Induction of Mutagenesis in *Erycina pusilla* by Ethyl Methanesulfonate and Sodium Azide

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Key word: *Erycina pusilla*, Chemical mutagenesis, EMS, Sodium Azide

Summary

The purposes of this study were to develop novel *Erycina pusilla* by EMS and sodium azide mutagenesis, and to determine the response of *Erycina pusilla* PLBs to EMS and sodium azide. *In vitro* PLBs of *Erycina pusilla* were exposed to various EMS treatments at 0, 0.1, 0.2, 0.4 and 0.8% for 0.5, 1 and 2 hour, and were exposed to various NaN_3 treatments at 0 , 0.5 , 1, 2, and 4 mM for 0.5, 1 and 2 hour. The survival percentages, number of plant regeneration and flow cytometric analysis were conducted after EMS and NaN_3 treatments. One month after EMS and NaN_3 treatments, some PLBs shriveled and started to turn brown. The results revealed that treatments of 0.1% EMS for 1 hour and 0.5 mM NaN_3 for 0.5 to 2 hour had highly survival percentages and plant numbers of *Erycina pusilla* plantlets. In general, EMS treatments were more effective in reducing survival percentages and numbers of plant regeneration as compared to those of NaN_3 treatments. The present study indicated that the diffusion of EMS and NaN_3 into *Erycina pusilla* PLBs was successful as evidenced by the gradual loss of explant viability with increasing EMS and NaN_3 exposure time and concentration. Lower concentration of EMS and NaN_3 treatments have less influenced biological damage and would be suitable for inducing desirable mutations in *Erycina pusilla*. Nevertheless, changes in profiles of flow cytometric histograms were found in several regenerated plants of EMS and NaN_3 treated *Erycina pusilla*.

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Introduction

The technology for induction of mutations is a powerful tool for developing better varieties of food and industrial crops (Hassan *et al*., 2010). The numerous mutagenesis techniques available, including insertional mutagenesis, X-rays, gamma rays, and fast and thermal neutrons, can be categorized by their physical properties and mutagenic effect (Watanabe *et al*., 2007). Chemical mutagenesis and physical mutation are general methods for mutation induction and has been applied for plant breeding in many countries in the world. Physical and chemical mutagens induce physiological damages, gene mutations and chromosomal aberrations in the biological material in M1 generation (Bashir *et al*., 2013).

In mutation breeding studies, it is important to determine a suitable dose/concentration of mutagen for a crop plant which can be employed for inducing maximum variability through point mutations. Seed germination, seedling growth, pollen sterility and chromosomal aberration are the commonly used criteria for studying radio-sensitivity in plants (Sheikh *et al*., 2012). Physical mutation has been successful in creating many *Dendrobium* 'Sonia' mutant varieties. In *Dendrobium* 'Ekapol' and *Dendrobium* 'Sonia' irradiation resulted in changes of flower pigmentation and size (Sakinah and Mohd Nazir, 2002). More variations with attractive combinations of spray length, bud number, flower color and form are required to create commercially valuable varieties (Hassan *et al.*, 2010).

Ethyl methane sulphonate (EMS), a chemical mutagen of the alkylating group has been reported to be the most effective and powerful mutagen and usually causes high frequency of gene mutations and low frequency of chromosome aberrations in plants (Bashir *et al*., 2013). Ethyl methanesulfonate (EMS) is a popular chemical mutagen, and N-methyl-N-nitrosourea (MNU) is also used for this purpose.

Sodium azide (NaN_3) is the least dangerous and the most efficient mutagen and has been reported to be mutagenic in several crop species (Adamu and Aliyu, 2007; Mostafa, 2011). The mutagenicity of sodium azide is arbitrated through the formation of an organic metabolite which enters the nucleus, interacts with DNA and generates point mutations in the genome. According to Nilan *et al.* (1977), Sodium azide is relatively safe to handle, inexpensive and non-carcinogenic as compared to other mutagens (Bashir *et al*., 2013).

