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In vitro mutation induction on TCL explants of Lilium (*Lilium* spp.) with Ethyl Methane Sulfunate (EMS)

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ABSTRACT

In vitro mutagenesis is considered as an efficient approach for increasing the plant diversity. Lilium is one of the important bulbous flowers that distributed widely over the world. The present study was carried out to investigate the effect of Ethyl Methane Sulfunate (EMS) on *in vitro* mutagenesis of *Lilium* spp. (cv. OT Geel and Robina). After surface sterilization bulb scales, they were treated with EMS solution. Then, they were cut into 3mm thickness TCL explants and incubated on regeneration medium. Results indicated that 0.2% EMS was the most effective concentration in enhancement of shoots numbers in both cultivars. Molecular changes in OT Geel and Robina mutants showed that mutation can induce high polymorphism, so that in OT Geel, 11 treatments were classified into 4 groups with similarity level 0.87% and in Robina, 11 treatments were grouped into 2 groups with similarity level 0.86%. In all treatments genetic variations were observed, but higher concentrations of EMS were more effective for mutation induction. The results indicate that the efficiency of ISSR marker for detection of genetic variants is high and leads to early selection of them. This study is the first report to identify EMS treated mutants using ISSR marker in Lilium plants.

Key words: Lilium, Chemical mutagen, Molecular marker, Genetic variation, In vitro culture.

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1. INTRODUCTION

Ornamental plants contain great economic value and present increasing demand of them points out the necessity of alternative for increasing the diversity of ornamental species than conventional breeding methods (1). Biodiversity enhancement followed by selection, assessment and propagation of high performance varieties is the basis of plant breeding programs. Compared with conventional breeding, mutation is an alternative approach for this purpose by increasing the biodiversity in populations that obtained from breeding crosses (2). For breeding of ornamental plants, this method is used more due to most of ornamentals propagate vegetatively (3) and contain easy evaluation traits including color, size and shape of flowers and leaves (4). Mutation refers to spontaneous or induced genetic variation in already existing cultivars but low frequency of spontaneous mutation results in gradual decline in application of this method by plant breeders (5). In contrast, several studies dedicated that artificial induced mutation by exposing plant materials to physical or chemical mutagens is so efficient to achieve new commercialized cultivars. All types of mutations makes changes in the chromosomes randomly so breeders don't observe any changes in genetic material even with the application of mutagens. In addition, most of mutations that occur in DNA are harmful and decrease plant efficiency (6). So, breeders must treat a large population of plants to achieve some useful mutants (2). Also it is essential to evaluate the effects of mutagen on morphological traits in next generations. Indeed in some species with sexual propagation, identification and selection of recessive mutations is possible in followed second and third generations of treated plants. With the development of *in vitro* techniques, mutation efficiency for breeding of vegetative or sexual propagative plants is increased. Therefore *in vitro* mutation induction can be a useful tool in improvement of ornamental features (7).

Despite the advantages of *in vitro* techniques for mutation breeding purposes, selected mutants need *in vitro* growth followed by *in vivo* acclimization and finally detection of mutants in a large plant population, but regards to low mutation frequency in plant cells, most of regenerated plants is not affected by mutagen treatment. So, maintenance of these plants until removing of them in final step causes increasing in costs of breeding program (8, 9). Nowadays, molecular markers facilitate *in vitro* mutation breeding by selection of mutants and putting down non-mutated plants in early steps of experiment. Therefore, this procedure leads to enhance *in vitro* mutation breeding efficiency (10). Lilium (*Lilium* spp.) is one of the commercially ornamental bulbous plants. Production of this plant is an important global industry (11). Owning the big and beautiful flowers and floral longevity makes this plant as one of the top ten ornamentals and takes place in 4th among cut flowers (12). Lilium species are native to some regions of northern hemisphere including Asia (China and Japan), northern America, Mediterranean regions of Europe and north of Africa. Lilies are usually propagated by scaling or seed dispersal. But due to dealing of sexual hybridization with many impediments and long duration of seed propagation, these species are usually propagated vegetatively (10). So, regards to advantages of molecular markers and application of *in vitro* techniques for plant mutation breeding, this study was conducted to evaluate the effects of Ethyl Methane Sulfunate (EMS) on *in vitro* mutation induction of *Lilium* spp. and evaluation of them with ISSR molecular marker.

