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Comparative Analysis of Physical and Chemical Mutagenesis in Chrysanthemum cv. 'Candid': Assessing Genetic Variation and Breeding Potential

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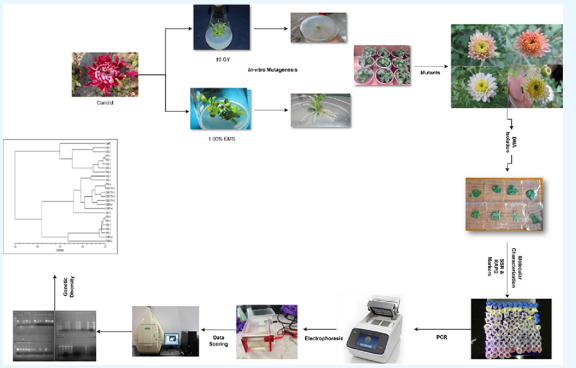
ABSTRACT: In this study, we developed a mutagenesis protocol specifically designed for chrysanthemum cv. "Candid" in order to introduce genetic variation. By subjecting chrysanthemum shoots to different doses of physical and chemical mutagens, we successfully generated a total of 24 mutants, each with unique genetic compositions. We observed that the mortality rate was lowest when the shoots were exposed to 10 Gy gamma irradiation and 1.00% EMS. To assess the diversity and relatedness among the mutants, we employed RAPD and SSR markers. The combination of these markers allowed us to construct a dendrogram that effectively categorized the mutant population into distinct clusters based on the specific mutagen treatments. Interestingly, the mutants induced by 10 Gy gamma irradiation exhibited greater genetic diversity in terms of flower colors. On the other hand, mutants created with 1.00% EMS displayed a higher level of variation and yielded more viable mutants. To determine the optimal markers for studying genetic diversity, we analyzed the polymorphic information content (PIC) of different markers. Among the tested markers, OPA-07 (RAPD) and JH47 (SSR) showed the highest PIC values, indicating their effectiveness in capturing genetic variability within the mutant population. Conversely, the PIC values of OPD-07 and JH20 demonstrated the lowest among the markers tested. Our results revealed a percentage of polymorphism ranging from 81.81% to 100% for RAPD markers and 66.66% to 100% for SSR markers. These findings indicate that physical mutation induced by 10 Gy gamma irradiation can be clearly distinguished from chemical mutation induced by EMS at concentrations of 1% and 0.75% in chrysanthemum cv. "Candid." Overall, this study provides valuable insights into the genetic composition of the generated mutants and highlights their potential for enhancing chrysanthemum-breeding programs. The identified markers, particularly, OPA-07 and JH47, can serve as valuable tools for future studies aimed at exploring and exploiting the genetic diversity within the chrysanthemum population.



INTRODUCTION

The chrysanthemum is a highly sought-after and valued cut flower crop that is grown in numerous countries, including Japan, China, the United States, France, the United Kingdom, and India. This versatile and important horticultural crop is considered the second largest, after roses, in the global flower market.[1] One of the most remarkable features of domesticated chrysanthemum is its intricate genetic heterozygosity. This characteristic allows for an endless array of unique flower forms and cultivars to be produced, making it an invaluable resource for breeders and scientists alike. In addition to its genetic diversity, the chrysanthemum is a hexaploid plant with 54 chromosomes, making it even more complex and fascinating for researchers to study.[2] It is propagated vegetatively and has a strong system of self-incompatibility,[3] making it difficult to obtain new cultivars through the crossing. Certain variants are more persistent than others[4] traditionally, and new cultivars have been derived through spontaneous mutations in vegetative reproduction. In recent years, tissue culture-derived induced mutations and somaclonal variants have been used as novel sources of variation.[5−9] Although substantial work has been performed to produce novelties in chrysanthemum by the use of physical and chemical mutagens, there is always a need to investigate the possibility of a new variation for the floriculture trade. Radiation-induced mutation breeding has been widely employed to improve well-adapted plant types and

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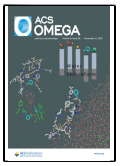

