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Ethyl Methane Sulphonate (EMS) Stimulates The Callus Diversity, Shape, and The Number of Leaves from Shoot Explant Derived Seed of Three Chilli Pepper (*Capsicum annuum* L.) In Vitro

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Abstract: *Chili (Capsicum annuum L.) is one of the crops that are important and widely cultivated in Indonesia. Limited superrior varieties of chili is still generally not able to increase productivity. Wide genetic variability of chili is needed as a basis for breeding of chili. The aim of the study was to improve morphological variability from shoot derived seeds induced by Ethyl Methane Sulphonate (EMS). This experiment was carried out in the tissue culture technology laboratory, Faculty of Agriculture, Universitas Padjadjaran. Descriptive method was used in this experiment and repeated three times. Student's T-Test was applied to evaluate different samples between 0% EMS concentration with 0.5%, 0.75% and 1% EMS concentration in three chilli plantlets namely Unpad CB2, Unpad CK5 and Hot Beauty. Seeds were soaked in EMS for six hours and used as explants source.Seeds were planted on medium MS wirhout Plant Growth Regulator.Shoots from seedling were existed and sub-cultured on basic medium MS supplemented with 2ppm BA + 0.2ppm IAA. The results showed that EMS concentration of 1% has decreasedon the character of germination percentage, callus size, plant height, number of leaves, and chlorophyll content. Induction of CB2 cultivar by 0.5% EMS increased morphological variation in leaf shape.*

Keywords:*Capsicum annuum, EMS, morphology, mutation, tissue culture*

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I. Introduction

The pepper (Capsicum sp.) plant is a shrub plant from the Solanaceae family. The pepper are thought to be originated from the American continent, specifically in Peru. The Capsicum are then brought to Europe by Colombus in 1492. Portugese traders are thought to first introduced peppers to India in 1542, which then widely spreads and finally came to Indonesia[1].

The pepper, specifically the chilli pepper, became an important commodity in Indonesia because of its usage in various settings, such as daily needs, food industry, and even pharmacy. The needs for chilli pepper increases every year. To fully satisfy the needs for chilli pepper, plant breeders are developing chilli peppers that have a high production rate and resistant towards pests.

The chilli pepper's diversity became a basis for improving the plant's character [2]. The limited diversity of chilli pepper in Indonesia became an obstacle in developing plants. Because of this, to obtain the plant's character that we need, such as pests resistant, is quite difficult.

One of the method to increase diversity is with mutation induction. Mutation induction is an alternative to increase diversity in a short time. The mutation process could break down/modify chemical chains in the DNA molecule so that the synthesized protein change, which in turn cause changes in the physiology and the morphology of the plant[3].

There are two kinds of mutagen that could be used for mutation induction, which are chemical and physiology. The chemical mutagen that is very popular for inducting diversity is Ethyl Methane Sulfonate (EMS). EMS was chosen because it could induct higher mutation points when compared to other chemical mutagens. The effectiveness of EMS has been proven on morphology enhancement of chilli pepper plant such as its height, number of petals, roots, branch patterns, age, and the number of fruits[4].

The purpose of this study is to identify the chilli pepper cultivar that is responsive towards EMS to increase morphological diversity from sprout from seed explant that are soaked in various EMS concentration. Through chilli pepper mutation induction by using EMS, genetic material that could be used as superior cultivar, or as hybridization parent, will be obtained. And finally, a chilli pepper cultivar that could be the solution for chilli pepper cultivation in Indonesia will be obtained.

II. Material And Methods

2.1 Material

The materials that are used in this study are Unpad CB 2, Unpad CK 5, and Hot Beauty cultivar seeds, EMS mutagen, MS medium [5], 2 mL/L of BA and 0.2 mL/L of IAA, gelatin, sucrose, 70% and 95% alcohol, 0.20 µm syringe filter, chlorophyll meter (Opti-Sciences CCM-200 plus), 1% detergent, 1% $HgCl_2$, 1% fungicide, 1% bactericide, dan 1% clorox.

2.2 Explants preparation and sterilization

The seeds are sterilized by soaking it in 1% $HgCl_2$, 1% fungicide, 1% bactericide, and 1% Clorox.Each solution was applied 4 minutesexcluding for clorox which is only 15 seconds. Finally, the seeds were washed with sterile aquadest three times and soaked in sterile aquadest for six hours.