2. MATERIALS AND METHODS

2.1. Plant material

Bulbs of two different cultivars of *Lilium* spp. cv. OT Geel and Robina were kept at 4^{0}C for a month to break their probable dormancy.

2.2. Explant preparation and sterilization

Bulb scales of 2 different Lilium cultivars were used for the experiment picked up from the internal part of the bulbs and separately washed with tap water to remove the pollutants. Then they were washed continuously with running tap water for 30 minutes. The bulb scales were surface sterilized in 2% (v/v) NaOCl for 15 min and then in 0.1% $HgCl_2$ (w/v) for 10 min. Explants were rinsed with sterile distilled water for 3 times under sterile conditions in a laminar flow hood. After every step of the sterilization process, bulb scales washed once with sterile distilled water to remove previous disinfectants.

2.3. Mutation treatment

Liquid stock solution of Ethyl Methane Sulfunate (EMS) was prepared by dissolving it in sterile distilled water with the concentration of 1% (v/v). With this stock solution and 0.1 M phosphate buffer, 0.2, 0.4, 0.6 and 0.8% (v/v) concentrations of EMS solutions were provided and sterile-filtered with a 0.2 µm pore filter under sterile conditions. For mutation treatment, all bulb scales of Lilium cultivars were soaked into different concentrations of EMS solutions for 30, 60 and 120 minutes and then rinsed with sterile distilled water. The upper half of bulb scales were cut and removed and the lower portion of them were cut into 3mm thickness TCL explants, so that each explant had the basal plate.

2.4. Plant regeneration and root induction

For adventitious bud induction, Murashige and Skoog (MS) basal medium (13) containing 1 mg/l 6-Benzylaminopurine (BA) and 0.5 mg/l a-Naphthalene Acetic Acid (NAA), 3% (w/v) sucrose and 0.7% (w/v) agar was used. The medium was adjusted to pH 5.70-5.80 prior to autoclaving at 121^{0}C for 15 min. All cultures were incubated at 25 ± 2^{0}C with a 16 h photoperiod illumination under cool white fluorescent lights (30µmol/m^2). For root induction, bulblets were transferred to half strength of MS medium for 2 weeks. 1.5-2 months after EMS treatments, growth characteristics such as percentage of regeneration and number of shoots per explant were recorded. The experiment was arranged in a completely randomized design with 3 replicates. Analysis of variance followed by the Least Significant Differences Test (LSD) with 95% probability. JMP8.0. The statistical computer program was used in all analyses.

2.5. Molecular markers

2.5.1. DNA extraction

Fresh leaves of regenerated plantlets (about 1 g) were ground to powder with pestle and mortar in liquid nitrogen and poured into 1.5 ml sterile microtubes. The procedure was followed by DENA ZIST Asia® column DNA isolation kit (S-1030) according to its instruction. Absorbance of samples at 260 and 280 nm is used to assess the purity of DNA and then 260/280 ratio was measured using the Nanodrop to determine possible sources of DNA contamination.

2.5.2. ISSR marker analysis

13 primers which were used in the previous ISSR analysis of lilies and some other ornamental plants (14, 15), were synthesized by DENA ZIST Asia® and were used for the experiment (Table 1).

2.5.3. PCR analysis

The PCR was carried out using 13 primers. The amplification reaction consisted of initial denaturation step at 94℃ for 5 min, followed by 40 cycles of 30 s at 94℃, primer annealing at specific annealing temperature (Table 1) for 1 min, extension step at 72 ℃ for 1 min and final extension at 72 ℃ for 10 min. The PCR products were electrophoresed on 1.7% agarose gel with TAE buffer at 75V for 2 hours and then the bands were visualized under UV light.

