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Effects of Ethyl Methanesulfonate on Mutation Induction in *Chrysanthemum spp.*

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Abstract

Mutation induction using EMS agent has been widely and successfully used for variation enhancement. In this study, the cultured plantlets from leaves of 7 chrysanthemum varieties were treated with 0, 1, 1.5, and 2 % ethyl methanesulfonate (EMS) solution for investigation of its efficacy for mutation induction. All treated explants were cultured on MS media supplemented with 2 mg/L 2,4D and 1 mg/L kinetin for callus induction and shoot regeneration. After the plants were cultured for 4 weeks, the percentage of shoot and root initiation and callus formation were observed. The varieties of M07-4, D27, 11-4 and 12-10 showed shoot/root regeneration and callus formation while the M07-14, D23 and 11-8 varieties showed shoot/root regeneration in some bottles. The surviving explants were transferred into the new MS medium supplemented with 1 mg/L BA and 0.1 mg/L NAA to obtain healthy plants ready for planting. The healthy plantlets were transferred to soil pot plants, and grown at Doi Khun Wang, Chiang Mai province, Thailand, for acclimatization. Morphological differentiation was evaluated after 16 weeks of planting in greenhouse. M07-14 varieties showed a maximum of 5 mutant characters. 11-8 varieties showed 4 mutant types while D23, D27, and 12-10 varieties produced 2 mutant types and 11-4 varieties showed only 1 mutant type. EMS proved to be effective in producing mutated chrysanthemum flowering plants that can lead to changes the color and form of flower from the original.

Keywords: Chrysanthemum, Ethyl methanesulfonate, Tissue culture, Mutation breeding

Introduction

Mutation breeding techniques are powerful tools for inducing genetic variation in plants and enhance agricultural biodiversity which can potentially lead to further genetic improvement. Plant parts such as tubers, bulbs or corms, dormant cuttings, grafts, bud wood, stolon, and rhizomes are the most commonly used parts for mutation breeding [1]. Induced mutations have already been known and successfully used in plant variety improvement and production of new varieties. For example, mutation breeding could induce salt tolerance in sweet potato [2], improve seed yield in fenugreek [3]. In vegetatively propagated plants, the major advantage of mutation induction is the ability to change some characters of an outstanding cultivar without altering the remaining genotype. Chemical agents can also be used for plant mutation induction [4]. The most chemical mutagens frequently applied in generating plant mutant species are EMS (ethyl methanesulfonate), NEU (nitroso ethyl urea), MNU (N-methyl N-nitrosourea), EI (ethyl eneimine), and colchicine. There are a lot of plant varieties officially released and registered as mutants via chemical mutagenesis such as rice, barley, wheat, maize, and ornamentals [5].

EMS is one of the chemical mutagens that has the efficacy to induce plant mutation. Alkylating agents are strong mutagenic compounds that can lead to DNA breakage [6], and can be applied in plant mutation breeding. EMS, which is an important alkylating agent, causes mispairing or mismatch pairing in the DNA. EMS can lead the alkylation of guanine (G) at O6 position; therefore, the alkylated G can pair with thymine (T) in place of cytosine (C), resulting in GC-AT transitions, which can lead to single-nucleotide mutations [7-10]. EMS-induced rice mutants were found to have a strong local sequence context bias specifically

targeting the second guanine of the RGCG sequence [11]. Several studies have shown that EMS-induced mutations are distributed randomly across the genome with high mutation density [12,13].

The previous studies showed that using EMS could induce mutation in many ornamental plants such as rose [14], African violet [15], gerbera [16], *Silene* species [17], marigold [18] and chrysanthemum [19] also. Chrysanthemum is one of the most economically important cut flowers and ornamental plants across the global flower market. Some research results showed that EMS was used to induce the desired characteristics in chrysanthemum. EMS-derived mutation in chrysanthemum which enhance salinity tolerance in *Chrysanthemum morifolium* Ramat when exposed to 0.025 % EMS in cultured media supplemented with NaCl [20]. Another report on using EMS in *in vitro* chrysanthemum showed that 48 mutants of chrysanthemum were obtained from EMS treatment and most of them were phenotypically uniform [21]. Another study found that EMS-induced mutation in chrysanthemum (*Dendranthemum grandiflora*) 'vivic' can change the color and shape of a flower [22]. The exposure of EMS can produce mutants with different colors and inflorescence shapes in 4 chrysanthemum cultivars [23]. In Thailand, many varieties of chrysanthemum *in vitro* were treated by EMS, resulting in many mutants. More than 20 chrysanthemum mutant varieties were in the registration process at the Plant Varieties Protection Office, Department of Agriculture, Thailand [24].