2.2 Mutation induction

The seeds were inducted by EMS. EMS was sterilized by syringe filter 0,22 µM on Laminar Air Flow (LAF). The EMS was applied consisted of four concentration whichwere 0%, 0.5%, 0.75% and 1% (v/v). The seeds are soaked in each of the concentration for 6 hours. After being inducted by the EMS, the seeds were washed with sterile aquadest and dried on filter paper. The seeds were planted in MS_0 media. After 4 weeks, the sprouts are subcultured in MS media containing 2mL/L BA and 0.2mL/L IAA, 3% sucrose, and 0.8% of agar. Every treatment consisted of six culture bottles. The culture weretransfered into the culture room with photoperiodicity of 16 hours of light, the room temperature is kept on ±22°C and relative humidity of ±70%.

2.3 Measurement variables

The observation was done on the chilli pepper plantlets using the following observation parameters: the callus size, the amount of leaves, the number of chlorophyll, and the shape of leaves. The callus size are measured by using clay models[6]. The sprout height are measured by putting a ruler in the side of the bottle and then measure from the stem base to the sprout's growing point. The number of chlorophyll are measured with the chlorophyll meter. The blossomed leaves are counted and the ones that fall are not counted, to identify the leave shape differences, descriptor from IPGRI (2010) is used [7] .

2.4 Data Analysis

The design that was based on descriptive methods without replicated design.The cultivars consist of three levels: Unpad CB2, Unpad CK5, and Hot Beauty, and the concentration consists of four levels: 0%, 0.5%, 0.75%, and 1% (v/v). Statistical analysis was usedStudent's T-Test to evaluated difference of diferrent samples between 0% EMS concentration with 0.5%, 0.75% and 1% EMS concentration in three chilli planlets. SPSS Statistics version 23 was used to execute the calculations.

III. Result

3.1 Germination

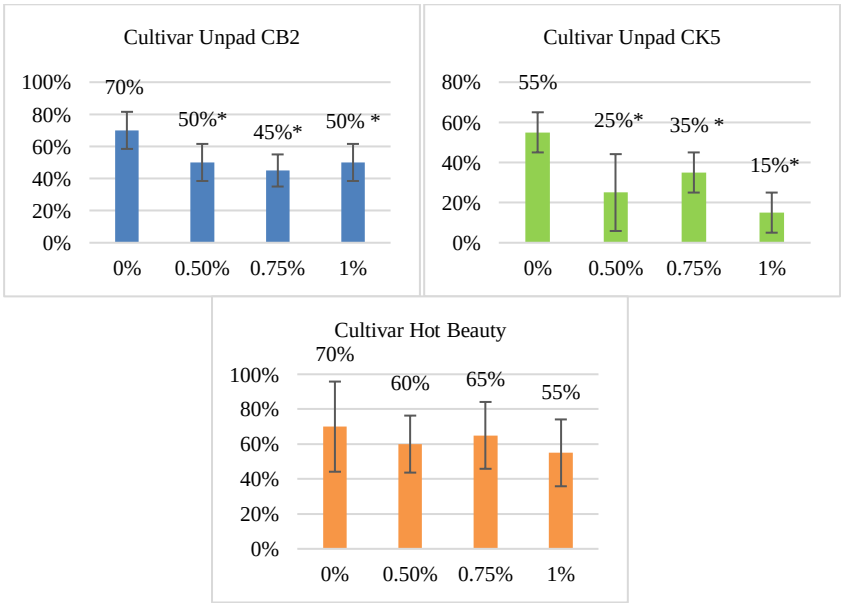

Figure no 1: Percent germinated three chili cultivars with EMS concentration treatment. Average value ± Standard Deviation (SD) which is followed by a symbol asterix showed a significant compared to control according to the Student's T-Test at the level p <0.05.

Based onfigure no 1, the analysis of the Student's T-Test of Unpad CB 2 cultivars, the percentage germinated at all concentrations compared to the control was significant. The Unpad CK5 cultivar also showed asignificantgermination percentage at all concentrations. While the Hot Beauty cultivar showed a percentage of germination that was not significantly in all concentrations. The percentage of germination that was not significant in Hot Beauty was planted because Hot Beauty is a hybrid plant that has resistance to abiotic stress so it needs a higher concentration to be able to influence this cultivar.

