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# *In vitro* Mutation Induction of *Hoya kerrii* with Ethyl Methane Sulfonate and Analysis of DNA Polymorphism using RAPD Markers

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## Abstract

The effects of Ethyl Methanesulfonate (EMS), a known alkylating agent that damages DNA, on the ornamental plant *Hoya kerrii in vitro* have not been extensively studied. **Aim:** The study aimed to evaluate the concentration- and time-dependent effects of EMS on the survival and morphological alterations in *Hoya kerrii* under *in vitro* conditions and to analyze the Deoxyribonucleic Acid (DNA) polymorphism using Random Amplified Polymorphic DNA (RAPD) markers. **Results:** The results indicated that EMS treatment led to several morphological alterations, including callus formation, reduced shoot size, leaf curling, and variations in pigmentation. As the EMS dosage increased, the shoot regeneration rate decreased, reaching its lowest at 0.8% EMS for 60 min (20% regeneration). DNA polymorphism analysis revealed that all 12 RAPD markers used were specific and stable across both replicates, with a Polymorphism Index (PIC) ranging from 0.27 to 0.37. The number of new bands appearing or disappearing increased with higher EMS dosages, resulting in DNA polymorphism rates between 35.16% and 55.16%. Phylogenetic analysis categorized the EMS dosages into three main groups: 0.2% EMS for 30 min clustered with the control group in cluster I, while 0.8% EMS for 60 min formed its distinct cluster (cluster III). Cluster II consists of a separate subcluster containing 0.4% EMS for 30 min, which is distinct from the other dose treatments. These findings suggest that 0.4% EMS for 30 min represents an optimal balance that produces significant genetic and morphological variation while maintaining high regeneration rates, making it the ideal condition.

**Major Findings:** This study provides a helpful reference for EMS optimization and supports the use of RAPD markers for early mutation screening in *Hoya kerrii* and other ornamental plants.

**Keywords:** Ethyl Methane Sulfonate, *Hoya kerrii*, Mutant *in vitro*, Random Amplified Polymorphic DNA

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## 1. Introduction

Hoya is a diverse genus, predominantly epiphytic, belonging to the tribe Marsdenieae (Apocynaceae), and is distributed across tropical and subtropical forests of the Indo-Australian Archipelago[1]. Hoya species are now widespread throughout the world and are regarded as ornamental plants due to their distinctive and lovely leaf and flower forms[2]. One well-liked climbing plant among them is *Hoya kerrii*, which is renowned for its heart-shaped leaves[3], varied flower hues, and distinctive scent[4]. Common hoya species are usually propagated asexually by selecting old leaves or stems with roots to plant in the ground, as they rarely mature enough to harvest seeds in the wild[5]. However, asexual reproduction of hoya plants is not efficient[5], reducing genetic diversity, thereby affecting commercial value and the ability to create new varieties. Since agricultural progress relies on plant breeding, a broad genetic base with valuable traits is essential for sustained improvement[6].

One of the main forces behind species diversity and evolution is mutation, which is a heritable alteration in

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the genetic material of living[7]. Mutation breeding has proven highly effective in enhancing crop traits, offering a faster improvement compared to conventional breeding methods[8]. However, spontaneous mutations occur at very low frequencies in nature, whereas chemical mutagens can cause mutations at higher frequencies[8,9]. Ethyl methanesulfonate (EMS) is an alkylating agent that converts guanine bases (G > A or C > T) in DNA and is commonly used in mutation breeding programs as a highly effective mutagen[10]. Furthermore, the rapid development of *in vitro* propagation techniques for a variety of plant species has made it possible to shorten the generation cycle in vegetatively propagated species and manage large numbers of mutant populations in a small amount of culture space[11]. According to earlier research, higher concentrations of EMS induce greater levels of genotoxic change, thereby increasing the likelihood of genetic variation *in vitro* conditions[12]. Numerous studies have demonstrated that *in vitro* mutation induction through EMS treatment is a feasible method to enhance genetic diversity in a range of ornamental plants, including Curcuma, gloxinia, rose, and African violet, to produce mutant lines with superior traits in terms of growth, morphology, and the aesthetic value of leaves and flowers[9,13-15]. There might be a threshold between mutagenesis duration and concentration, though, because prolonged exposure can cause severe cytotoxicity, reduced survival, and instability in the plant genome[9,16-18]. Nevertheless, *Hoya kerrii in vitro* genotoxic and cytotoxic reactions to EMS exposure have not yet been the subject of any published research.

DNA molecular markers have long helped identify potential crop lines precisely and expand selection opportunities for them[11], as well as in helping to better understand the nature of genetic instability[16]. Even though microsatellite-based techniques (SSR and ISSR) are preferred because of their high information diversity, RAPD is still used because it allows the simultaneous detection of polymorphisms at multiple locations throughout the genome[19]. Moreover, RAPD stands out due to its affordability, speed, ease of use, and lack of requirement for prior knowledge of the gene sequence[20,21]. While locus-specific markers like SSR, which require prior sequence information, and ISSR markers preferentially target regions near microsatellite sites[22], may have difficulty identifying dispersed nucleotide changes throughout the genome, because EMS primarily results in point mutations[23]. While RAPD markers use random primers to amplify different regions of the genome[24], determine the degree of polymorphism by looking at how DNA bands appear, vanish, or change in intensity[25]. This marker has been successfully applied in genetic diversity analysis, especially in early detection and selection of mutations in mutation breeding in various plant species, including Hoya[4,13,26,27]. This study aimed to induce *in vitro* mutations in *Hoya kerrii* using EMS and apply RAPD molecular markers for genetic polymorphism analysis, providing a scientific basis for future experiments.