The global ornamental plant market has been growing rapidly in both production and exporting and becoming an important part of the economy in many countries, including Thailand. The global ornamental plant market growth is expected to continue to increase more than 6 % during the period of 2022 - 2029 [25]. So, mutation induction will help to enhance genetic variability in ornamental plants with different characteristics from wild plants. Therefore, the goal of the present research is to investigate the effects of EMS for mutation induction in chrysanthemum.

Materials and methods

The cultured plantlets from leaves of 7 chrysanthemum varieties; M07-4, M07-14, 11-4, 11-8, 12-10, D23, and D27 were cut into 1×1 cm^2 and soaked in 0, 1, 1.5, and 2 % EMS solution for 60 min with 100 bottles (1 plantlet per 1 bottle) per treatment. After the treatments, the explants were washed with sterile distilled water. After that, all treated explants were cultured on MS media supplemented with 2 mg/L 2,4D and 1 mg/L kinetin for callus induction and shoot regeneration [4]. After 4 weeks, the effects of EMS-induced mutation were investigated.

The surviving plantlets and calluses from EMS treatment were transferred into the new MS medium supplemented with 1 mg/L BA and 0.1 mg/L NAA to obtain healthy plants ready for planting. The explants were transferred to fresh medium every month until 12 weeks. Healthy chrysanthemum plantlets *in vitro* were carefully taken out of the medium and washed with water for the attached medium removal and then transplanted into plant trays in the nursery at Doi Khun Wang Agricultural Research Center (DKW) Royal Project, Chiang Mai province, Thailand. The trays were kept in greenhouses with a misting system for 8 weeks. After that, young chrysanthemum plants were transferred to an experimental field and taken care of as pot plants for 8 weeks. The shape, color, and size of the flower and plant height were recorded compared with the control for mutation selection and further commercial potential testing.

The experimental design was a completely randomized design (CRD) with 3 replications. Statistical Tool for Agricultural Research (STAR) program was used for all statistical analyses [26].

Results and discussion

The *in vitro* 7 varieties of chrysanthemum were exposed to various concentrations of EMS. The results showed that each variety has different sensitivity to EMS. The various concentration of EMS affect survival, callus formation and shoot/root initiation (**Table 1**, **Figure 1**). The results showed that 4 varieties, M07-4, D27, 11-4 and 12-10 showed shoot/root regeneration and callus formation while the other 3 varieties showed shoot/root regeneration in some bottles. The calculation of shoot/root initiation and callus formation percentage were calculated based on their own survival. Some explants of 3 varieties, M07-14, D23 and 11-8, have stunted growth and development. They survived in medium without any change in form or growth which are indicated as "unchanged" in **Table 1**. The M07-14, D23 and 11-8 varieties were more sensitive to EMS treatment compared with the other varieties based on the change of cultured plantlet formation. The percentage of the unchanged formation tended to increase when exposed to the high concentration of EMS. For the other varieties, all of the cultured plantlet was induced into callus and regenerated into shoot or root. At 4 weeks after EMS exposure, the unchanged explants were also included in the survival percentage calculation. However, they had low growth rate; therefore, they were not used in the next step of experiment.

Table 1 Rates (in percent) of survival, shoot/root initiation and callus formation after being treated with EMS for 4 weeks.

