ASEAN
Journal of Scientific and Technological Reports
Online ISSN:2773-8752

Research article

Mutation Induction in Toothbrush Orchids Using Ethylmethane Sulphonate (EMS) and Detection of Genetic Variation by SSR (Simple Sequence Repeat) Marker

Yaowamarn Keawsaard[1], Parita Pentem[2], Phakarat Rotduang[3], Muntaka Weeraphong[4], and Supawadee Ramasoot[5]*

[1] Department of Agricultural, Faculty of Science and Technology, Nakhon Si Thammarat Rajabhat University, Tumbon Thayew, Mueang district, Nakhon Si Thammarat Province, 80280, Thailand
[2] Department of Biology, Faculty of Science and Technology, Nakhon Si Thammarat Rajabhat University, Tumbon Thayew, Mueang district, Nakhon Si Thammarat Province, 80280, Thailand
[3] Department of General Science, Faculty of Education, Nakhon Si ThammaratRajabhat University, Tumbon Thayew, Mueang district, Nakhon Si Thammarat Province, 80280, 80280, Thailand
[4] Department of Education in Science (Biology), Faculty of Science and Technology, Nakhon Si Thammarat Rajabhat University, Tumbon Thayew, Mueang district, Nakhon Si Thammarat Province, 80280, 80280, Thailand
[5] Department of Education in Science (Biology), Faculty of Science and Technology, Nakhon Si Thammarat Rajabhat University, Tumbon Thayew, Mueang district, Nakhon Si Thammarat Province, 80280, 80280, Thailand
* Correspondence: supawadee_ram@nstru.ac.th

Citation:
Keawsaard, Y.; Pentem, P.; Rotduang, P.; Weerapong, M.; Ramasoot, S. Induction of mutation in yoothbrush 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.

Article history:
Received: July 22, 2024
Revised: October 24, 2024
Accepted: November 18, 2024
Available online: December 14, 2024

Abstract: *Dendrobium secundum* is a monocotyledonous plant. It is classified in the *Dendrobium* genus, an important and outstanding economic flowering plant. It is an orchid sold domestically and internationally due to its overall toothbrush-like design, both in the flower arrangement and stem. In recent years, the number of toothbrush orchids has decreased. Therefore, this study aimed to study the effects of ethylmethane sulfonate (EMS) and the detection of genetic variation in toothbrush orchids using simple sequence repeat (SSR) markers. EMS solution was used to soak 0.5x0.5 cm in diameter of the protocorm-like bodies (PLBs) at concentrations of 0.5, 1.0, and 2.0%. After that, they were cultured on Vacin and Went (VW) medium supplemented with 1 mg/l BA and 20 g/l sucrose, adjusted to pH 5.7, and solidified with 0.2 % phytagel. The cultures were maintained at 26 ±2°C under light at 3,000 lux for 14 hours daily. After culturing for 30 days, the results showed that PLBs immersed in EMS solution at 1.7% for 90 minutes gave a survival rate of 50% (Lethal dose; LD_{50}). For the development of PBLs, PBLs immersed in 0.5 % EMS for 60 minutes gave the highest PLB induction (7.37%) and average number of PLBs (1.41 PLBs/explant) after culturing for 8 weeks. For the detection of genetic variation, a total of 9 SSR primers were used, including EgCIR0409, EgCIR0905, EgCIR0781, EgCIR0446, EgCIR1772, EgCIR0337, EgCIR0337, EgCIR0243, mEgCIR0465, and mEgCIR008. The results found that only one primer gave polymorphic banding. EgCIR0905 primer gave polymorphic banding at 50% and specific DNA banding size at 200 bp.

Keywords: Toothbrush orchid; Mutation; Ethylmethane sulphonate; Genetic variation; Simple sequence repeat marker

