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Mutation Induction in *Chrysanthemum* Cut Flowers Using Gamma Irradiation Method

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Abstract

Chrysanthemum is one of the ornamental plants that cultivated widely in worldwide. This flower is one of the most important cut and pot flowers in the world due to its high diversity of colors and shapes. The creation of mutation is an important method for the production of new cultivars, and many cultivars have been produced through spontaneous and induced mutations. In this study, explants of leaf pieces from three most cultivated chrysanthemum varieties were irradiated with different gamma rays. This experiment was conducted as factorial in randomized complete block design with three replications. The results showed that an appropriate dose of gamma ray to produce a mutation in the varieties used in this experiment, is 25 G-ray. The obtained results showed that the 25 G-ray in purple cultivar produced the greatest change in petal color with a mutation rate of 54.56%. Meanwhile, the maximum number of new colors belonged to the purple group. Also, in the pink cultivar, the highest number of colored flowers was observed with a change of 32.11% in the 25 G-ray treatment. Based on the results of this study, four new cultivars will be introduced to the Iranian flower industry as a new cultivar.

Keywords: Chrysanthemum, Gamma ray, Mutagenesis, Radiation.

INTRODUCTION

In most countries, chrysanthemum is one of the most important cut flowers and has a lot of commercial importance in the flowering industry. *Chrysanthemum morifolium* belongs to the Asteraceae family and has more than 200 species. Approximately, 20% of the world's floral market belongs to chrysanthemum trade. Many cultivars with white, pink, red, orange and yellow colors are produced by breeders. Flower color is one of the most important characteristics of plant breeding in ornamental plants. Also, flower color is an important customer-friendly feature in greenhouse products, and breeders are always looking for a new color. Breeding by hybridization or mutation has been successfully used to produce new flower color. For example, the yellow cultivated roses (*Rosa hybrida*) have been produced by continuous hybridization with the Persian wild yellow-flowerd *R. foetida*. However, breeders are faced by limited genetic pool of a species. Biotechnology liberates the breeders from this restriction, and the first transgenic plant that changed its flower color, red/orange petunia, was reported about 30 years ago. These petunias transgenic plants accumulated pelargonidin-based anthocyanins by expression of the corn dihydro-flavonol 4-reductase (DFR) gene, which non-transgenic plants do not have DFR gene. Since then, a number of plants with modified flower-color, such as carnations and roses with violet flowers have been generated, while other carnations and roses do not have. Chrysanthemum was the target species for blue color engineering, but the successful color change in this species was done after carnation and rose which was due to the hardness expression of the transgene and the limitation of the promoters that had the required efficacy (Okitsu *et al*., 2018).

Breeding by mutation induction has been done successfully to improve many plant species. About 70% of mutated varieties are produced by gamma rays. There are two pathways for gamma rays, in the field (chronic) or in the laboratory (acute) (Nagatomi and Degi, 2009). The main issue in any breeding program with mutation is the discovery of new and superior genotypes through genetic changes in the available germplasm. The main advantage of mutagenesis in chrysanthemum is the ability to change one or more finite features of a great variety without altering its other traits. Gamma rays have been effectively used to induce mutations in chrysanthemum (Patil *et al*., 2017). Chrysanthemum is one of the cultivars of ornamental plants, which is cultivated extensively in the world and Iran. The purchase and sale of this flower in the world in 2017 was 366 million dollars (Azadi *et al*., 2016). In this era, modern breeding is a fundamental part of the new plant-breeding technologies that have changed traditional breeding methods and become an inevitable breeding tool. The mutation is an important tool for the breeding of new cultivars, and many cultivars have been produced using spontaneous or induced mutations. Gamma and X rays have been widely used to induce mutations in chrysanthemum. However, in addition to mutation, irradiation treatment causes genetic damage such as chromosomal aberration in plants. Reducing in the number of chromosomes is one of the chromosomal aberrations reported in chrysanthemum which irradiated by gamma or X-ray (Dowrick and El-Bayoumi, 1966). In addition, decreasing the number of chromosomes is correlated with the decrease in the diameter of the flowers in chrysanthemum (Ichikawa *et al.*, 1970). Therefore, reducing the number of chromosomes is an unwanted effect that is caused by irradiation in order to produce mutants with desired and improved traits. Therefore, irradiation with the least damage is considered. The dose ratio used for mutagenesis is an important factor in irradiation treatment and its effects have been evaluated by survey of various traits including mortality, growth and fertility. Therefore, the used dose ratio has been shown to affect the damage caused by irradiation in chrysanthemum. Also, the dose ratio affects the somatic mutations. It was reported that flower-colors mutants are effectively derived from gamma rays (Nagatomi *et al*., 2000). However, no information is available on the effect of the total radiation dose and dose rate on mutation induction in chrysanthemum.