Erycina pusilla (*Erycina pusilla* (L.) N. H. Williams & M.W. Chase) used to be known as *Oncidium pusillum* or *Psygmorchis pusilla* is so unique appearance that it has miniature size with bright yellow flowers on a green fan of leaves (Chase *et al*., 2005). *Erycina pusilla* is a very small size of orchids, lack pseudo-bulbs which can storage water and nutrient, monopodial growth, the

leaves laterally flattened and grow in one direction only can grow up to 5 cm long and 1 cm wide (Felix and Guerra, 2010). The miniature plants are odd looking and have a disproportionate large single flower. The flower is yellow color, size about 1.5 to 4 cm long and 1 to 2 cm wide. The chromosome number of *Erycina pusilla* is 2n = 12 (Felix and Guerra, 1999), and it's the smallest known in orchids. *Erycina pusilla* is a tiny miniature orchid, with lovely flowers blooming all year. *Erycina pusilla* have potentially formed an emerging flora industry.

However, there are several aspects still need to be improved in *Erycina pusilla*, such as only yellow color flower, single flower spike, few variation in cultivar, period of flowering. The aims of present study were to develop novel *Erycina pusilla* by EMS and sodium azide mutagenesis, and to determine the response of *Erycina pusilla* PLBs to EMS and sodium azide treatments.

Materials and Methods

1. Plant materials

Erycina pusilla plants were purchased from Flower Space Orchids Company, Changhua, Taiwan. The seed capsules after self-pollination were collected and used as experimental material.

2. Induction of protocorm-like bodies (PLBs) and proliferation

The seed capsules of *Erycina pusilla* after selfing were dissected aseptically. Mature seeds of *Erycina pusilla* were sterilized with a solution of NaOCl (1% available chlorine) that shaking by vertex 15 min, and then washed three to five time with sterilized distilled water. The seeds were sown in plastic petri dishes containing sterile 1/2 MS medium (2.2 g/L Murashige and Skoog salts, 30 g/L sucrose, 8 g/L agar, pH 5.7) (Murashige and Skoog, 1962). The plastic petri dishes were cultivated in a growth chamber at 25 ℃ with under 8 hours/16 hours dark/light photoperiod. After 6 months of induction, PLBs formed from embryoid bodies *in vitro* were used as materials for EMS and sodium azide treatments.

3. Experimental methods

(1). Induction of mutation by EMS treatment

An orthogonal experiment design was employed in order to test various treatment regimens encompassing four EMS concentrations include 0, 0.2, 0.4 and 0.8, and three treatment durations include 1/2, 1, and 2 hour. EMS stock solution was sterilized through 0.45 μm sterile millipore filtration in a laminar flow chamber. EMS stock solution was diluted with sterilize water to final 0.2%, 0.4% and 0.8% solution.

Fifteen PLBs were immersed in 15 ml of EMS solution in a 125 ml flask for 1/2, 1, and 2 hour with shaking at 100 rpm. After EMS treatment, the PLBs were washed with sterilized water

three times and then placed on their 1/4 MS medium, cultivated at 25±2 ºC, 35 μmol $m^{-2}s^{-1}$ PPFD, and 16 hour photoperiod. The PLBs were subcultured every 1 month and maintained for 6 months. After 6 months of culture, the explants were changed to CN medium (containing sucrose 20 g/l, 2.2 g MS vitamins, 1 g/l tryptone, 170 mg/l $Na.H_2PO_4.H_2O$, 100 mg/l myo-Inositol, 200 ml coconut water, 1 g/l charcoal and 8 g/l agar at pH 5.2). After another 6 months of culture, regenerated plants about 2-3 cm in height with 6-8 leaves and 4-8 roots were transferred to pots containing sphagnum moss and acclimatized under greenhouse conditions.

(2). Induction of mutation by sodium azide treatment

An orthogonal experiment design, was employed in order to test various treatment regimens encompassing five sodium azide concentrations include 0, 0.5, 1, 2, and 4 mM, and three treatment durations include 1/2, 1, and 2 hour. Sodium azide stock solution (0.1 N) was prepared by dissolved 0.65 sodium azide in phosphate (pH 3) to enhance efficiency. The phosphate buffer consisted of 79.45 ml of 0.1 M citric acid (2.1 g/100 ml) and 0.2 M 20.55 ml Na_2HPO_4 (3.5 g/100 ml). Sodium azide stock solution was sterilized through 0.45 μm sterile millipore filtration in a laminar flow chamber. Sodium azide stock solution was diluted with sterilize water to final 0.5, 1, 2 and 4 mM solution.

