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In Vitro Mutagenesis of *Dendrobium Gabriella Suryajaya* Using *Ethyl Methane Sulfonate* (EMS) and Plantlet Regeneration

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Abstract. Plant breeding through mutation techniques has the main purpose to explore genetic diversity in the presence of useful traits for developing new plants. *Ethyl Methane Sulfonate* (EMS) is a widely used chemical to induce mutations in plants focused on obtaining genetic variation. EMS can induce random points of mutations and some of which can create new stop codons in the desired gene. EMS has been successfully used to generate morphological diversity and encourage the improvement of desired traits. The 3-month-old *D. Gabriella Suryajaya* Orchid protocorm-like bodies (PLB) treatment with EMS concentrations of 0.025%, 0.05%, and 0.075% can change genetic diversity, especially in leaf morphology. A total of 105 orchids were soaked in EMS solution at concentrations for 12 and 24 hours respectively. The phenotypic variations observed in this population include changes in leaf color and the number of buds. This EMS mutant population will be used for further studies including screening for various traits such as through ISSR analysis to determine the level of diversity. This research shows that mutagenesis using EMS can produce the amount of variability in *Dendrobium*. The generation of variability for desired traits resulted in the identification of several mutants with important agronomic characteristics that can be used as germplasm for improvement.

Keywords: diversity, *Gabriella Suryajaya*, leaf, phenotype, variation.

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INTRODUCTION

The *D. Gabriella Suryajaya* is a type of plant that is included in the Orchidaceae family and is widely recognized in Indonesia or the world because it has a unique and beautiful color, shape, type, and variety of flowers, also has a high aesthetic and economic value (Puspitaningtyas, 2017). In producing a variety of orchids and varieties that are superior to this species, better plant quality is needed through plant propagation. According to (Sarmah *et al.*, 2017), the high demand for orchid plants encourages a process of developing varieties of orchid diversity, especially to produce plants that outplay attractive and good-quality color and shape variations. (Hinsley et al., 2018) states that the higher the level of genetic diversity of plants, the greater the opportunity to obtain quality and superior genotypes.

Efforts can be made to focus and improve plants, especially on the color, shape, variety of flowers, and plant size and resistance to pests and diseases, one of which is to carry out plant breeding techniques. Plant breeding through mutation is an effort that can improve the genetic diversity of a plant (Espina et al., 2018). The method used to quantitatively improve plant characteristics is the mutation method. The development of the *Dendrobium* orchid plant by the chemical mutation method using *Ethyl Methane Sulfonate* (EMS) can influence a characteristic and morphology of the plant (Romiyadi dkk., 2018). This study used EMS which can produce different leaf color variations when compared to controls and causes shoot

multiplication. But research on *D. Gabriella Suryajaya* is still rare.

Ethyl Methane Sulphonate (EMS) compounds are the most commonly used compounds in plants for induction of plant mutations because they cause high frequencies in nucleotide substitution and allow it to be the mutagen of choice in plant induction targets (Talebi *et al*., 2012). Mutations due to the influence of EMS cause guanine to experience substitution, where guanine which should be paired with cytosine, will pair with thymine so that there will be changes in amino acids which then change protein composition (Chopra, 2005). Based on research results Qosim *et al.*, (2012) showed that EMS mutagen treatment could affect meristem growth in *Phalaenopsis* hybrid orchids in shoot formation at EMS concentrations of 0.025% and 0.05%. The use of EMS solution at a concentration of 0.05% also affected the appearance of *Phalaenopsis* orchid plantlets from protocorm (Romiyadi *et al.,* 2018). The purpose of this research was to determine the best concentration for the growth of *D. Gabriella Suryajaya*. after mutation induction by EMS.

METHODS

This research was conducted from April 2022 to July 2022 at the Center for Development of Advanced Science and Technology Laboratory (CDAST), University of Jember, Jember Regency, East Java Province. The 3-month-old protocorm-like bodies (plb) *D. Gabriella Suryajaya* from in vitro seedlings, which has a dwarf (1 cm) and compact growth habit, was used to create a mutant population by EMS. Two soaking times (12 and 24 h) and four concentrations of EMS (0%, 0.025%, 0.05%, and 0.075%) were tested to optimize the EMS concentrations. For each treatment, three replicates of 105 plb were planted in 21 bottles of culture (each bottle amounted to 5 plb). Each plb was treated with EMS in 10 ml falcon at room temperature. The tools used in this study were glassware, tweezers, falcon, tube, petri-dish, beaker glass, and standard tools for tissue culture.