to create new variants with enhanced agricultural traits.[10] Since the majority of cultivated chrysanthemums are polyploids with significant genetic variability, mutants with allied flower color, form, floral size, and shape are frequently recovered.[11] Radiation can easily elicit complementary floral hues in chimera tissue, which can then be isolated using in vitro techniques. Variation in genes is vital for crop development in every plant breeding operation. Particularly for flower color, mutation breeding is an effective method for producing heritable alteration.[10] Growing demand for a new type of chrysanthemum has spurred the development of new cultivars. Radiation-induced mutation breeding, an application of nuclear technology in agriculture, has been widely employed to improve well-adapted plant types by one or a few essential features.[12−14] Several physical mutagens have transformed in a beneficial way commercially significant characteristics of horticultural plants. γ-rays are among the most extensively employed physical mutagens for generating mutations in flowering plants due to their ease of application and potency. The optimal dose for inducing mutations in chrysanthemums ranges from 10 to 30 Gy, depending on the genotype.[15] In addition to considerations like the choice of material, the trait to be enhanced, the type of mutagens to be utilized, and their dosage, experimental procedures should also be addressed. Consequently, by mutation breeding, it is possible to create a heritable genetic variation of adequate magnitude and frequency for quantitative and qualitative traits of relevance to the breeding program. Consequently, the genetic variability caused by mutation was explored in order to establish a new cultivar of chrysanthemums that are favored by consumers. Physical and Chemical mutagens have been investigated with success in order to develop new fewer kinds rapidly and with more efficacy, particularly in the absence of UV light.[16−19] EMS has proven to be one of the most successful chemical mutagens for developing new cultivars of ornamental plants, as it generates a large number of point mutations in plant genomes. EMS induces low rates of chromosomal abnormalities during mutagenesis in addition to large levels of gene mutation.[20,21] In many nations, chrysanthemums are becoming more prevalent due to their qualities of enormous, spectacular flowers, vibrant floral colors, diverse cultivars with different variations, robust resilience, and abundant cultural connotation. In recent years, numerous studies on chrysanthemums have been undertaken, with a primary focus on morphology,[22] cytology,[23] biochemistry,[24] and physiology.[26,25] Conversely, studies on the molecular genetics of chrysanthemums are uncommon due to the aneuploidy of the chrysanthemum at the genomic level, which reveals nonexponential gain or loss of chromosomes caused by the transmutation and hybridization of chrysanthemum varieties. Consequently, there are relatively little genetic data available for this plant. In addition, chrysanthemum cultivars have been examined for numerous morphological features and morphometric criteria, but the molecular characterization of the chrysanthemum genome has received little attention. To encourage the maintenance and justifiable use of chrysanthemum cultivars, it is required to develop and deploy techniques for analyzing the genomic assortment of chrysanthemum cultivars. The use of codominant markers in chrysanthemums is currently restricted. Due to their multiallelic nature, hypervariability, codominant legacy, reproducibility, comparative copiousness, wide-ranging genome coverage (comprising organelle genomes), chromosome-specific position, mechanization approachability, and extraordinary output genotyping, simple sequence repeat markers (SSR) have added admiration in plant genetics and breeding.[27] The majority of large-flowered chrysanthemum cultivars are hexaploidy-based aneuploids, reflecting a complicated genetic background,[23] thus making the use of SSR markers for the chrysanthemum genome challenging. Therefore, for the SSR study of chrysanthemums, the read and data processing provide a substantial obstacle. Fortunately, Esselink et al.[28] established a sophisticated technique known as microsatellite DNA allele counting-peak ratios (MAC-PR) for computing the ratios between the peak regions for two alleles in all trials in which these two alleles co-occurred. The identified allele peak ratios are then intrigued on a histogram, and histograms that create at least two clearly identifiable groups are chosen for additional analysis. In addition, these classifications need to be secluded de novo from the accessions being investigated for the first time, which is a time-consuming process. This disadvantage has been overcome as a result of the current usage of library amelioration and mechanized sequencing.[29] Microsatellites can now be isolated by using a variety of methods that have recently been developed. It takes time and money to screen genomic libraries with microsatellite probes and sequence the clones that test positive.[30] The screening of microsatellite enriched, small insert libraries, on the other hand, can considerably cut the time and cost involved.[30] Flower color was measured as the variance between the parent flower cultivar and mutants developed, and mutation frequency was estimated as a proportion of desirable or undesirable color mutants among the total number of plants irradiated with each physical or chemical mutation dose in this study.

In recent years, molecular markers have become essential tools in the field of mutagenesis breeding. By using these markers, researchers can accurately detect true mutants while eliminating nonmutated plants in the early stages of the experiment. This ability to quickly identify mutants has greatly improved the efficiency of mutagenesis breeding and reduced the time required to identify desired traits. In addition, molecular markers have the added benefit of allowing researchers to determine the genetic relationship between the mutants and their original mother plants.[31] The use of molecular markers in mutagenesis breeding has proven to be an effective approach for enhancing the genetic diversity of crops. This is particularly important for crop improvement, as it allows breeders to introduce new traits into plants, such as disease resistance or improved yield. With the help of molecular markers, researchers can also track the inheritance of specific traits across generations, which is vital in understanding the genetic basis of these traits. Overall, the use of molecular markers in mutagenesis breeding has revolutionized the field by allowing for more precise and efficient identification of true mutants while also enabling the discovery of new genetic traits in plants. This has greatly enhanced our ability to improve the quality and productivity of crops, which is essential for meeting the growing demands of a rapidly expanding global population. The objective was to assess the genetic diversity between the parents and mutants produced at the molecular level. Understanding the link between species under consideration might provide further support for the development and planning of breeding strategies. Such findings could pave the way for further investigation into the genetic divergence and the possibility of

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discovering the gene(s) responsible for variance in color features.

MATERIAL AND METHODS

In this study, we aimed to investigate the effects of cobalt-60 (^{60}Co) gamma irradiation and ethyl methyl sulfonate (EMS) treatment on in vitro grown microstalks of chrysanthemum cv.'Candid'. The micro shoots were obtained from the Plant Introduction Centre in Srinagar, J & K. To begin the experiment, uniform-sized shoots were extracted from in vitro grown clumps of the same age and transferred to flasks containing fresh Murashige and Skoog medium supplemented with BAP (benzylaminopurine) 0.25 mg/L and GA3 (gibberellic acid) 0.25 mg/L. The shoots were then exposed to different doses of gamma irradiation (0, 10, 20, 30, and 40 Gy) at a rate of 100 Gy per minute using a Panoramic Batch Irradiator (PANBIT) at Baba Atomic Research Station in Zakura, Srinagar, J & K, India. A week later, the in vitro micro shoots were immersed in various percentage doses of EMS solution for 1 h and 45 min. The entire procedure, including the EMS treatment, was conducted under laminar airflow conditions to ensure sterility. The experiment was carried out in a laminar airflow environment, with five sets of replicates for each treatment. Each set consisted of three to seven individual shoots in a flask. The shoots were allowed to grow vegetatively for two generations (VM1 and VM2) at 5 week intervals. The researchers recorded the mortality of the shoots at 1 and 2 weeks after irradiation and EMS exposure. They also calculated the LD50 value using the probit model. To assess the frequency of mutation, the researchers examined the flower color of the chrysanthemum mutants obtained. They compared the color of the parent flower with that of the mutants and calculated the ratio of desired or undesired color mutants to the total number of plants irradiated with each gamma irradiation dose. For molecular analysis of the physical and chemical mutants, genomic DNA was isolated and quantified from 24 mutants of chrysanthemum cv.'Candid'. The DNA was then amplified using PCR (polymerase chain reaction) with the use of 6 RAPD (random amplified polymorphic DNA) markers and 6 SSR (simple sequence repeat) markers. The amplified bands were scored using a binary system, and a dendrogram was constructed to assess the genetic diversity of the mutants.

