Malays. Appl. Biol. (2014) 43(1): 91–96

THE EFFECT OF ETHYL METHANE SULFONATE (EMS) ON REGENERATION AND SOMACLONAL VARIATIONS OF PETUNIA (*Petunia hybrida* VILM.)

SAHI, N.[1] and EHSANPOUR, A.A.[2*]

[1,2]Department of Biology, Faculty of Science, University of Isfahan, Isfahan, Iran
[*]E-mail: ehsanpou@sci.ui.ac.ir

ABSTRACT

In order to study the effect of EMS (Ethyl Methane Sulfonate) on plant regeneration of *Petunia* (*Petunia hybrida* Vilm.), sterile seeds were cultured in MS medium. Leaf segments from *in vitro* grown plants were treated with 1% and 2% EMS for 15, 30 and 60 min and were then transferred to plant regeneration medium (MS and BAP, 05 mg/l). No growth was observed in leaf explants treated with 2% EMS for 30 and 60 min. Regenerated shoots from samples treated with 1 or 2% for 15 min were then transferred to MS medium and were sub cultured after rooting. Plants treated with 2% EMS for 15 min showed a significant reduction in the dry and fresh weight. RAPD-PCR analysis using OPAA6 and FPK101 primers showed few differences among treated and untreated plants with EMS.

Key words: *Petunia hybrida*, Ethyl Methane Sulfonate, Regeneration

INTRODUCTION

Plant regeneration is one of the basic techniques of tissue culture in which a complete plant having root and stem is produced from an undeveloped plant cell. Plant regeneration using *in vitro* culture is an indication of the flexibility of plant tissues. This phenomenon occurs in response to specific culture conditions. The two primary pathways leading to whole plant regeneration is a prerequisite for most plant breeding, genetic and transformation applications under *in vitro* conditions either through somatic embryogenesis or shoot organogenesis followed by root organogenesis (Miguel and Marum, 2011). Both developmental pathways can occur either without a callus intermediate stage or by callus formation. A few plant species regenerate using both organogenesis and somatic embryogenic pathways, but many plant species regenerate using either of these pathways (Miguel and Marum, 2011).

Breeding has been practiced since the early human civilization and selection was the first method of breeding, adding the criterian of suitability for man's use, e.g. larger seed, better taste, easier harvest ability to those of natural adaptation, fitness and offspring. It has been said that the ultimate source of all heritable variations to select from are mutations. Using mutants in cross breeding does not require in-depth knowledge on mutations, as mutanted traits are the object of interest. But when the mutant trait is not inherited as expected, the breeder may begin to think about the actual mutational event that led to the mutant phenotype.

Mutations can artificially be induced. Induced mutations are the plant breeder's one hope for freedom from complete dependence on nature as the only source of the genetic variants necessary in plant improvement (Hayes *et al.,* 1955). These mutations typically occur at much higher frequencies than spontaneous mutations (Satoh *et al.,* 1982). Recently, addition of chemical mutagens has opened up a new era in mutation research. Ethyl methane sulfonate (EMS), a compound of the alkaline sulfonate series is widely used to induce higher frequencies of mutations in microorganisms (Kolmark, 1957) and higher plants (Minocha, and Arnason, 1962; Hajra, 1979). In plants, EMS usually causes point mutations, but loss of a chromosome segment or deletion can also occur (Okagaki *et al.,* 1991). Therefore, EMS has the potential of altering loci of particular interest without inducing a great number of closely linked mutations.

* To whom correspondence should be addressed.

The application of mutants in general, biochemical or physiological studies suggest that such mutants can be selected from a mixed population. *In vitro* techniques are widely exploited for such selection procedures. There is only a handful of chemical mutagens which have been applied with any frequency to plant cell cultures. Ethyl methane sulphonate (EMS) has by far been the most commonly used. So far some phenotypes have been selected after EMS treatment (Kumar, and Yadav, 2010).

Petunia hybrida Vilm. is an economically important ornamental species with a sweet smell (Berenschot *et al.,* 2008; Nakamura *et al.,* 2006). Different environmental factors have been found to influence Petunia's morphogenesis under *in vitro* culture condition. Results have proved that new phenotypes of *Petunia hybrida* can be established through tissue culture (Abu-Qaoud *et al.,* 2010). The aim of this study was to evaluate the changes of regenerated *Petunia* plants after treatment with EMS.

