In vitro mutagenesis of pruacan (Pimpinella pruatjan Molk): effect of chemical mutagen EMS and lethal dose determination

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Abstract. Pruacan is a rare medicinal plant endemic to the Dieng Plateau of the Central Java, Indonesia which has properties as an aphrodisiac. Increasing genetic variability of this plant in order to support the breeding programs and increase the productivity and active ingredients of this plant is important. This study is aimed to evaluate the effect of chemical mutagen EMS on the growth and development of in vitro culture of pruacan and lethal dose determination. Embryogenic callus were soaked in 0, 0.1, 0.3, 0.5 and 0.7% EMS for 2 hours and then cultured on regeneration medium containing DKW basal salt medium + IBA 5 mg/l. EMS suppressed the growth and development of pruacan somatic embryos culture. The lowest explant fresh weight, percentage of explants forming plantlets and number of plantlets per explant were obtained from 0.7 % EMS (2.38 g, 91.67 % and 6.22, respectively). Lethal dose 50 was obtained from EMS 0.61, 0.645 and 0.53% based on the explants fresh weight, the percentage of explants forming plantlets. And the number of plantlets per explant, respectively. EMS 0.53% can then be applied to induce mutation in the in vitro culture of pruacan.

1. Introduction
Pruacan (Pimpinella pruatjan Molk, or P. alpina KDS.) Is an Indonesian medicinal plant known to have aphrodisiac, diuretic and tonic properties and has long been used in traditional medicinal herbs [1]. Pruacan spreads limited in the Dieng Plateau with a limited area at an altitude of 1,850 - 2,050 m above sea level (asl) [1]. Harvesting from nature without any conservation and cultivation efforts causes this plant to be endangered and has now been declared a rare plant [1]. Increasing genetic diversity in this plant is needed to support its breeding program. One of the efforts that can be made to increase the genetic diversity of pruacan is through induced mutation. Mutations are a major source of variability of organisms, and mutation-assisted breeding plays an important role in crop improvement either directly or by complementing conventional breeding [2]. Induced mutations can give rise to many different mutant alleles with different degrees of trait modification [3], therefore induced mutations are expected to increase the genetic diversity of pruacan. According to [4], mutation breeding plays a major role in the development of superior plant varieties. along with recombinant and transgenetic breeding.

Induced mutation combined with in vitro technique is a beneficial method because it can increase the frequency of variation. Technically, in vitro culture can produce somaclonal variations and these variations can be increased by using mutagens. There are several advantages of the in vitro mutation
method compared to conventional (in vivo) methods, including: 1) mutations occur at the cell level so that the possibility of chimeras is smaller, 2) mutation rates are higher because each cell has direct contact with mutagens, 3) can followed by in vitro selection where thousands of prospective plant cells can be selected on a laboratory scale so that the selection of mutants becomes more efficient.

EMS (ethyl methanesulphonate, CH₃SO₂OC₂H₅) is one of the most effective and widely used chemical mutagens, and one of the most potent mutagen [3]. EMS has been used in in vitro mutation to induce diversity and increase agronomic properties in grapes [2,5], increasing genetic variability in *Lycopersicon esculentum* [6] and *Capsicum annuum* [7]. However, there is no report yet the use EMS in pruacan plant. Initial studies on induced mutations are important to find out the optimum dose to elicit the best response. This study aims to 1) determine the effect of EMS on the growth and development of pruacan embryogenic culture, 2) determine the optimum dose (lethal dose 50) of EMS on embryogenic culture of pruacan.

2. **Materials and methods**

One hundred and forty-four pieces of 0.5 x 0.5 x 0.5 cm³ embryogenic callus of pruacan were immersed in 0, 0.1, 0.3, 0.5 and 0.7% (v/v) EMS solution for 2 hours. Sodium phosphate buffer solution pH 7 0.1M and DMSO (dimethyl sulfoxide) 4% were used as a solvent (EMS 0%). After EMS treatment, explants were rinsed 3 times with sterile aquades, then placed in a petri dish with sterile filter paper. The explants were planted on regeneration medium containing DKW basal medium with the addition of IBA 5 mg/l, sugar 30 g/l and phytagel 2 g/l with pH medium 5.8. The cultures were then incubated at 16-18°C under TL lamps for 16 hours a day to form plantlets. This research was arranged in a completely randomized environmental design with 4 replications, each replication consisting of 3 bottles and each bottle consisting of 3 explants.

