Comparison of Particle Bombardment and Silicon Carbide Whisker Vortex Mixing Methods As Mediators for NMU Based Mutagenesis Agent on *Aglaonema*

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ABSTRACT – ***Mutagenesis of cultured Aglaonema axillary buds on Murashige-Skoog medium was performed using mutagen Nitrosomethylurea (NMU). The process was performed with 2 different mediation methods: vacuum-gunpowder powered particle gun with tungsten microprojectile, and silicon carbide whisker added into direct mutagen chemical under utilization of vortex shaker mixing (vortexing) method. Particle bombardment approach was used under 3 different levels of diluted concentrations: 1, 10, 100, and 1000 times of dilution, while for vortexing only used 1000 times of dilution (10, 25, and 50 µM) under 1, 3, and 5 minutes Results of these 2 methods were production of green, pale green, white, and dead shoots. Applying of vortexing with silicone carbide provides optimum mutagenesis only in lower concentration as dead samples occurred in higher level of NMU concentration beyond 25 µM with lethal dosage of 75% (LD_{75}) is 25 µM with 1 minute of duration. Using particle bombardment mediation for mutagenesis, wider range and diverged mutants were produced. Particle bombardment is concluded to be safer mediation method even in least concentration of NMU dilution. Vortexing mediation offered optimization for lower maximum range of concentration, 25 µM in less than 3 minutes of mixing duration.***

Keywords – *Aglaonema*, NMU, mutagenesis, variegated, particle bombardment, silicon carbide

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1.INTRODUCTION

Cultivation of ornamental plants is one of many important aspects in Indonesian agricultural sector development. *Aglaonema* is one of the ornamental plants which leaf possesses varied combinations of colors that so unique it attract some horticulturists and hobbyists to collect and grow these plants. Like every ornamental plants, diversity of colors is very important attribute in breeding programs in order to keep the plant value up in the market. The colour in the Aglaonema leaf such as the anthocyanine could be used as ingredient for medicine. Possible conventional way to produce diversity of plant phenotypes is by doing hybridization. Unfortunately, this way takes 1 plant generation periods in order to cross the genetic materials. Later, proposed by doing tissue culture combined with approach of biotechnology, the process can be shortened.

One of those biotechnology approaches is by using the principle of mutagenesis to produce mutants. Mutagenesis causes changes in DNA caused by rearrangement of nucleobase sequence and the impact is a permanent inheritable change. Mutagenesis uses inducing agent called mutagen, including physical agents like high energy and ionizing radiation, and chemical agents. In this study, Nitrosomethylurea (NMU) serves as chemical mutagenic compound, which causes alkylation in DNA and suspected to causing changes in transcriptional process [1].

Conventional way to apply chemical based mutagens is by soaking the plant specimen tissue for a certain time and specified concentration. On this study, two novel ways for mutagenesis will be introduced: particle bombardment with tungsten microprojectiles, and vortex shaker mixing or vortexing method, assisted with addition of silicon carbide

whisker. Application of biolistic approach will utilize its principle to randomly scattered the mutagen with microprojectile mediation to the sample. However, aside of its randomness of the result on the plant tissue, this method has been regarded as one of the choice for DNA transfer [2]. In the other side, utilization of silicon carbide has been reported successfully performed in DNA transfer in creating a transgenic maize plant [3]. By simple words, biolistic approach imply the random, dispersed, and small amount of induction on tissue in contact of mutagen carried by the microprojectile, while silicon carbide is used to induced scar in the tissue, allowing the direct interaction between cells with the mutagen in larger probability; hypothetically will boost the chance of mutation.

Using these techniques, one method that producing optimum result with more mutant variations and lethal dose (LD) will be observed.

2.RESULTS

2.1 Lethal Dose Comparison

Aglaonema tissue samples after the mutagenesis between mediation of particle bombardment and vortexing with silicone carbide have shown differences in graphic of lethal dosage under less than 3 weeks periods in the photoperiodic shelves.