Spontaneous mutations occur in ornamental plants with a low rate, which is usually referred to as Sports or somatic mutation. The percentage of occurrence of these mutations in chrysanthemum, carnation, rose and begonia are 30, 25, 40, and 45, respectively, and has long been a favorite for flower breeders (Nakagawa, 2009). Many consumers of flowers, prefer shapes and colors of wild flowers, but due to the fact that different varieties require different nutritious environments, they aren't reluctant to reproduce a large number of these varieties. Therefore, by using breeding methods through mutagenesis, new cultivars can be created with favorable genetic variation of flower consumers, which require growth conditions such as wild cultivars (Schum, 2003). In this purpose, physical mutagen such as X-rays and gamma rays have been used which have led to increased diversity in ornamental plants (Schum, 2003; Datta *et al*., 2005). Recently, 50 new cultivars have been registered in chrysanthemum through the creation of a mutation by irradiation method (Datta *et al*., 2005). Considering that Iran is not a member of the International Union for the Protection of New Varieties of Plants (UPOV), the new varieties are slowly import to the country and there is no permission for mass reproduction as well as the problems of some imported varieties that are not compatible with the climate of Iran and, on the other hand, the susceptibility of some cultivars to fungal and viral diseases, the diversified flower and plant market, and the increasing need for new cultivars, led to perform this study to aim breeding and introducing new chrysanthemum varieties.

MATERIALS AND METHODS

Plant materials

In this research, three important cultivars of chrysanthemum on the Iranian flower market (Bella Rosa, Rambla, Bonfire Yellow) were selected and provided from the National Institute of Flowers and Ornamental Plants. *In vitro* seedlings were used to increase the possibility of genetic mutations. Stem nodes from 1 to 3 cm length were used for seedling production. Sterilization of explants were taken with sodium hypochlorite 1% for 5 to 10 minutes. The basic MS medium with 30 g of sucrose, 100 mg of myoinositol, 5 mg of thiamine HCL and 8 g per liter of plant agar was prepared and its pH adjusted to 7.5. This medium was sterilized with autoclave at 121 ˚C and then BAP, Kn and IAA growth regulators were added to the medium according to the purpose of the experiment (Yesmin *et al*., 2014). Stem node explants were sterilized and placed in supplemented medium to grown and sterile seedlings were obtained. From these sterile seedlings, 1 × 1 cm leaf explants were prepared and placed in regeneration media. The explants were irradiated after 1 to 2 weeks after emergence of callus or shoot production symptoms.

Finding optimal dose

To determine the appropriate dosage of radiation, 100 seedlings of each cultivar were tested in a Randomized Complete Block Design. These seedlings were irradiated with gamma rays ranging from 15 to 35 G-ray/hour at the Research Institute of Nuclear Farming. Then the leaf explants were transferred to regeneration medium. Irradiated samples were evaluated for 3 months.

Characteristics measurement

After mutagenesis, the selection of mutants that have the potential to be introduced as a cultivar was done based on the measurement of some traits. Among mutated chrysanthemum, the main color characteristics, flowering start, end of flowering, flower diameter, plant height, expansion rate and flower density were measured.

Experimental design and data analysis

This experiment was conducted as factorial in Randomized Complete Block Design with three replications. Finally, the statistical analysis of the obtained data from the measured traits was done using SAS software (9.1) and also the comparison of the meanings was done based on the LSD test at 1% probability level.

RESULTS AND DISCUSSION

After transferring the irradiated samples to the regeneration medium, seedling regeneration percentage was investigated. The highest percentage of regeneration in all three cultivars was obtained from explants irradiated with 20 G-ray per hour (25% regeneration) and with increasing irradiation up to 35 G-ray, all of the explants lost their regeneration ability (Table 1). In all of explants, with increasing radiation dose, no significant increase or decrease was observed in the regeneration rate (Table 1), which is probably due to the stability of the callus stem cells for the division and specialization or because of low dose of radiation. But in a similar study, Yamaguchi *et al*. (2008) concluded that regeneration rates could be influenced by radiation dose and dose rate.

Table 1. Regeneration rate of various varieties after irradiation.

Irradiation dose (G-ray)	Regeneration percentage		
	Bella Rosa	Rambla	Bonfire Yellow
15	18%	12.5%	18%
20	25%	25%	25%
25	19%	18.7%	6.2%
30	6.2%	6%	0
35	0	0	0

Variance analysis of different irradiation treatments (doses) on Rambla chrysanthemum did not show a significant difference in measured traits (Table 2). The physiological conditions of the explants, such as stem diameter or the age of the donor seedlings, and possibly the low rate of the dose, can be due to the non-significant effect of irradiation on the regeneration of explants. Nagatomi and Degi (2009) reported that an effective dose that eliminates 50% of the germplasm varies according to plant tissue. They estimate this value 100 G-ray for the main stems, but other tissues such as leaves, buds and petals were destroyed relatively less dose of radiation. In general, in vegetative propagated plants separation of mutants from mutated parts are very difficult, while in these plants, keeping mutant genotypes is easier than maintenance of species which is propagated by seed. It has been shown that continuous combination of irradiation with gamma irradiation and tissue culture is very effective in solving this problem. In a study by tissue culture of flower organs of *Chrysanthemum morifolium* Ramat., which was continuously irradiated from germination to flowering with gamma rays, non-chimerical mutants with a variety of color and shape of the flower was obtained (Nakagawa, 2009).