## 2. Materials and Methods

### 2.1 Materials

Samples of 1-year-old *Hoya kerrii* stem segments with buds that were 2-3 cm in size were cleaned for five minutes under running tap water, surface sterilized for 30 seconds with 70% alcohol, and then rinsed three times with sterile distilled water. The sample was then soaked in 5% NaClO solution with 3 drops of dishwashing liquid added for 15 minutes, washed with sterile distilled water 3-5 times[4]. After sterilization, the shoot samples were cultured in Murashige and Skoog medium (MS)[28] supplemented with 0.8 mg/L NAA + 1.5 mg/L BAP + 1 mg/L $GA_3$, 30 g/L sucrose, and 7 g/L agar[29], for initial culture. The study was conducted at the Plant Gene Technology Laboratory, Institute of Biotechnology and Food Technology, Can Tho University, Vietnam. The culture medium was adjusted to pH 5.7-5.8, dispensed 50 mL into each jar, and sterilized by wet heat autoclaving at 121°C, 1 atm pressure for 15 minutes before use. The tissue culture incubator was maintained at 25 ± 2°C, with a lighting period of 16 h/day and a light intensity of 2000 - 2500 lux.

### 2.2 Methods

#### *2.2.1 Evaluation of Shoot Survival Responses to EMS Dosage*

A 1% EMS stock solution was first prepared using distilled water. This stock was subsequently diluted with 0.1 M phosphate buffer (pH 7.2)[9] to obtain different concentrations of EMS (0%, 0.2%, 0.4%, 0.6%, and 0.8%), followed by sterilization[30]. *In vitro* stem segments were then exposed to the EMS solutions for either 30 or 60 minutes under continuous shaking at 80 rpm[13]. After treatment, the explants were rinsed three times with sterile distilled water, blotted dry with sterile paper[13,31], and transferred to MS basal medium containing 1 mg/L

IBA, 3.5 mg/L BAP, 30 g/L sucrose, and 7 g/L agar[4] for shoot regeneration. The experiment was laid out in a completely randomized design with ten replicates at each of the various EMS treatment doses. Each replication consisted of ten jars, each of which contained ten stem segments. The regeneration rate was determined after three weeks of culture, based on the number of shoot-forming stem segments[26]. One-way ANOVA was used to analyze the data, and Turkey's HSD test was used to compare means at the 5% significance level in Minitab software version 16. To investigate the relationship between EMS dose and regeneration rate, a linear regression model was performed. Regeneration rate was treated as the dependent variable, while treatment duration and EMS concentration were included as the independent variables. Regression coefficient and model significance were assessed at the 5% level using Microsoft Excel version 2013.

### 2.2.2 *Evaluation of Shoot Morphological Responses to EMS Dosage*

Regenerated shoots were observed morphologically after three weeks of *in vitro* culture[26]. Shoot regeneration was classified into three main forms: direct shoot clusters are a collection of shoots that originate directly from the parent tissue without the use of callus tissue as an intermediary; indirect shoot clusters are a cluster of shoots formed from intermediate callus tissue; and single shoots are individual shoots that emerge independently from the explant. In addition, factors such as the appearance of callus, number of shoots per sample, shoot color and size, and leaf shape were recorded to assess the level of shoot differentiation.

### 2.2.3 *Evaluation of DNA Polymorphism by RAPD Markers*

Leaf samples from regenerated shoots at different doses (each dose selected a sample with different morphology compared to the control) were used to extract total DNA by the Cetyltrimethylammonium Bromide method (CTAB)[32]. The qualified DNA was then exposed to 12 RAPD markers for a Polymerase Chain Reaction (PCR), which included: OPA 14, OPB 11, OPC 05, OPF 01, OPF 02, OPF 03, OPF 04, OPG 04, OPK 14, OPS 11, and OPT 05[4], produced at the Phu Sa Biochemical Company, Can Tho City, Vietnam. The components of a PCR reaction include: MasterMix 5X (3µL), primer 10 µL (1.2 µL), Taq DNA polymerase 5 unit/µL (0.15 µL), DNA template 30 ng/µl, and $BiH_2O$ (12.5 µL)[20]. Despite being widely used, RAPD markers have certain drawbacks. One is that they are susceptible to variations in reaction conditions, which may lead to inconsistent results across different labs[19]. PCR amplifications were meticulously standardized and carried out in two separate replicates, each using a different thermal cycler, to reduce the limitations of RAPD analysis. The thermal cycling was performed for a total of 45 cycles including:

(1) denaturation at 94°C for 5 min; (2) denaturation at 94°C for 1 min for 20 s; (3) primer annealing at 35°C for 35 s; (4) annealing at 72°C for 1 min 20 s; (5) stabilization at 72°C for 10 min; (6) storage at 4°C for ∞ time. PCR products were electrophoresed in 2% agarose gel at 50 volts for 60 min using Tris-Borate-EDTA buffer (TBE) and compared with a 100 bp standard ladder (Bioline)[4,14].

Images of DNA bands after electrophoresis were recorded using GelAnalyzer v2019 software[33] and encoded in binary (0 and 1)[14] and genetic grouping, determining the similarity matrix using NTSYS 2.1 software according to the Unweighted Pair Group Method with Arithmetic Mean (UPGMA)[34]. DNA band polymorphisms observed included the disappearance of normal bands and the appearance of new bands in treated samples when compared with control samples[35]. Then, the Polymorphism Information Content (PIC) value of primers[34] and Genomic Polymorphism rates (GP)[16] of each EMS dose is calculated using the following formula:

$$PIC = 2fi \times (1\text{-}fi)$$

$$PG = (a/n) \times 100$$

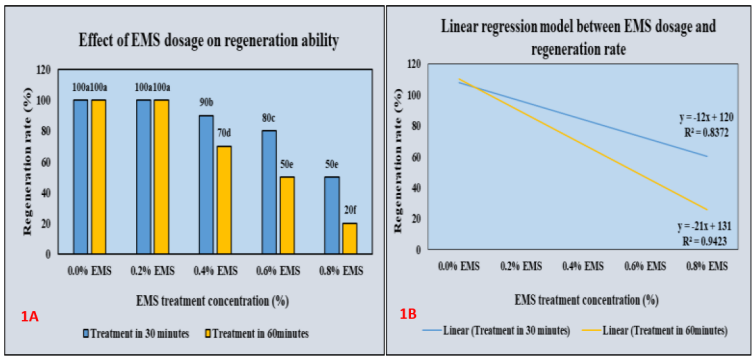
where (i): the locus order is calculated, (fi): the frequency of alleles present, (1-fi): the frequency of alleles absent, (a): the count of polymorphic bands detected in samples treated with EMS, and (n): the total number of bands observed in the control.