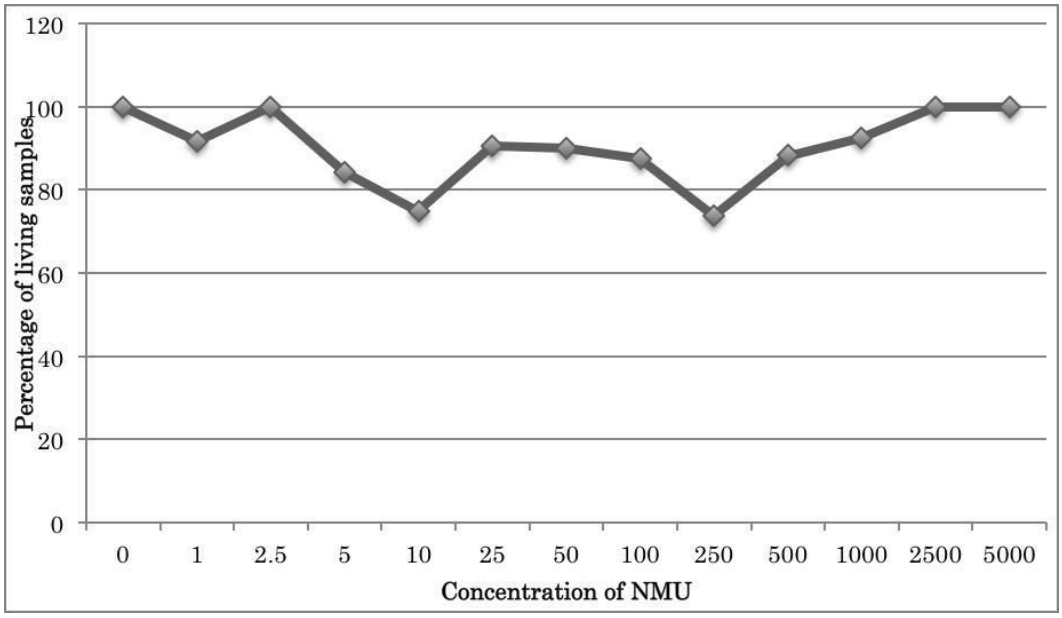

Figure 1: Result of particle bombardment mediated mutagenesis. Concentration of NMU is on µM.

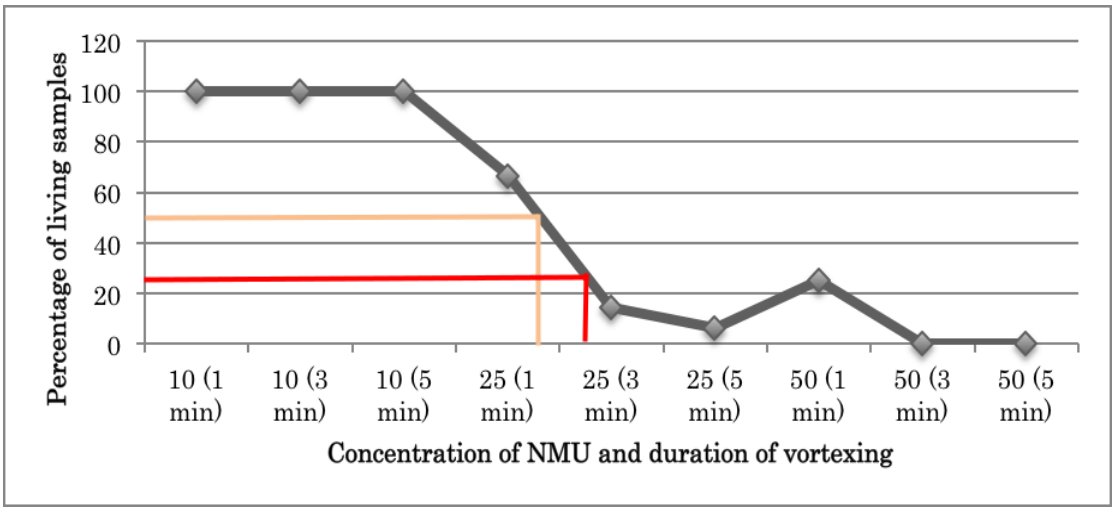

Figure 2: Result of vortex mixing (vortexing) mediated mutagenesis. Concentration of NMU is on µM. Lethal dosage of 75% (LD_{75}) is shown in red lines, while lethal dosage of 50% (LD_{50}) is shown in orange lines.