Table 2. Analysis of variance of various gamma ray doses on Rambla variety.

SoV	df	Regeneration	Viability	Growth ability
Treatment	4	0.039^{ns}	0.143^{ns}	0.093^{ns}
Error	15	0.043	0.140	0.188
CV (%)		167	142	192

^{ns}: insignificant at Duncan’s test.

After regeneration of the explants, to prevent repeated proliferation of mutated branches from single callus cells, only one branch was selected from each callus and then transferred to the next stage. The regenerated seedlings were transferred to the pot and then transferred to the greenhouse after the adaptation period. After the flowering of plants, the number of flowers with change in petals color, counting and taking notes. Plants with new colors were classified as qualitative in a range of groups. The frequency of mutations as a proportion of flowers with a new color to the total analyzed plants for each cultivar was calculated separately (Table 3). In the cultivar with yellow flower, the formed flowers were classified into pale-yellow and dark-yellow categories. In this variety, the highest frequency of mutation (4.85%) was attributed to explants that were treated with a 25 G-ray. In cultivar with purple flowers (Rambla), the most change in the color of petals with a rate of mutation of 26.54 % belonged to seedlings regenerated from explants treated with 25 G-ray. The highest number of new colors belonged to the pale-purple group, while in the samples that were treated with a dose of 30 G-ray, the number of plants belonging to purple was dominant. In pink variety (Bella Rosa), the highest number (32.11%) of flowers with a change in color, was related to 25 G-ray treatment (Table 3). According to the results, the optimal dose rate for induction of mutation in these three chrysanthemum varieties was estimated 25 G-ray. In another study with aim of change the color of the chrysanthemum from among the 3688 regenerated plants, 15% of the plants were mutated, of which 79% were in the same group as the parent, and only 21% of the plants had different colors (Nagatomi and Degi, 2009). Fig. 1 shows a number of flowers with new colors.

In cultivar with purple, four new colors were created (Fig. 1). The selections 1 and 4 were different in terms of plant expansion and flower density from donor cultivar, so that plant expansion and flower density were higher than the native plant, and in these selections the diameter of flower was more than in mother plant. From the beginning and end of flowering, there was no significant difference between the selections and the mother plant. The most interesting thing about these choices was the darker color of choices 1 and 4 than the native plant, but the choices 2 and 3, which did not differ from the morphological traits with the mother plant (Table 4), compared to the native plant had a light color.

Fig. 1. Examples of flowers with change in color by gamma irradiation in variety with purple flowers. A: native plant with purple flower; B, C, D and E, selections 1 to 4, respectively.

Table 3. Number of flowers with new colors derived from three chrysanthemum varieties after irradiation with different doses of gamma ray.

Dose	Variety	Investigated plants number	Light pink	Dark pink	Pink with yellow disks	Pink with yellow disks	Light purple	Dark purple	Red - purple	orange	Purple with yellow disks	Light yellow	Dark yellow
15	Bonfire Yellow	210	0	0	0	0	0	0	0	0	0	2	0
20	Bonfire Yellow	332	0	0	0	0	0	0	0	0	0	6	1
25	Bonfire Yellow	268	0	0	0	0	0	0	0	0	0	11	2
30	Bonfire Yellow	79	0	0	0	0	0	0	0	0	0	3	0
35	Bonfire Yellow	0	0	0	0	0	0	0	0	0	0	0	0
15	Rambla	106	0	0	0	0	3	1	0	0	2	0	0
20	Rambla	217	0	0	0	0	11	6	2	1	3	0	0
25	Rambla	162	0	0	0	0	25	10	4	1	3	0	0
30	Rambla	89	0	0	0	0	2	7	1	0	0	0	0
35	Rambla	0	0	0	0	0	0	0	0	0	0	0	0
15	Bella Rosa	155	10	6	1	1	0	0	0	0	0	0	0
20	Bella Rosa	258	12	9	3	3	0	0	0	0	0	0	0
25	Bella Rosa	109	17	9	3	3	0	0	0	0	0	0	0
30	Bella Rosa	62	5	3	0	0	0	0	0	0	0	0	0
35	Bella Rosa	0	0	0	0	0	0	0	0	0	0	0	0