Genetic Diversity Based on DNA Molecular Studies. Table 1 contains a listing of the various buffers and stock solutions (along with their composition and formulation) utilized in the current investigation. Solutions and buffers were kept at the appropriate pH, autoclaved and stockpiled at 4 °C.

Table 1. List of Simple Sequence Repeats Primers[a]

name of primer	sequence	ref
JH04	F: TCTCCACTCCCTCATTTTCACTC R: CAACTCGTACACCAATACCACGA	Zhang et al. 23
JH09	F: TTCGCCCTCTGCTGCTCTTGTAA R: CCATTTTCTTGGCTTCTTGTGCT	
JH20	F:CACTTTCTTCTACAACCATCTTTACA R:CATGTGCGAGTGAATGTGAGTAGT	
JH28	F: CGATGTTTTAGTTGATTATGTGGA R: GCTTATGGAGACCTTTCTTTATTT	
JH31	F: CTCTTTTGGCTGCTCTAACATATC R: CAAGTTTGACACTGTCACGGAC	
JH47	F: CTTCTTATCTCCTAACATTCCCA R: ATGTGATATGGAGGAGCCTTT	

[a]F: forward; R: reverse.

Genomic DNA Isolation. The genomic DNA of all 24 mutants (physical and chemical) was extracted using the CTAB method.[67] When immature leaves were used, the concentration of extracted DNA ranged from 227.4 to 2070.2 ng/μL.

DNA Quantification. The quantity and quality of DNA were assessed to confirm the DNA concentration and presence of contamination. It was accomplished by utilizing two approaches.

Agarose Gel Electrophoresis. Using the intensity of intact bands, the quality and content of DNA were estimated. The casting tray and combs were meticulously cleaned and placed in the electrophoresis apparatus. 100 mL of TAE (1×) buffer was placed into a conical flask, 3/1.5 g of agarose was added, and the mixture was microwaved for 2−3 min to melt the agarose. After a few minutes, 4 μL of EtBr was added, mixed, and then poured into the mold. The gel was permitted to be set for approximately 30 min before the combs were removed. The electrophoresis buffer was TAE buffer (1×), and PCR products comprising the bromphenol blue dye were placed in the wells. At least one well of each lane was loaded with 2 μL of DNA molecular ladder (100 bp) and electrophoresed at 80 V for several hours. The gel image was then captured utilizing a gel documentation method (Biorad).

Genomic DNA Isolation. The genomic DNA of all 24 mutants, including both physical and chemical mutants, was extracted using the CTAB method (68). In this method, immature leaves were used as the source of DNA, resulting in extracted DNA concentrations ranging from 227.4 to 2070.2 ng/μL.

DNA Quantification. To verify the DNA concentration and check for contamination, two different approaches were employed to assess the quantity and quality of DNA.

Agarose Gel Electrophoresis. Agarose gel electrophoresis was utilized to estimate the quality and content of DNA based on the intensity of intact bands. The casting tray and combs were meticulously cleaned and placed in an electrophoresis apparatus. A conical flask was used to prepare 100 mL of TAE (1×) buffer, into which 3/1.5 g of agarose was added. The mixture was microwaved for two-three min to melt the agarose, and after a brief cooling period, 4 μL of EtBr (ethidium bromide) was added and thoroughly mixed. The agarose gel was poured into the mold and allowed to set for approximately 30 min before removing the combs. TAE buffer (1×) was employed as the electrophoresis buffer, and PCR products containing bromophenol blue dye were loaded into the wells. For reference, at least one well of each lane was loaded with 2 μL of DNA molecular ladder (100 bp). The gel was then subjected to electrophoresis at 80 V for several hours, and a gel documentation method (Biorad) was used to capture the resulting gel image. By analysis of the intensity and integrity of the bands on the gel, the quality and content of DNA can be estimated. This information is critical in determining the suitability of the DNA samples for further analysis and experimentation.

UV-Spectrophotometric Method. Estimation of DNA concentration in various samples was performed using the formula:

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$$\text{DNA concentration}(\mu g/mL) = \text{OD260} \times 50 \times \text{dilution factor}$$

In order to dilute the concentrated DNA, sterile MQ water was employed. About 250 μL of each sample at a final concentration of 25 ng/μL was produced. PCR was then used to amplify the DNA that had been diluted.

SSR and RAPD Assay. Six SSR primers and 6 random decamer primers (specified in Tables 1 and 2) were utilized to amplify genomic DNA of 24 mutants (physical and chemical) by adjusting the proportion of PCR mixture components in the final PCR reaction as defined in the Material and Methods section (Table 3). The choice of SSR and RAPD markers was based on previously published findings. Integrated DNA technologies (IDTs) were responsible for the synthesis of the primers. The six SSR markers with high PIC (polymorphic information content) values based on flower traits were selected in this study.