Table 1. The list of used ISSR primers for identification of Lilium mutants

	Primer no	Sequence	Annealing temperature (°C)		Primer no	Sequence	Annealing temperature (°C)
1	UBC-824	$(TC)_8G$	52.4	8	3A21	$(TG)_7ACC$	**52.4**
2	UBC-853	$(TC)_8RT$	51.6	9	3A39	$(CA)_7GTA$	**50**
3	3A01	$(GA)_8TC$	53.9	10	3A42	$(GACA)_4C$	**52.4**
4	3A07	$(AG)_7CTT$	50	11	UBC-873	$(GACA)_4$	**48.2**
5	3A62	$(TG)_7ACT$	50	12	UBC-815	$(CT)_8G$	**52.4**
6	UBC 843	$(CT)_8RA$	54	13	UBC 844	$(CT)_8RC$	**52**
7	UBC 845	$(CT)_8RG$	52			R: A or G	

2.5.4. Genetic distance estimation

To determine the genetic distance between mutants, bands were scored as either present (1) or absent (0) using Total Lab software. Then a matrix of genetic similarity or distance among genotypes according to Jaccard's similarity coefficient was used in a cluster analysis to generate UPGMA dendrograms using NTSYS.2 analysis software.

3. RESULTS AND DISCUSSION

3.1. Effect of EMS treatments on in vitro regeneration of Lilium

Nearly 100% of explants in all treatments in two different Lilium cultivars were regenerated after 1.5-2 months. Morphological changes had shown in some of bulblets (Figure 1).

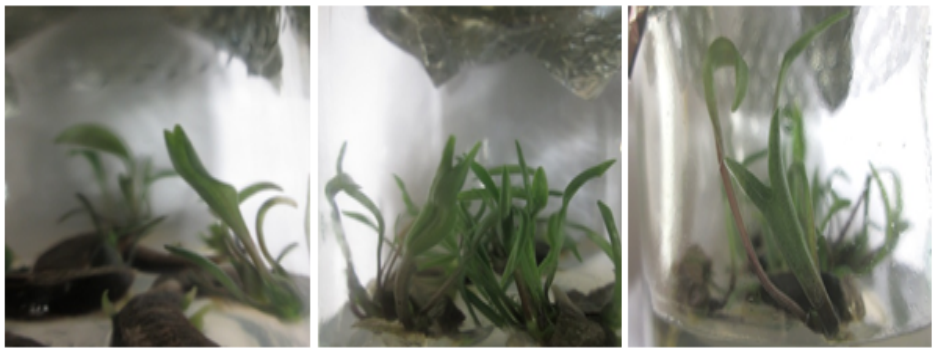


Figure 1. Observed morphological changes in TCL explants of Lilium after treatment with %0. 2 EMS. Some of the shoots were divaricated in both cultivars

Despite regeneration percentage, number of bulblet per explant showed high significant differences in different concentrations and durations of EMS treatments (Table 2). Number of bulblet per explant varied between two cultivars. In Robina, the highest number of bulblet (1.79 bulblets per explant) was obtained with 0.2% EMS for 120 min. While in OT Geel, the highest and lowest number of bulblet (3.05 and 0.49 bulblets in explant) was derived from the 0.2 and 0.8% EMS concentrations, respectively.

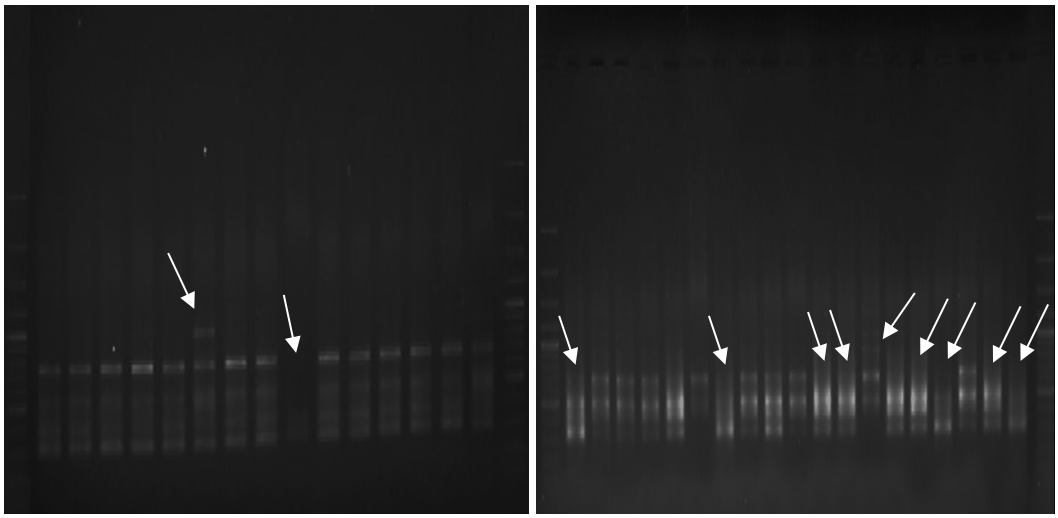
Table 2. The effect of concentration and duration of EMS exposure on bulblet regenerated number of Lilium cv. OT Geel and Robina