Graphics in figure 1 and 2 shown that amount of living samples are dropping significantly as increasing of NMU concentration from 10 μM to 25 μM of vortexing result. In other hands, results of particle bombardment showing several different impact on the samples. Least number of result by particle bombardment is on 250 μM, it's 73.7%, following by 75% in 10 μM. Lower than 10 μM, the number of living samples is dropping after 2.5 μM of concentration. Between these concentration, number is increasing between 10 μM and 25 μM but gradually get decreased between 25 μM to 100 μM, followed by significant drop between 100 μM to 250 μM, and inclining again after 250 μM.

Comparing to vortexing result in figure 2, graphic lines are showing declining in number of percentage after 10 μM. Lethal dosage of 50% (LD_{50}) approached in around 25 μM with 1 minutes of mixing, while LD_{75} whereas 25% of survivability of samples hitted in circa 25 μM with 3 minutes of mixing period.

2.2 Mutation Result Comparison

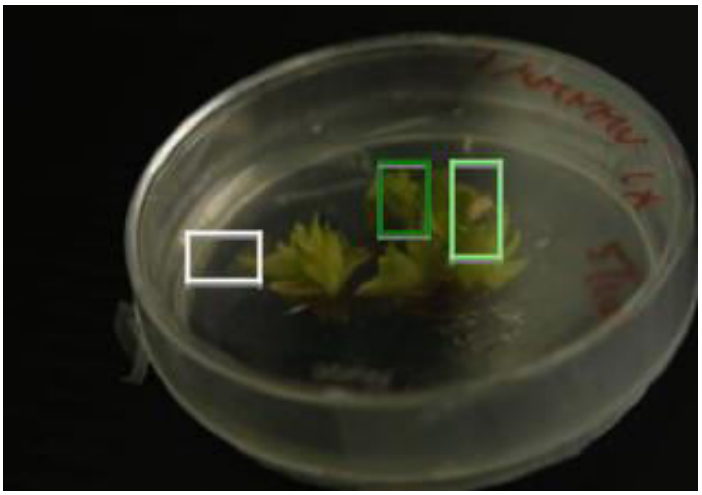

Figure 3: Shoots of *Aglaonema* and color classification for mutant identification: white, green, and pale green.

Reference for color result can be seen in figure 3. According to research data by Mariani, *et al*. [5], the normal coloration of the 3 weeks old shoots is green. It's also described that the white shoots percentage is at the highest number at 5000 μM of NMU.Pale green coloration is probably the result of somaclonal variation caused by the multiple subcultures, which is 5 times on this study. Somaclonal variation may appear due large amount of subculture [4]. However, color change of shoots still not able to determine the color of leaves inside it until it grown enough. The variagated white albino coloration might be resulted in gene change due the mutation [5].

The mutated *Aglaonema* plants were changed into cheerful colors after acclimatization (Fig. 4). In addition, the green and white mutated *Aglaonema* were also existed after acclimatization.

Figure 4: Cheerful colors of mutated *Aglaonema* after acclimatization

In contrast with result of vortexing, due to number of death plant followed the process, percentage of resulted mutants are low in number in higher concentration of NMU. Result can be seen in figure 5, 6, and 7.