Table 2. List of RAPD Primers

name of primer	sequence
OPA-02	TGCCGAGCTG
OPA-07	GAAACGGGTG
OPA-11	CAATCGCCGT
OPC-08	TGGACCGGTG
OPD-07	TTGGCACGGG
OPE −01	CCCAAGGTCC

Table 3. Particulars of the PCR Mixture Constituents and Thermal Cycling Profile Used for DNA Amplification Reactions

reaction constituents		SSR	RAPD
DNA template		2 μL	1.5 μL
primer		1.4 μL	1.5 μL
DNTPs		0.6 μL	1.0 μL
PCR buffer		2.0 μL	2.0 μL
$MgCl_2$		1.0 μL	1.0 μL
ddH_2O		2.8 μL	5.2 μL
Taq polymerase		0.2 μL	0.3 μL
thermal profile			
initial denaturation		94 °C for 4 min	94 °C for 4 min
denaturation	30 cycles	94 °C for 30 s	94 °C for 1 min
annealing		50−57 °C for 30 s	37 °C for 1 min
extension		72 °C for 30 s	72 °C for 2 min
final extension		72 °C for 10 min	72 °C for 15 min
store		4 °C	4 °C

Primer Dilutions. The concentration of lyophilized primers was raised to 100 M stock solution or 100 pmol/μL by multiplying the number of nanomoles of primer (provided on the vial) by 10 and adding the corresponding quantity of sterile MQ H_2O. The vials were centrifuged for one min at 8000 rpm and left at room temperature for one to 2 h. The stock was diluted to a 10 μM solution, or 10 pmol/μL, with sterile MQ water and kept at −20 °C.

UV-Spectrophotometric Method. To estimate the DNA concentration in various samples, we employed a UV-spectrophotometric method was employed. The formula used for this estimation was

$$\text{DNA concentration}(\mu g/mL) = \text{OD260} \times 50 \times \text{dilution factor}$$

In order to dilute concentrated DNA, sterile MQ water was used. Each sample was diluted to a final concentration of 25 ng/μL, resulting in a volume of approximately 250 μL. The diluted DNA samples were then subjected to PCR amplification.

SSR and RAPD Assay. For the amplification of genomic DNA from the 24 mutants (physical and chemical), a total of 6 SSR primers and 6 random decamer primers were utilized. The specific primers used in the assay can be found in Tables 1 and 2. The proportions of PCR mixture components for the final PCR reaction were adjusted as described in the "Materials and Methods" section (Table 3). The selection of SSR and RAPD markers was based on previously published findings, and the primers were synthesized by Integrated DNA Technologies (IDT). In this study, six SSR markers with high PIC (polymorphic information content) values, based on flower traits, were chosen.

Primer Dilutions. To prepare the primers for use in the experiment, the lyophilized primers were reconstituted to a concentration of 100 M stock solution or 100 pmol/μL. This was achieved by multiplying the number of nanomoles of primer indicated on the vial by 10 and adding the corresponding quantity of sterile MQ H_2O. The vials containing the reconstituted primers were then centrifuged for 1 min at 8000 rpm and left at room temperature for 1 to 2 h. The stock solution was further diluted to a 10 μM solution, or 10 pmol/μL, using sterile MQ water and stored at −20 °C for future use.

PCR Amplification. The DNA was amplified in accordance with the thermal profile. The DNA amplification product was separated by using horizontal 1.5% agarose gel electrophoresis for RAPD and 3% agarose gel electrophoresis for SSR resolution. To get a final concentration of 1×, 4 μL of loading dye (6×) was applied to tubes. All of the gels were then photographed by using a UV transilluminator.

Statistical Analysis. The binary data of SSR and RAPD markers were used for the estimation of Jaccard's similarity coefficient[76] using NTSYS-pc version 2.02e[32] software, and this similarity matrix was used for cluster analysis using the unweighted pair-group technique with arithmetic averages (UPGMA) and sequential, agglomerative, hierarchical and

Table 4. Mortality of ^{60}Co Gamma Irradiated *In Vitro* Shoot tips of Chrysanthemum Cv. Candid at the 2nd Week[a]

					95% confidence interval	
parameter	estimate	Std. error	Z	sig.	lower bound	upper bound
dose	1.770	0.637	2.778	**0.005**	0.521	3.019
intercept	−2.417	0.873	−2.768	0.006	−3.290	−1.544

[a]LD_{50}= 23.19.

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Table 5. Mortality of EMS Exposed to *In Vitro* Shoot Tips of Chrysanthemum Cv. Candid at the 2nd Week[a]

					95% confidence interval	
parameter	estimate	std. error	Z	sig.	lower bound	upper bound
dose	−3.947	1.378	−2.865	**0.004**	−6.647	−1.247
intercept	−0.896	0.271	−3.312	0.001	−1.167	−0.626

[a]LD_{50}= 0.593.

nested (SAHN) clustering algorithms to obtain dendrogram using NTSYS-pc version 2.02e.[32]

RESULTS

Effect of ^{60}CO Gamma Irradiation and Ethylmethane Sulfonate (EMS) on Survival (%) of *In Vitro* Shoots. The death rates of tissue-cultivated shoots of chrysanthemum cv. "Candid" following gamma irradiation and EMS treatment were recorded at the end of the second week. The results are presented in Tables 4 and 5. There was a consistent and clear decline in the percentage of surviving shoots as the radiation dosage increased. Among the gamma-irradiated shoots, the highest survival rate was observed in those exposed to a dose of 10 Gy (60.71%) followed by those exposed to doses of 20 Gy (60.71%) and finally those exposed to a dose of 30 Gy (40.47%). Shoots exposed to a dose of 40 Gy had a death rate of 35.71% by the end of the second week (Figure 1b−e). The