Varieties	EMS concentration (%)	Survival (%)*	Shoot/root Initiation (%)*	Callus formation (%)*	Unchanged (%)*
M07-4	0	100a	63.99a	36.01c	0
	1	100a	7.99c	92.01a	0
	1.5	92b	36.01b	63.99b	0
	2	88c	7.99c	92.01c	0
	F-test	**	**	**	ns
M07-14	0	92b	39.99b	0	60.01c
	1	92b	44.00a	0	56.00d
	1.5	64c	15.98c	0	84.02b
	2	100a	0d	0	100.00a
	F-test	**	**	ns	**
D23	0	100a	48.01a	0	51.99d
	1	96b	15.98c	0	84.02b
	1.5	100a	12.00d	0	88.00a
	2	100a	39.99b	0	60.01c
	F-test	*	**	ns	**
D27	0	100a	63.99a	36.01d	0
	1	48c	56.00b	44.00c	0
	1.5	84b	12.98d	87.02a	0
	2	100a	36.01c	63.99b	0
	F-test	**	**	**	ns
11-4	0	100a	84.02a	15.98d	0
	1	92c	56.00d	44.00a	0
	1.5	92c	60.01c	39.99b	0
	2	96b	76.00b	24.00c	0
	F-test	**	**	**	ns
11-8	0	100a	92.01a	0	7.99c
	1	92b	48.01b	0	51.99b
	1.5	100a	19.99c	0	80.01a
	2	100a	48.01b	0	51.99b
	F-test	*	**	ns	**
12-10	0	100a	88.00a	21.98c	0
	1	100a	76.00b	24.00c	0
	1.5	80b	72.01c	27.99b	0
	2	60c	44.00d	56.00a	0
	F-test	**	**	**	ns

*Mean values within the column followed by the different letter were significantly different according to Duncan's multiple range test ($p < 0.05$)

Figure 1 Chrysanthemum explants after 4 weeks of EMS exposure; A) initiated shoot, B) formed callus, C) unchanged explants and D) dead explants.

The surviving plantlets and calluses from EMS treatment were cultured in tissue laboratory for 12 weeks. Then, all healthy chrysanthemum plantlets with the complete shoot and root formation were transplanted to the natural environment for acclimatization. Because the actual natural environment is very different from the environment in tissue culture bottles, therefore; the acclimatization process is a necessary step to help plants adapt to the new environment. Mutation selection was investigated at 16 weeks after planting. Due to the rust disease destroyed all treatment of M07-4 variety and 0, 1, and 1.5 % EMS treatment of 11-4 variety, so they could not evaluate the experimental results (**Table 2**). All progenies of variety M07-14 had the highest average height (22.2 - 33.2 cm), and the flowers were similarly large (4.7 - 5.9 cm). The varieties D23 and D27 showed the same height (8.0 - 9.4 cm), but flower size varied from small to large flowers (2.8 - 7.9 cm), while 11-8 and 12-10 varieties had medium height (16.0 - 23.0 cm) and had relatively small flower size (3.3 - 4.0 cm) (**Table 2**).

Table 2 The average growth rates of chrysanthemum plants under different EMS treatments after 16 weeks of planting in the greenhouse.

Line no.	EMS concentration (%)	Height* (cm)	Flower width* (cm)	Note
No. M07-4	0, 1, 1.5, 2	-	-	died from rust disease
No. M07-14	0	23.20c	4.74cd	-
	1	22.20d	4.90bc	-
	1.5	27.60b	5.14b	-
	2	33.20a	5.90a	-
	F-test	**	*	
No. D23	0	9.40b	7.40b	-
	1	9.60a	7.90a	-
	1.5	9.20c	4.50d	-
	2	7.80d	6.50c	-
	F-test	**	**	

Line no.	EMS concentration (%)	Height* (cm)	Flower width* (cm)	Note
No. D27	0	9.40a	7.50a	-
	1	8.00b	3.90b	-
	1.5	8.40c	3.60b	-
	2	9.20a	2.80c	-
	F-test	*	**	
No. 11-4	0, 1, 1.5	-	-	died from rust disease
	2	27.4	6	-
	F-test	-	-	
No. 11-8	0	23.00a	3.32bc	-
	1	21.40b	3.26c	-
	1.5	18.60c	3.54b	-
	2	18.60c	4.02a	-
	F-test	**	*	
No. 12-10	0	16.80a	3.70c	-
	1	15.00c	3.85bc	-
	1.5	16.60a	4.00a	-
	2	16.00b	3.90ab	-
	F-test	*	*	

*Mean values within the column followed by the different letter were significantly different according to Duncan's multiple range test ($p < 0.05$)

The mutant chrysanthemum flower characteristics, which are considered the most important part of this research, were found as follows:

1) Variety D23 produced 2 mutant types as shown in **Figure 2**. **Figure 2B** shows no blooming variety from 1.5 % EMS treatment and **Figure 2C** shows a paler yellow shade from 2 % EMS compared with the control (**Figure 2A**).