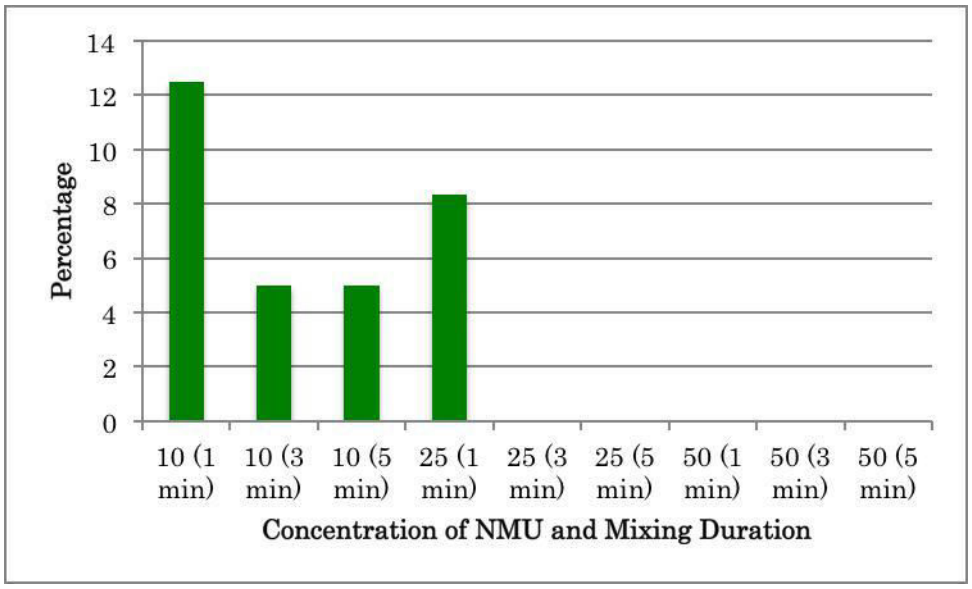

Figure 5: Comparison of percentage of green colored shoots appeared after 3 weeks of vortex mixing. Concentration is stated in μM.

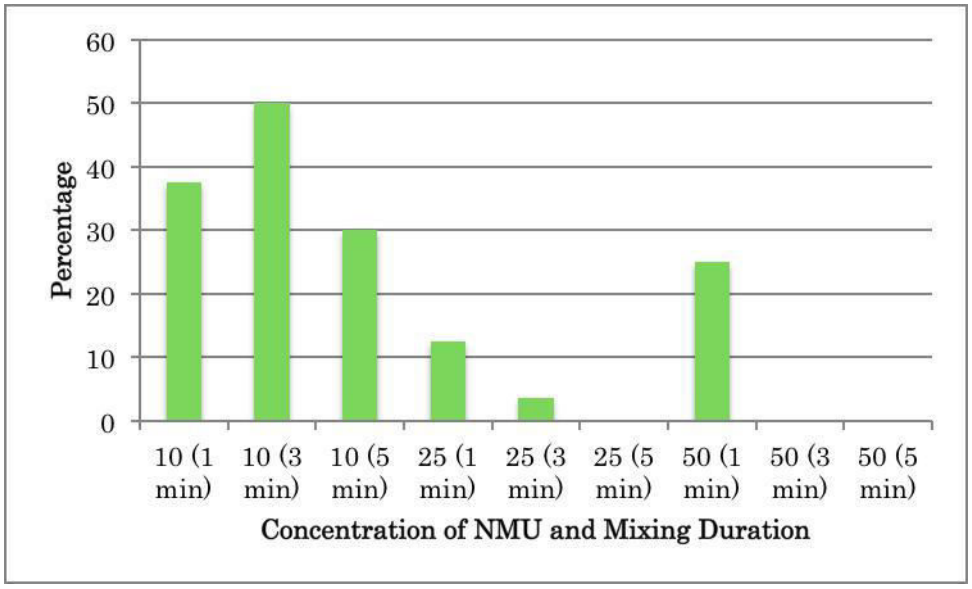

Figure 6: Comparison of percentage of pale green colored shoots appeared after 3 weeks of vortex mixing. Concentration is stated in μM.