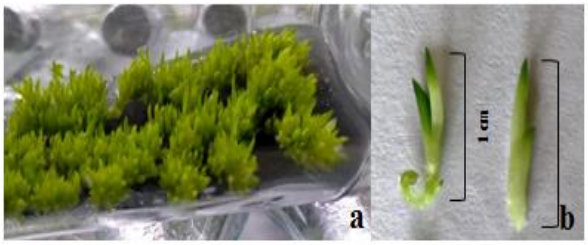


Figure 1. Planting material for mutation with chemical mutagens a) a group of 3-month-old plb orchid plants, b) plb that is ready to be used as a mutation material

Regeneration Media Preparation

To induce mutagenesis, the EMS-treated plb were cultured on ½ MS (Murashige and Skoog, 1962) medium containing 0,1 ppm NAA and 1 ppm BAP. All media were prepared using 20 g sucrose, and 150 ml coconut water, and the media were solidified with 1,5 g gelrite for 1 L aquadest at pH 5.8. The coconut water complex is mainly the content the hormones auxin and cytokinins has have a major impact on growth in vitro explants (Ambarwati *et al.*, 2021)

In Vitro Induction of Mutation

For the mutation treatment, 1% EMS stock solution from sigma was used to prepare 0.025%, 0.05%, and 0.075% solutions using 0.1 M phosphate buffer (pH 7.2) which were then filter-sterilized with an SFCA-PF 0.2 µm filter under aseptic conditions. The plb was immersed in 10 ml EMS solutions with concentrations 0.025%, 0.05%, and 0.075% in a 10 ml tube (each tube contains 10 plb) for 12 and 24 hours. After the mutagenic treatment, all explants were rinsed with sterile aquadest 3 times, dried in sterile filter paper, and transferred to regeneration media.

Orchids Plantlet Observation

Observations were made after the plantlet regeneration process for 12 weeks. Some of the observational variables to be observed include plantlet height, number of leaves, number of shoots, number of roots, percentage of live plantlets, and leaf color. The entire observation process is carried out in LAF (Laminar Air Flow) to avoid contamination. Observation of leaf color was carried out by

looking at the oldest leaf color in the explants. Leaf color visuals were seen using the Munsell Plant Tissue Color Chartbook. This study uses the *Standard Error Of Mean* (SEM). This method is used to determine the difference in error from the data obtained and the level of variance in each sample.

RESULTS AND DISCUSSION

Number of shoots and leaves

The effects of EMS and their combinations were primarily evaluated under in vitro conditions after 12 weeks. The treatment using the EMS chemical mutagen which produced the highest number of shoots was EMS 0.05% with an average of 4.07 shoots. In the 0.05% EMS treatment for 24 hours soaked produced the highest number of shoots and leaves. Based on the results of SEM analysis, the number of shoots EMS 0.05% and without EMS or EMS 0% were not significantly different. In this case, plb in the presence of EMS 0.05% can form shoots normally because the nutrients available in MS media can stimulate plb growth, and the addition of 1 ppm BAP can stimulate shoot formation. What differentiates this is the number of EMS concentrations used and the soaking time, which causes different growth of shoots. Giving EMS to orchids, especially the variable number of shoots, gives the result that EMS can stimulate shoot growth or inhibit shoot growth. The treatment that produced the lowest shoots was EMS 0.050% soaking for 12 hours with an average of 2.53, but soaking for 24 hours produced the most shoots.

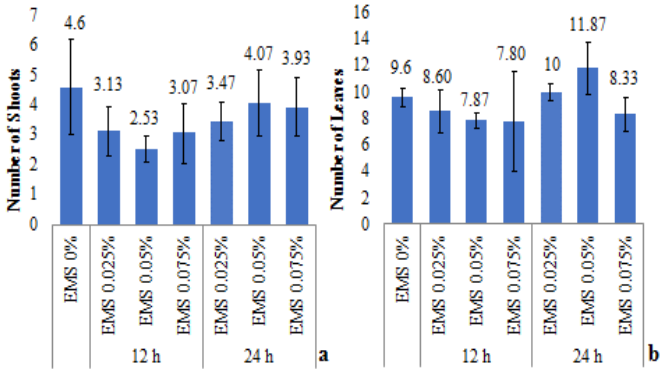


Figure 2. EMS Concentration and Soaking Duration on *D. Gabriella Suryajaya* a) Number of Shoots, b) Number of Leaves