1. Introduction

Dendrobium secundum is a significant and exceptional economic plant, better known as the toothbrush orchid. It is an orchid sold domestically and

internationally due to its overall toothbrush-like design, both in the flower arrangement and stem. Consequently, there are now fewer toothbrush orchids. The orchids mentioned above also lack variety [1]. One method to alter the characteristics of orchids, from their appearance to their growth potential, is in vitro plant breeding and propagation. In vitro, mutation treatment can be used to create new, improved orchid varieties by using both chemical and physical mutagens, which can broaden the possibilities for orchid variants while accelerating the generation of mutants. [2]. The resulting mutations are expected to increase the diversity and value of orchids. There are numerous methods for breeding. Both chemical and physical methods can be used to accomplish this. The physical ones are ultraviolet, gram, X-rays, and so forth. Mutagenic compounds are used as part of the chemical side. Numerous chemical species can induce mutations, each with a unique set of consequences. Gene mutations will likely result from EMS, a chemical mutagen that can cause alkylation. [3]. According to Li et al. (2021) [4], there is no research on the mutations that lead to increased heterozygosity in orchids due to chemical mutagens that are part of alkylating agents. In orchids such as *Cymbidium*, *Dendrobium*, *Oncidium*, and *Phalaenopsis*, mutation induction—which has been successfully applied in multiple studies—uses the polyploidization breeding technique in combination with colchicine and nitric oxide (NO) mutagens. [4]. Using EMS concentrations, Romiyadi et al. (2018) [5] generated mutations and illustrated interactions with three hybrid Phalaenopsis species on root variables by employing EMS concentrations. Qosim et al. (2016) [6] showed that the development of shoots from hybrid Phalaenopsis spp. was impacted by immersing EMS in a 0.025% and 0.05% concentration for 12 hours. EMS enhances the genetics of other crops, such as sugarcane, to achieve high yields. [7]. EMS induces random point mutations in the plant genome [8,9]. Therefore, molecular analysis must be performed. A sequence repeat (SSR) molecular marker is ideal for genome-level research [10]. Co-dominant, repeatable, easily distinct alleles, high degree of polymorphism, and easily detectable by PCR technology are some characteristics of a simple sequence repeat [11,12]. Microsatellite-specific primer development is relatively expensive and time-consuming to acquire DNA sequence information. [13]. However, no study has been done on applying EMS materials to toothbrush orchids. Therefore, this research aims to study and determine the effect of various concentrations of EMS and durations of time on mutation and investigate genetic variation in toothbrush orchids with SSR markers for various morphology in this orchid and some commercial cultivars.

2. Materials and Methods

2.1 Plant materials

Protocorm-like bodies (PLBs) of toothbrush orchids at 0.5x0.5 cm in diameter were cultured on VW medium supplemented with 1 mg/l BA, 0.2% phytagel and 20 g/l sucrose [14]. The pH was adjusted to 5.7. The culture condition was maintained at 25±2 °C and 3000 lux light intensity for 14 hours of photoperiod.

.

2.2 Effect of various concentrations of EMS and duration time on the development of PLBs

PLBs at 0.5x0.5 cm in diameter were soaked with various concentrations of EMS at 0, 0.5, 1.0, and 2.0% for 60 and 90 min by incubating on a shaker at 100 rpm. After that, filter to separate PLBs from EMS solution, wash with sterile distilled water 2-3 times, and blot with sterile filter paper. PLBs were cultured on VW medium supplemented with 1 mg/l BA, 0.2% phytagel, and 20 g/l sucrose. The culture was subcultured on the same medium component every four weeks for three months. The percentage of survival rate (lethal dose: LD_{50}), the proliferation of PLBs induction (%), and the number of PLBs (PLBs/explant) were recorded.

2.3 Detection of genetic variation of PLBs by SSR markers

2.3.1 DNA Extraction

DNA from each PLBs was extracted using the cetyltrimethyl ammonium bromide (CTAB) method by Doyle and Doyle (1990) with some modification [15] with some modification. In brief, 100 mg of explants were ground to a fine powder in approximately 500 µl of CTAB buffer. The plant extract mixtures were put into microcentrifuge tubes and incubated for 45 minutes at 60°C in a water bath. Following incubation, the extract mixtures were centrifuged for 15 minutes at 12,000 rpm, and the supernatant was then moved to a fresh, clean microcentrifuge tube. 500 µl of chloroform was added to each tube, and the mixture was mixed by inverting

the tube. The tube was thoroughly mixed and then centrifuged for 10 minutes at 13,000 rpm to extract the upper aqueous phase containing the DNA and transfer it to a fresh microcentrifuge tube. To precipitate the DNA, 750 µl of isopropanol was added to the solution, and the tube was slowly inverted several times. Following precipitation, the DNA pellet was dried in a temperature-controlled environment and twice washed with 70% ethanol. In TE buffer, the DNA pellet was dissolved [20 mM Tris-HCl (pH 8.0) and 0.1 M EDTA (pH 8.0)], electrophoresed on 0.75% agarose gel, explored in UV light, and photographed. The amount of DNA was qualitatively measured by comparison with the known quality of λDNA.