Cultivar	Concentration (%)	Duration (min)			
OT Geel	0.2	2.50 abc	2.72 ab	3.05 a	2.10 bcdef
	0.4	2.50 abc	2.83 ab	0.98 ghijk	2.38 abcd
	0.6	2.50 abc	2.16 bcdef	1.11 ghijk	1.30 fghijk
	0.8	2.50 abc	1.70 cdefgh	0.49 k	2.21 abcde
Robina	0.2	0.80 ijk	0.81 ijk	0.54 jk	1.79 cdefg
	0.4	0.80 ijk	0.95 ghijk	0.83 hijk	1.38 efghij
	0.6	0.80 ijk	1.58 defghi	1.10 ghijk	1.50 efghi
	0.8	0.80 ijk	0.97 ghijk	0.79 ijk	0.95 ghijk

*Data with the similar letters are not significant based on Least Significant Differences Test (LSD) (p≤0.05).

3.2. ISSR analysis

In treated plantlets with EMS, 66 putative mutants in both cultivars were obtained which with 3 controls were analyzed using 13 ISSR primers. For ISSR analysis, 8 primers of 13 primers showed polymorphic bands so that between 44 scorable bands, 24 bands were polymorphic. Number of bands varied from 8 bands for 3A01 and 3A49 primers to 4 bands for UBC 824, UBC 873 and UBC 853 primers. This demonstrates the ability of ISSR marker for detection of Lilium mutants (Figure 2).



A B

Figure 2. ISSR patterns of Lilium cv. (A) OT Geel and (B) Robina mutants amplified using 3A01 primer

Polymorphism percentage varied from 25% for UBC 853 primer to 87% for 3A01 primer, and the average of polymorphism rate was calculated 45% (Table 3). Grouping of putative mutants and control in dendrograms showed genetic differentiation between them.

Table 3. List of used ISSR primers in treated Lilium plants with EMS and their polymorphic information

Primer no	Number of bands	Number of polymorphic bands	Frequency of polymorphic bands
3A01	8	7	87%
3A39	5	3	60%
3A49	8	3	37%
UBC 824	4	3	75%
UBC 873	4	3	75%
UBC 843	5	2	40%
UBC 853	4	1	25%
UBC 845	6	2	33%
Total	44	24	-

3.3. Cluster analysis in Lilium spp. cv. OT Geel

In this cultivar, the dendrograms were classified in two groups and 5 separated individuals at 0.87 genetic similarty level (Table 4) (Figure 3).

Table 4. Putative mutants of Lilium spp. cv. OT Geel treated with different concentrations of EMS and different times

EMS treatment	Putative mutants concerning each treatment
0	Control 1-3
0.2% 30 min	M1
0.2% 60 min	M2-M3
0.2% 120min	M4-M6
0.4% 30min	M7-M9
0.4% 60min	M10-M12
0.4% 120min	M13-M15
0.6% 30min	M31-M33
0.6% 60min	M16-M18
0.6%120min	M28-M30
0.8% 30min	M19-M21
0.8% 60min	M25-M27
0.8% 120min	M22-M24

Most of the putative mutants (19) and control were classified in group I. Group I subdivided into 3 subgroups A1, A2 and A3. A1 included the three controls and M33, M27, M28, M18, M10, M14 and M29 putative mutants. The lowest genetic difference was found in this subgroup. M8, M9, M11, M13, M15, M16, M20 and M32 putative mutants were classified in A2 subgroup. A3 subgroup contains 4 putative mutants M1, M3, M5 and M7. The second group (II) was included M17, M19, M21, M22, M23, M24 and M25 mutants. M4 and M6, M2, M30 and M31 mutants were not grouped within any clusters. These five mutants showed more genetic variation compared to other groups of mutants.