Fifteen PLBs were immersed in 15 ml of sodium azide solution in a 125 ml flask for 1/2, 1, and 2 hour with shaking at 100 rpm. After sodium azide treatment, the PLBs were washed and cultivated in the same condition as mentioned above

4. Flow cytometer analysis

Approximately 0.5 cm^2 of young leaf tissue from each sample was taken, and the nuclei were extracted and then stained, using the reagent kit Partec CyStain UV precise P (Paetec, Münster, Germany). In order to extract cell nuclei, HR-A extraction buffer (400 μl) containing 1% w/v PVP was added to the leaf tissue which was then macerated with a razor blade. The homogenate was incubated for one minute and then filtered through a Partec 30 μm Cell-Trics disposable filter in order to remover cell debris. HR-B staining solution (1.6 ml) was added to the suspension of nuclei and sample were analyzed immediately using a PA-I flow cytometer (Partec) equipped with a 100 W high-pressure mercury arc lamp. Leaf sample from three untreated plant of *Erycina pusilla* were used as control

5. Statistical analysis

Comparisons of quantitative data between two variable groups were made using Statistic version 8 and morphological variation rates analyzed using ANOVA and multiply comparison among levels of each factor and among combination were analyzed using Duncan's test. Percentage data were transformed *via* arcsine before analysis.

Results

1. Effects of EMS on *Erycina pusilla* PLBs

(1). Effects of EMS on shoot regeneration of *Erycina pusilla* PLBs

The percentage of survival rate of *Erycina pusilla* PLBs after treated with various concentrations of EMS for different times and followed by cultivation for 6 months was shown in Fig 1. The percentage of survival rate of PLBs treated with 0.1 % EMS was higher than those of 0.2, 0.4, and 0.8% EMS treatments (Fig. 1A). Results indicated that percentage of survival rate decreased with increasing EMS concentration. Likewise, percentage of survival rate decreased with increasing incubation time of EMS (Fig. 1B). Results of combination of concentrations and incubation time for EMS treatment was shown in Fig. 1C. Highest percentage of survival rate was obtained in 0.1% EMS treated for 1 hr., lowest percentage of survival rate was found in 0.8 % EMS treated for 2 hr. (Fig. 1C).

(2). Morphological characteristics of EMS treated plants

The morphological characteristics of mutagenic plants were compared with those of corresponding control plants. The vegetative characteristics traits of mutagenic plants as compared with those of the corresponding control plant were shown in Fig. 1D. Changes in plant form were observed in several EMS treated plants (Fig. 1D). Changes in flower morphology were also observed in regenerated *Erycina pusilla* plants treated with 0.1% EMS for 1 hr (Fig. 2). Thus, number of plant regenerated from *Erycina pusilla* PLBs after EMS treatment was highest in 0.1% EMS treated for 1 hr (108 regenerated plants). Apparently, EMS cause the death of *Erycina pusilla* PLBs, especially in treatments of 0.4% and 0.8% concentrations (Data not shown).

(3). Analysis of EMS treated plants by flow cytometry

Attempts had been made to screen the putative *Erycina pusilla* mutants in the early stage at DNA level with the aid of flow cytometry. Flow cytometric histograms of 11 regenerated *Erycina* plants by EMS mutagenesis were examined. Several unique peaks in 11 putative mutants as compared with those of the corresponding control plant were observed (Fig. 3). Whether these differences could reflect DNA variation need to be further verified.

2. Effects of sodium azide on *Erycina pusilla* PLBs

(1). Effects of sodium azide on shoot regeneration of *Erycina pusilla* PLBs

The percentage of survival rate of *Erycina pusilla* PLBs after treated with various concentrations of sodium azide for different incubation times and followed by cultivation for 6 months was shown in Fig 4. The percentage of survival rate of PLBs treated with 0.5 mM sodium azide was higher than those of 1, 2, and 4 mM sodium azide treatments (Fig. 4A).