2.3.2 SSR Analysis

The Thawaro method [16] was used to analyze DNA differences using the SSR technique. Nine primer pairs (EgCIR0008, EgCIR0243, EgCIR0337, EgCIR0409, EgCIR0446, EgCIR0465, EgCIR0781, EgCIR0905, and EgCIR1772) were used for PCR amplification of DNA. Twenty ng of template DNA, 1.5 units of Tag polymerase, 0.3 mM primer, 10X Tag buffer, 2.5 mM MgCl2, and 100 µl of each dNTP were included in each 10 µl amplification mixture. A thermocycler was used to perform PCR amplification. Using the following program: one minute of denature at 95°C; thirty seconds at 94°C, sixty seconds at 52°C, and one minute at 72°C; and eight minutes of final elongation at 72°C. Afterward, the amplified products were denatured at 94°C for 5 minutes before adding an equal volume of loading buffer (98% formamide, 0.025% bromophenol blue, 0.05% xylene cyanol). The PCR products were then electrophoresed at 150 V in 1%X TAE buffer on 2.0% (w/v) agarose gels. Gel documentation was used to view the gels under ultraviolet light after they had been stained with ethidium bromide for 15 minutes. The reproducibility of the amplification patterns was verified by using different DNA preparations from the mother plant.

2.4 Statistical analysis

A completely randomized design with three replicates, each containing ten explants, was used for the experimental design and statistical analysis. Duncan's Multiple Range Test (DMRT) was used to compare the mean differences. Data was analyzed using ANOVA.

3. Results and Discussion

3.1 Effect of various concentrations of EMS and duration time on the survival rate of PLBs

After immersing the PLBs in EMS solution at different concentrations and durations, they were grown on VW medium and added 1.0 mg/l BA and 0.2% phytagel. The results showed that PLBs immersed in EMS solution at 1.7% for 90 minutes gave the lethal dose at 50% (LD_{50}) after culturing for 1 month (Figure 1). In contrast, all EMS solutions (0, 0.5, 1, and 2 %) gave the highest average survival rates at 93.33, 70, 63.33 and 53.33%, respectively (Figure 2). According to Sirinya and Te-chato (2008) [17], it was found that the survival rate of *Dendrobium friedericksianum* tends to decrease with a longer immersion time in each concentration of EMS.

For the effect of EMS solution on proliferation of PLBs induction, the result found that PLBs were soaked in 0.5% EMS for 60 minutes gave the highest PLBs induction at 7.37% and average number of PLBs at 1.41 PLBs/explant, significant difference with other treatments ($p \leq 0.05$) (Table 1). Samala et al. (2014) [18] found that PLBs immersed in EMS at 0 and 0.75% concentrations for 60 and 90 minutes gave a more than 50% survival rate. In addition, PLBs immersed in EMS at a concentration of 0.75% for 90 minutes showed dark green and succulent PLBs, while control treatment (without EMS) gave light green and no succulent PLBs. Exposure to many substances can disrupt the germination process's enzymes and prevent auxin production [19]. It is a reaction to cell damage and will show PLB growth. For characteristics of PLBs, the result found that PLBs have a light green and pale yellow (Figure 3). Romeida et al. (2012) [20] described that in every area of the cell exposed to the mutagen, PLBs either lose their capacity to produce chlorophyll or cannot do so.