## 3. Results and Discussion

### 3.1 Evaluation of the Effect of EMS Treatment Dosage on the Shoot Survival

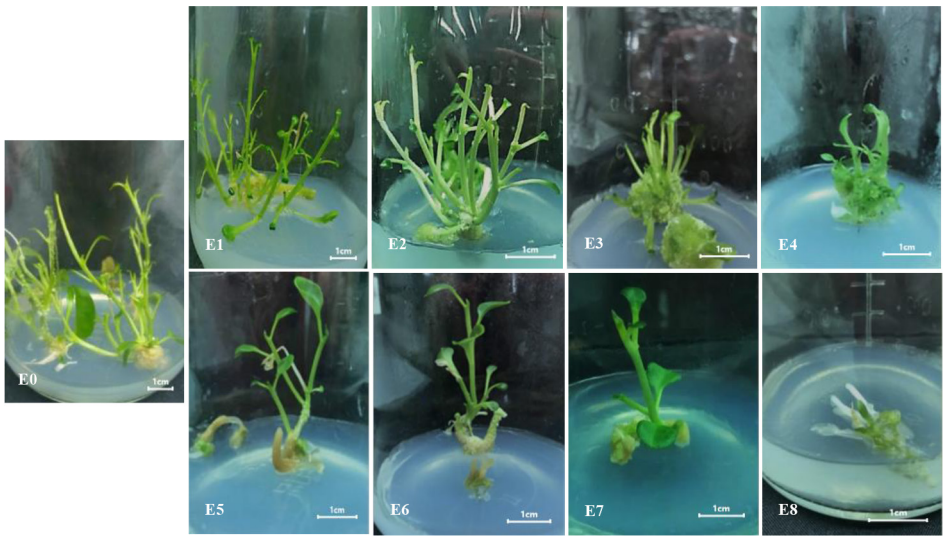
The treatment dose, which includes both concentration and exposure duration, determines the success of chemical mutation induction[16]. There is a strong correlation between EMS dosage and mortality rate[18]. The results of recording the regeneration rate of the stem segment are presented in Figure 1A, showing that at a concentration of

0.2% EMS, the regeneration rate reached 100% at both 30- and 60-min treatment times, equivalent to the control. At concentrations of 0.4, 0.6, and 0.8% EMS, the regeneration rate was maintained at a medium-high level of 90%, 80%, and 50% respectively, after 30 min of treatment, while at the same concentration but increased to 60 min of treatment, the regeneration rate was low, reaching only 70%, 50% and 20% respectively. The regression model analysis results, shown in Figure 1B, indicate that the coefficient of determination $R^2$ reached high values in both equations, reflecting a linear and stable relationship between EMS treatment dosage and regeneration rate. In general, the rate of stem regeneration tended to decrease as the EMS concentration increased, with the decline becoming more pronounced as the EMS treatment time was prolonged. Specifically, the slope of the regression line for the 60-minute treatment was greater with the equation y = -21x + 131, indicating that the rate of decline in the survival rate occurred more rapidly than in the 30-minute treatment with the equation y = -12x + 120. This result is similar to the study on *Saintpaulia* cv. Crystobal, showed that there was no notable difference in shoot survival among the treatment durations of 30, 60, 120, and 240 min at an EMS concentration of 0.2%, with all treatments resulting in a 100% survival rate. However, when increased to 0.6% EMS for 60 min, the survival rate decreased to 50%, and also at the same concentration, when increased to 120 minutes, it led to the complete death of the shoots[9]. The cause may be that EMS damages DNA by adding methyl groups (-$CH_3$) to the oxygen and nitrogen atoms of nucleotide bases, and occurs at multiple locations in the genome, causing cell-death mutations[17].




*Note: In Figure 1A, the mean values following the same letter were not statistically significantly different (p-value < 0.05).*

**Figure 1.** Effect of EMS treatment dosage on the regeneration ability of stem segments.




*Note: E0 = 0% EMS (control); E1 = 0.2% EMS for 30 min; E2 = 0.2% EMS for 60 min; E3 = 0.4% EMS for 30 min; E4 = 0.4% EMS for 60 min; E5 = 0.6% EMS for 30 min; E6 = 0.6% EMS for 60 min; E7 = 0.8% EMS for 30 min; E8 = 0.8% EMS for 60 min. The bar measures 1 cm.*

**Figure 2.** Effect of EMS treatment dosage on shoot morphology of *Hoya kerrii.*

**Table 1.** Effect of EMS on shoot morphology

| Treatment | Regenerated shoot form | Callus | Shoot number | Shoot color | Shoot size | Leaf size | Leaf form |
|---|---|---|---|---|---|---|---|
| E0 (Control) | Direct shoot cluster | No | 6 - 8 | Fresh green | High | Large | Heart |
| E1 | Direct shoot cluster | No | 7 - 10 | Fresh green | High | Very tiny | Pointed |
| E3 | Indirect shoot cluster | Large | 5 - 7 | Green white | Low | Very tiny | Curled |
| E5 | Direct shoot cluster | No | 2 - 3 | Dark green | Medium | Medium | Elongated |
| E7 | Single shoot | No | 1 | Dark green | Medium | Medium | Rounded |
| E2 | Direct shoot cluster | No | 5 - 7 | Fresh green | Medium | Very tiny | Pointed |
| E4 | Indirect shoot cluster | Large | 3 - 4 | Light green | Low | Very tiny | Curled |
| E6 | Single shoot | No | 1 | Dark green | Medium | Medium | Rounded |
| E8 | Single shoot | Small | 1 | Yellow green | Very low | Very tiny | Curled |

*Note: E0 = 0% EMS (control); E1 = 0.2% EMS for 30 min; E2 = 0.2% EMS for 60 min; E3 = 0.4% EMS for 30 min; E4 = 0.4% EMS for 60 min; E5 = 0.6% EMS for 30 min; E6 = 0.6% EMS for 60 min; E7 = 0.8% EMS for 30 min; E8 = 0.8% EMS for 60 min.*

## 3.2 Evaluation of the Effect of EMS Treatment Dosage on Shoot Morphology

Before beginning large-scale mutagenesis, the ideal dose of the mutagen should always be established[15]. Besides the regeneration ability, the morphological characteristics of the shoots are also an indicator of interest in many studies on *in vitro* mutation induction[4,31,36]. The abnormal morphology suggested EMS penetrance[9], leading to phenotypic screening and the use of selective molecular markers at an early stage[26,27]. The morphology of regenerated shoots under various EMS treatments is shown in Figure 2 and Table 1.