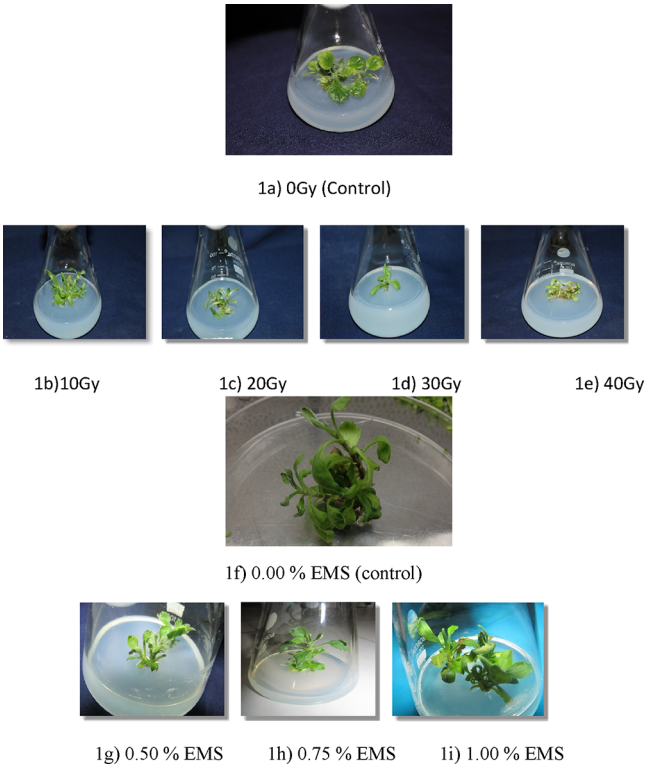

Figure 1. Survival of ^{60}Co gamma irradiated and EMS exposed shoots at the 2nd week.

estimated LD50 at the end of the second week was approximately 23.19 Gy (Figure 2a). Significant variation was also observed among the different EMS treatments. The lowest survival percentage was observed at a dose of 0.50% EMS, while the highest survival percentage was observed at a dose of 1.00% EMS. Shoots treated with 1.00% EMS had the highest survival rate at 80.95% followed by those treated with 0.75% EMS at 66.66%. Treatment with 0.50% EMS resulted in the lowest shoot survival rate at 39.28% after 2 weeks (Figure 1g−i). The LD50 after 2 weeks of EMS treatment was calculated to be 0.59% (Figure 2b).

Effect of ^{60}CO Gamma Irradiation and Ethylmethane Sulfonate (EMS) on Leaf Count and Leaf Area. Gamma irradiation treatments significantly recorded a decline in the leaf number on the plant^{-1} and the leaf size in both intervals, i.e., after 4 and 8 weeks, as compared to control. At the end of the fourth week, the minimum leaf number on the plant^{-1} and the size were registered under the highest dose of 40 Gy followed by 30 and 20 Gy, and the lowest gamma irradiation dose 10 Gy recorded a minimum decrease in the leaf number and size, as compared to the control. At the end of the eighth week, both the leaf number and leaf size improved in all the gamma irradiation doses, including the control plants, but recorded a similar trend of decline in both the parameters, as in the fourth week interval with the successive gamma irradiation doses (Figure 3a,b). As compared to the control group, plants exposed to EMS treatments lost significantly more leaves per plant at the 4 week and 8 week time points, while at the same time gaining size at the leaf level. All three EMS mutagens varied significantly from one another in recording both the leaf number and size plant^{-1}. Both growth metrics were shown to increase with each successive concentration dose in these treatments, echoing the pattern seen in the earlier ones. Regardless of the concentration of the mutagen, by the eighth week, the number of leaves and the average size of those leaves had both increased. Although the increasing trend of both the parameters under successive doses of treatments continued in this interval also. Data analysis shows that after 8 weeks, the minimum number of leaves per plant was 9.50 when treated with 0.50% EMS, which is a decrease of 39.68% compared to the control plants (15.75) leaves. Subsequent treatments with EMS doses of 0.75% and 1.00% resulted in leaf counts of 12.75 and 14.00, respectively. These treatments showed a decrease of 19.04 and 11.11%, respectively, against control. On the other hand, leaf size recorded an increasing trend with each increment of the EMS dose over control (Figure 4a,b). Further perusal of data reveals that a maximum leaf size of 42.62 cm^2 was recorded by the highest dose of 1.00% EMS, which corresponded to an increase of 49.43% leaf size over the control (28.52 cm^2). The other two treatments, i.e., EMS 0.50 and 0.75% doses, recorded 31.03 and 34.44 cm^2 leaf size, respectively. When compared to the control, these treatments corresponded to an increase of 8.80 and 20.75% over the control.

Effect of ^{60}CO Gamma Irradiation and Ethylmethane Sulfonate (EMS) on the Number of Days to Floral Bud Appearance. With the increment of each dose of irradiation (Figure 3c), there was a significant delay in the days to bud appearance in comparison to control plants (23.50). Under 10, 20, and 30 Gy doses, days to bud appearance were recorded as 27.25, 37.00, and 39.25, respectively. On the other hand, days to bud appearance under the last dose of 40 Gy recorded the