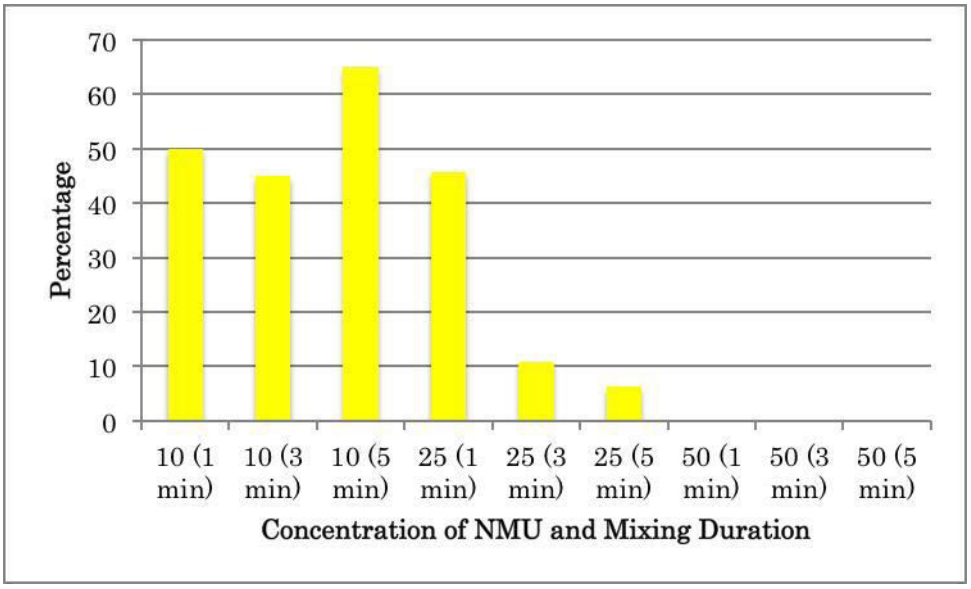

Figure 7: Comparison of percentage of white colored shoots appeared after 3 weeks of vortex mixing. Concentration is stated in μM.

3.DISCUSSION

Shoots were rested for 3 weeks in order to optimize the impact of mutation induction by NMU compound on tissue explants. Usage of particle bombardment and silicone carbide whiskers is based from DNA plant transformation techniques [2].

Utilization of vortexing by silicon carbide whisker here is basically modified technique of common chemical soaking whereas explants are simply put inside the mutagen for some certain of time, some mechanical shaking (or usage of shaker machine) can be used, then followed by post-treatment of recovery by sterile aquadest [6]. Operation of vortex shaker here is to allow the whiskers to penetrate and causing scars to the tissue, enabling the mutagen (NMU) to enter the samples. This silicon carbide and vortexing was done in experiment by Frame, *et al*. [3] to produced transgenic maize plants, the distinction here is addition of a mutagen to apply on plant samples instead of DNA molecules to transfer.

This study results indicated that applying particle bombardment as mutagen mediator provides bigger potential to produce plant mutants compared to vortexing with silicon carbide whisker in the context of survivability and diverging results of mutants. NMU itself is an alkylating mutagen compound causing damages to gene, allowing transcriptional error of the genomic DNA during gene expression. In large concentration, this compound is known to be lethal due damage it caused to the gene. It is suspected that adding extra mechanical damage on the tissue that performed to add bigger surface area and holes on tissues making interaction of the living cell with the mutagen will be easier so optimum mutation progress can be achieved.

According to data shown by figure 2, LD_{75} the maximum range of desirable result approached 25 μM in later stage of 1-minute vortexing. While level of LD_{50} is 25 μM on earlier 1 minutes. By knowing this lethal dosage for mutation using this method, possible further study for mutagenesis on for this vortexing with silicone carbide whiskers can be performed to improve this study using the vortexing method in only circa 25 μM of concentration, with time limit of the process is between 1 to 3 minutes.