2) Variety D27 produced 2 mutant types as shown in **Figure 3**. **Figure 3B** shows a tiny no blooming chrysanthemum variety from 1 % EMS and **Figure 3C** shows a small flower which fewer petals from 2 % EMS compared with the control (**Figure 3A**).

3) Variety M07-14 produced 5 mutant characters as shown in **Figure 4**. The 2 mutant characters (**Figures 4B** and **4C**) came from 0 % EMS (**Figure 4A**). **Figure 4B** shows smaller and had fewer pale pink petals than the wild type plant, and **Figure 4C** shows more numerous small petals than their parent line caused of their receptacle to disappear and changed to petals. The mutant of 1 % EMS was shown in **Figure 4D** which has larger petal and more compact flower form than control. At 1.5 % EMS showed 2 mutant lines (see **Figures 4E** and **4F**): **Figure 4E** is similar to control (**Figure 4A)**, but they had darker pink petals than control and **Figure 4F** shows more compact flower form than control. Mutant from 2 % EMS as shown in **Figure 4G** produced asymmetrical numerous more petals compared with control.

4) Variety 11-8 produced 4 mutant characters as shown in **Figure 5**. At 1 % EMS, the first characteristic mutant was shown in **Figure 5B** which had paler orange petals and another characteristic mutant was shown in **Figure 5C** which had darker orange until near pink shade and fewer petals than the control. **Figure 5D** was shown 1.5 % EMS induced-mutants that flower color changed to be a light pink shade which similar forms to control. While 2 % EMS induced-mutants was shown that petal color was change to the darker orange until near pink shade and petal number was less than the control.

5) Variety 12-10 produced 2 mutant characters as shown in **Figure 6**. At 1.5 % EMS produced a paler pink petal shade (**Figure 6B**) and at 2 % EMS had a little bit darker shade of pink (**Figure 6C**) than 1.5 % EMS mutant but still paler than when compared with the control.

6) Variety 11-4 produced 1 mutant character from 2 % EMS as shown in **Figure 7**. The mutant produced a very pale pink petal shade compared with the control pink shade.

All mutants and control were carried on after they flowered until they finished the blooming period. The interesting chrysanthemum lines were kept and sent back to the tissue culture laboratory to preserve the mutant lines for mutant phenotype stability confirming and commercial market testing in the next step.

Figure 2 Chrysanthemum Variety D 23 A) control, B) 1.5 % EMS mutant, and C) 2 % EMS mutant.

Figure 3 Chrysanthemum Variety D 27 A) control, B) 1 % EMS mutant, and C) 2 % EMS mutant.

Figure 4 Chrysanthemum Variety M07-14 A) control, B) - C) 2 mutants from control, D) 1 % EMS mutant, E) - F) 1.5 % EMS mutant, and F) 2 % EMS mutant.

Figure 5 Chrysanthemum variety 11-8 A) control, B) - C) 1 % EMS 2 mutants, D) 1.5 % EMS mutant (D), and E) 2 % EMS mutant.

Figure 6 Chrysanthemum Variety 12-10 A) control, B) 1.5 % EMS, and C) 2 % EMS mutant.

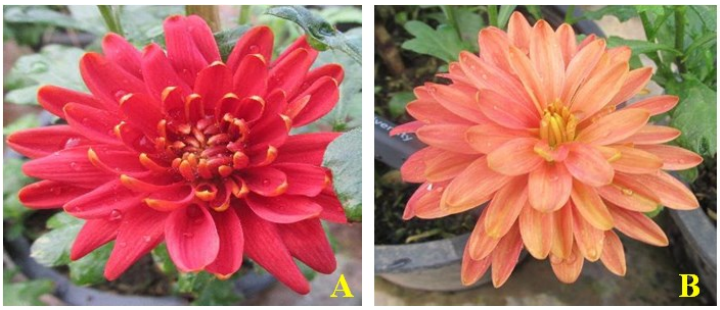

Figure 7 Chrysanthemum Variety 11-4 A) control and B) 2 % EMS mutant.