MATERIALS AND METHODS

Seeds of *Petunia* were obtained from Natural Resource Center of Isfahan, Iran. Seeds were surface sterilized for 1–2 min in 95% ethanol, followed by treatment in 15% sodium hypochlorite (v/v) with 3 drops per liter of Tween-20 for 20 min. This was followed by 3 washes with sterile distilled water. Sterile seeds were then placed on 30 ml full strength MS (Murashige and Skoog, 1962) medium in 250 ml culture containers.

For plant regeneration and treatment with EMS, leaf segments (Approx. 1.5 cm^2) from fully grown *in vitro* plants were treated with 1% and 2% EMS for 15, 30 and 60 min, respectively (EMS concentration was selected based on the preliminary experiments, data not shown). The EMS treated leaf segments were then transferred to plant regeneration medium (MS medium supplemented with 0.5 mg/lit BAP). After 60 days, the number and percentage of plants regenerated from leaf segments were recorded.

PCR Amplification:

PCR was performed using universal RAPD primers in a Thermo Cycler (Eppendorf Netheler-Hinz, Hamburg, Germany). The amplification conditions include 1 cycle at 94ºC for 5 min, 35 cycles at 94ºC for 1 min, 35ºC for 30 sec, and 72ºC for 30 sec. PCR reactions were carried out in a 25 µl reaction volume containing 30 mg of genomic DNA, 1 U of *Taq* polymerase, dNTP (0.2 mM), Tris–HCl (10 mM) pH 8.3, and $MgCl_2$ (2.5 mM), and 2 µM RAPD primers: RAPD Primers were used as a single primer to identify somaclonal variations.

FPK105 (5'-ACTTGGCGGCCT-3'),
FPK101 (5'-ACACGGACGTCA-3'),
OPAA14 (5'-AACCGGCCAA-3'),
OPAA06 (5'-GTGGGTGCCA-3'),
OPA10 (5'-GTGATCGCAG-3').

After amplification, PCR products were separated on 1% agarose gel, stained with 0.5 µg/ml ethidium bromide and visualized by illumination under UV light (UVP BioDOC-It^{TM} system).

All experiments were carried out with 3 replications and the data was analyzed using the ANOVA based on Tukey Test at $p<0.05$.

RESULTS

Figure 1 shows the regeneration of leaf without EMS treatment. After two weeks postculture on MS supplemented with BAP (1mg/l), small granular callus and shoot buds were initiated on the wounded edges of the leaves as shown in Figure 1B. After 60 days, mature shoots were emerged on the leaves. (Fig.1D).

Results of leaf segments treated with different concentrations and time course of EMS are presented in Figure 2. Leaf explants treated with 1% EMS either for 15 or 30 min initiated shoot buds after 2-3 weeks. It was also found that 1% EMS treatment for 60 min kept the explants alive but shoot regeneration from this treatment was very poor (Fig. 2). In comparison 1 and 2 % EMS, either for 30 or 60 min, killed the leaf explants except treatment for 15 min.

The survived leaf segments after EMS treatment are also observed as shown in Figure 3. All leaf samples treated with 1% EMS survived and were greenish up to 30 min after EMS application, while increasing treatment time (60 min) reduced the viability of leaf segments dramatically. Application of 2% EMS on leaf segments of *Petunia* for either 30 or 60 min produced necrosis. In this case, only 2% EMS for 15 min kept the leaf explants alive significantly. However, the regeneration potential of explants in this treatment was very low.

Fresh and dry weight of regenerated shoots from EMS treated and untreated explants were also measured. Treatment of leaf with 2% EMS for 15 min showed significantly lower fresh weight and dry weight ($p<0.05$) compared with other treatments as shown in Figure 4.