Table 4. The events selected based on morphological traits require to introduce new variety

Plant	Grade	Maincolor	Flowering start	Flowering peak	Floweringend	Petal style	Flower diameter	Height	Expansion	Petal density
Native	1	Dark purple	30 Oct.	3 Nov.	25 Nov.	Double	5.4 mm	50	Medium	Medium
Selection1	1	Dark red	30 Oct.	3 Nov.	26 Nov.	Double	6.5 mm	62	High	High
Selection2	1	Pale red	30 Oct.	3 Nov.	12 Nov.	Double	5.5 mm	57	Medium	Medium
Selection3	1	Light purple	30 Oct.	2 Nov.	22 Nov.	Double	5.7 mm	61	Medium	Medium
Selection4	1	Dark red	30 Oct.	2 Nov.	29 Nov.	Double	6.2 mm	65	High	High

Analysis of variance of different irradiation treatments on Bonifire Yellow cultivar, showed a significant effect of different doses on the viability of explants. By increasing the radiation dose from 15 up to 35 G-ray, there was a significant decrease in the survival rate of the explants (Table 5). However, there was no significant effect on regeneration or growth potential traits. Species of a genus and even varieties of a species due to different genetic structures show a different sensitivity to irradiation, this sensitivity has depends on genotype, tissue type, age of tissue or plant, developmental stage, intensity and duration of radiation and other factors, which excessive increase of radiation caused to growth of mortality rate. The optimal treatment of radiation is usually determined by criteria such as LD50 (Datta, 2005).

Table 5. Analysis of variance of various gamma ray doses on Bonfire Yellow variety.

SoV	df	Regeneration	Viability	Growth ability
Treatment	4	0.051^{ns}	0.46^{*}	0.144^{ns}
Error	15	0.031	0.101	0.061
CV (%)		167	72	179

* and ^{ns}: Significant at $P < 0.01$ and insignificant respectively.

There was no significant change in the measured traits in Bella Rosa variety (Table 6) indicating the genetic stability of the explants after applying gamma rays. Knowing the response of the plant to the induction of mutation in terms of the dose and the duration of the radiation treatment has a significant role in the success of the creation of diversity through the mutagenesis. Because the frequency and type of mutations depends to the plant variety, mutagenicity, treatment conditions and explants conditions before and after stress applying (Szarejko and Forster, 2007). Patil *et al*. (2017) studied gamma-ray effects on suckers of chrysanthemum and observed that the survival rate of the explants was reduced by increasing gamma radiation dose rate. Similar results were obtained in the Banerji and Datta (2002) studies, which showed that with increasing dose of gamma rays on the Jaya and Lalima cultivars, the survival rate of the explants was significantly lower.

Table 6. Analysis of variance of various gamma ray doses on Bella Rosa variety.

SoV	df	Regeneration	Viability	Growth ability
Treatment	4	0.042^{ns}	0.231^{ns}	0.144^{ns}
Error	15	0.89	0.104	0.207
CV (%)		177	117	209

* and ^{ns}: Significant at $P < 0.01$ and insignificant respectively.

In a similar report, the average of mutation rate in regenerated seedlings from non-irradiation treatment in all studied chrysanthemum cultivars was less than 0.5%, which indicates the presence of somaclonal variation. Somaclonal variation is often found in regenerated seedlings from callus, but this amount is less than that which has a significant effect on the control treatments (Nagatomi and Degi, 2009). Therefore, appropriate mutagenic treatment is necessary to achieve effective mutation induction. Compared with other plant breeding methods, there are special advantages in breeding by mutagenesis in plants that reproduce vegetatively, such as the frequency of diversity that can be 100 or 1000 times more than the frequency of natural mutation and its diversity spectrum is also increased, which among of these mutation, the amount of useful mutations increases significantly. Even some of the rare mutations that not occur in the nature and it does not happen through crossing, happens by this method. Among the techniques and diversity creating sources, physical mutagens such as gamma rays, have shown a high potential for plant breeding (Predieri and Virgilio, 2007).

CONCLUSION

Flower color is one of the most important factors in ornamental plants. The chrysanthemum breeding goal should focus on the rapid production potential of new cultivars, in order to meet market demand and be competitive. The results of this study indicated that low doses do not have the required efficacy to produce useful mutations, and high doses cause chromosomal aberrations and loss of explants. In this study, a suitable dose for gamma rays was determined for three cultivars and it was 25 G-ray. Two to five new colors were created from these cultivars. Based on the results obtained from the mutants and based on the measured traits, four new cultivars will be introduced to the Iranian flower industry as new cultivars. Investigating and continuing the research with other cultivars, different parts of the chrysanthemum as an explant and a wider range of gamma rays will be very effective in achieving new varieties with greater diversity. Also, morphological and genetical complementary analyses are needed to identify and record new cultivars.

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