Based on the graph above, it can be seen that the number of leaves formed with the highest yield was the EMS concentration of 0.05% (E2) with a soaking duration of 24 hours, with a result of 11.87. The number of leaves that showed the lowest yield with a total of 7.80 leaves was at 0.075% EMS concentration (E3) with a soaking duration of 12 hours. Based on the graph results, soaking for 12 hours at the highest EMS concentration, 0.075% can reduce the number of leaves. At the concentration that gave the lowest plant height, EMS 0.075%, it also produced the lowest number of leaves. This proves that the physiological changes that occur as a result of mutations occur randomly and depend on the response of the genetics of the plant itself. EMS immersion duration did not give a linear response to the growth of the number of leaves. In the treatment without EMS (EMS 0%) the number of leaves was 9.6 leaves. Differences in explant responses due to the administration of mutagens depend on the sensitivity of the cell's meristem composing explants.

Figure 3. Multiplication of *D. Gabriella Suryajaya* shoots a. EMS 0% b. EMS 0.05% 12 hours c. EMS 0.05% 24 hours

Giving EMS to the *D. Gabriella Suryajaya* orchid on the variable number of shoots gave results as a stimulant for shoot growth or as a shoot inhibitor. At 12 weeks, the highest number of shoots produced was at 0% EMS. The following research has been conducted by Kamila dkk. (2022), showed that the treatment which produced the highest number of shoots on the *Macodes Petola* orchid was without 0% EMS treatment, which resulted in 5 shoots. The different EMS concentrations given resulted in different responses to each plant, especially in the character of the number of leaves and multiplication (Sari dkk., 2016). According to (Zhang & Gao, 2020), the use of semi-solid media containing ½ MS basal is an effective medium for regenerating plb resulting from mutations.

The regeneration rate of the number of shoots developed from plb indicates the existence of shoot multiplication. The development of more and more shoots was significantly affected by an increase in EMS concentration (Nasri *et al.* 2022). This study showed that the form of shoot multiplication in control plantlets and EMS treatment had different forms. In the control plantlets, it was seen that the number of shoots was less and not in groups, and the roots were elongated. Whereas in plantlets treated with EMS 0.05%, the number of buds was greater and in groups and in contact with each other.

Number of roots and plantlet height

Based on the results of the graph above, it can be seen that the optimum concentration to stimulate root growth is EMS 0.025% (E1). It is because at this concentration the number of roots has the highest yield. This is supported by the decrease in yield at the next increase in concentration, 0.05% EMS. At 12 hours of EMS, 0.05% and 0.075% immersion had the same number of roots, while at 24 hours immersion of EMS 0.05% and 0.075% had a different number of roots but decreased. The lowest number of roots was 3.33 at an EMS concentration of 0.05% (E2) soaking for 12 hours where this concentration gave the lowest number of shoots and also produced the lowest number of roots. It is suspected that the concentration and duration of soaking can inhibit root growth and shoot growth in plantlets. Roots can absorb nutrients in the media, so the more the number of roots, the more optimal the absorption of nutrients for plant growth (Aqidah dkk., 2022).

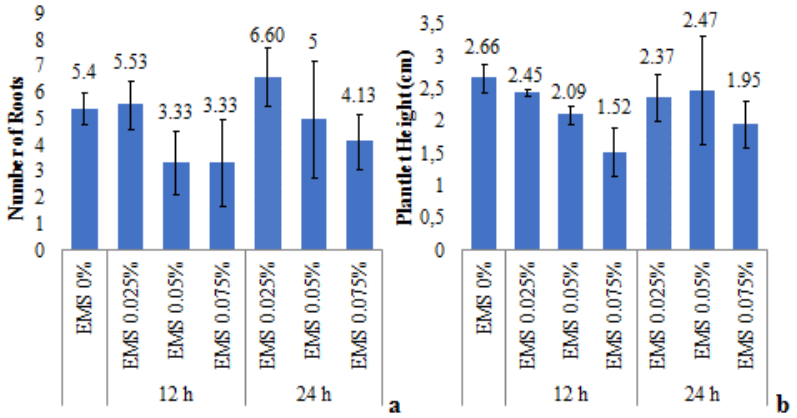


Figure 4. EMS Concentration and Soaking Duration on *D. Gabriella Suryajaya* a) Number of Roots, b) Plantlet Height