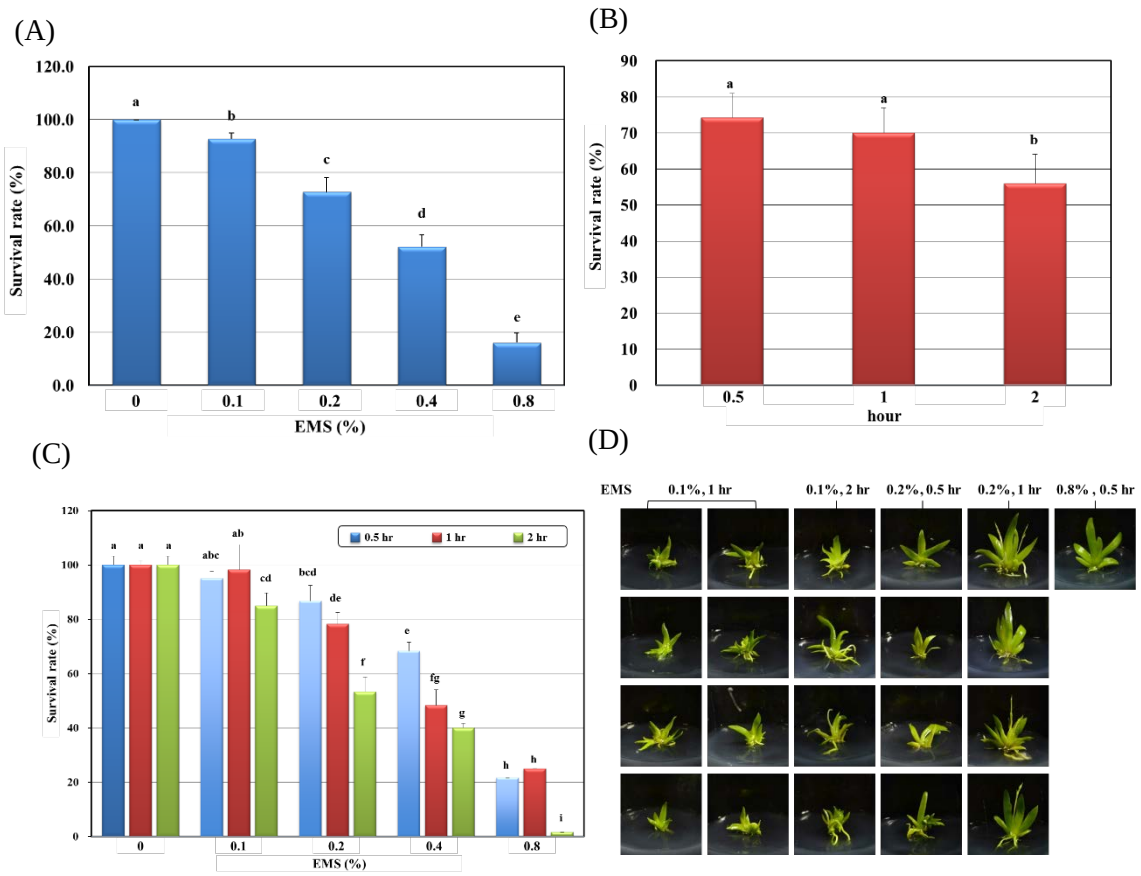

Fig. 1. Effects of concentrations (A), incubation times (B), and concentrations x incubation times (C) of EMS treatments on the survival rate of *Erycina pusilla* PLBs after cultivation in 1/4 MS medium for 6 months, and appearances of representative regenerated *Erycina pusilla* plantlets after EMS treatments (D).

Fig. 2. Changes in flower morphology of *Erycina pusilla* by EMS mutagenesis. A: untreated; B, C, D: treated with 0.1% EMS for 1 hr.