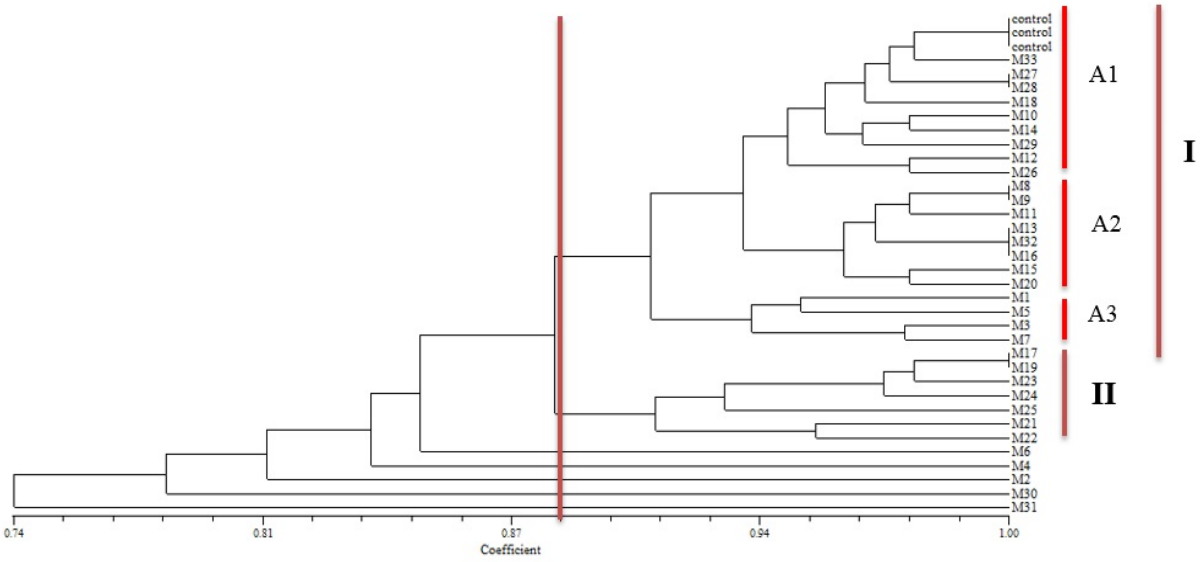


Figure 3. UPGMA dendrograms obtained from ISSR analysis between control and 33 putative mutants of *Lilium* spp. cv. OT Geel

As can be seen from Figure 4, in most treatments of OT Geel, the generated variation were similar and there are no significant differences between them ($p \leq 0.05$). Among all the variant lines, putative mutants of 0.2% EMS treatment for 60 and 120 min and 0.6% EMS treatment for 30 min were observed the lowest similarity level that equal to 0.885, 0.858 and 0.883 compared to the control (1), respectively.