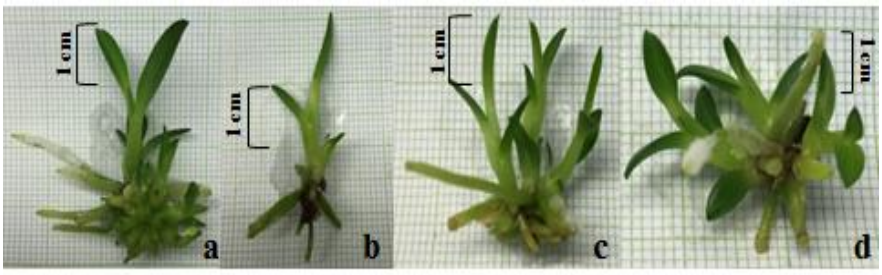


Figure 5. Orchid plantlets 12 weeks after being regenerated. (a) Plantlets from 0% EMS concentration. (b) Planlets from 0.025% EMS concentration. (c) Planlets from 0.050% EMS concentration. (d) Planlets from 0.075% EMS concentration

Plantlet height at each immersion duration at different EMS concentrations resulted in differences in each treatment. Treatment without EMS (EMS 0%) produced the highest plantlet, which was 2.66 cm. Based on SEM analysis, the highest yield in the EMS treatment was at a concentration of 0.05%, soaking for 24 hours, which was 2.47 cm with a high level of diversity and higher than the control, which has a difference of 0.19 cm. While the lowest plantlet height yield was at 0.075% EMS concentration of 1.52 cm in 12 hours of immersion and 1.95 cm in 24 hours of immersion. This shows that the administration of the highest concentration of EMS will experience growth inhibition to reduce the average height of orchid plantlets. The higher concentration caused a decrease in plantlet height, namely at EMS concentrations of 0.025% (E1), EMS (0.05%), and EMS 0.075% (E3). This is aligned with research conducted by Pratiwi dkk. (2013), on Marigold plants which shows that EMS concentrations can inhibit plant growth. The stunted plantlets are caused by EMS which is a toxic compound, thereby inhibiting plantlet growth and causing chromosomal aberrations (Rustini & Pharmawati, 2014).

Percentage of live plantlets

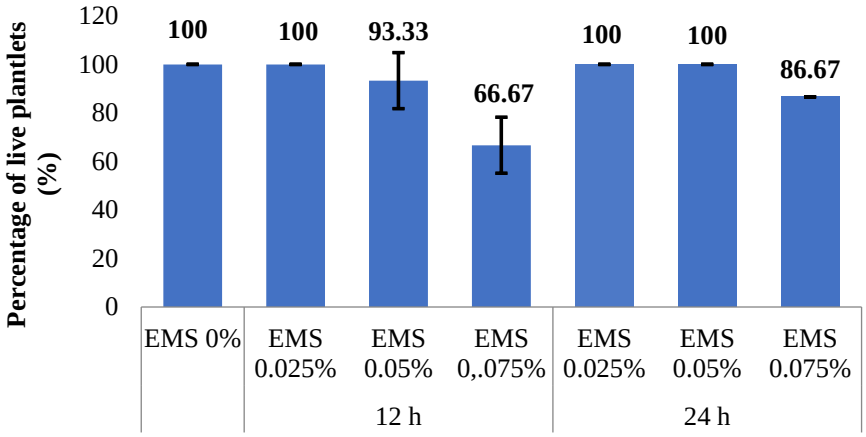


Figure 6. EMS Concentration and Soaking Duration on the Life Percentage of *D. Gabriella Suryajaya* Orchids

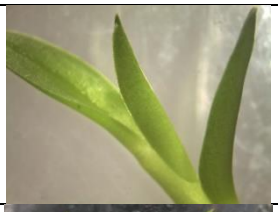
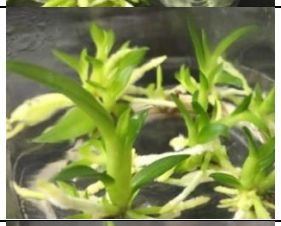
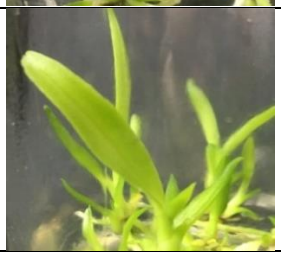
Based on the results of the graph above, it can be seen that the highest percentage of plantlet survival was at several concentrations, 0% EMS, 0.025% 12-hour immersion, 0.025% concentration, and 0.05% 24-hour immersion of 100% plantlet survival. The concentration that produced the lowest percentage was at the highest concentration, EMS 0.075% by 66.67% at 12 hours of immersion, while at 24 hours of immersion, it was 86.67% of live plantlets. EMS mutagen concentration can affect the percentage of viable plantlets. The highest EMS concentration of 0.075% can reduce the percentage of the life of orchid plantlets.