RAPD analysis

In order to check the risk of variation within the genome of EMS treated plants regenerated from leaf segments, PCR was carried out using universal RAPD primers. Among five different primers tested, only OPAA6 and FPK101 showed different pattern

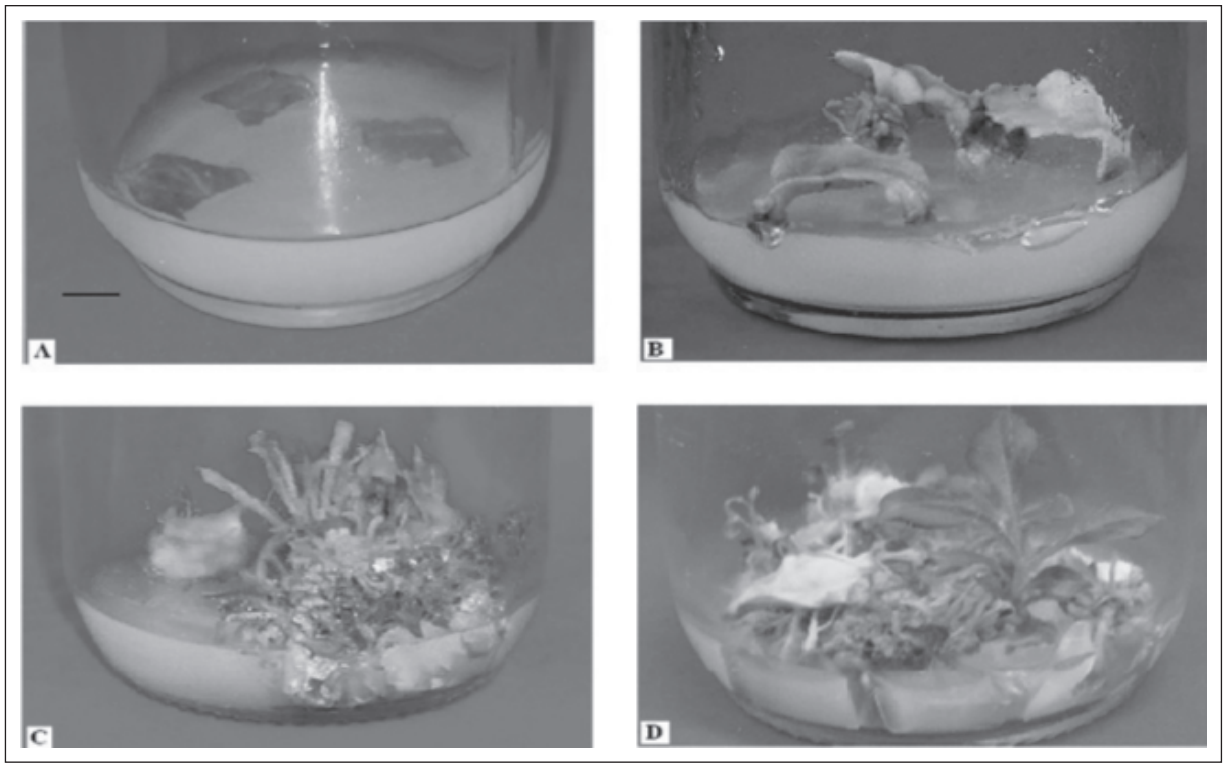

Fig. 1. Plant regeneration steps from leaf explants of *Petunia* either untreated or treated with 1% EMS for 15 min. (A) leaf at day 0, (B) shoot bud initiation after 2 weeks, (C & D) mature shoots regenerated within 60 days. (Scale bar indicates 1cm).