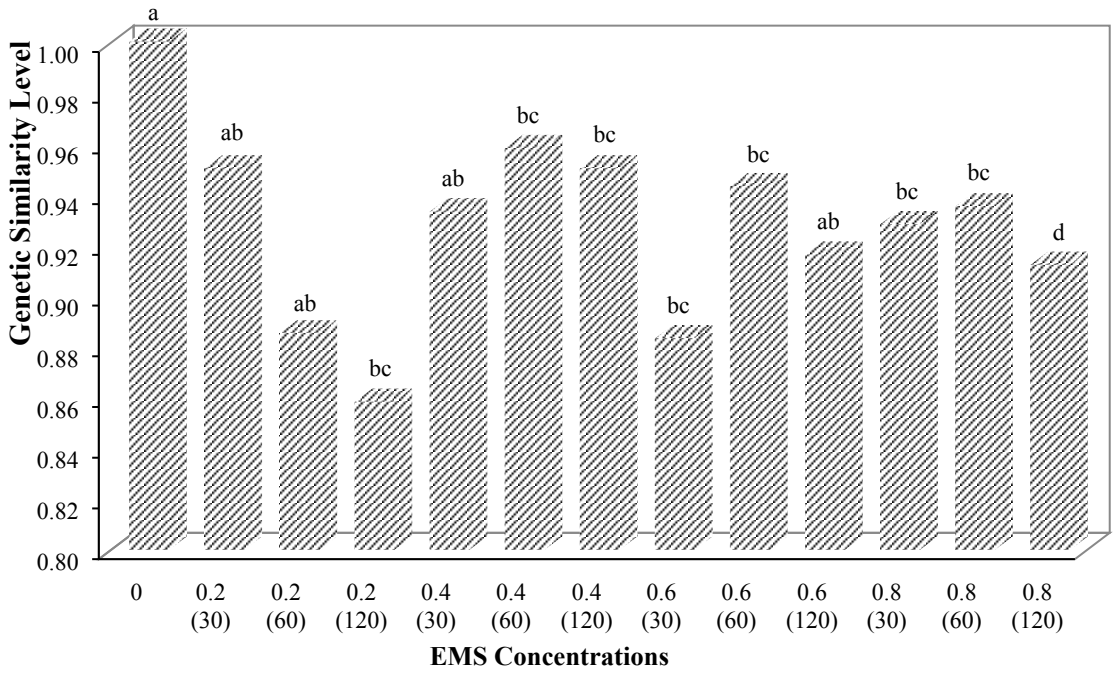


Figure 4. Similarity level of putative mutants for *Lilium* spp. cv. OT Geel exposed to various EMS treatments in different time

3.4. Cluster analysis in Lilium spp. cv. Robina

The dendrograms of Robina mutants showed two groups and 3 separated individuals at 0.86 genetic similarity levels (Table 5).

Table 5. Putative mutants of *Lilium* spp. cv. Robina treated with different concentrations of EMS and different times

EMS treatment	Putative mutants concerning each treatment
0	Control 1-3
0.2% 30min	M1-M3
0.2% 60 min	M10-M12
0.2% 120min	M22-M24
0.4% 30min	M13
0.4% 60min	M14-M15
0.4% 120min	M25-M27
0.6% 30min	M4-M6
0.6% 60min	M16-M18
0.6%120min	M28-M30
0.8% 30min	M7-M9
0.8% 60min	M19-M21
0.8% 120min	M31-M33

Group I was divided into two groups, A1 and A2. The lowest genetic diversity was occurred in group A1 including M2, M11, M18, M29, M10, M14, M12, M26, M27, M28, M8, M9, M20, M13, M16, M1, M5, M6, M3, M7, M23 and M30. Subgroup II was comprised M17, M19, M24, M25, M21 and M22 mutants. Group II consisted of M15 and M32 mutants. M4, M31 and M33 mutants were not grouped within any clusters (Figure 5).

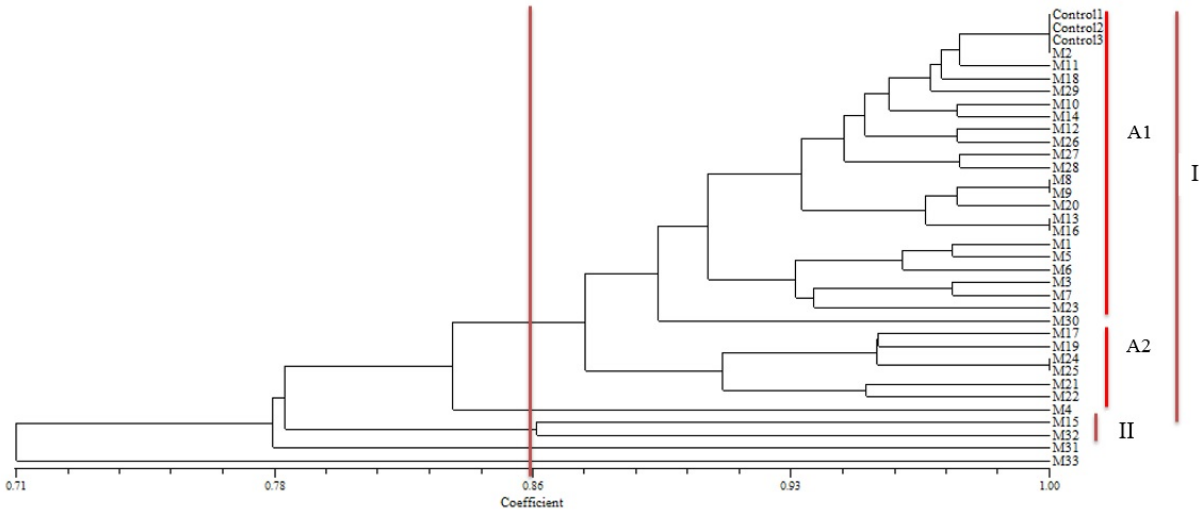


Figure 5. UPGMA dendrograms obtained from ISSR analysis between control and 33 putative mutants of *Lilium* spp. cv. Robina