Compared to vortexing, particle bombardment mediation provides steadier result with survivability of samples hit maximum of 73.9% as shown on figure 1. Therefore, this concluded that particle bombardment mediated mutagenesis serves better result than vortexing with silicone carbide for mass mutant production. Lowering graphic for the result between green shoots and white shoots demonstrated the effectiveness of mutation level as the NMU concentrations get higher. This effect is resulted by the random scatter impact of the tungsten microprojectile bombardment toward the sample tissues, and unlike the vortexing that happened in the chemical itself, the bombardment occurred between particle gun and samples performed in vacuum. The result probably similar to spraying, with assistant of microprojectiles that serves to inject holes to the tissue, and making surface area to contact are wider. Also in random impact, holes in the cell walls making the mutagenesis more effective as NMU can enter faster to cells.

Vortexing result showing that in only usage of 10 μM of NMU with range between 1 to 3 minutes, production of white shoots already reached 65% (figure 7) while pale green shoots (figure 6) appeared more than green shoots (figure

5), 50% against 12.5% on the following range of 3 durations. The quantity however, is insignificant to death samples caused by this project.

4. CONCLUSION

Conclusion of this project according to the large quantity and diverged result of mutation, mediation of mutagenesis by particle bombardment is capable to produce safer result to the samples rather than vortexing method using silicon carbide whiskers. While on the other side, in matter of efficiency by impact using lower concentration of mutagen, silicon carbide utilization is still considerable for higher effect of mutation.

5.MATERIAL AND METHODS

5.1 Preparation of NMU Stock Solution and Aglaonema Shoots

Solid NMU powder was diluted in sterile distilled water until the concentration is 1 M that served as stock solution. This study is using the same methods as the earlier research done by Mariani, *et al*. [7], using the *Aglaonema* auxiliary shoots as explants, and 5 times of subculture per 2 weeks in MS solid medium with NAA for auxin and TDZ as kinetin. At the end of subculture processes, 1000 cloned shoots were produced. 500 shoots were used for particle bombardment, and other 500 was for vortexing. For easier mobilization to apply mutagenesis, *Aglaonema* samples were put in 2-3 tissues on small plastic Petri dish as shown in figure 3.

5.2 Particle Bombardment

Earlier study from [2]Mariani, *et al*., 2011 supplies data for this research. This particle bombardment was performed with custom-built gunpowder-bullet powered particle gun and done inside a sterile laminar airflow table. First, nylon pellets, tungsten microprojectile powder and gunpowder bullets with the range of 0.76 to 0.82 grams were prepared. Then, solution of NMU was partitioned into several concentrations with different dilution level as described in table 1.

Table 1: Dilution and Concentration of NMU

Level of Dilution (times)	NMU Concentration (μM)
10000	1 2.5 5
1000	10 25 50
100	100 250 500
10	1000 2500 5000

1.5 mL of NMU and tungsten 2.5 mg microprojectile powder inserted into a microfuge tube, lid closed and tube tapped to mix and allowing NMU to adhere to the powdery projectiles. Later, 6 μL of microprojectile-mutagen mix added by using micropipette to the nylon pellet. Petri dish containing the samples was set to bombarding table and as nylon and bullet was placed. This set was put inside bombardment canister

Air inside canister was sucked using vacuum pump before trigger was pulled. Following the bombardment, air regulation valve was turned back to atmospheric pressure, samples removed from the table, and the Petri dish sealed before moved into photoperiodic shelves.

5.3 Vortex Mixing With Silicon Carbide Whiskers

Differed from particle bombardment method, this procedure will use only 1 level, which is 1000 times of NMU dilution with 3 set of duration of time as enlisted in table 2.

Table 2. Mixing Duration and Concentration of NMU

Duration (minute)	NMU Concentration (μM)
1	10 25 50
3	10 25 50
5	10 25 50

On sterile laminar airflow table, 2.5 mg of silicone carbide whisker was added into NMU solution inside a centrifuge tube. Then *Aglaonema* samples were put inside the mix and vortexing was performed. Later, samples were put into sterile aquadest to rinse of excess mutagen before put back into the Petri dish and being sealed inside, and placed into photoperiodic shelves.

6.ACKNOWLEDGEMENT

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