At 0.025% EMS concentration soaking for 12 hours and 24 hours influences the success rate of a mutation process in plants. Based on the results of the research conducted, 98 mutant orchid plantlets could survive the 105 mutant orchid plantlets that were planted. The lethality level of the mutated orchid plantlets was affected by increasing the concentration of the EMS mutagen used. The EMS concentration greatly affects the success rate of the mutation process in plants, the use of high EMS concentrations can increase the mutation process but will have an impact on the death rate of plants (Yadav *et al*., 2016). The use of EMS with concentrations that are too high can result in a decrease in the level of efficiency and effectiveness of the mutation process. According to (Yoosumran *et al*. 2018) The lethal dose (LD 50) of *Dendranthemum* from EMS was 1.22% EMS for 60 minutes and 0.72% EMS for 120 minutes.

Leaf Color

Observation of leaf color was carried out 12 weeks after planting by observing the oldest leaf color on the explants. Leaf color visuals use the Munsell Plant Tissue Color Chart-book. Based on observations, there are 8 leaf color categories with dark green, light green, and white visual colors. The most dominating leaf color was 5GY 5/10 with a dark green visual of 23 explants.

Table 1. Effect of EMS on Leaf Color of *D. Gabriella Suryajaya* in vitro

Treatment/number of samples)	Munsell Color	Picture
EMS 0% (15/15)	Munsell value: 7.5 GY 6/10	
EMS 0.05% 12 h (1/15) EMS 0.025% 24 h (4/15) EMS 0.05%, 24 h (1/15) EMS 0.075%, 24 h (4/15)	Munsell value: 5 GY 5/8	
EMS 0.025%, 12 h (1/15) EMS 0.05%, 12 h (2/15) EMS 0.025%, 24 h (2/15) EMS 0.05%, 24 h (2/15) EMS 0.075%, 24 h (2/15)	Munsell value: 5 GY 6/8	
EMS 0.025%, 12 h (2/15) EMS 0.05%, 12 h (1/15) EMS 0.05%, 24 h (1/15)	Munsell value: 5 GY 7/8	
EMS 0.025%, 12 h (4/15) EMS 0.05%, 12 h (4/15) EMS 0.075%, 12 h (1/11) EMS 0.025%, 24 h (5/15) EMS 0.05%, 24 h (7/15) EMS 0.075%, 24 h (2/15)	Munsell value: 5 GY 5/10	
EMS 0.025%, 12 h (5/15) EMS 0.05%, 12 h (4/15) EMS 0.075%, 12 h (5/11) EMS 0.025%, 24 h (3/15) EMS 0.05%, 24 h (3/15) EMS 0.075%, 24 h (3/15)	Munsell value: 5 GY 6/10	
EMS 0.025%, 12 h (2/15) EMS 0.05%, 12 h (2/15) EMS 0.075%, 12 h (2/11) EMS 0.025%, 24 h (1/15) EMS 0.05%, 24 h (1/15) EMS 0.075%, 24 h (1/15)	Munsell value: 5 GY 7/10	
EMS 0.025%, 12 h (1/15)	Munsell value: 2.5 GY 8/4	

Based on table 1, it can be seen that the color of the shoots in the EMS 0% (E0) control treatment looks dark green and light green with the color category 7.5GY 6/10 and 7.5GY 7/10. Visually, 105 plants with 8 color categories are different from the control leaf color (EMS 0%, namely dark green and light green with color categories 5GY 5/8, 5GY 6/8, 5GY 7 /8, 5GY 5/10, 5GY 6/10, 5GY 7/10, and white with categories 2.5GY 8/4. In each treatment, not all samples gave the same leaf color. The leaf color category with the most plant samples was light green with 28 samples. Color indicators can determine the age and quality of plants because the color that is formed will determine whether the cell is actively dividing or dying (Restanto dkk., 2021).

Leaf color analysis showed that 0% EMS concentration produced the best dark green leaf color with a value of 7.5 GY 6/10. In the EMS treatment 0.025%, 0.05%, and 0.075% showed leaf color with hue values of 5 GY and 2.5 GY where there were differences in each value and chroma values. Leaf color indicates the presence of chlorophyll in the plantlet tissue, the greener the leaf color indicates the more chlorophyll it contains and vice versa if the green color is lighter, the chlorophyll content is less. The color with the lowest chlorophyll level is found in E1T1 with a value of 2.5 GY 8/4.