3.2 Plantlets Morphology

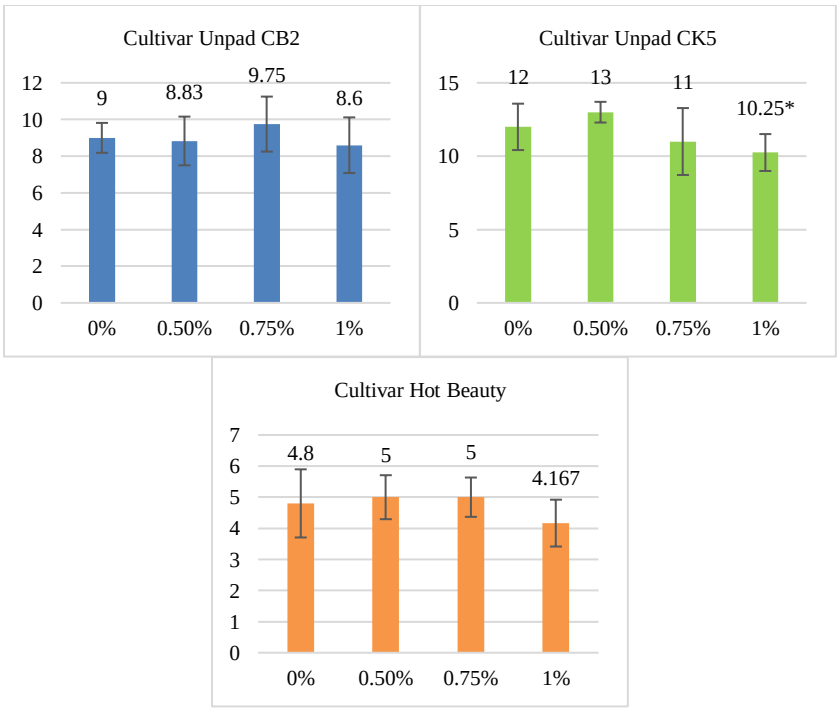

Figure no 2: Callus size three chili cultivars with EMS concentration treatment. Average value ± Standard Deviation (SD) which is followed by a symbol asterix showed a significant compared to control according to the Student's T-Test at the level p<0.05.

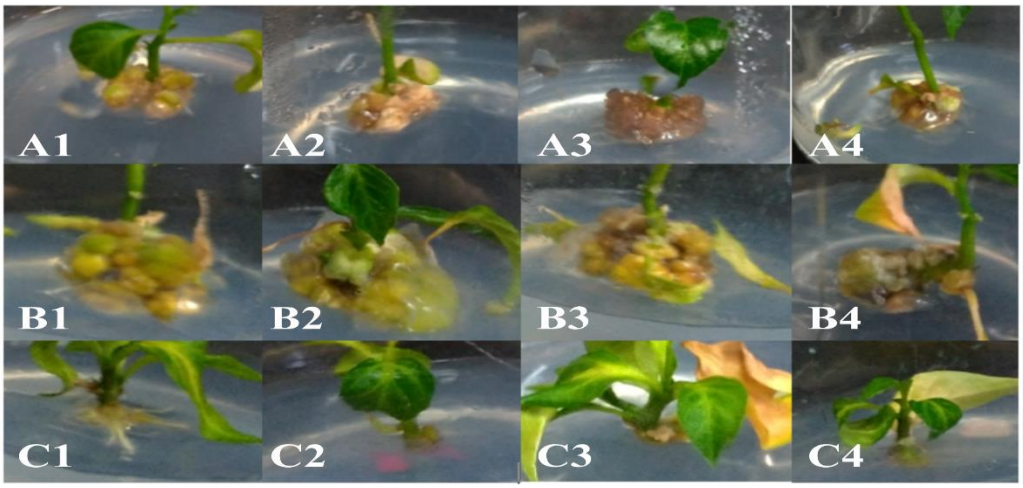

Figure no 3: Callus formation each treatments, A1)Unpad CB2 with 0%, A2) Unpad CB2 with 0.5%, A3) Unpad CB2 with 0,75%, A4) Unpad CB2 with 1%, B1)Unpad CB2 with 0%, B2) Unpad CB2 with 0.5%, B3) Unpad CB2 with 0,75%, B4) Unpad CB2 with 1%,C1)Unpad CB2 with 0%, C2) Unpad CB2 with 0.5%, C3) Unpad CB2 with 0,75%, C4) Unpad CB2 with 1%