The stem segment in the control generated a direct shoot cluster that grew straight from the dormant axillary bud without callus. These shoot clusters ranged from 6 to 8 shoots, were high and fresh green in color, and the size large, heart-shaped leaves. The direct shoot clusters were also observed at 0.2% EMS for 30 min (7-10 shoots) and 0.2% EMS for 60 min (5-7 shoots) doses; the shoots remained fresh green color but slightly reduced in size, the leaves formed were very tiny and pointed (Figure 2E1-E2). Indirect shoot clusters arising from the callus of 0.4% EMS for 30 min (5-7 shoots) had a healthier white-green color than those of 0.4% EMS for 60 min (3-4 shoots), which had a light green color. The shoot cluster size in these two doses was small, with tiny and curly leaves, and callus formation was more dominant than shoot formation (Figure 2E3-E4). Darker green shoots and fewer shoots than the control were detected at doses of 0.6% EMS for 30 min (2-3 shoots), 0.6% EMS for 60 min (1 shoot), and 0.8% EMS for 30 min (1 shoot); shoots and leaves at these doses were of medium size. In contrast to the 0.6% EMS for 30 min treatments, which had an elongated form, the doses of 0.6% EMS for 60 min and 0.8% EMS for 30 min showed a rounder leaf form (Figure 2E5-E7). Yellowish green shoots, very low shoot size, curled leaves, or unclear leaf form were also observed at 0.8% EMS for 60 min (Figure 2E8). The morphological changes observed in the regenerated *Hoya kerrii* shoots in this study are representative of the developmental disturbances induced by the EMS agent under *in vitro* conditions. This result is consistent with the previous finding that EMS induced physiological changes in *Atropa acuminata,* resulting in increased shoot numbers at low concentrations and a decrease as the concentration increased to medium levels[17]. Changes in shoot size, leaf curl, and leaf pigmentation were also observed in cultivars of *Sinningia speciosa* and *Curcuma hybrid* cv. Sweet memory after EMS treatment[13,14]. The abnormal growth-related reactions observed in plants may result from EMS's known effects on physiology, anatomy, biochemistry, and molecular gene expression regulatory processes[37,38]. This disruption may interfere with tissue organization, cell division, and differentiation during regeneration[39]. These mutations may initiate morphometric and reproductive alterations in plants[40].

## 3.3 Evaluation of the Effect of EMS Treatment Dose on DNA Polymorphism using RAPD Markers

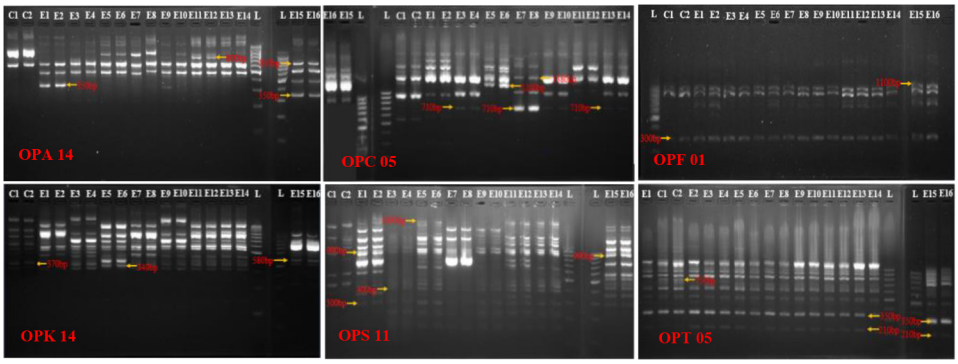
### *3.3.1 RAPD Reproducibility and Amplification Profile*

Underlying DNA sequence differences determine the level of phenotypic variation[38]. However, only a

few mutations manifest in the phenotype in the first generation (M1) because most are recessive mutations and will usually manifest in the next generation[41]. To explore whether morphology variations observed in *Hoya kerrii* under EMS treatment were associated with genetic alterations, RAPD markers were employed to examine DNA polymorphisms in both mutant and control samples. Since RAPD markers are known to be sensitive to PCR conditions, this reproducibility is especially crucial. Carefully standardizing DNA extraction and amplification processes can effectively lessen this restriction[19]. The results in this study indicated that all 12 RAPD markers used were specific, capable of pairing and amplifying products from template DNA, and consistent in two repetitions (Figure 3). An average of 85.72 bands/marker was observed, with the greatest number of DNA bands (167 bands) recorded at primer OPT 05, while the smallest number of bands (16 bands) was recorded at marker OPG 04 (Figure 4A). In comparison to the control sample of 108 bands, the highest number of bands (118) was recorded at 0.4% EMS for 30 min, and the lowest number of bands (93) was recorded at 0.2% EMS for 60 min (Figure 4B). Variation in the number of bands reflects differences in RAPD amplification profiles among treatments, as short sequences of random primers are used to boost the probability, and they can identify homologous sequences that are appropriate for annealing[42]. To further evaluate the informativeness of the RAPD marker used, polymorphic information content (PIC) was determined, as PIC value reflects the effectiveness of a molecular marker in identifying polymorphisms and plays a crucial role in the selection of markers for genetic research[43]. The results presented in Figure 4C show that the PIC values of the different RAPD primers were in the average range, with the highest value in primer OPC 05 (0.37) and the lowest in primer OPF 01 (0.27). The average PIC value range is also widely used in DNA polymorphism analysis in many species[14,25,27].