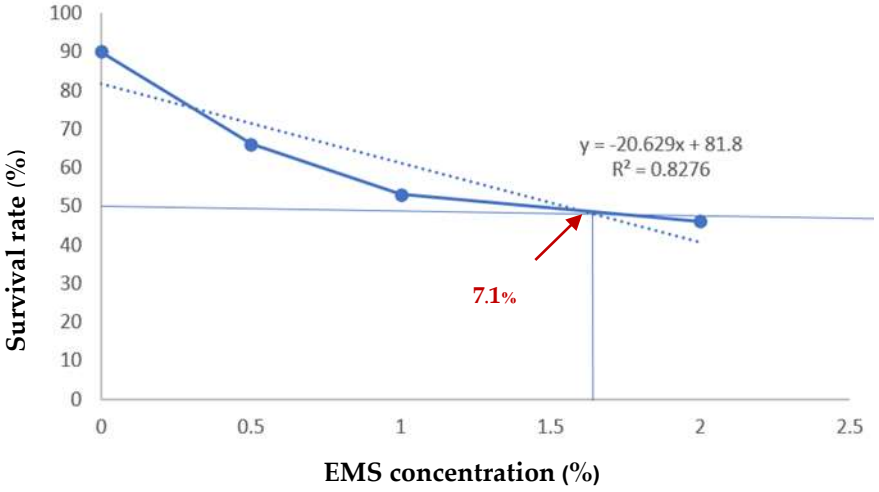

Figure 1 Survival rate of PLBs of toothbrush orchid after having been treated with 0, 0.5, 1, and 2 % for 90 minutes and cultured on VW medium with 1.0 mg/l BA and 0.2% phytagel for 1 month.

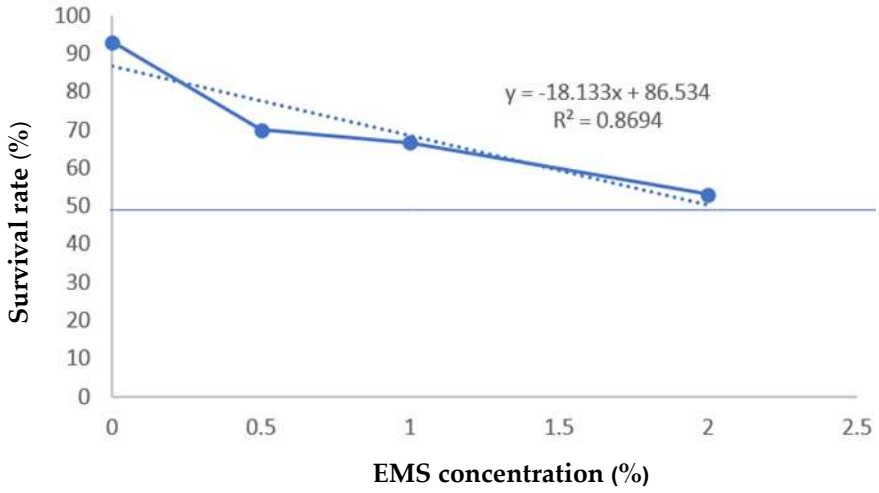

Figure 2 Survival rate of PLBs of toothbrush orchid after being treated with 0, 0.5, 1, and 2 % for 60 minutes and cultured on VW medium with 1.0 mg/l BA and 0.2% phytagel for 1 month.

Table 1 Effect of EMS solution concentration and immersion time on proliferation of PLBs after 2 months of culture

EMS concentrations (%)	Duration time (minute)	PLBs proliferation (%)	No. of PLBs (PLBs/explant)
0	60	3.73abc	0.75abc
0.5	60	7.37a	1.41a
1	60	3.63abc	0.73abc
2	60	0.93c	0.19c
0	90	2.57bc	0.51bc
0.5	90	5.53ab	1.11ab
1	90	3.67abc	0.73abc
2	90	1.80bc	0.37bc
F-test		*	*
C.V.(%)		19.68	19.22

* significant difference at $p \leq 0.05$ means that the different letters in the same column are significantly different by DMRT