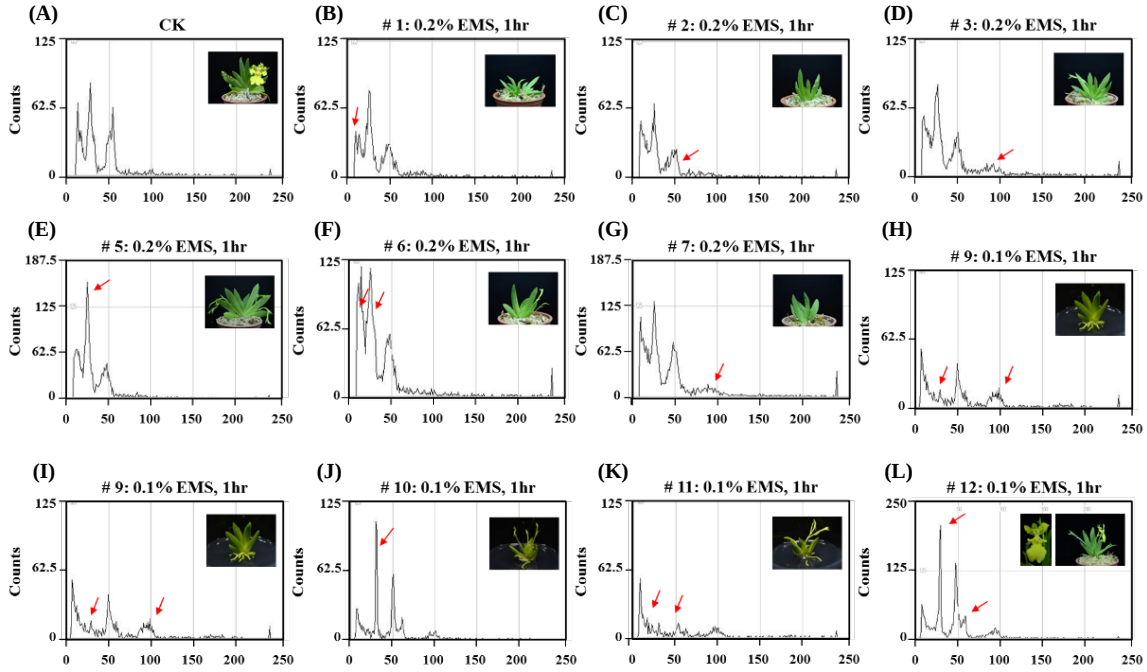

Fig. 3. Flow cytometric histograms of regenerated plants of *Erycina pusilla* by EMS mutagenesis. A red arrow indicated the different peak in putative mutants from the untreated plants. On the X-axis, the channel numbers of the flow cytometric distribution analyses are plotted, the Y-axis represents the number of fluorescence signals recorded per channel. A: untreated (CK); B~G: treated with 0.2% EMS for 1 hr; H~L: treated with 0.1% EMS for 1 hr.

Results indicated that percentage of survival rate decreased with increasing sodium azide concentrations. Interesting, percentage of survival rate decreased was not corresponding with increasing incubation time of sodium azide (Fig. 4B). The combination of concentrations and incubation time for sodium azide treatment was shown in Fig. 4C. All the PLBs were survived in 0.5 mM sodium azide treated for 0.5, 1 or 2 hr, and. lowest percentage of survival rate was found in 4 mM sodium azide treated for 1 hr (Fig 4C).

(2). Morphological characteristics of sodium azide treated plants and analysis of sodium azide treated plants by flow cytometry

The number of plant regenerated from *Erycina pusilla* PLBs after sodium azide treatment was highest in 0.5 mM sodium azide treatments (Data not shown.). Besides, sodium azide was less toxicity to *Erycina pusilla* PLBs in comparison to EMS treatments. The morphological characteristics of sodium azide treated mutagenic plant were compared with those of