### 3.3.2 *EMS-induced Genetic Polymorphism*

Newly appearing or disappearing bands were identified to reflect the polymorphism in the genome of the EMS treatment doses compared to the control. The results presented in Figures 5A show that the total number of new bands appearing in the 30 minutes treatment time group was not completely linear with concentration, with 47 bands at 0.2% EMS concentration, increasing to a maximum of 68 bands at 0.4% EMS concentration,




*Note: L = Leader 100 bp; C1 and C2 = 0% EMS (control); E1 and E2 = 0.2% EMS for 30 min; E3 and E4 = 0.2% EMS for 60 min; E5 and E6 = 0.4% EMS for 30 min; E7 and E8 = 0.4% EMS for 60 min; E9 and E10 = 0.6% EMS for 30 min; E11 and E12 = 0.6% EMS for 60 min; E13 and E14 = 0.8% EMS for 30 min; E15 and E16 = 0.8% EMS for 60 min.*

**Figure 3.** Electrophoresis results of PCR products with some RAPD markers.

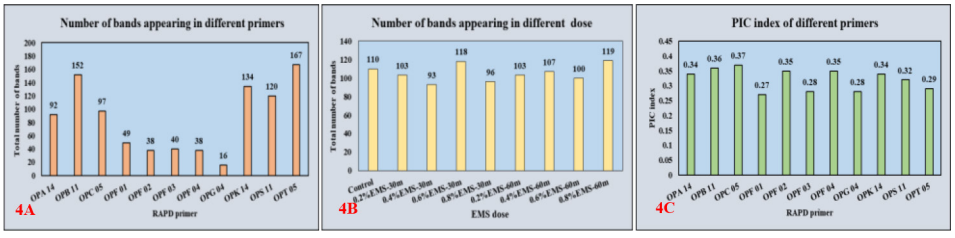



**Figure 4.** PIC results and the number of DNA bands detected in different primers and samples.

then decreasing and stabilizing at 0.6 and 0.8% EMS concentrations (59 and 60 new bands). Meanwhile, in the 60-minute treatment time group, the number of newly appearing bands increased more clearly, from 52 bands at a 0.2% EMS concentration to 85 bands at a 0.8% EMS concentration. New band products demonstrate how specific oligonucleotide priming sites can be altered by mutations, large deletions, and homologous recombination[35]. Many new mutant alleles form a source of genetic diversity, and help study the function of the target gene[6]. Observed DNA alterations could result from EMS-generated point mutations and subsequent frameshifts in coding and non-coding regions[16]. EMS is a well-known alkylating agent, mainly causing G/C to A/T transition mutations during DNA replication by ethylating guanine bases[10]. Similar to the previous trend, the total number of lost bands at both treatment times increased linearly with EMS concentration (Figure 5B). The number of lost bands relative to the control was highest at 0.8% EMS for 60 min (84 bands) and lowest at 0.2% EMS for 30 min (62 bands). The loss of DNA bands may be related to the EMS mechanism of creating strand instability in the DNA, which, during replication, can cause strand breaks; cross-linking between strands of one or different DNA molecules can also cause loss of nucleotides[39].

In fact, the emergence of novel bands can be linked to genetic mutations, whereas their disappearance may result from DNA damage, methylation, or chromosomal alterations, all of which play a role in creating DNA polymorphisms[16]. The results of the polymorphism rate analysis showed that the lowest value was recorded at a dose of 0.2% EMS for 30 min (35.16%), and the highest value was recorded at a dose of 0.8% EMS for 60 min (55.16%) (Figure 5C). Overall, the polymorphism rate tends to increase proportionally with both the concentration and duration of EMS treatment. The results align with previous findings regarding the effects of EMS on genome polymorphism and instability in regenerated wheat and *Musa laterita*[12,38]. Notably, the rate of genomic polymorphism in *Hoya kerrii* was higher than that in *Cordyline fruticosa*, with the maximum rate recorded being 31.8% at 1% EMS for 10 min[44]. However, in this study, no significant differences were detected among the treatments at 0.4, 0.6 (30 and 60 min), and 0.8% EMS for 30 min, all of which exhibited polymorphism rates above 42%. This study suggests that EMS treatment at or above 0.4% EMS for 30 min may approach a saturation threshold for genetic polymorphism in *Hoya kerrii*.

Overall, the observed variation in RAPD banding profiles demonstrates the suitability of RAPD markers for detecting EMS-induced, treatment-dependent genetic changes in regenerated *Hoya kerrii* shoots. These genome-wide sensitivities make RAPD markers invaluable for early mutation screening, as they capture general genomic responses. In contrast, more locus-restricted markers, such as SSRs, are better suited for targeted analyses in subsequent generations[45]. The present findings support previous studies showing the effectiveness of RAPD markers in detecting polymorphism in rose and cowpea affected by EMS mutations[15,40].

### 3.3.3 *Genetic Similarity and Cluster Analysis*

The results of the analysis of genetic similarity coefficients of EMS treatment doses presented in Table 2 show that the similarity coefficients ranged from 0.45 to 0.65 compared to the control. Within the same group of EMS doses, the highest coefficient of similarity of 0.87 was recorded at doses of 0.6% EMS for 60 min and 0.8% EMS for 30 min. If the similarity coefficient is about 0.61, the phylogenetic tree diagram (Figure 6) can be divided into 3 clusters: Cluster I includes the control and the 0.2% EMS for 30 min, with the highest similarity coefficient (0.65), and less variation in morphological characteristics than the other clusters. While the 0.8% EMS for 60 min separates into a separate cluster (Cluster III) with the lowest similarity coefficient when compared to the control sample (0.45), and is characterized by a low regeneration rate, yellowish-green shoots, and weakness. In comparison to the control,