In this study, EMS-induced mutation allowed various new characteristics of flower form and color of chrysanthemums. The inflorescence diameter was tended to decrease under high EMS concentration. The higher mutagenic concentrations may cause adversely affect physiological and molecular effects on overall of plant growth and development which resulting in change of inflorescence size and form of flower. In addition, this observation is probably due to chromosomal damages and extrachromosomal origins [27]. From the present study, the EMS-induced variation of M07-14, 11-8, 12-10 and 11-4 variety flower color were change to lighter or darker compared to control. While D23 and D27 variety, which have yellow

petals, were not shown the modification of flower color, because the yellow chrysanthemum varieties are tend to be more stable to mutagens treatment than others color varieties [28]. The change in flower color may be caused by the mutation in gene involving in biosynthesis of anthocyanin or carotenoid which are the main pigments contributing to variety of flower color [21,29,30].

The 2 most commonly used experimental variables to describe the effects of chemical mutagenesis are the concentration of chemical mutagen solution and the duration of treatment. When applied in combination with mutation induction and plant tissue culture increases the overall efficiency of the mutagenic treatments. This combination allows the effective creation and *in vitro* selection of new chrysanthemum variations. Plant tissue culture also provides the ability to handle these large mutagenized populations on a laboratory scale, thus allowing the development and implementation of efficient and reliable methodologies to screen for flower color and formation responses in the field experiment [31]. In this research, the results indicated that there were a lot of EMS effects to induce mutation in 7 varieties of Chrysanthemum *in vitro*. Notice that M 07-14 variety produced 5 mutant characters. The 2 mutants came from the control treatment, which is possible because of 2 major reasons. The first reason is a spontaneous mutation which can occur in the natural environment. The second reason was that the chrysanthemum varieties in this experiment were cultured *in vitro*, that can induce genetic and epigenetic instability, which can cause phenotypic changes known as somaclonal variation [32-34]. Normally, the occurrence of spontaneous mutation is very low and the appearance of somaclonal variation is sometimes unstable and nonheritable [35]. Therefore, induced mutation will help increase mutation rate. Furthermore, the combination of *in vitro* culture and induced mutagenesis can enhance the genetic variability.

Conclusions

The cultured explants of 7 chrysanthemum varieties after EMS exposure were shown that M07-4, D27, 11-4 and 12-10 varieties showed shoot/root regeneration and callus formation. The others 3 varieties, M07-14, D23 and 11-8, showed shoot/root regeneration in some bottles and did not change in form or growth rate of explant in some bottles. After planting the healthy seedlings derived from surviving plantlets and calluses from EMS treatment, all treatment of M07-4 variety and 0, 1, and 1.5 % EMS treatment of 11-4 variety were severely damaged by rust fungi so they could not be evaluated in the final experimental results. The results of EMS showed 5 mutant characters from variety M07-14 and 4 mutant types from variety 11-8. While variety D23, D27, and 12-10 produced 2 mutant types each, and variety 11-4 showed only one mutant type. All interesting chrysanthemum lines were sent back to the tissue culture laboratory and prepared for commercial market testing in the further experiment. The findings of the present research supported that EMS can induce new traits of chrysanthemums that derive new shapes and colors of flowers in various types. The new traits depend on EMS concentration and the response of each variety.

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References

[1] C Broertjes. Improvement of vegetatively propagated plants by ionizing radiation. *In*: Proceedings of FAO/IAEA Sponsored Latin-American Study Group Meeting on Induced Mutations and Plant Improvement, Buenos Aires, Argentina. 1972, p. 293-9.

[2] YS Luan, J Zhang, XR Gao and LJ An. Mutation induced by ethylmethanesulphonate (EMS), *in vitro* screening for salt tolerance and plant regeneration of sweet potato (*Ipomoea batatas* L.). *Plant Cell Tissue Organ Cult.* 2007; **88**, 77-81.