Based onfigure no 2, the results of the analysis showed that Unpad CB 2 cultivars had average callus size that werenot significant at all concentrations. Unpad CK5 cultivar showed that callus size was significant at 1%concentrationbut not significant at concentrations of 0.5% and 0.75%. The Hot Beauty cultivar showed that callus size at all concentrations were not significant.Hot Beauty Cultivar has a low callus size, the media used is thought to be unresponsive to this cultivar, because each cultivar has a response to their respective media[8].EMS can stimulates callus formations such as different callus sizes and types(compact and viable) (figure no 3).

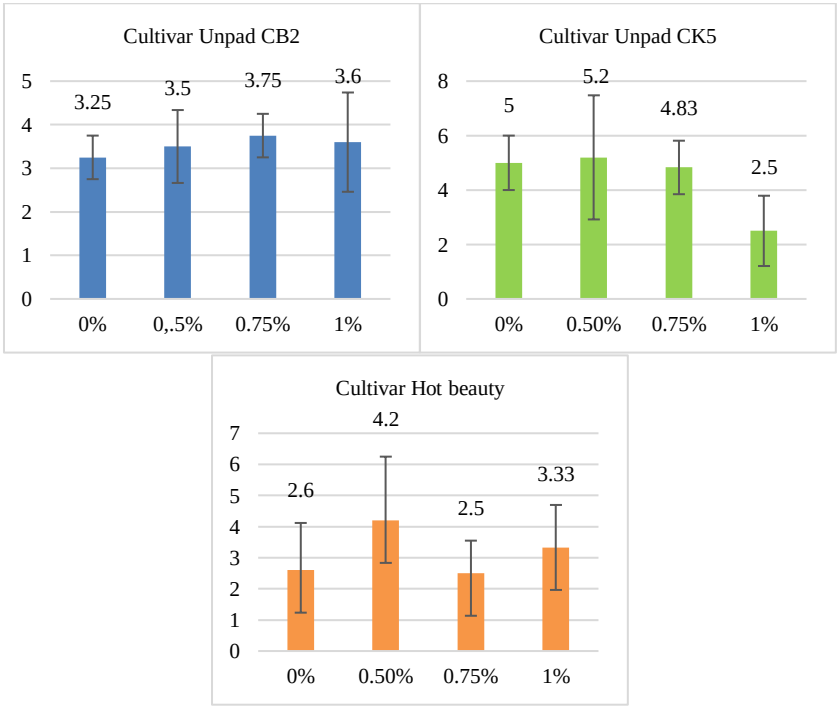

Figure no 4: Number of leaves three chili cultivars with EMS concentration treatment. Average value ± Standard Deviation (SD) which is followed by a symbol asterix showed a significant compared to control according to the Student's T-Test at the level $p<0.05$.

Based on figure no 4, the analysis of the Student's T-Test of Unpad CB 2 cultivars, the number of leaves at all concentrations compared to the control was not significant. The Unpad CK5 cultivar also showed a not significant number leaves at all concentrations. While the Hot Beauty cultivar showed a number of leaves that was not significantly in all concentrations. the number of leaves in all cultivars was not significant because it was suspected that the mutation induction was not able to mutate quantitative characters such as the number of leaves.

