1–D3), the similarity values further increased, ranging from 0.63 to 0.82. This Jaccard similarity pattern indicates that an increase in EMS concentration is inversely proportional to the level of induced genetic change. In the 0.4% EMS treatment group (E1–E2), the similarity values varied

widely, ranging from 0.33 to 0.86. The similarity values increased again in the 0.5% EMS treatment group (F1–F2), indicating a high level of genetic similarity compared to other treatment groups, with values ranging from 0.73 to 0.91. Overall, similarity values tended to increase with increasing EMS concentration. This pattern may be attributed to the use of RAPD markers, which employ random primers; therefore, amplification can occur at multiple genomic locations simultaneously and is dominant, resulting in polymorphic DNA band patterns that may be inconsistent (Probojati et al., 2019). However, the 0.2% EMS treatment group (C1 and C2) was considered the most optimal treatment, as it showed clear cluster separation from the control group without inducing excessively extreme genetic changes.

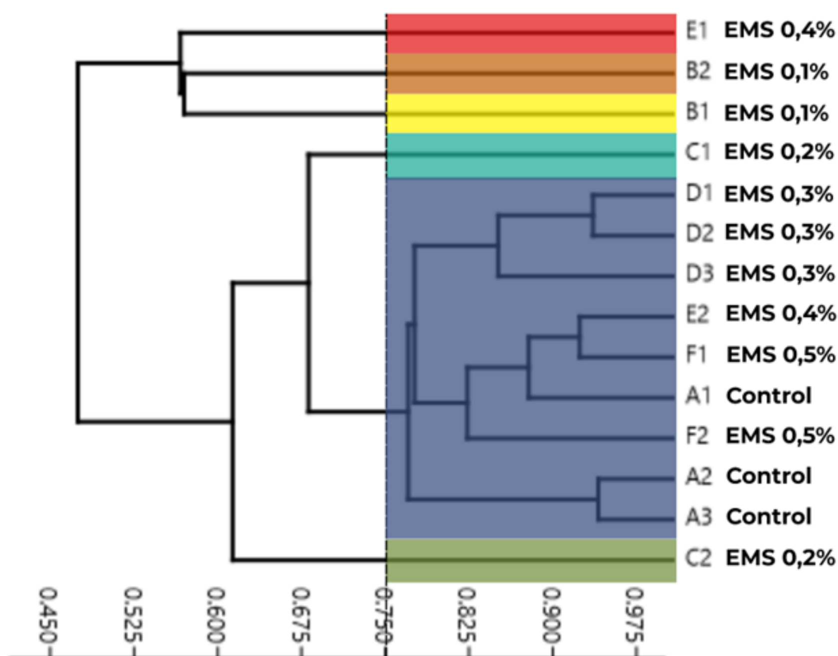

Figure 1. Cluster analysis of porang plants after EMS mutation induction

Table 4. Jaccard similarity coefficient between normal and mutated porang

	A1	A2	A3	B1	B2	C1	C2	D1	D2	D3	E1	E2	F1	F2
A1	1.00													
A2	0.71	1.00												
A3	0.67	0.93	1.00											
B1	0.31	0.56	0.63	1.00										
B2	0.33	0.47	0.53	0.56	1.00									
C1	0.63	0.58	0.63	0.42	0.58	1.00								
C2	0.69	0.53	0.50	0.21	0.44	0.65	1.00							
D1	0.77	0.80	0.75	0.41	0.50	0.71	0.67	1.00						
D2	0.71	0.75	0.71	0.47	0.56	0.76	0.63	0.93	1.00					
D3	0.63	0.76	0.82	0.59	0.67	0.78	0.56	0.81	0.88	1.00				
E1	0.33	0.47	0.53	0.56	0.56	0.58	0.30	0.50	0.56	0.67	1.00			
E2	0.83	0.86	0.80	0.44	0.44	0.65	0.60	0.92	0.86	0.75	0.44	1.00		
F1	0.91	0.79	0.73	0.38	0.39	0.69	0.64	0.85	0.79	0.69	0.39	0.92	1.00	
F2	0.83	0.73	0.80	0.44	0.44	0.65	0.60	0.79	0.73	0.75	0.44	0.85	0.77	1.00

Note: A1: Control (A1-A3) EMS 0.1% (B1-B2), EMS 0.2%(C1-C2), EMS 0.3% (D1-D3): EMS 0.4% (E1-E2), EMS 0.5% (F1-F2).

3. Evaluation of RAPD Marker Efficiency

Six of the 20 primers were found to successfully amplify polymorphic DNA bands (Table 5). OPA-2, OPA-9, OPA-11, and OPA-13 produced the highest number of DNA bands (four bands each), while OPA-

3 produced the lowest number of DNA bands (two bands). The total number of bands consisted of 17 polymorphic bands (80.95%) and 4 monomorphic bands (19.05%). Analysis of several primers indicated that primer effectiveness in detecting mutations is not determined only by the number of bands produced, but also by its ability to show differences in DNA band patterns between control and treatment samples. OPA-11 showed a high total number of bands, a high level of polymorphism, and high informative parameter values, including PIC, EMR, and MI (Table 5 and Figure 2C). However, the DNA band patterns produced by OPA-11 could not clearly distinguish between control and treatment samples, unlike those produced by OPA-2 and OPA-9 (Figures 2A and 2B).