There was no significant difference in similarity level of putative mutants compared to control ($p \leq 0.05$) (Figure 6). In Robina, the lowest similarity level were observed in putative mutants resulting from 0.8% EMS treatment for 120 min which had the most genetic diversity. In general, all used EMS treatments in this experiment were effective for the variation induction in samples. Since high concentrations of EMS have not been affected on explants regeneration and growth them, thus the use of EMS high concentrations (0.8%) and longer duration exposure of EMS is recommended in order to induce more diversity. Pair wise analysis of mutants such as M9 and M8, M16 and M12, M19 and M16, M25 and M1, M28 and M26, M32 and M12, M32 and M15 had genetic similarity coefficient equal to 1, but they were different compared to the control. In *Lilium* spp. cv. Robina, Jaccard's genetic similarity coefficients pair wise of genotypes differed from 0.585 to 1. The greatest and lowest similarity compared to control was observed in M2, M19 and M32 mutants with genetic similarity coefficient equal to 1 and 0.585, respectively. Pair wise analysis of mutants such as M9 and M8, M16 and M13, had genetic similarity coefficient equal to 1 within each pair wise, but they were different compared to the control.

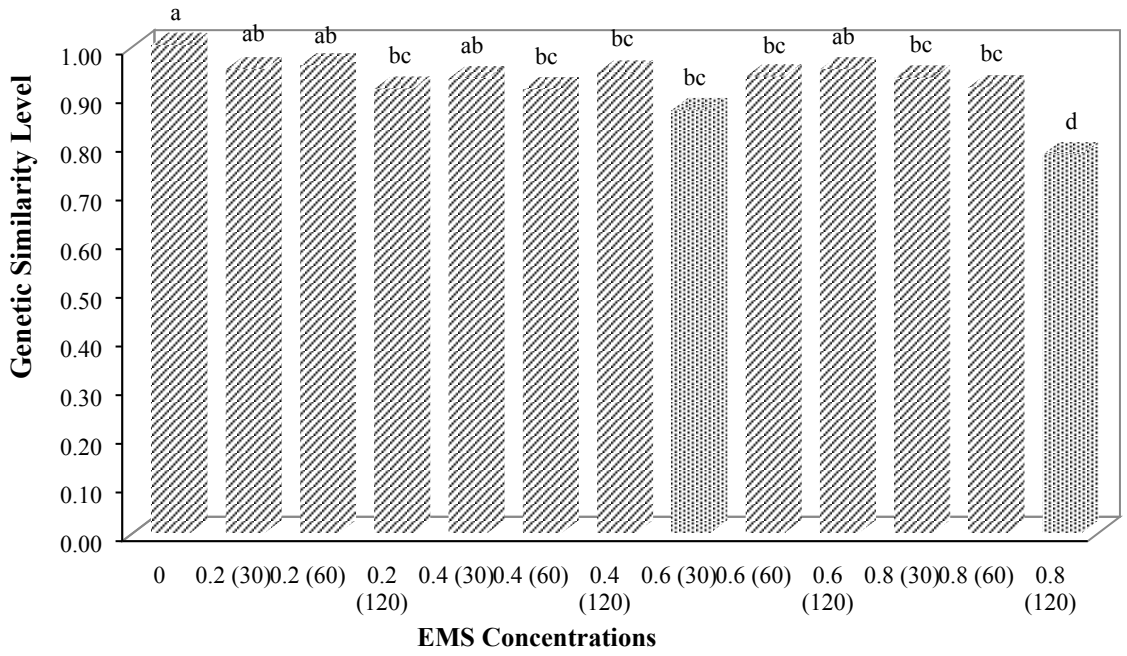


Figure 6. Similarity level of putative mutants for *Lilium* spp. cv. Robina exposed to various EMS treatments in different time