Variables observed include the addition of fresh weight of explants, the percentage of explants forming plantlets and the number of plantlets per explant. Observations were made every month until the age of 3 months. Determination of lethal dose 50 was done using the curveexpect 1.3 program. Lethal dose 50 is the EMS dose which is caused 50% reduction in growth and development of explant compared to control or EMS 0%.

3. **Results and discussion**

The results showed the addition of explant fresh weight was decreased with an increase in the EMS dosage at the age of 1 and 3 months.

The results of this study are in line with those from previous studies. Increasing the EMS concentration reduced seed germination, plant viability and pollen fertility in two varieties of *Vicia vaba* [8,9]. Increasing the concentration of mutagenic agent in the medium resulting in reduction the sprouting of shoot and root in in vitro culture of grape [2]. As increasing of EMS concentration also decreased the germination of *Lycopersicon esculentum* seeds [6] *Capsicum annum* seeds [7]. The survival rate of explants, number of shoots, number of roots, root length and number of strawberry leaves decreased when EMS concentration and duration were increased [10]. The inhibitory effect of EMS is thought to be due to DNA damage in mutagen-treated cells. DNA damage increased in a concentration-dependent manner in nuclei from root and leaf tissues of plant, and induced genomic DNA damage versus induced somatic mutation in leaf nuclei were highly correlated [8]. The use of high concentration of EMS (5.0mM) resulted in cell damage in callus culture of petunia [4]. The meiotic abnormalities were found along with increasing EMS concentration in two varieties of *V. vaba* [9].
Figure 1. The effect of EMS on: (a) the average of explant fresh weight, (b) the percentage of explants forming plantlets, (c) the number of plantlets per explant.

Figure 2. Comparison of number of plantlets aged 3 months in control aquadest (left) and EMS 0.7% for 2 hours (right)

The mutagen dose is the result of multiplying the concentration and length of treatment period. The higher the concentration and the length of the treatment period, the greater the mutagen dose received by the plant. The higher the mutagen dose, the higher the inhibitory effect on explant growth and development. However, the sensitivity of each plant material to mutagens varies depending on genetic, physiological and environmental conditions during and after mutagen application. Early studies of induced mutations were primarily aimed at finding the optimal mutagen and dosage combination for the best response. [3]. In this study, we tried to find out the lethal dose 50 (LD50) of EMS on in vitro culture of pruacan. The result shows that the LD 50 of EMS was different depending on the parameters observed. LD 50 was 0.61, 0.645 and 0.53 % based on the addition of explant fresh weight,
percentage of explants forming plantlets and number of plantlets per explant, respectively (figure 2a, b and c). The difference in LD 50 values between the three parameters was caused by differences in the sensitivity of each parameter to EMS treatment. This study shows that the parameter number of plantlets per explant is more sensitive to EMS treatment compared to the addition of explants fresh weight and the percentage of explants forming explant. For further study, EMS 0.5% for two hours was recommended used in induced mutation of in vitro culture of pruacan using embryogenic culture as explants.

![Figure 3a](image1.png)  
**Figure 3a.** Regression analysis for determine of lethal dose 50 based on the addition of explant fresh weight

![Figure 3b](image2.png)  
**Figure 3b.** Percentage of explants forming plantlet

![Figure 3c](image3.png)  
**Figure 3c.** Number of plantlets per explant (C)

4. Conclusions
Increasing of EMS concentration in the medium decreased the addition of explants fresh weight, the percentage of explants forming plantlet and the number of plantlets per explant in embryogenic culture of pruacan. The lowest the addition of explants fresh weight, the percentage of explants forming plantlet and the number of plantlets per explant were obtained from 0.7% EMS, while the LD50 was obtained from 0.5% EMS.

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