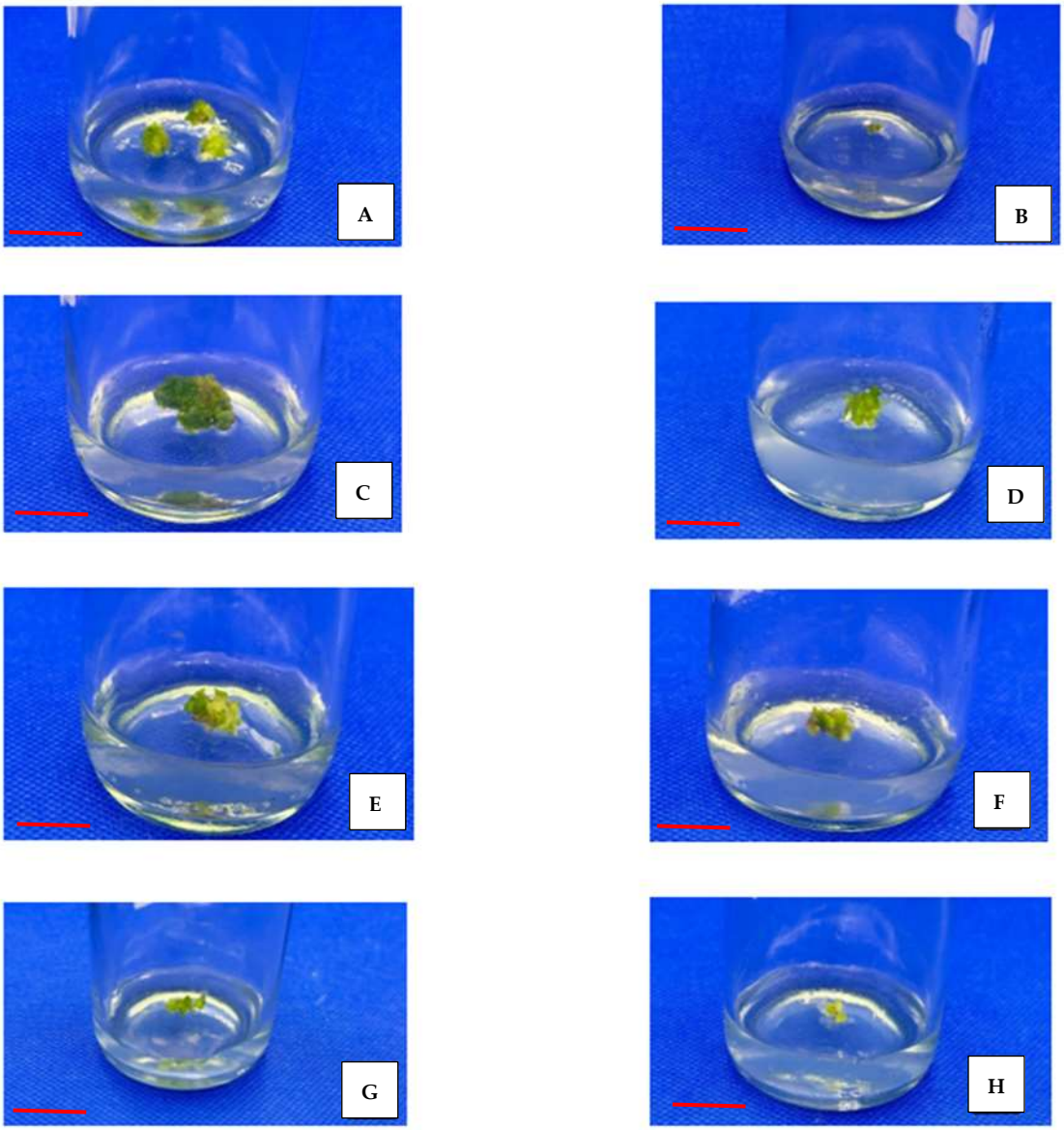

Figure 3 Characteristics of PLBs derived from soaking in various EMS and duration time concentrations and were cultured on VW medium with 1.0 mg/l BA and 0.2% phytagel after 8 weeks of culture. (Bar = 1 cm.)

A. 0% EMS + 60 minute duration time
B. 0.5% EMS + 60 minute duration time
C. 1% EMS + 60 minute duration time
D. 2% EMS + 60 minute duration time
E. 0% EMS + 90 minute duration time
F. 0.5% EMS + 90 minute duration time
G. 1% EMS + 90 minute duration time
H. 2% EMS + 90 minute duration time

3.2 Detection of genetic variation of PLBs by SSR markers

In this present study work, nine SSR-primers tested (EgCIR0008, EgCIR0243, EgCIR0337, EgCIR0409, EgCIR0446, EgCIR0465, EgCIR0781, EgCIR0905 and EgCIR1772) only one primer (EgCIR0905) could provide polymorphic patterns of DNA among treated PLBs with EMS solutions. The EgCIR0905 primer gave a DNA banding with polymorphism, and a specific DNA band at 200 bp of PLBs was treated with 2% EMS for 60 minutes (Figure 4). In this study, EMS-induced mutation induction resulted in genomic change, as evidenced by the appearance of new alleles or the loss of alleles in specific sizes compared to the control plant. Depending on the plant's genotype, EMS treatment can cause different amounts of genomic change. DNA damage,

nucleotide modification, DNA fragment breakage, and chromosome rearrangement brought on by EMS treatment can all result in the disappearance of an allele. Multiple nucleotide changes in the mutant plant's primer binding region can result in the emergence of new alleles [21]. A mutagenesis mechanism that changes the G/C to A/T nucleotides in the primer binding regions of the SSR marker can cause genomic alterations in plant-induced mutations with EMS [22, 23, 24]. Furthermore, nucleotide insertions or deletions in mutant plant DNA sequences cause the microsatellite marker's repeat region to lengthen or shorten [25, 26, 27]. Boonsrangsom et al. (2008) [28] found that Eight primer pairs could be used to amplify the products giving the expected sizes and detect genetic polymorphism in the population with the allele numbers ranging from 4 to 7 (averaged 5.25 alleles per locus).