[3] SK Basu, SN Achaya and JE Thomas. Genetic improvement of fenugreek (*Trigonella foenum-graecum* L.) through EMS induced mutation breeding for higher seed yield under western Canada prairie conditions. *Euphytica* 2008; **160**, 249-58.

[4] V Puripunyavanich, A Piriyaphattarakit, N Chanchula and T Taychasinpita. Mutation induction of *in vitro* Chrysanthemum by gamma irradiation. *Chiang Mai J. Sci*. 2019; **46**, 609-17.

[5] International Atomic Energy Agency. *Manual on mutation breeding*. 3rd ed. International Atomic Energy Agency. Vienna, Austria, 2018.

[6] C Auerbach and JM Robson. Chemical production of mutation. *Nature* 1946; **157**, 302.
[7] LS Lee, BJ Till, H Hill, OA Huyn and J Jankowicz-Cieslak. *Mutation and mutation screening*. *In*: R Henry, and A Furtado (Eds.). Cereal genomics: Methods in molecular biology. Humana Press, New Jersey, 2014, p. 77 -95.
[8] J Jankowicz-Cieslak and BJ Till. *Forward and reverse genetics in crop breeding*. *In*: JM Al-Khayri, SM Jain and DV Johnson (Eds.). Advances in plant breeding strategies: Breeding, biotechnology, and molecular tools. Springer Cham, Switzerland, 2015, p. 215-40.
[9] S Henikoff and L Comai. Single-nucleotide mutations for plant functional genomics. *Annu. Rev. Plant Biol*. 2003; **54**, 375-401.
[10] S Gnanamurthy and D Dhanavel. Effect of EMS on induced morphological mutants and chromosomal variation in Cowpea (*Vigna unguiculata* (L.) Walp). *Int. Lett. Nat. Sci*. 2014; **17**, 33-43.
[11] IM Henry, U Nagalakshmi, MC Lieberman, KJ Ngo, KV Krasilev, H Vasquez-Gross, A Akhunova, E Akhunov, J Dubcovsky, TH Tai and L Comai. Efficient genome-wide detection and cataloging of EMS-induced mutation using exome capture and next-generation sequencing. *Plant Cell* 2014; **26**, 1382-97.
[12] EA Greene, CA Codomo, NE Taylor, JG Henikoff, BJ Till, SH Reynolds, LC Enns, C Burtner, JE Johnson, AR Odden, L Comai and S Henikoff. Spectrum of chemically induced mutations from a large-scale reverse-genetic screen in Arabidopsis. *Genetics* 2003; **164**, 731-40.
[13] BJ Till, SH Reynolds, EA Greene, CA Codomo, LC Enns, JE Johnson, C Burtner, AR Odden, K Young, NE Taylor, JG Henikoff, L Comai and S Henikoff. Large-scale discovery of induced point mutations with high-throughput TILLING. *Genome Res.* 2003; **13**, 524-30.
[14] SK Senapati and GR Rout. *In vitro* mutagenesis of rose with ethylmethanesulphonate (EMS) and early selection using RAPD markers. *Adv. Horticult. Sci.* 2008; **22**, 218-22.
[15] J Fang and S Traore. *In Vitro* mutation induction of *Saintpaulia* using ethyl methanesulfonate. *HortScience* 2011; **46**, 981-4.
[16] M Ghani, S Kumar and M Thakur. Induction of novel variants through physical and chemical mutagenesis in Barbeton daisy (*Gerbera jamesonii* Hook.). *J. Horticult. Sci. Biotechnol*. 2013; **88**, 585-90.
[17] L Jiang and BL Dunn. Ethyl methanesulfonate and caffeine mutagenetic treatment to four ornamental *Silene* species. *J. Environ. Horticult.* 2016; **34**, 95-100.
[18] DY Lenawaty, D Sukma, M Syukur, DN Suprapta, W Nurcholis and SL Aisyah. Increasing the diversity of marigold (*Tagetes* sp.) by acute and chronic chemical induced mutation of EMS (Ethyl Methane Sulfonate). *Biodiversitas* 2022; **23**, 1399-407.
[19] S Rahmah. Induksi keragaman dua varietas krisan (*Dendranthema grandiflora* Tzvelev) dengan etil metana sulfonate (EMS) secara *in vitro*. Scientific Repository, IPB University, Bogor, Indonesia, 2011.
[20] Z Hossain, AKA Mandal, SK Datta and AK Biswas. Development of NaCl tolerant strain in *Chrysanthemum morifolium* 'Ram throung' *in vitro* Mutagenesis. *Plant Biol.* 2006; **8**, 450-61.
[21] RR Latado, AH Adames and AT Neto. *In vitro* mutation of chrysanthemum (*Dendranthema grandiflora* Tzvelev) with Ethyl Methanesulphonate (EMS) in immature floral pedicels. *Plant Cell Tissue Organ Cult*. 2004; **77**, 103-6.
[22] V Yoosumran, S Ruamrungsri, W Duangkongsan and S Kanjana. Induced Mutation of *Dendranthemum grandiflora* through tissue culture by Ethyl Methanesulphonate (EMS). *Int. J. Agr. Tech.* 2018; **14**, 73-82.
[23] F Nasri, H Zakizadeh, Y Vafaee and AA Mozafari. *In vitro* mutagenesis of *Chrysanthemum morifolium* cultivars using ethylmethanesulphonate (EMS) and mutation assessment by ISSR and IRAP markers. *Plant Cell Tissue Organ Cult*. 2021; **149**, 17.
[24] S Chaidaroon and I Ungvichien. *Research and development in producing disease-free chrysanthemums for promotion and technology transfer*. Thailand Institute of Scientific and Technological Research, Pathum Thani, Thailand, 2016.
[25] Data Bridge Market Research. Global flowers and ornamental plants market - industry trends and forecast to 2029, Available at: https://www.databridgemarketresearch.com/reports/global-flowers-and-ornamental-plants-market, accessed on May 2023.
[26] International Rice Research Institute. *STAR - Statistic tool for agricultural research. Star 2.0.1. Software. Biometrics and breeding informatics*. Plant Breeding, Genetics and Biotechnology Division, Laguna, Philippines, 2013.
[27] D Kapadiya, S Chawla, A Patel and T Ahlawat. Exploitation of variability through mutagenesis in Chrysanthemum (*Chrysanthemum morifolium* Ramat) var Maghi. *Bioscan* 2014; **9**, 1799-804.