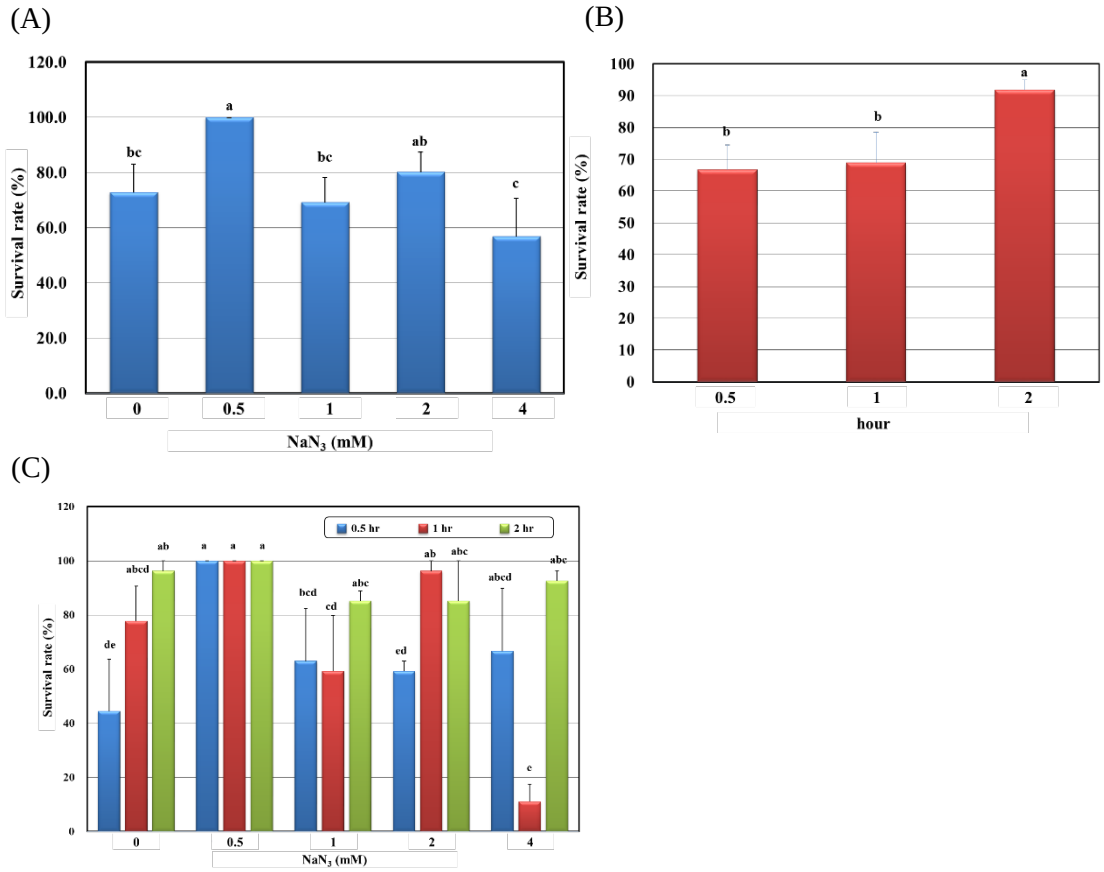

Fig. 4. Effects of concentrations (A), incubation times (B), and concentrations x incubation times (C) of sodium azide treatments on the survival rate of *Erycina pusilla* PLBs after cultivation in 1/4 MS medium for 6 months.

corresponding control plants. The vegetative characteristics traits of mutagenic plants as compared with those of the corresponding control plant were shown in top-right corner of Fig. 5. Changes in plant form were observed in several sodium azide treated plant (Fig. 5 B-K) as compared with those of the corresponding control plant (Fig. 5A). Attempts had been made to screen the putative *Erycina pusilla* mutants in the early stage at DNA level with the aid of flow cytometry. Flow cytometric histograms of 10 putative *Erycina pusilla* plants by sodium azide mutagenesis were examined. Several unique peaks were detected in 10 putative mutants as compared with those of the corresponding control plant (Fig. 5A). Whether these differences could reflect DNA variation need to be further verified.