Figure 7. Response of *D. Gabriella Suryajaya* leaf color a. EMS 0.05% 12 hours b. EMS 0.05% 24 hours c. EMS 0%

At 12 weeks the results of the orchid plantlets experienced a response to differences in leaf color where there were leaves that experienced color gradations from white to dark green at EMS concentrations of 0.05%, soaking 12 hours and 24 hours. The response to color differences that occurred looked different when compared to the control plantlets where the leaf color on the control plantlets was even green and did not have white gradations. According to Widiarsih & Dwimahyani (2013), the appearance of variegata leaves or leaves with a mixture of the normal color of green leaves with other colors, either white (albino) or yellow (viridis) indicates the occurrence of a chlorophyll mutation process. Chlorophyll mutations occur in chloroplasts which cause defects in mutant genes (defective mutant genes) which can then interfere with the process of photosynthesis in leaves. Thus, the impact of mutations in the chloroplast gene is characterized by the appearance of symptoms of striped color on plant leaves. Mutations outside the cell nucleus cause stunted growth, changes in flower morphology, and other morphological deviations that occur in genes in mitochondria (Lestari, 2016).

The death of explants

Figure 8. The death of explants *D. Gabriella Suryajaya* caused by EMS compared to control. a.Control b. EMS 0.075% 12 h c. EMS 0.075% 12 h d. EMS 0.05% 12 h

EMS mutagen concentration causes different growth and development responses in explants. In figure a, the explants grow normally, i.e. remain green and produce green leaves and shoots. This condition occurs because there is no physiological damage in the plant so the plant can grow normally. Figures 8b, 8c, and 8d are some of the explants that were not able to survive so they could not give rise to new shoots. The response of explants with brown or drying stems can occur due to damage to the cells in the plant due to EMS and the mutated gene is lethal so that the symptoms of the mutation can be observed because the explants die before maturity (Lestari, 2016). In this study, explants treated with EMS produced various mutants which were the result of the pleiotropic effect of mutated genes or mutations at different loci in the genome (Basu *et al.* 2008). However, according to Sasmita dkk. (2022), the brown explants look like browning. browning occurs due to compounds phenol in explants being too high, causing explants to change color from brown to black.

One of the successes in propagation by mutation is the suitability of using mutagen concentrations and the accuracy of mutation techniques. The use of a mutagen with a concentration of 0.075% can cause the explants to turn brown, unable to form buds then die. According to (Qosim dkk., 2012) this is due to the influence of high EMS concentrations (> 0.15%) that can inhibit the growth of cells in plants. At 0.025% EMS concentration, it was able to give a white leaf response and did not produce buds at 12 weeks. This is because the leaves that are formed have mutations due to EMS administration. After all, they have a different appearance and response when compared to the control leaves (EMS 0%) (Kamila dkk., 2022).

The combination of higher doses and soaking time for plants treated with EMS mutagen showed a higher mortality rate and decreased yield in plants (Basu *et al.*, 2008). EMS immersion time did not significantly affect the survival percentage of orchid plantlets, this is by following research conducted (Kamila dkk., 2022) that soaking time using EMS had no significant effect on reducing the plant height of Macodes petola orchids. The response given to EMS soaking time on Macodes petola plant height gave different results. This difference in response may occur due to genetic factors per individual plant. EMS chemical mutagens are very effective because they can induce many point mutations in the plant genome. In addition, EMS can cause a low level of chromosomal aberrations in the process of mutagenesis (Nasri *et al*., 2022). This research shows that mutagenesis using EMS can produce the amount of variability in *D. Gabriella Suryajaya* orchid plantlets. Generation of variability and selection for desired traits resulted in the identification of several mutants with important agronomic characteristics that can be used as germplasm for the improvement of these crops.

CONCLUSION

Mutations using EMS can produce variations in leaf morphology and different leaf colors when compared to controls. The EMS concentration of 0.025% produced the highest number of roots and there were albino plantlets with chlorophyll deficiency. The EMS concentration of 0.05% gave the most color variations in the leaves and the highest number of leaf blades. EMS concentration of 0.075% resulted in the lowest percentage of plantlet survival, plantlet height, and the lowest number of roots. The highest percentage of plantlet multiplication was found in the treatment without EMS or 0% EMS and 0.05% EMS 24 hours immersion of 12 buds.

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