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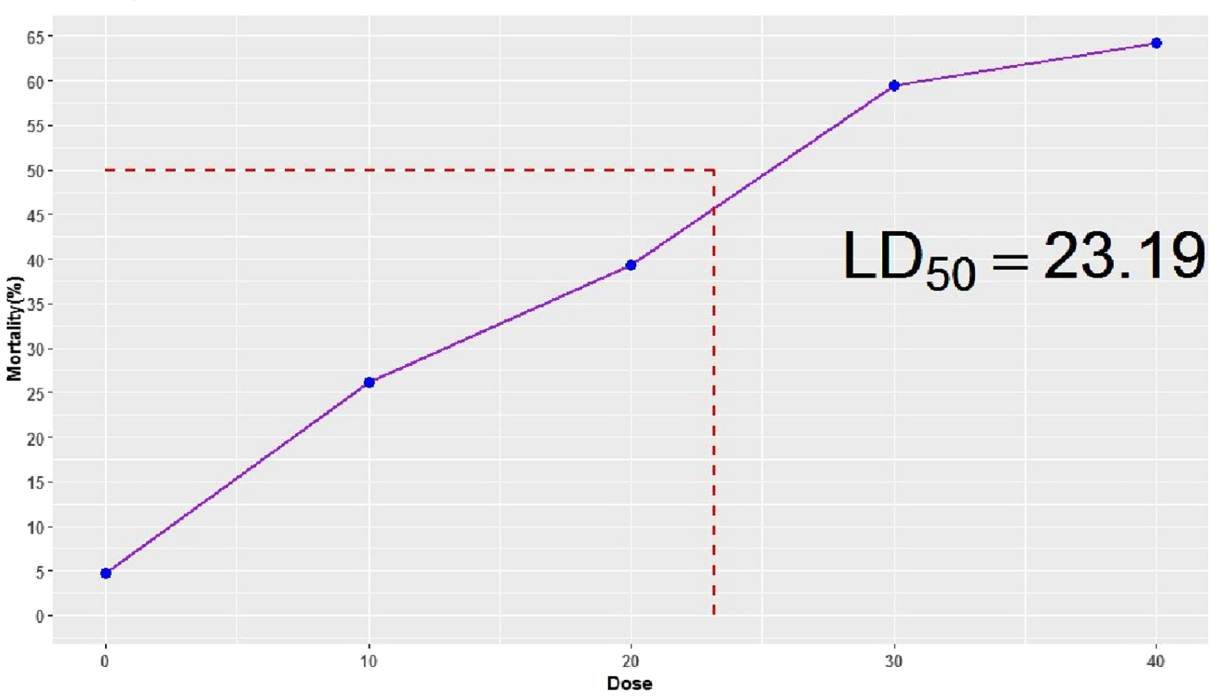
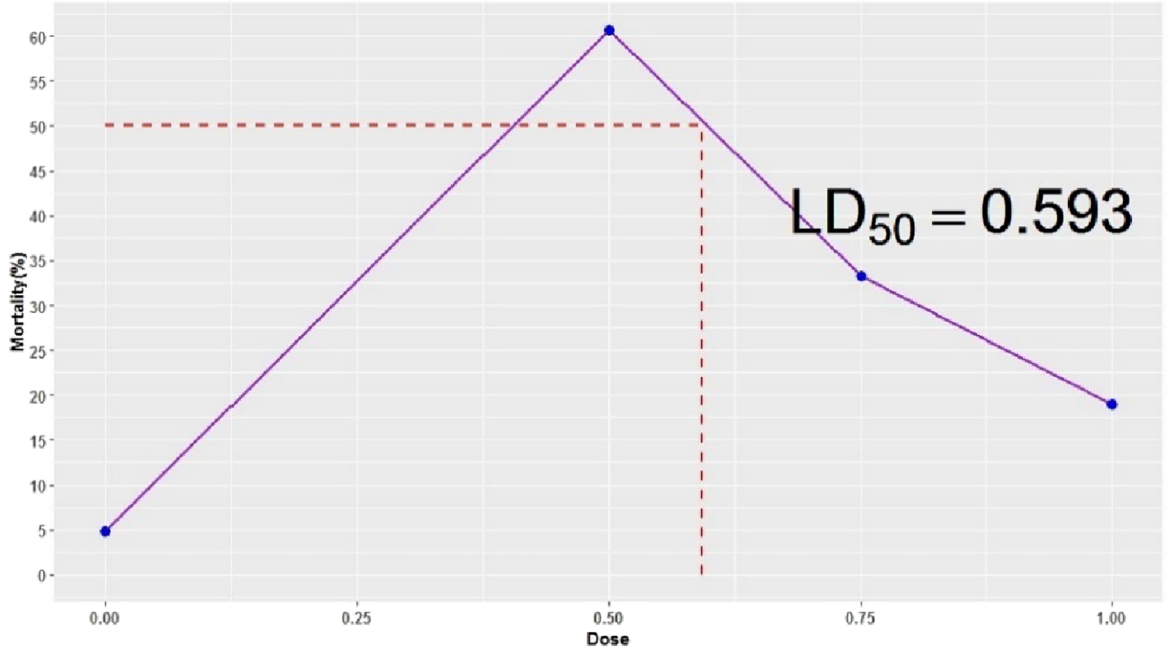


2a) (LD $_{50}$) for ^{60}Co gamma irradiated micro shoots



2b) (LD $_{50}$) for EMS treated micro shoots

Figure 2. Mortality rate (LD $_{50}$) for ^{60}Co gamma irradiated and EMS treated micro shoots of chrysanthemum cultivar candid at the 2nd week.

highest at 40.75, which represented a maximum delay as compared to control. In cv. "Candid" regarding days to flower bud appearance, perusal of data (Figure. 4c), reveal that with the increment of each dose of EMS there was a significant increase in days to flower bud appearance in comparison to control plants (23.50). Data showed that the significantly maximum number of days (28.00) taken to bud appearance was recorded under 0.50% EMS dose followed by 0.75% EMS (26.25), corresponding to an increase of 19.14 and 11.70% over control. Meanwhile, significantly minimum days taken to bud appearance were recorded under 1.00% EMS (25.25), representing a minimum decline of 7.44% over control.

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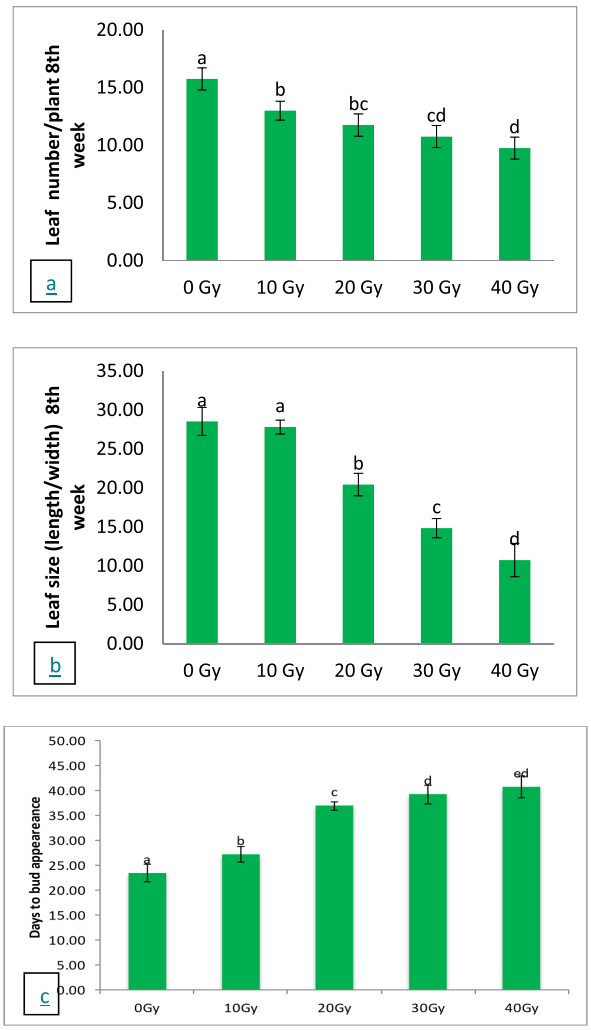