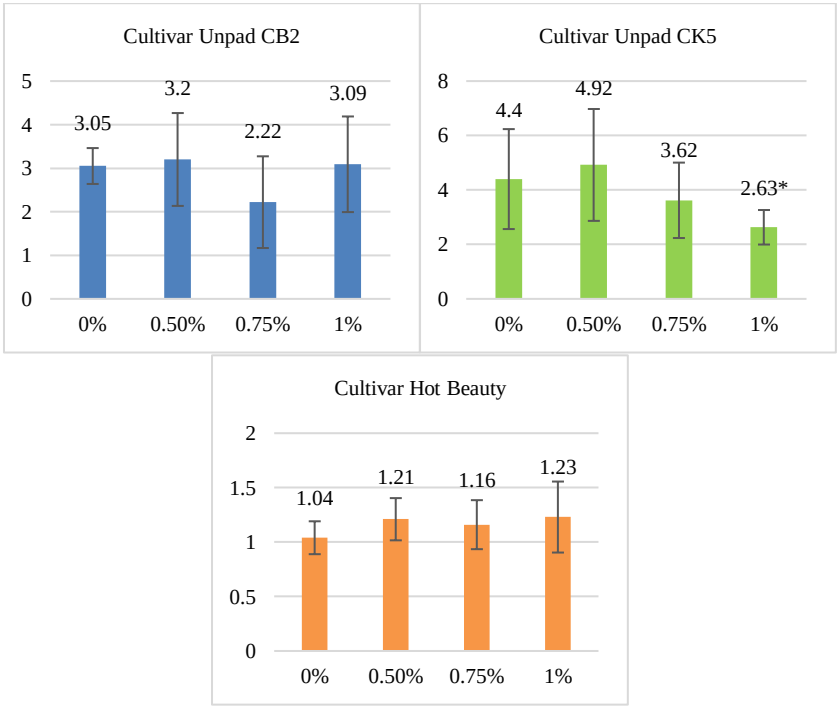

Figure no 5: Plant Height three chili cultivars with EMS concentration treatment. Average value ± Standard Deviation (SD) which is followed by a symbol asterix showed a significant compared to control according to the Student's T-Test at the level p<0.05.

Based on table no 4, the results of the analysis showed that Unpad CB 2 cultivars had average plant height that werenot significant at all concentrations. Unpad CK5 cultivar showed that plant height was significant at 1%concentrationbut not significant at concentrations of 0.5% and 0.75%. The Hot Beauty cultivar showed that callus size at all concentrations were not significant.

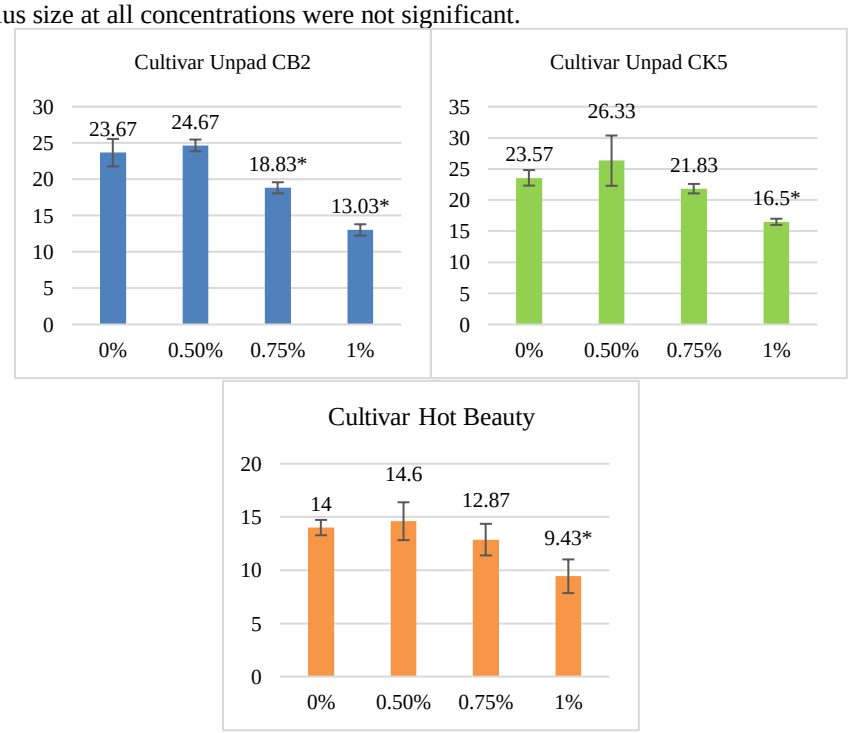

Figure no 6: Chlorophyll Content Indexthree chili cultivars with EMS concentration treatment. Average value ± Standard Deviation (SD) which is followed by a symbol asterix showed a significant compared to control according to the Student's T-Test at the level p<0.05

Based on figure no 6, the analysis of Student's T-Test of Unpad CB 2 cultivar, the chlorophyll content was significantly at 0.75% and 1%concentrations. Unpad CK5 and Hot Beuaty cultivars showed significantly chlorophyll content at a concentration of 1%. Unpad CB2 cultivar was the most susceptible cultivar to EMS because at a concentration of 0.75% the leaves had shown a decrease in chlorophyll content.