OPA-9 and OPA-2 produced clearer and more consistent DNA band patterns. These primers showed differences in band profiles, where the DNA band at the 2000 bp locus was absent in all control samples and in several treatment samples (B1, E2, F1, and F2). Meanwhile, other treatment samples (B2, C1, C2, D1, D2, D3, and E1) showed diversitys in DNA band patterns (Figure 2B). A similar trend was also observed with the OPA-2, in which the DNA band at the 1000 bp locus was not amplified in all control samples (A1–A3) and several treatment samples (C2, D1, E2, F1, and F2), but appeared in other treatment samples (B1, B2, C1, D2, D3, and E1). The appearance or loss of DNA bands following EMS mutation induction is thought to result from random point mutations in nucleotide sequences, leading to changes in amplification patterns and generating diversity in DNA bands detected by the marker (Shah et al., 2016). In addition, EMS induction may influence primer annealing efficiency, resulting in the disappearance of existing bands or the emergence of new bands due to altered amplification patterns (Poerba et al., 2009; Suteja et al., 2019). Therefore, primers OPA-2 and OPA-9 are recommended as potential biomarkers for evaluating the success of EMS mutation induction in porang.

Table 5. Polymorphism analysis of RAPD molecular markers

No	Primer	TNB	NPB	PB (%)	PIC	EMR	MI
1	OPA-2	4	3	75	0.31	2.25	0.71
2	OPA-3	2	2	100	0.29	2.00	0.58
3	OPA-9	4	4	100	0.33	4.00	1.33
4	OPA-11	4	4	100	0.43	4.00	1.73
5	OPA-13	4	4	100	0.27	4.00	1.07
6	OPA-16	3	0	0.0	0.00	0.00	0.00
	Total	21	17	475.00	1.64	16.25	5.42
	Mean	3.50	2.83	79.17	0.27	2.71	0.90

Note: total number of bands (TNB), number of polymorphic bands (NPB), polymorphic band percentage (PB%), polymorphic information content (PIC), effective multiplex ratio (EMR), marker index (MI).

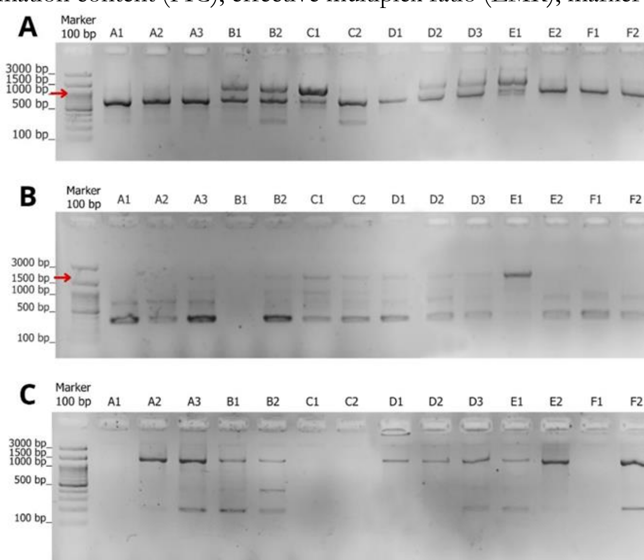

Figure 2. Polymorphic bands after mutation induction using EMS. (A) OPA-2 (B) OPA-9 (C) OPA-11. Arrows indicate polymorphic bands where bands appear in some loci but do not appear in others. A1: Control (A1-A3) EMS 0.1% (B1-B2), EMS 0.2%(C1-C2), EMS 0.3% (D1-D3): EMS 0.4% (E1-E2), EMS 0.5% (F1-F2)

Mutation induction using EMS in porang results in the formation of new alleles and alterations in DNA banding patterns, reflecting an increase in genetic diversity at the molecular level. This result is consistent with the findings of Wahyudi et al. (2020) in soybean, which reported that EMS effectively increase genetic diversity as detected by RAPD molecular markers. Diversitys in DNA banding patterns observed in mutant plants indicate that random point mutations induced by EMS can affect genome structure through changes in nucleotide sequences, leading to the loss or emergence of specific alleles. At the molecular level, EMS is known to induce G/C to A/T base substitutions, causing errors in complementary base pairing and increasing the frequency of gene mutations (Sikora et al., 2011; Savitri & Fauziah, 2018), thereby confirming the potential of EMS as an effective mutagen.

Conclusion

Mutagenesis using EMS has been shown to effectively increase genetic diversity in porang, as indicated by a 61.9% increase in genetic diversity in EMS-treated porang compared with the control. Primers OPA-2 and OPA-9 are recommended as potential biomarkers for detecting the success of EMS-induced mutagenesis in porang. The 0.2% EMS treatment was identified as the optimal concentration because it produced clear cluster separation from the control group without causing extreme genetic changes, thus showing strong potential as a germplasm source for porang breeding programs.

Author Statements

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Generative AI: The authors affirm that only OpenAI was used in the preparation of this manuscript for language refinement and grammar correction. No artificial intelligence tools were used to generate content or interpret data in this manuscript.

Data availability: The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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