In this experiment, all explants in both cultivars were regenerated. Despite some reports concerning the effect of EMS on frequency of explant survival in *Saintpaulia* spp. (16) and *Petunia hybrida* (17), there was no significant difference among different durations and concentrations of EMS application in explant survival. All explants in different treatments of EMS were survived. In Saintpaulia in 0.2% EMS treatments, there was no significant difference between the different treatment durations in terms of explant survival; nearly 100% of explants survived the treatments. A similar survival rate was recorded for the 0.4% EMS treatments for 30, 60, and 120 min. However, a significantly lower survival rate was observed when the explants were treated for 240 min (e.g. 85%). With the 0.6% EMS treatments, there was a gradual reduction in the survival rate of the explants as the duration of EMS exposure increased. No survival was recorded when the leaves were treated for more than 120 min (16). Also, in number of bulblets per explant were observed significant difference in various treatments of EMS. Latado et al. (2004) (18) studied the effect of EMS treatments on flowering stem of *Dendranthema grandiflora*. They reported that the number of bulblets was decreased with enhancement of EMS concentration. Also shoot tip culture of banana (*Musa* spp.) in exposure to high concentrations of EMS showed a significant reduction in shoot number (19). It can be interpreted as toxicity of high concentrations of EMS as Sahi and Ehsanpour (2014) (17) reported. Chemical mutagens have been applied to numerous plants to induce mutations. For instance, *Kohleria eriantha* internodes treated with N-Nitroso-N-Methylurea have resulted in a mutant with shorter internodes and smaller leaves (20). Two cultivars of *Ipomoea purpurea* treated with EMS, N-methyl-N-nitro-N-nitrosoguanidine and NaN_3, showed corolla whorl-specific characteristics (21). Rodrigo et al. (2004) (18) obtained chrysanthemum mutants with various petal colors (i.e. pink–salmon, light pink, bronze, white, yellow, and salmon) by means of EMS treatment. However, the low penetration into vegetative tissues of chemical mutagens is a major concern in chemical mutagenesis because this might lead to low mutation efficiency and difficulties in reproducing the experiment (3). This problem can nevertheless be circumvented by performing *in vitro* mutagenesis in which mutations were reported to occur more uniformly compared with the in vivo treatments (22). The EMS concentrations used in the present study were hence effective in inducing mutations in Lilium, and they were within the range (0.5% to 2%) tested for *in vitro* mutagenesis of other plants (16, 18, 23). Results in the present study showed that all treatments were induced genetic variation but higher concentrations of EMS were more effective for mutation induction. Also previous studies have suggested that the rate of chromosomal abnormalities in *Sainpaulia* sp. and *Dendrobium* sp. increases by enhancement of concentration or duration of mutagenic agent's exposure (16, 24). Molecular changes in OT Geel mutants with ISSR marker showed that mutation could provide high polymorphism, so that 12 treatments were classified into 4 groups with similarity level of 0.87% and genetic similarity ranging from 0.708 to 1. In Robina, 12 treatments were grouped into 2 groups with similarity level of 0.86% and genetic similarity ranging from 0.585 to 1. The results of this study revealed that ISSR marker has a good differentiation ability to determine genetic diversity. ISSR markers involve the amplification of DNA segments present at an amplifiable distance between two identical microsatellite repeat regions oriented in opposite direction. ISSRs exhibit the specificity of microsatellite markers, but need no sequence information for primer synthesis enjoying the advantage of random markers (25). The present study showed that the new variants developed

through sports and identify as new variants using ISSR markers. ISSR markers usually show high polymorphism although the level of polymorphism has been shown to vary with the detection method. The genetic variation through molecular markers has been highlighted in a number of plant species including ornamental plants (26, 27).

4. CONCLUSION

The results of this experiment indicated the efficiency of EMS application to create genetic variation. So this method can be used to assist breeding programs for improvement of ornamental crops. Also, EMS induced mutants could be identified at the molecular level with ISSR markers. Application of ISSR marker can reduce the size of mutant population with the possibility of early selection of them. This study is the first report to identify EMS treated mutants using ISSR marker in Lilium plants. The system described in this study is easy and fast to perform. It is hoped that the *in vitro* EMS-induced mutations can open up a new approach for the breeding of different cultivar of Lilium. Also, our results indicate the effectiveness of ISSR markers for the identification of Lilium mutants obtained from EMS that could allow earlier selection and reduction of the mutant population size. The combined utilization of ISSR analysis together with *in vitro* mutagenesis as a source for inducing geneticK variation provides a useful tool for future improvement of Lilium.

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AUTHORS CONTRIBUTION

This work was carried out in collaboration among all authors.

CONFLICT OF INTEREST

The authors declared no potential conflicts of interests with respect to the authorship and/or publication of this paper.

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