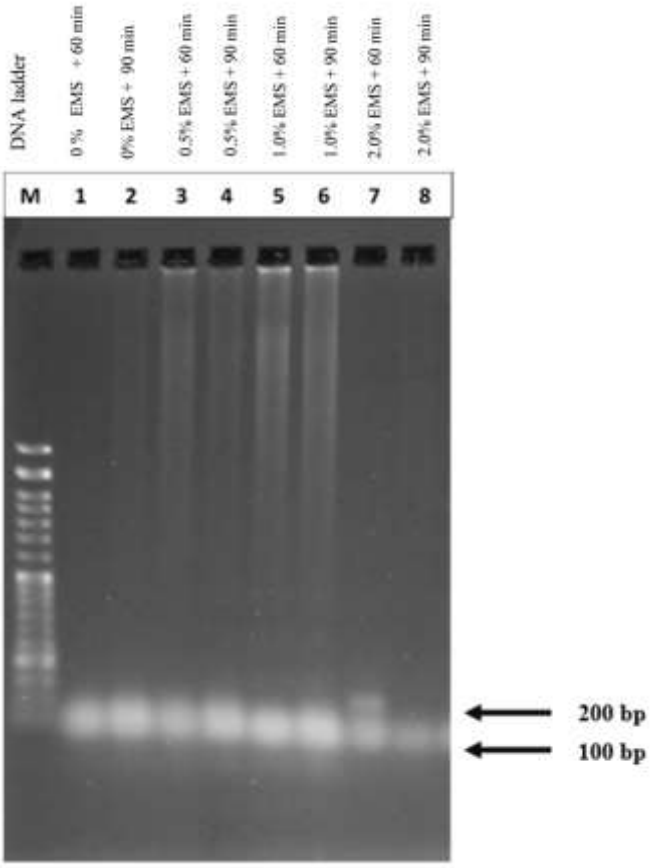

Figure 4 DNA band patterns of PLBs of toothbrush orchids immersed in EMS solution concentrations were examined with SSR markers using the primer EgCIR0905.

4. Conclusions

The effect of EMS on the characteristics of PLBs and their genetic variability after verification with SSR markers was investigated. EMS solution was used to soak a 0.5 cm piece of the protocorm-like bodies (PLBs) at concentrations of 0.5, 1.0, and 2.0%. After that, they were cultured on VW medium supplemented with 1 mg/l BA and 20 g/l sucrose, adjusted to pH 5.7, and solidified with 0.2 % phytagel. The cultures were maintained at 26 ±2°C under light at 3,000 lux for 14 hours daily. After culturing for 30 days, the results showed that PLBs immersed in EMS solution at 1.7% for 90 minutes gave the highest average survival rates at 50% (LD_{50}). For the development of PBLs, PBLs were derived and immersed in 0.5 % EMS for 60 minutes, giving the highest PLB induction (7.37%) and average number of PLBs (1.41 PLBs/explant) after culturing for 8 weeks. A total of 9 SSR primers were used to detect genetic variation. The results found that EgCIR0905 primer gave polymorphic banding at 50% and specific DNA banding size at 200 bp. The present study recorded 90 days of interval observation and suggests that further studies need to obtain more compressive mutation.

5. Acknowledgements

The authors thank the Science Center in Faculty of Science and Technology for the research laboratory. Agricultural Diversity and Biotechnology Research Unit, Research and development institute, Nakhon Si Thammarat Rajabhat University partially supports this research.

Author Contributions: Conceptualization, P.R. and S.R.; methodology, P.R.; software, S.R.; validation, P.R., S.R., and T.K.; formal analysis, P.R.; investigation, P.R.; resources, P.R. and T.K..; data curation, P.R..; writing—original draft preparation, P.R.; writing—review and editing, S.R.; visualization, T.K.; supervision, S.R..; project administration, P.R. and S.R All authors have read and agreed to the published version of the manuscript." Please turn to the CRediT taxonomy for the term explanation. Authorship must be limited to those who have contributed substantially to the work reported.