Figure 3. Influence of γ rays on (a) the leaf number per plant at the eighth week, (b) the leaf size per plant at the eighth week, and (c) the days to flower bud appearance.

Effect of ^{60}CO Gamma Irradiation and Ethylmethane Sulfonate (EMS) on Floral Color and Probability of Mutation. Regarding the color of flowers following gamma irradiation, only plants irradiated with a 10 Gy dosage were used to select mutants with the desired color, which evolved 60% pink, 15% orange pink, 10% white, 5% light yellow, and 10% the same color as the control, i.e., a red hue (Figure 5a–e). Larger dosages of 20, 30, or 40 Gy led to either distorted red buds or distorted red flowers (Figure 5f,g). Under 20, 30, and 40 Gy, it was undesirable to have color mutations. Regarding the mutation frequency in chrysanthemum flowers based on flower color, data revealed that there was a highly desired mutation frequency amounting to 90% when the plants were irradiated with a 10 Gy dose. Meanwhile, under 20, 30, and 40 Gy doses, flower mutation frequency, although recorded in percent, produced undesirable mutants. Only plants given a dose of 1.00% EMS were selected for the intended color mutations, which evolved 60% light pink, 10% white, and 30% that had no color and were identical to control flowers, i.e., retaining their original red color. In contrast, flower color mutants exposed to 0.75% EMS evolved light pink (55%), white (10%), and 35% retained their original red hue, whereas only 20% of mutants exposed to 0.5% EMS evolved

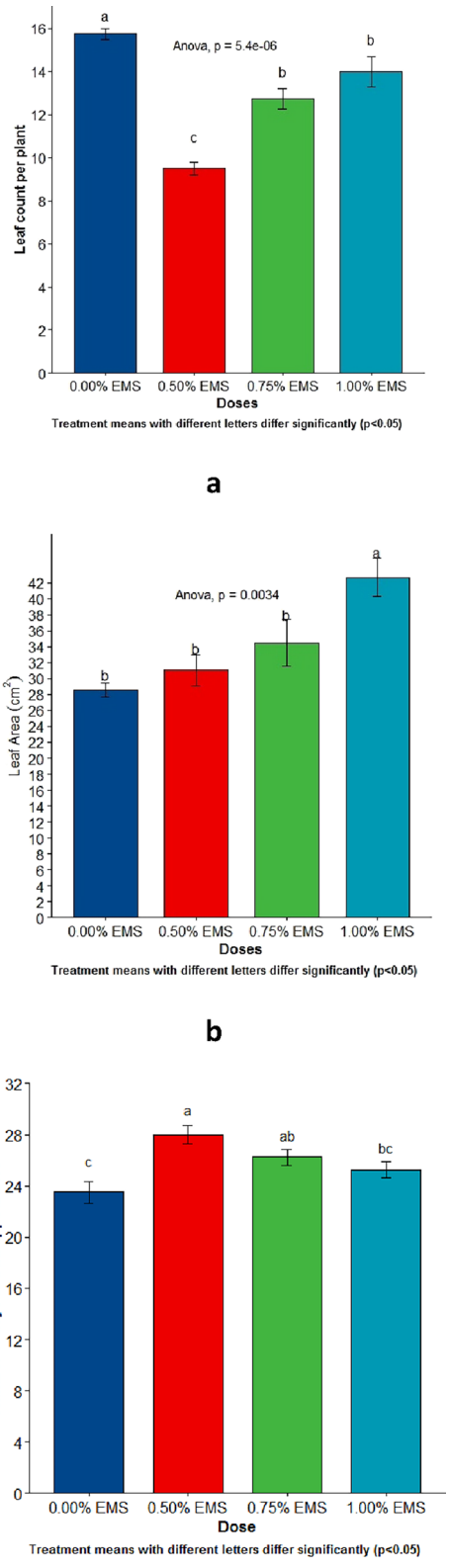

Figure 4. Influence of EMS (ethylmethanesulfonate) on (a) leaf count, (b) leaf area, and (c) days until the development of the floral bud

light pink, while the remaining 80% of plants remained unchanged (Figure5h,i). Data regarding the percent mutation

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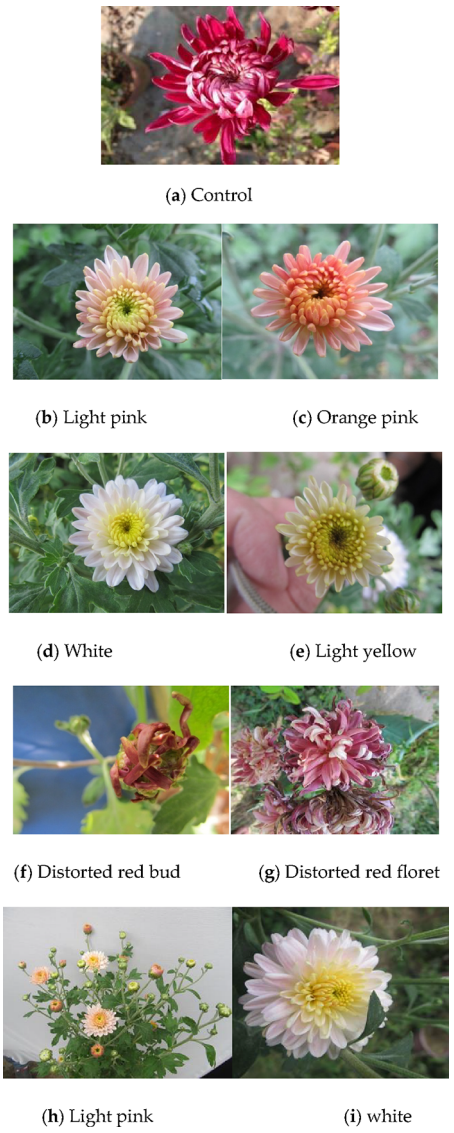