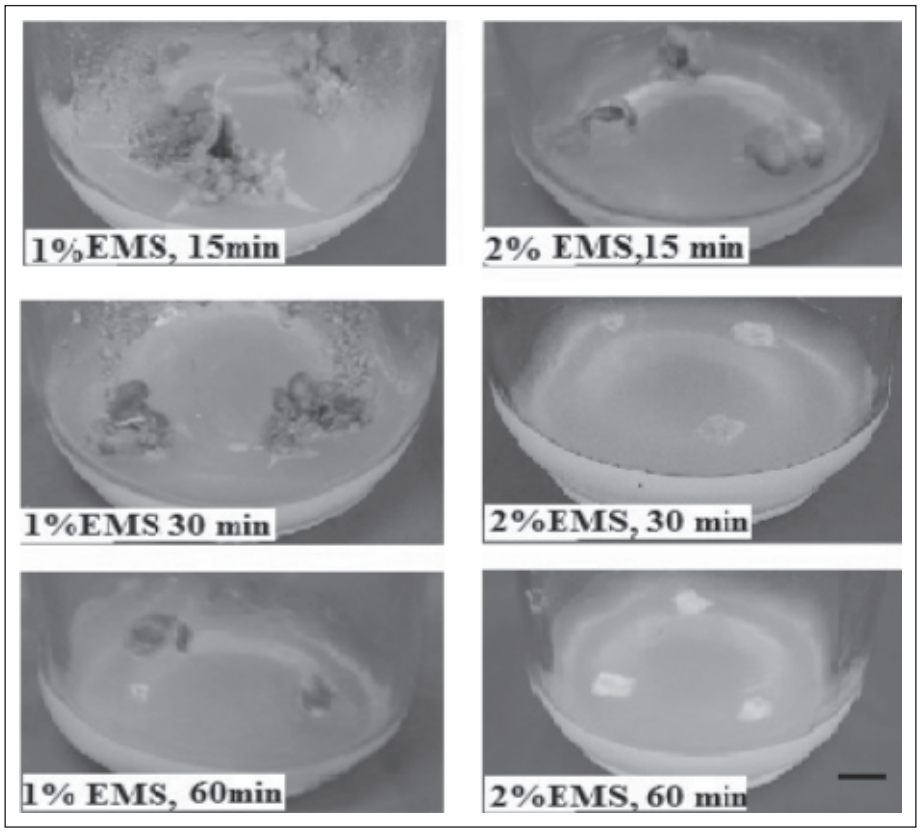

Fig. 2. Effect of duration and concentration of EMS on leaf explants of *Petunia*. (Scale bar indicates 1 cm).

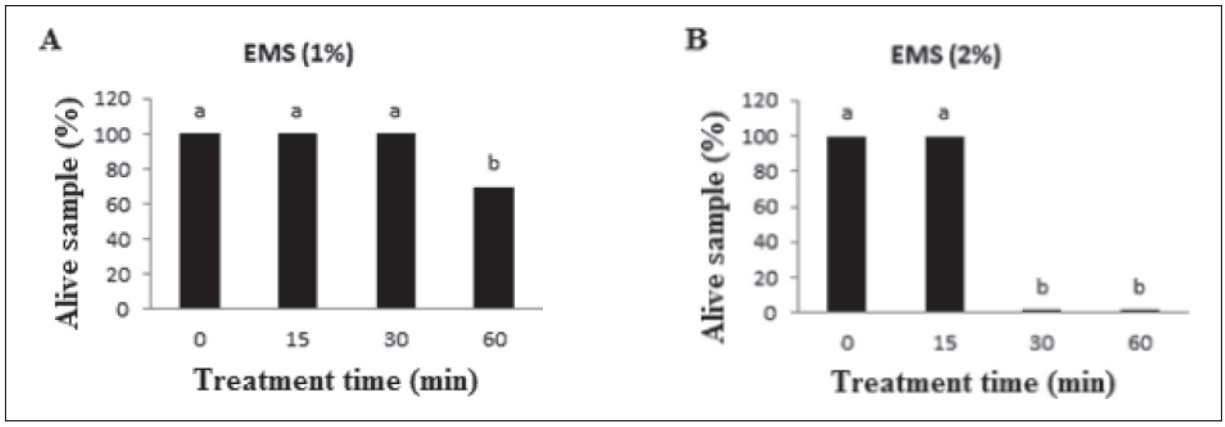

Fig. 3. The effect of EMS on *Petunia* alive sample. A: EMS 1%, B: EMS 2%. Data are average of 3 replications ±SD and dissimilar letters are significant based on Tukey Multiple Range Test ($p<0.05$).