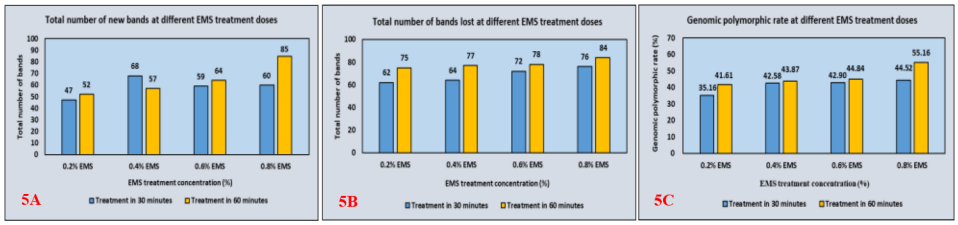
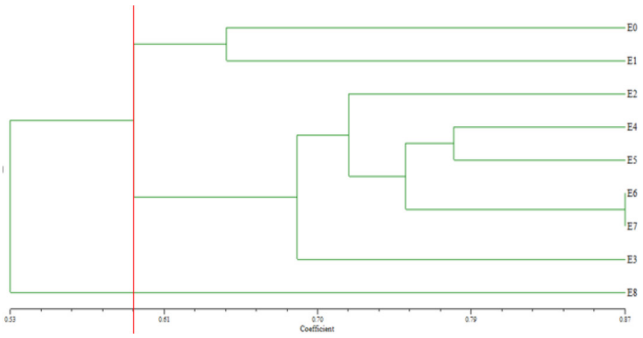



**Figure 5.** Variations in DNA bands and genomic polymorphism at varying dosages of EMS.

**Table 2.** Genetic similarity coefficients between EMS treatment doses and control

| Treatment | E0 | E1 | E2 | E3 | E4 | E5 | E6 | E7 | E8 |
|---|---|---|---|---|---|---|---|---|---|
| E0 | 1.00 | | | | | | | | |
| E1 | 0.65 | 1.00 | | | | | | | |
| E2 | 0.58 | 0.68 | 1.00 | | | | | | |
| E3 | 0.56 | 0.63 | 0.71 | 1.00 | | | | | |
| E4 | 0.56 | 0.62 | 0.73 | 0.73 | 1.00 | | | | |
| E5 | 0.57 | 0.59 | 0.74 | 0.68 | 0.78 | 1.00 | | | |
| E6 | 0.55 | 0.62 | 0.69 | 0.65 | 0.74 | 0.74 | 1.00 | | |
| E7 | 0.55 | 0.62 | 0.51 | 0.57 | 0.76 | 0.76 | 0.87 | 1.00 | |
| E8 | 0.45 | 0.54 | 0.50 | 0.47 | 0.56 | 0.54 | 0.55 | 0.58 | 1.00 |




*Note: E0 = 0% EMS (control); E1 = 0.2% EMS for 30 min; E2 = 0.2% EMS for 60 min; E3 = 0.4% EMS for 30 min; E4 = 0.4% EMS for 60 min; E5 = 0.6% EMS for 30 min; E6 = 0.6% EMS for 60 min; E7 = 0.8% EMS for 30 min; E8 = 0.8% EMS for 60 min.*

**Figure 6.** Phylogenetic tree diagram comparing control and various EMS treatment dosages.

all of the EMS treatments at the average level fell into cluster II, with similarity coefficients ranging from 0.55 to 0.58, showing diversity in shoot morphology and regeneration ability. In general, most of the doses in this group belonged to subcluster IIa, except for 0.4% EMS for 30 min, forming subcluster IIb. The smaller the similarity, the greater the genetic difference between the treatments and the control sample, and the higher the possibility of variation occurring[14]. However, when the similarity coefficient is too low, it also has the potential to cause genetic instability or affect survival due to the potential for harmful mutations. Previous studies have shown that strong mutagenic pressure often increases the frequency of harmful mutations, which can lead to reduced regeneration or even lethality[4]. As evidence, the regeneration rate at 0.8% EMS for 60 min was very low, only 20%, as shown in Figure 1A. The balance between the increase in genetic diversity and the population's mortality rate determines the effectiveness of a mutagenesis process, as both beneficial and lethal mutations happen at random[46].

## 4. Conclusion

EMS was able to induce a mutation in *Hoya kerrii*, and its penetration reduced the cell tissue's capacity to regenerate. At varying EMS dosages, morphological changes were also noted, including the development of secondary shoot clusters from callus, a decrease in the number of shoots, and modifications to the color and shape of the shoot and leaves. RAPD markers are effective tools for early detection of genetic changes in *Hoya kerrii* shoots regenerated under different EMS treatment doses. The degree of genetic material damage was reflected in the frequency of appearance and loss of DNA bands, which increased with EMS dose. This underscores the importance of molecular toxicology in evaluating the effects of mutagens and directing the utilization of advantageous morphological variation in plants. A distinct genetic relationship between EMS doses in relation to one another and the control was also evident in the phylogenetic tree created using the UPGMA method. Remarkably, compared to the control, the 0.4% EMS concentration treated for 30 min showed a moderate polymorphism rate of 42.58% and a similarity coefficient of 0.56. However, compared to the average treatment dosage cluster, it created a separate subcluster. In addition, a high survival rate of 90% and the appearance of numerous new bands (68 bands) at this dose demonstrated the capacity to produce high variation without causing genomic instability. These results indicate that the treatment is optimal for *in vitro* mutagenesis of *Hoya kerrii*. The mutant clones

produced could serve as valuable genetic resources for breeding programs focused on ornamental plants. Additionally, they may contribute to the development of new phenotypic variations with potential commercial and horticultural value.