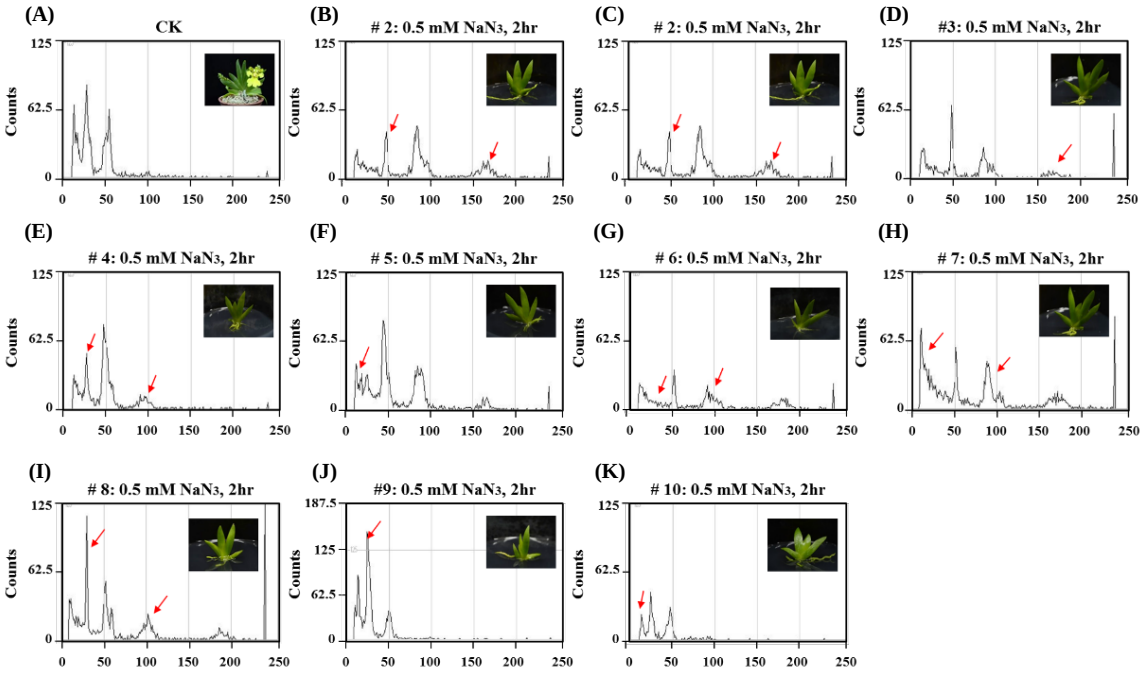

Fig. 5. Flow cytometric histograms of regenerated plants of *Erycina pusilla* by sodium azide mutagenesis. A red arrow indicated the different peak in putative mutants from the untreated plants. On the X-axis, the channel numbers of the flow cytometric distribution analyses are plotted, the Y-axis represents the number of fluorescence signals recorded per channel. A: untreated (CK); B~K: treated with 0.5 mM NaN_3 for 2 hr.

Discussion

Many studies had reported the adverse effect of physical and chemical mutagens on various biological parameters (Bashir *et al*., 2013). The orchids flower industry thrives on novelty and traits such as flower color, flower form, flower size, maturity time and scent are primary novelty markers because they are key determinants in consumer choice of ornamental plants. For *Erycina pusilla*, a species that is mostly appreciated for its small plants and small flower.

Mutation breeding, which leads to altered phenotypes after permanent heritable change in the structure of the genetic material, is now established as a time-saving and inexpensive approach for plant growth and flower improvement (Rego and Faria, 2001; Fang, 2011). The present study explores the possibility of adopting mutation breeding in generating new *Erycina pusilla* cultivars. Chemical mutagens have been applied to numerous plants to induce mutations. The observations on the survival rate of *Erycina pusilla* in the present study revealed that EMS were more superior

to NaN_3 in reducing the survival rate of *Erycina pusilla* plants. Rodrigo *et al*. (2004) obtained chrysanthemum mutants with various petal colors for example pink–salmon, light pink, bronze, white, yellow, and salmon by means of EMS treatment.

The present study showed that the diffusion of EMS into *Erycina pusilla* PLBs was successful as evidenced by the gradual loss of explant viability with increasing EMS exposure time and concentration. The EMS concentrations used in the present study were hence effective in inducing mutations in *Erycina pusilla*, and the concentrations were within the range between 0.5% to 2% which was similar to that of *in vitro* mutagenesis for other plants (Latado *et al*., 2004; Luan *et al*., 2007; Fang, 2011).