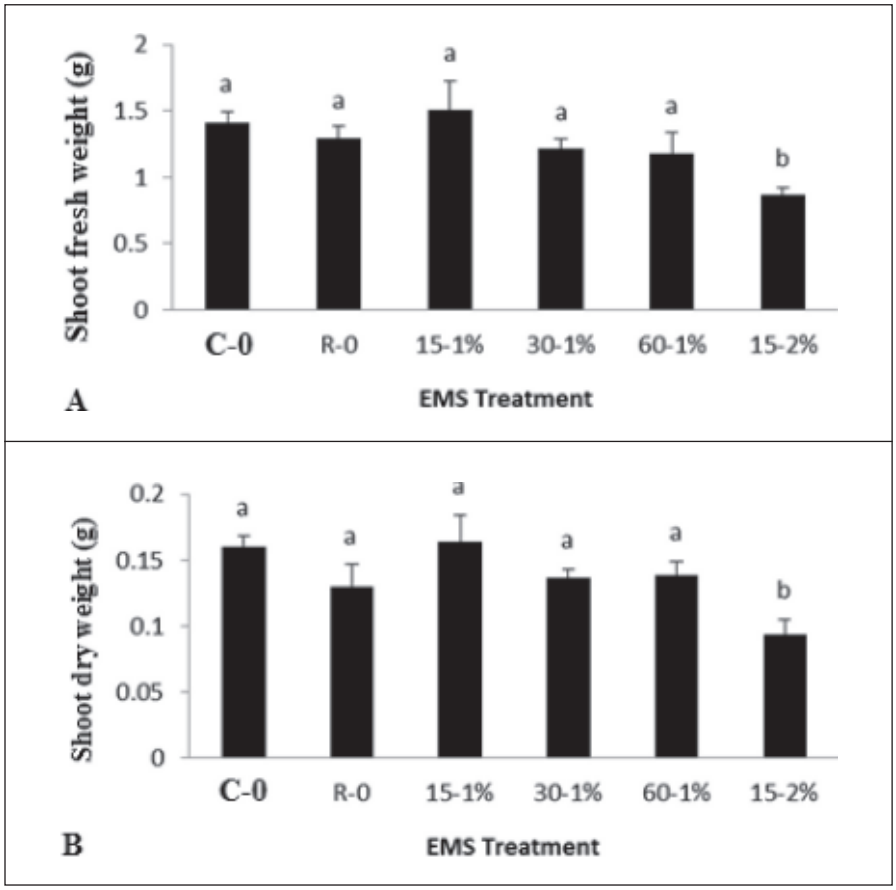

Fig. 4. The effect of EMS on *Petunia* leaf segment (A) fresh weight, (B) dry weight. (C-0): untreated control plant, (R-0): regenerated plant from untreated leaf, (15-1%): 15 min EMS concentration 1%, (30-1%): 30 min EMS concentration 1%, (60-1%): 60 min EMS concentration 1%, (15-2%): 15 min EMS concentration 2%. Data are average of 3 replications ±SD and dissimilar letters are significant based on Tukey Multiple range Test ($p<0.05$).

of DNA bands. Treatment of leaf segments with EMS for 30 min revealed two DNA bands of approximately 700 and 1000 bp, which were missing in other EMS treatments. Treatment with EMS for 60 min also showed 3 bands with 450, 700 and 1000 bp. These DNA bands were absent in this treatment (Fig. 5B). FPK101 also showed changes in the pattern of DNA bands in 2% EMS for 15 min when compared with untreated and non-regenerated plants (Fig. 5A).