Funding: This research received no external funding

Conflicts of Interest: The authors declare no conflict of interest.

References

[1] Orchidtropical. Available online: https://www.orchidtropical.com/. (access on 7 October 2022).
[2] Agatha, S.; Fenny, I.; Sulistyo, E.; Popy, H. Induction of protocorm-like bodies (PLBs) Phalaenopsis spp. hybrids mutation through ultraviolet irradiation (UV254) and ethyl methane sulfonate (EMS). *Journal of Applied Agricultural Sciences*. **2023**, *7*(1), 1-15. DOI: 10.25047/agriprima.v7i1.512.
[3] Talebi, A.B.; Talebi, A.B.; Shahrokhifar, B. Ethyl methane sulphonate (EMS) induced mutagenesis in Malaysian rice (cv.MR219) for lethal dose determination. *American Journal of Plant Sciences*. **2012**, *3*. 1661-1665. http://dx.doi.org/10.4236/ajps.2012.312202.
[4] Li, C.; Dong, N.; Zhao, Y.; Wu, S.; Liu, Z.; Zhai, J. A review for the breeding of orchids: Current achievements and prospects. *Horticultural Plant Journal*. **2021**, *7*(5), 380-392. https://doi.org/10.1016/j.hpj.2021.02.006
[5] Romiyadi, R.; Komariah, A.; Amien, S. Keragaan tiga jenis planletanggrek Phalaenopsis asal Protocorm yang diinduksi Ethyl Methyl Sulfonate (EMS) secara *in vitro*. *Kultivasi*. **2018**, *17*(1), 596–607. https://doi.org/10.24198/kultivasi.v17i1.16077
[6] Qosim, W. A.; Istifadah, N.; Djatnika, I.; Y. Pengaruh. Mutagen Etil Metan Sulfonat terhadap Kapasitas Regenerasi Tunas Hibrida Phalaenopsis, *in vitro*. *Jurnal Hortikultura*. **2016**, *22*(4), 360-365. https://doi.org/10.21082/jhort.v22n4.2012.
[7] Avivi, S.; Hariyono, K.; Hartatik, S.; Pertanian, F.; Jember, U. Analisis Pendugaan Parameter Genetik pada Genotipe Tebu Mutan. Agriprima: *Journal of Applied Agricultural Sciences*, **2022**, *26*(2), 124-134. https://agriprima.polije.ac.id/index.php/journal/article/view/v6i2-c
[8] Arisha, M, H.; Shah, S.N.M.; Gong, Z. Ethyl methane sulfonate induced mutations in M2 gene ratio and physiological variations in M1 generation of peppers (*Capsicum annum* L.). *Fronteirs in Plant Science*, **2025**, 6(399), 1-7. doi:10.3389/fpls.2015.00399.
[9] Tadmor, Y.; Katzir, N.; Meir, A. Induced mutagenesis to augment the natural genetic variability of melon (Cucumismelo L.). *Israel Journal of Plant Sciences*, **2007**, *55*(2), 159-169. doi: 10.1560/IJPS.55.2.159
[10] Temnykh, S.; Park, W.D.; Ayers, N. Mapping and genome organization of microsatellite sequence in rice (*Oryza sativa* L.). *Theoretical and Applied Genetics*, **2000,** *100*(5), 697-712. doi: 0.1007/s001220051342.
[11] Prasanna, B.M; Pixley, K.; Warburton, M.L.; Xie, C.X. Molecular marker-assisted breeding options for maize improvement in Asia. *Molecular Breeding*, **2010**, *26*(2), 339–356. doi:10.1007/2Fs11032-009-9387-3.
[12] Ciampi, A.Y.; Azevedo, V.C.R.; Gaiotto, F.A. Isolation and characterization of microsatellite loci for Hymenaea courbaril and transferability to *Hymenaea stigonocarpa*, two tropical timber species. *Molecular Ecology Resources*, **2008**, *8*(5), 1074-1077. doi: 10.1111/j.1755-0998.2008.02159.x
[13] Lorieux, M.; Ndjiondjop, M.N.; Ghesquiere, A. A first interespecific *Oryza sativa* x *Oryza glaberrima* microsatellite based genetic linkage map. *Theoretical and Applied Genetics*, **2000**, *100*(3–4), 593-601. doi: 10.1007/2Fs001229900061.
[14] Ramasoot, S.; Weerapong, M.; Keawsaard, Y.; Ritchuay, S.; Rotduang, P. Enhance efficiency propagation and conservation of toothbrush orchid *in vitro*. *Songklanakarin Journal of Plant Science*, **2022**, *9*(1), 15-23.