Seeds of two cultivar i.e. Unpad CB2 and Unpad CK5 of inducting by 0.5% EMS cause mutagenetic effect formation of three foliage leaves (figure no 7). Addition three foliage leaves showed potential mutant for accelerating photosynthesis.

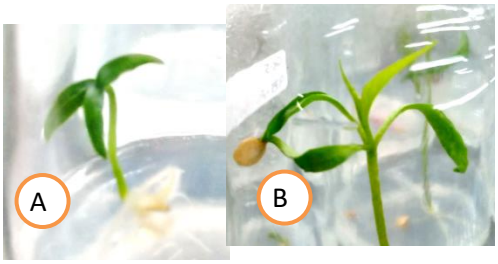

Figure no7:Three Foliage Leaves on M_0Generation
A (k_1e_2 treatment on 2 MST), B (k_2e_2 treatment on 4 MST)

Table no 6: The Observation Data of Chilli Pepper Leaf Shape of Every Treatment

Cultivar	EMS	Genotipe	Leaf Shape	Profil in cross section	Filotaksis
k_1 (Unpad CB2)	e_1 (0%)	4	Ovate	Flat	fibonacci spiral
		8	Ovate	Flat	fibonacci spiral
		1.1	Ovate	Flat	fibonacci spiral
	e_2 (0.5%)	1.2	Ovate	Flat	fibonacci spiral
		9.1	Ovate	Flat	fibonacci spiral
		1.1	Deltoid	Flat	fibonacci spiral
	e_3 (0.75%)	11	Ovate	Moderately concave	fibonacci spiral
		1	Ovate	Flat	fibonacci spiral
		7	Deltoid	Flat	fibonacci spiral
	e_4 (1%)	9	Lanceolate	Flat	fibonacci spiral
		10	Ovate	Flat	fibonacci spiral
		3	Ovate	Flat	fibonacci spiral
k_2 (Unpad CK5)	e_1 (0%)	11.1	Ovate	Flat	fibonacci spiral
		11.2	Ovate	Flat	fibonacci spiral
		9.2	Ovate	Flat	fibonacci spiral
	e_2 (0.5%)	2	Ovate	Moderately concave	fibonacci spiral
		4	Ovate	Flat	fibonacci spiral
		8	Ovate	Flat	fibonacci spiral
	e_3 (0,.5%)	3.2	Ovate	Flat	fibonacci spiral
		1.2	Ovate	Flat	fibonacci spiral
		8	Ovate	Moderately concave	fibonacci spiral
	e_4 (1%)	11	Lanceolate	Flat	fibonacci spiral
		3	Ovate	Flat	fibonacci spiral
		7	Ovate	Flat	fibonacci spiral
k_3 (Hot Beauty)	e_1 (0%)	7.2	Lanceolate	Flat	fibonacci spiral
		12	Lanceolate	Flat	fibonacci spiral
		9	Lanceolate	Flat	fibonacci spiral
	e_2 (0.5%)	9	Lanceolate	Flat	fibonacci spiral
		2	Ovate	Flat	fibonacci spiral
		6.1	Lanceolate	Flat	fibonacci spiral
	e_3 (0.75%)	1.1	Lanceolate	Flat	fibonacci spiral
		5.2	Lanceolate	Flat	fibonacci spiral
		5.1	Lanceolate	Flat	fibonacci spiral
	e_4 (1%)	9	Lanceolate	Flat	fibonacci spiral
		10	Lanceolate	Flat	fibonacci spiral
		6.1	Lanceolate	Flat	fibonacci spiral

Note : k_1 = Unpad CB2, k_2 = Unpad CK5, k_3 = Hot Beauty, e_1 = 0% EMS (Control), e_2 = 0,5% EMS, e_3 = 0,75% EMS, e_4 = 1% EMS.