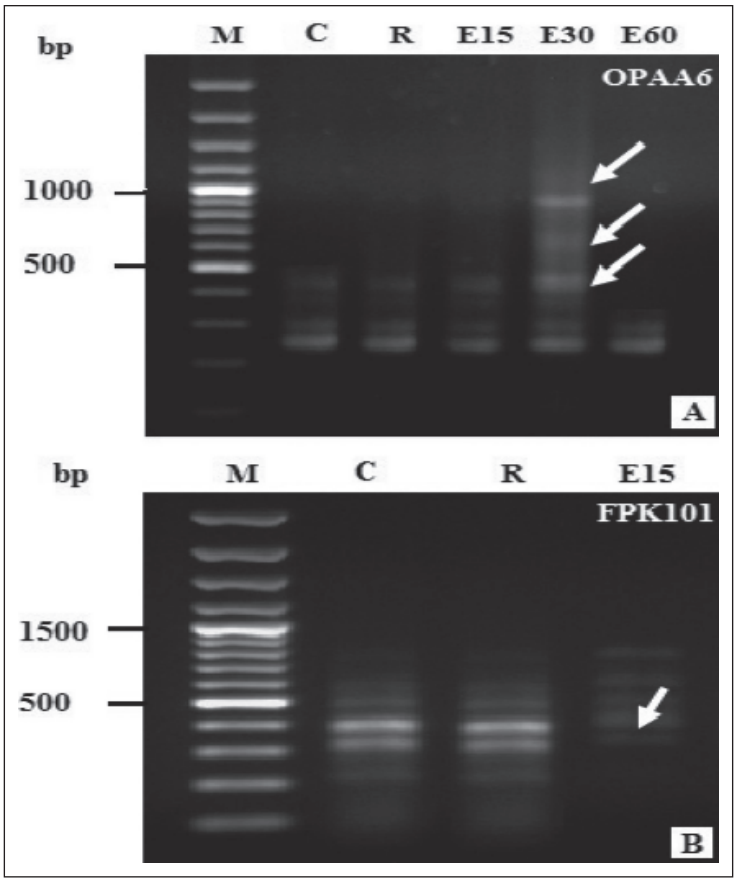

Fig. 5. Results of RAPD-PCR using OPAA6 and FK101 primers. **A:** (M): Marker, (C): Untreated control, (R): Untreated regenerated plant (E15): 1% EMS treatment for 15 min, (E30): 1% EMS treatment for 30 min, (E60): 1% EMS for 60 min. **B:** (M): DNA Marker, (C): Untreated control, (R): Untreated regenerated plant (E15): 2% EMS treatment for 15 min.

DISCUSSION

The performance of all biological parameters measured in the present study revealed some changes in physiological parameters with increasing concentration and duration of EMS treatment. Application of 1% EMS for either 15 or 30 min showed viable regenerated plants. The morphological study of regenerated plants after either EMS treatment or untreated showed no significant difference. This might be due to suitable treatment at 1% EMS for 15-30 min for plant regeneration from leaf segments. However further study is needed to evaluate possible genetic or epigenetic changes occurred in regenerated plants. An increase in EMS concentration by 2% showed obvious toxicity in regenerated plants. Similar finding was already reported by Wattoo *et al.* (2012) in rice.

Using 2% and 1% EMS within 60 min at all times significantly decreased the fresh and dry weights. Previous study indicated that high concentration of EMS decreased some physiological parameters including number of branches, fruit weight and fruit size of eggplant. It can be interpretated as toxicity of high concentration of EMS as Aruna *et al.* (2010) reported. Similar finding has also been reported by Saba and Mirza (2002), where 1% EMS treatment for 6 hours decreased the stem and root length, fruit number and average fruit weight in *Lycopersicon esculentum* and 3-5 hours duration of EMS treatment in sesame reported by Kumar and Yadav (2010).

Somaclonal variation is a relatively popular phenomenon that occurs under plant tissue culture conditions and it can be detected using RAPD-PCR technique (Williams *et al.,* 1990). In the present study, we have applied 5 different primers for detecting any genomic variation in regenerated plants treated with EMS. Only APAA6 and FPK101 revealed a few variations in DNA bands. Two specific DNA bands were observed in explants treated with 1% EMS for 30 min while these bands were absent in the other treatments. One DNA band with approx. 450bp was observed in all treated plants except for 1% EMS with 60 min duration using APAA6 and a DNA band with approx. 350 bp observed in leaf samples treated with 2% EMS for

15 min. Changes in DNA pattern similar to our study caused by somacolonal variation (due to the application of EMS) was also reported earlier (Anwar *et al.,* 2010; De Klerk, 1990; Jain, 2001; and Smy´kal *et al.,* 2007). Another source of variation known as somaclonal variation might be methylation in regenerated plants caused by EMS as reported in pea (Cecchini *et al.,* 1992) and rice plants (Muller *et al.,* 1990).

In the present study, EMS revealed some variations in genomic as well as epigenetic changes and these changes might be applied to obtain plants with higher quality products.

ACKNOWLEDGEMENT

Authors wish to thank University of Isfahan for their support.

REFERENCES

Abu-Qaoud, H., Abu-Rayya, A. and Yaish, S. 2010. *In vitro* regeneration somaclonal variation of *Petunia hybrid. Journal of Fruit and Ornamental Plant Research*, **18(1)**: 71-81.

Anwar, N., Kikuchi, A. and Watanabe, K.N. 2010. Assessment of somaclonal variation for salinity tolerance in sweet potato regenerated plants. *African Journal of Biotechnology*, **9(43)**: 7256-7265.