[28] A Schum. Mutation breeding in ornamentals: An efficient breeding method? *Acta Horticult.* 2003; **612**, 47-60.
[29] H Liu, C Luo, D Chen, Y Wang, S Guo, X Chen, J Bai, M Li, X Huang, X Cheng and C Huang. Whole-transcriptome analysis of differentially expressed genes in the mutant and normal capitula of *Chrysanthemum morifolium. BMC Genomic Data* 2021; **22**, 2.
[30] M Mekapogu, BMK Vasamsetti, OK Kwon, MS Ahn, SH Lim and JA Jung. Anthocyanins in floral colors: Biosynthesis and regulation in chrysanthemum flowers. *Int. J. Mol. Sci.* 2020; **21**, 6537.
[31] R Pathirana. Plant mutation breeding in agriculture. *CABI Rev.* 2011, https://doi.org/10.1079/PAVSNNR20116032.
[32] B McClintock. The significance of responses of the genome to challenge. *Science* 1984; **226**, 792-801.
[33] PJ Larkin and WR Scowcroft. Somaclonal variation - a novel source of variability from cell cultures for plant improvement. *Theor. Appl. Genet.* 1981; **60**, 197-214.
[34] X Wang, R Wu, X Lin, Y Bai, C Song, X Yu, C Xu, N Zhao, Y Dong and B Liu. Tissue culture-induced genetic and epigenetic alterations in rice pure-lines, F1 hybrids and polyploids. *BMC Plant Biol.* 2003; **13**, 77.
[35] G Duta-Cornescu, N Constantin, DM Pojoga, D Nicuta and A Simon-Gruita. Somaclonal variation - advantage or disadvantage in micropropagation of the medicinal plants. *Int. J. Mole. Sci.* 2023; **24**, 838.