The Unpad CB2 cultivar in all four of the EMS concentration could produce 3 mutants with differing leaf shape. The 0.5% EMS could produce 2 mutants with differing leaf shape on The Unpad CB2 and Hot Beauty cultivar. The 1% EMS could produce 2 mutants with differing leaf shape on The Unpad CB2 and CK5 cultivar.

IV. Discussion

4.1 Germination

The EMS concentration treatments gave differing effect towards each of the chilli pepper cultivar germination percentage (Table o 1). As the EMS concentration increases, the germination rate of the seed decreases[9]. This result supports The chilli pepper seeds that has been inducted with 2.5% EMS concentration, which suggests that such concentration level is 100% lethal for sepal [10]. This inhibition is thought to be caused by the inhibition of physiological process in plants, enzyme activities [11], hormone imbalance and mitosis inhibition [12]. The EMS could decrease the α-amylase enzyme activities caused by the structural changes in the active side [13]. This enzyme is used for breaking down the α-1.4 glycosidic bond on amylum, which resulted in monomer glucose. This disturbance of the amylase enzyme activities causes the disturbance of the energy supply for germination.

4.2 Plantlet Morphology

Based on Table no2, the 0.5% EMS concentration has the biggest callus size at Unpad CK5. On the other hand, the 1% concentration has the smallest callus size. These results showedIncrease of EMS concentration could make decrease the callus scale, with the decrease differs in each of the cultivar. The success of mutation induction greatly depends on the genotype that was used. The genotype is one of the reason why the plants have different responses [14].

Base on table no 3, in the treatment of 0.5% EMS concentration induced in Unpad CK5 and Hot Beauty cultivars can increases the number of leaves more than other treatments. This increase of leaves is due to the usage of low concentration EMS that could acts as auxin so that it could stimulate sprout growth [15]. In other concentration the decrease of leaves is due to the given mutagen that could stimulate several amino acid biosynthesis that would increase numerous enzymes such as polyphenol oxidase, catalase, and pyroxidase which in turn would inhibit leaf growth [16]. The increase of EMS concentration, the plant height, leaf size, number of leaves, and plant weight would decrease [11].

The Unpad CK5 cultivar have the best response plant height on the 0,5%concentration, but is not significantly different with the 0% and 0.75% concentration. The KV. Gelora Chilli Pepper that was inducted in 0.5% EMS has no significant difference in planlet height with the control treatment [17]. The results showed that the plant height decreases with the increase of EMS concentration. The 1% concentration has the lowest plant height average than other concentration. The decrease of vegetative plant character could be linked with the decrease of auxin rate, auxin synthesis inhibition, and chromosome deviations [18].

The 1% EMS concentration had a effect on all cultivarstowards the chlorophyll content index. An increase in EMS concentration of up to 1.5% can cause chlorophyll deficiency [14]. Chlorophyll deficiency induction is closely related to chromosomal aberration that occurs in plant cells. This chlorophyll mutation can be used as an indicator of mutations because chlorophyll mutations often occur in seeds or leaves that are treated by EMS [19].

The changes on the leaf shape are due to the changing genetic constitution of the plants. The changing genetic constitution is expressed through the plant's morphology. The mutagen became one of the factors of diversity in the plants. The types and concentration that was used would affect the mutagen effectiveness in increasing diversity. chilli pepper seeds that was soaked for 6 hours in 0.5% EMS concentration produced a mutant with differing flower morphology from the control [20]. Marigold seeds that was soaked for 4 hours in 0.9% EMS produced plants with chimera and dwarf leaves [21].

The leaf shape changes in planlets differ based on the concentration. The leaf shape character are not affected by the EMS concentration alone. Genotype also affected the genetic variation. The 5 chilli pepper genotype that was inducted in EMS to increase their resistance from ChiVMV disease gave differing responses, the gelora genotype is the only genotype that increases resistance from ChiVMV disease [17].

V. Conclusion

EMS concentration of 1% has decreased on the character of germination percentage, callus size, plant height, number of leaves, and chlorophyll content. Induction of CB2 cultivar by 0.5% EMS increased morphological variation in leaf shape.

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