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## 6. References

1. Wanntorp L, Grudinski M, Forster PI, Muellner-Riehl AN, Grimm GW. Wax plants (*Hoya*, *Apocynaceae*) evolution: Epiphytism drives successful radiation. Taxon. 2014; 63(1):89-102. https://doi.org/10.12705/631.3
2. Alam N, Siddique W, Mishra MK, Pandey A, Purshottam DK, Singh KJ, *et al.* Micropropagation of *Hoya carnosa*, *H. kerrii*, *H. parasitica*, and *H. longifolia* using tray-based floating and stationary hydroponic systems. Sci Hortic. 2023; 311:111804. https://doi.org/10.1016/j.scienta.2022.111804
3. Kim HJ, Jeong RD. First report of *Hoya* necrotic spot virus in *Hoya kerrii* in Korea. Plant Pathol J. 2024; 106(3):1419. https://doi.org/10.1007/s42161-024-01666-4
4. Tri NM, Nguyen TL, Tien TTM, Truc LNK, Yen PH, Anh DNM, *et al.* Effects of 2,4-dichlorophenoxyacetic acid, α-naphthylacetic acid on the variant formation of *Valentine Hoya* (*Hoya kerrii*). VN J Sci Tech. 2023; 65(9):63-68. https://doi.org/10.31276/VJST.65(9).63-68
5. Nga NTT, Soneseo S, Hong TT. Research on *in vitro* culture of *Hoya multiflora* species. TNU J Sci Tech. 2021; 226(10):154-162. https://doi.org/10.34238/tnu-jst.4571
6. Yali W, Mitiku T. Mutation breeding and its importance in modern plant breeding. J Plant Sci. 2022; 10(2):64-70. https://doi.org/10.11648/j.jps.20221002.13
7. Bado S, Forster BP, Nielen S, Ali AM, Lagoda PJ, Till BJ, *et al.* Plant mutation breeding: current progress and future assessment. Plant Breed Rev. 2015; (3):23-88. https://doi.org/10.1002/9781119107743.ch02
8. Udage AC. Introduction to plant mutation breeding: Different approaches and mutagenic agents. J Agric Sci Sri Lanka. 2021; 16(03):466-483. https://doi.org/10.4038/jas.v16i03.9472
9. Fang JY, Traore S. In vitro mutation induction of *Saintpaulia* using ethyl methane sulfonate. Hortic Sci. 2011; 46(7): 981-984. https://doi.org/10.21273/HORTSCI.46.7.981
10. Khangura RS, Best NB, Dilkes BP. Uses and opportunities for ethyl methanesulfonate mutagenesis in maize. Cold Spring Harbor Protocols. 2025; Jun 3. Epub ahead of print. PMID: 40461284. https://doi.org/10.1101/pdb.top108504
11. Pathirana R. Plant mutation breeding in agriculture. CAB Rev. 2011; 1-20. https://doi.org/10.1079/PAVSNNR20116032
12. Sonsan L, Wiriyaampaiwong P, Tankrathok A, Srisamoot N. EMS-induced genetic variation and morphological changes in *Musa laterita*. Curr Appl Sci Technol. 2023; e0264707. https://doi.org/10.55003/cast.2025.264707
13. Yoosumran V, Saetiew K, Ruamrungsri S, Akarapisarn A, Teerarak M. Induced mutation of *Curcuma* hybrid cv. sweetmemory through tissue culture by ethyl methanesulphonate (EMS). Int J Agri Technol. 2025; 21(1):381-396. https://doi.org/10.55164/ajstr.v28i1.255125
14. Pha NT, Nhi NTH, Thao MT, Khang TD, Hien PTT. Effects of ethyl methane sulfonate on the variation of gloxinia (*Sinningia speciosa* (g. Lodd.) hiern) under *in vitro* conditions. J For Sci Technol. 2022; (6):21-28. https://doi.org/10.55250/jo.vnuf.2022.6.021-028
15. Senapati SK, Rout GR. *In vitro* mutagenesis of rose with ethyl methanesulphonate (EMS) and early selection using RAPD markers. Adv Hort Sci. 2008; 22(3):218-222. https://www.jstor.org/stable/42882643.
16. Türkoğlu A, Haliloğlu K, Tosun M, Bujak H, Eren B, Demirel F, *et al.* Ethyl methanesulfonate (EMS) mutagen toxicity-induced DNA damage, cytosine methylation alteration, and iPBS-retrotransposon polymorphisms in wheat (*Triticum aestivum* L.). Agronomy. 2023; 13(7):1767. https://doi.org/10.3390/agronomy13071767
17. Warren JJ, Forsberg LJ, Beese LS. The structural basis for the mutagenicity of O6-methyl-guanine lesions. Proc Natl Acad Sci. 2006; 103(52):19701-19706. https://doi.org/10.1073/pnas.0609580103
18. Chen L, Duan L, Sun M, Yang Z, Li H, Hu K, *et al.* Current trends and insights on EMS mutagenesis application to studies on plant abiotic stress tolerance and development. Front Plant Sci. 2023; 13:1052569. https://doi.org/10.3389/fpls.2022.1052569
19. Dhakshanamoorthy D, Selvaraj R, Chidambaram A. Utility of RAPD marker for genetic diversity analysis in gamma rays and ethyl methane sulphonate (EMS)-treated *Jatropha curcas* plants. C R Biol. 2015; 338(2):75-82. https://doi.org/10.1016/j.crvi.2014.12.002
20. Williams JG, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 1990; 18(22):6531-6535. https://doi.org/10.1093/nar/18.22.6531
21. Nurtjahjaningsih ILG, Haryanti T, Widyatmoko AYPBC, Indrioko S, Rimbawanto A. Genetic diversity of *Calophyllum inophyllum* revealed by RAPD (Random Amplification Polymorphism DNA. J Penelit Sos Ekon Kehutan. 2015; 9(2):91-102. https://doi.org/10.20886/jpth.2015.9.2.103-115