[15] Doyle, J.J.; Doyle, J.L. *Isolation of plant DNA from fresh tissue*. Focus. **1990**, pp.12- 15.
[16] Thawaro, S.; Te-chato, S. Application of molecular markers in the hybrid verification and assessment of somaclonal variation from oil palm propagated *in vitro*. *Science Asia*, **2009**, *35*, 142-149. http://dx.doi.org/10.2306/scienceasia1513-1874.2009.35.142
[17] Sirinya, M.; Te-chato, S. Use of ems to induce mutation in *Dendrobium friedericksianum* Rchb.f., *Journal of Agriculture*, **2008**, *24*(2), 153-164.
[18] Samala, S.; Te-chato, S.; Yenchon, S. Effect of ethyl methanesulphonate (EMS) on *Dendrobium Sonia*. *Khon kaen agr. Journal*, **2014**, *42*(3), 506-511.
[19] Arisha, M. H.; Liang, B.K.; Muhammad, S. S. N.; Gong, Z.H.; Li, D.W. Kill curve analysis and response of first-generation *Capsicum annuum* L. B12 cultivar to ethyl methane sulfonate. *Genetics and Molecular Research*, **2014**, *13*(4), 10049-10061. https://doi.org/10.4238/2014.November.28.9
[20] Romeida, A.; Stujahjo, S. H.; Purwito, A.; Sukma, D.; Rurstikawati, S. Induksi Mutasi Protocorm Like Bodies (PLBs) Anggrek Spathoglottis plicataBlume. Aksesi Bengkulu pada Sebelas Taraf Dosis Iradiasi Sinar Gamma. *Prosiding Simposium Dan Seminar Bersama PERAGI-PERHORTI-PERIPIHIGI Mendukung Kedaulatan Pangan Dan Energi Yang Berkelanjutan*, **2012**, 381-387. https://repository.ipb.ac.id/handle/123456789/59898
[21] Sharmin, A.; Hoque, M.E.; Haque, M.M.; Khatun, F. Molecular diversity analysis of some chilli (*Capsicum* spp.) genotypes using SSR markers. *American Journal of Plant Science*, **2018**, *9*(3), 368-379. doi: 10.4236/ajps.2018.93029.
[22] Botstein, D.; White, R.L.; Skolnik, M.; Davis, R.W. Construc-tion of a genetic linkage map in man using restriction fragment lenght polymorphisms. *American Journal of Human Genetics*, **1980**, *32*(3), 314–331.
[23] Greene, E.A.; Codomo, C.A.; Taylor, N.E. Spectrum of chemically induced mutations from a large scale reverse genetic screen in Arabidopsis. *Genetics*, **2003**, *164*(2), 731-740.
[24] Powell, W.; Morgante, M.; Andre, C. The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. *Molecular Breeding*, **1996**, *2*(3), 225–238. doi: 10.1007/BF00564200.
[25] Zhang, G.; Wang, Y.; Guo, Y. Characterization and mapping of QTLs on chromosome 2D for grain size and yield traits using a mutant line induced by EMS in wheat. *The Crop Journa*l, **2015**, *3*(2), 135-144. doi: 10.1016/j.cj.2014.11.002
[26] Tautz, D.; Renz, M. Simple sequences are ubiquitous repetitive components of eukaryotic genomes. *Nucleic Acids Research*, **1987**, *12*(10), 4127-4138. doi: 10.1093/nar/12.10.4127.
[27] Kim, Y.S.; Schumaker, K.S.; Zhu, J.K. EMS Mutagenesis of Arabidopsis. In: Salinas J, Sanchez-Serrano JJ (eds) Arabidop-sis Protocols. *Methods in Molecular Biology; New Jersey, Hu-mana Press*. **2006**. pp 101-103. doi: 10.1385/1-59745-003-0:101.
[28] Boonsrangsom, T.; Pongtongkam, P.; Masuthon, S.; Peyachoknagu, S. Development of microsatellite markers for Dendrobium orchids. Thai Journal of Genetics, **2008**, *1*(1), 47-56.