Aruna, J., Prakash, M. and Sunil Kumar, B. 2010. Studies on effect of mutagens in M1 population of Brinjal (*Solanummelongena* L). *Research Journal of Agricultural Sciences*, **1(4)**: 470-471.

Berenschot, A., Zucchi, M., Tulmann-Neto, A. and Quecini, V. 2008. Mutagenesis in *Petunia x hybrida Vilm* and isolation of a novel morphological mutant. *Brazilian Journal of Plant Physiology*, **20(2)**: 91-103.

Cecchini, E., Natali, L., Cavallini, A. and Durante, M. 1992. DNA variations in regenerated plantsof pea *(Pisumsativum*L.).*Theorical and Applied Genetics*, **84**: 874-879.

De Klerk, G.J. 1990. How to measure somaclonal variation. *Acta Botanica Neerlandica*, **39**: 129-144.

Ehsanpour, A.A. and Twell, D. 2005. Analysis of SFL1 and SFL2 promoter region in *Arabidopsis thaliana* using gateway cloning system. *Journal of Science*, **16**: 303-309.

Hajra, N.G. 1979. Induction of mutations by chemical mutagens in tall Indica rice. *Indian Agricalture*, **23**: 67-72.

Hayes, H.K., Immer, F.R. and Smith, D.C. 1955. Methods of Plant Breeding, 2nd Ed. Mcgraw Hill Book Company, New York. 210pp.

Jain, S.M. 2001. Tissue culture-derived variation in crop improvement. *Euphytica*, **118**: 153-166.

Kolmark, G. 1957. Mutagenic properties of certain esters of inorganic acids investigated by neurospora back mutation test. *Comp. Rend. Trac. Lab. Cartsberg, Ser. Physiol*., **26:** 205-220.

Kumar, G. and Yadav, R.S. 2010. EMS induced genomic disorders in sesame (*Sesamumindicum* L.). *Plant Biology*, **55(2)**: 97-104.

Miguel, C. and Marum, L. 2011. An epigenetic view of plant cells cultured *in vitro*: somaclonal variation and beyond. *Journal of Experimental Botany*, **10**: 1-13.

Minocha, J.L. and Arnason, T.J. 1962. Mutagenic effectiveness of ethyl methane sulfonate in barley. *Nature*, **196**: 499.

Muller, E., Brown, P.T.H., Hartke, S. and Lorz, H. 1990. DNA variation in tissue-culture-derived rice plants. *Theoretical Applide Genetics*, **80**: 673-679.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum*, **15(3)**: 473-497.

Nakamura, K., Matsubara, K., Watanabe, H., Kokubun, H., Ueda, Y., Oyama-Okubo, N., Nakayama, M. and Ando, T. 2006. Identification of *Petunia hybrida* cultivars that diurnally emit floral fragrances. *Scientia Horticulturae*, **108**: 61-65.

Okagaki, R.J., Neuffer, M.G. and Wessler, S.R. 1991. A deletion common to two independently derived waxy mutations of maize. *Genetics*, **128(2)**: 425-431.

Saba, N. and Mirza, B. 2002. Ethyl methane sulfonate induced genetic variability in *Lycopersiconesculentum. International Journal of Agriculture and Biology*, **4(1)**: 89-92.

Satoh, C., Awa, A.A., Neel, J.V., Schull, W.J., Kato, H., Hamilton, H.B., Otake, M. and Goriki, K. 1982. Genetic effects of atomic bombs: In Liss, A.R. (Ed.), Human Genetics, New York. 276 pp.

Smy'kal, P., Valledor, L., Rodríguez, R. and Griga, M. 2007. Assessment of genetic and epigenetic-stability in long-term *in vitro* culture of pea (*Pisumsativum* L.). *Plant Cell*, **26:** 1985-1998.

Wattoo, J.I., Aslam, K., Shah, S.M., Shabir, G., Sabar, M., Naveed, S.A., Waheed, R., Samiullah, Q., Muqaddasi, Q.H. and Arif, M. 2012. Ethylemethane sulphonate (EMS) induced mutagenic attempts to create genetic variability in basmati rice. *Journal of Plant Breeding and Crop Science*, **4(7)**: 101-105.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A. and Tingey, S.V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research*, **18**: 6531-6535.