22. Nadeem MA, Nawaz MA, Shahid MQ, Doğan Y, Comertpay G, Yıldız M, *et al*. DNA molecular markers in plant breeding: current status and recent advancements in genomic selection and genome editing. Biotechnol Biotechnol Equip. 2018; 32(2):261-285. https://doi.org/10.1080/13102818.2017.1400401
23. Gillmor CS, Lukowitz W. EMS mutagenesis of *Arabidopsis* seeds. Plant embryogenesis: Methods and protocols: Springer; 2020. p. 15-23. https://doi.org/10.1007/978-1-0716-0342-0_2
24. Babu KN, Sheeja TE, Minoo D, Rajesh MK, Samsudeen K, Suraby EJ, *et al*. Random amplified polymorphic DNA (RAPD) and derived techniques. 2021; 219-247. https://doi.org/10.1007/978-1-0716-0997-2_13
25. Danylchenko O, Sorochinsky B. Use of RAPD assay for the detection of mutation changes in plant DNA induced by UV-B and γ-rays. 2005; 5(1):S9. https://doi.org/10.1186/1471-2229-5-S1-S9
26. Murugan T, Awasthi OP, Chellapilla B, Shankar K. EMS induced *in vitro* mutagenesis for solid mutant induction and validation of mutants using morphological and molecular tools in *Kinnow mandarin* (*Citrus nobilis*× *Citrus deliciosa*). Indian J Agric Sci. 2024; 94(6):644-649. https://doi.org/10.56093/ijas.v94i6.145347
27. Kaur N, Sharma RK, Sharma M, Singh V, Ahuja P. Molecular evaluation and micropropagation of field selected elites of *R. damascena*. Gen Appl Plant Physiology. 2007; 33(3-4):171-186.
28. Murashige T, Skoog F. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant. 1962; 15(3):473 https://doi.org/10.1111/j.1399-3054.1962.tb08052.x
29. Jian Y, Zhimin Z, Jiangli L, Jian L, Kunhua L. Method for tissue culture of *Hoya kerrii*. In: techniques PfAH-Prbtc, editor. 2012.
30. Dar SA, Khan IS, Majeed G, Tyub S, Nawchoo IA, Kamili AN. Chemical-induced variations in callus regenerated *Atropa acuminata* plants: A study on ethyl methanesulfonate effects. Biotechnol Rep. 2025; 46:e00888. https://doi.org/10.1016/j.btre.2025.e00888
31. Keawsaard Y, Pentem P, Rotduang P, Weeraphong M, Ramasoot S. Induction of mutation in toothbrush orchids using ethylmethane sulphonate (EMS) and detection of genetic variation by SSR (simple sequence repeat) marker. ASEAN J Sci Tech Report. 2025; 28(1):e255125. https://doi.org/10.55164/ajstr.v28i1.255125
32. Rogers O, Bendich A. Extraction of DNA from plant tissue, plant molecular. Biol Manual A. 1988; 6:1-10. https://doi.org/10.1007/978-94-017-5294-7_6
33. Istvan Lazar P. Gel Analyzer (Version 19.1). http://www.gelanalyzer.com/index.htm 2019 [
34. Rohlf EJ. NTSYSpc - Numerical Taxonomy and Multivariate Analysis System (version 2.1).Exeter Software. 2000.
35. Atienzar FA, Conradi M, Evenden AJ, Jha AN, Depledge MH. Qualitative assessment of genotoxicity using random amplified polymorphic DNA: comparison of genomic template stability with key fitness parameters in *Daphnia magna* exposed to benzo [a] pyrene. Environ Toxicol Chem. 1999; 18(10):2275-82. https://doi.org/10.1002/etc.5620181023
36. Shalaby TA, El-Banna A. Molecular and horticultural characteristics of *in vitro* induced tomato mutants. J Agri Sci. 2013; 5(10):155-163. https://doi.org/10.5539/jas.v5n10p155
37. Purente N, Chen B, Liu X, Zhou Y, He M. Effect of ethyl methanesulfonate on induced morphological variation in M3 generation of *Chrysanthemum indicum* var. *aromaticum*. HortScience. 2020; 55(7):1099-104. https://doi.org/10.21273/HORTSCI15068-20
38. Türkoğlu A, Tosun M, Haliloğlu K. Evaluation of ethyl methanesulfonate-induced *in vitro* mutagenesis, polymorphism and genomic instability in wheat (*Triticum aestivum* L.). J Crop Sci Biotechnol. 2023; 26(2):199-213. https://doi.org/10.1007/s12892-022-00172-2
39. Thanh LD. Genetic principles of plant breeding. Hanoi: Science and Technology Publishing House; 2000.
40. Girija M, Gnanamurthy S, Dhanavel D. Genetic diversity analysis of cowpea mutant (*Vigna unguiculata* (L.) Walp) as revealed by RAPD marker. Int J Adv Res. 2013; 1:139-147.
41. Siddique MI, Back S, Lee J-H, Jo J, Jang S, Han K, *et al*. Development and characterization of an ethyl methane sulfonate (EMS) induced mutant population in *Capsicum annuum* L. Plants. 2020; 9(3):396. https://doi.org/10.3390/plants9030396
42. Shinde N, Bharose A, Sarode D, Swathi R, Pimpale P, Shinde S. Assessment of hybrid purity in maize (*Zea mays* L.) using RAPD and SSR markers. Pharma Innov J. 2021; 10: 870-874.
43. Serrote CML, Reiniger LRS, Silva KB, Rabaiolli SMS, Stefanel CM. Determining the polymorphism information content of a molecular marker. Gene. 2020; 726:144175. https://doi.org/10.1016/j.gene.2019.144175
44. Kondapperuma C, Hewagamage H, Pieris S. Detection of polymorphism in ethyl methyl sulfonate treated *Cordyline fruticosa* (L.) A. Chev. mutants using RAPD markers. International Conference on Sustainable Biotechnology. Sri Lanka Institute of Information Technology. 2025. p. 169-77. https://doi.org/10.54389/RHCM1030
45. Asfaw D, Jiru T. Yield evaluation and genetic variability assessment in Sesame (*Sesamum Indicum* L.) mutant population using morphological characters and simple sequence repeat (SSR) markers. Adv Crop Sci Tech. 2024; 12(656):2.
46. Yi Z, Su Y, Xu M, Bergmann A, Ingthorsson S, Rolfsson O, *et al*. Chemical mutagenesis and fluorescence-based

high-throughput screening for enhanced accumulation of carotenoids in a model marine diatom *Phaeodactylum tricornutum*. Mar drugs. 2018; 16(8):272. https://doi.org/10.3390/md16080272

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