Sodium azide was found as an effective mutagenic agent which presented useful to improve flower characteristics of ornamental species, for example in morning glory (Bhate, 2001), gerbera (Chang and Chu, 2005) and *Spathoglostis plicata* Blume (Roy and Biswas, 2005). Sodium azide was found to induce chromosomal aberration, with the most predominant anomalies were translocations, lagging chromosome, bridge formation and sticky chromosome, including decreased the mitotic index in a dose-dependent manner (Ragunathan and Panneerselvam, 2007; Srivastava and Kapoor, 2008). Türkan *et al*., (2006) reported that NaN_3 would penetrate into plant cell than destroyed or affected their growth by hampered metabolic function with resulting in reduced cell activity, and NaN_3 also hinders the repairing of the damaged DNA process thus accumulation of mutation and mis-incorporation of vase was occurred at a high frequency. Tejakhod (2009), the result presented in *Kalanchoe* found that aberrant growth mutants of 'Sunrise' could flower, and the flower character of these mutants were different from that of its original genotype and change a little colour (dull-red). Early flowering mutation, from the result of this study was found that early blooming and more flower spikes per plant when treated by low concentration of EMS or NaN_3.

Although flow cytometric assays involved the measurement of fluorescence associated with DNA to determine stages of cell cycle, apoptosis, gene transfections, chromosomal aberrations, polarity, endoreduplication, proliferation checkpoints, and doubling times, flow cytometry analysis had been used in the study of EMS-induced DNA damage (Wagner *et al*., 2003) and isolation of mutants induced by EMS (Doan and Obbard, 2012). Analysis of flow cytometric histograms of regenerated *Erycina pusilla* plants revealed that several unique peaks were detected in putative mutants as compared with those of the corresponding control plant. Whether these differences could reflect DNA variation need to be further verified.

Based on our finding, we suggested that 0.1% EMS treatment for 1 hour and 0.5 mM of NaN_3 for 0.5 to 2 hour, could be adopted for the *in vitro* mutagenic manipulation of *Erycina pusilla* to obtain mutant cultivars. The observations on biological parameters in the present study

revealed that EMS were more superior to NaN_3 in reducing survival rate and regenerated plantlets. In general, the reduction in survival rate and regeneration plantlets was more at the higher concentrations, which indicate the greater sensitivity of *Erycina pusilla* due to occurrence of more genic, chromosomal and physiological disturbances at these concentrations. The above findings indicate a general superiority of EMS than NaN_3 and provide a guideline for selecting suitable mutagenic treatments for inducing desirable mutations in *Erycina pusilla*.

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扇形文心蘭(*Erycina pusilla*)之 EMS 及疊氮化鈉誘變

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摘要：本研究目的為經由EMS和疊氮化鈉(sodium azide)等化學誘變方式創新扇形文心蘭(*Erycina pusilla*)品系。將扇形文心蘭PLB分別浸泡於0、0.1、0.2、0.4和0.8%的EMS或0、0.5 、1、2 和4 mM疊氮化鈉溶液0.5、1和2小時後，進行PLB培養及誘導再生，記錄培植體存活率與再生植株數目，並將再生葉片進行流式細胞儀分析。試驗結果顯示處理0.1% EMS 1個小時和0.5mM 疊氮化鈉0.5和2小時之扇形文心蘭培植體有較高的存活率。整體而言，EMS處理相較疊氮化鈉處理會大幅度降低培植體存活率且產生較少的再生植株。隨著處理EMS和疊氮化鈉的濃度增加，扇形文心蘭培植體的存活率下降，最終死亡，而低濃度下的傷害影響則較少，此可作為扇形文心蘭誘變的參考濃度。部份再生植株的葉片及花朵形態及樣式與未處理組比較有明顯的差異。流式細胞儀分析結果顯示經EMS和疊氮化鈉誘變的扇形文心蘭植株之DNA發生變化。

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