Figure 5. (a) Chrysanthemum cultivar Candid (control) mutants. (b–e) 10 Gy ^{60}Co gamma irradiation. (f, g) 30 and 40 Gy ^{60}Co gamma irradiation. (h, i) 1.00% ethylmethanesulfonate (EMS).

frequency in chrysanthemum flowers calculated based on flower color showed that there was a highly desired mutation frequency amounting to 70%, when the plants were treated with 1.00% EMS dose. Meanwhile, mutation frequency under 0.75% and 0.50% EMS doses decreased to 65.00 and 20.00%, respectively.

Influence of Markers on Polymorphism among Chrysanthemum Mutants and Parents. An average of 6.66 fragments/primer, a total of 80 fragments, were generated. Only 77 polymorphic bands with an average of 6.41 bands per primer were found. The number of amplified fragments ranged from 5 for OPD-07 to 18 for OPA-11, with amplicon sizes extending from 250 to 1900 bp for RAPD primers, and from 0 for (JH09, JH28, and JH31) to 6 for JH47, with amplicon sizes ranging from 50 to 950 bp for SSR primers. In terms of RAPD primers, the OPA-11 primer yielded the largest number of polymorphic bands. All 18 amplified bands were found to be polymorphic. A higher number of polymorphic bands was observed in the case of OPA-02 (13) and OPC-08 (11) and OPA-07(09), while the OPA-07 primer produced the lowest number of polymorphic bands, i.e., 09 out of 11 bands (Figure 6a–c), whereas for SSR primers, the JH47 primer produced the highest number of polymorphic bands. It amplified six bands, all of which were discovered to be polymorphic. JH04 (03) and JH20 (03) primers produced a greater number of polymorphic bands, whereas the JH04 primer produced the fewest polymorphic bands, consisting of only two out of three bands (Figure 7a–c). It was determined that the polymorphic information content of each primer extended from 0.08 (primer OPD-07) to 0.40 (primer OPA-07) and 0.12 (JH20) to 0.37 (JH47), with an average of 0.27 in RAPD and SSR markers. The majority of primers exhibited good values. OPE-01 (0.37) and JH28 (0.40) had a greater PIC value (0.32). In the case of RAPD, the percentage of polymorphism ranged from 81.81% (OPA-07) to 100% (OPC-08, OPD-07, OPE-01, OPA-11, and OPA-02), with an average of 96.96% per primer (Figure 6a–f), and from 66.66% (JH04) to 100% (JH09, JH20, JH28, JH31, and JH47) with an average of 94.44% per primer in SSR (Figure 7a–f). Values of these parameters for all of the primers are presented in Table 6. OPA-07 and JH47 were deemed the most efficient primers for the genetic diversity study of physical and chemical mutants of chrysanthemum cv. "Candid" based on their high PIC values, whereas OPD-07 and JH20 were deemed the least efficient.

Genetic Divergence Analysis. The dendrogram generated by combining RAPD and SSR marker data split the population exposed to various treatments into two primary clusters that were further subdivided. 10Gy1 was reported to be completely distinct from 3EMS 1%, 1EMS 0.75%, 2EMS 0.75%, and 4EMS 0.75%; however, 20Gy1 was found to be extremely similar to 30Gy1 with a similarity value of 1 (Figure 8).

DISCUSSION

Increased radiation-induced mortality has been linked to the blocking of mitosis and growth at the growing tips.[33] There is some evidence that irradiation kills cells by inhibiting their ability to produce essential substances, including vitamins, hormones, ATP, and other molecules. The breakdown of endoplasmic reticulum and plasma membrane integrity is the primary cause of the injury. Premature differentiation or cell death during late interphase was described by Evans[34] as a primary reason for diminished growth induced by exposure to sublethal radiation doses, although mitotic cell delay and chromosomal abnormalities had a small effect. Prior research on in vitro somatic mutagenesis in gerbera has shown that 20 Gy of X-rays applied to *in vitro*-produced shoots causes 10% phenotypic alterations.[35] Walther and Sauer[35] conducted their research on gerberas with gamma irradiation at doses ranging from 10 to 25 Gy, and they found an LD50 value of about 20 Gy. A single dose of 30 at 600 Gy h^{-1} or 50 at 8.4 Gy h^{-1}, as determined elsewhere,[36] is optimal for reasonable survival and growth of irradiated shoots. In addition, Kumari et al.[37] found that exposing rooted cuttings of the chrysanthemum variety "Otome Pink" to 0, 10, 15, and 20 Gy of γ-rays significantly reduced their survival. An ideal protocol for the use of 3000–4000 kR of gamma irradiation in chrysanthemum petal explants was devised by Wang et al.[38] Microshoots of in vitro-cultured *Chrysanthemum morifolium* cv. "Qiuzhishan" were exposed to 5–30 Gy of 60Co γ-rays in another study; Riviello-Flores et al.[14] found that a dose of 20 Gy was lethal for the plants, while a dose of 10 Gy was optimal for inducing mutations. Reduced plant viability in irradiated plant material

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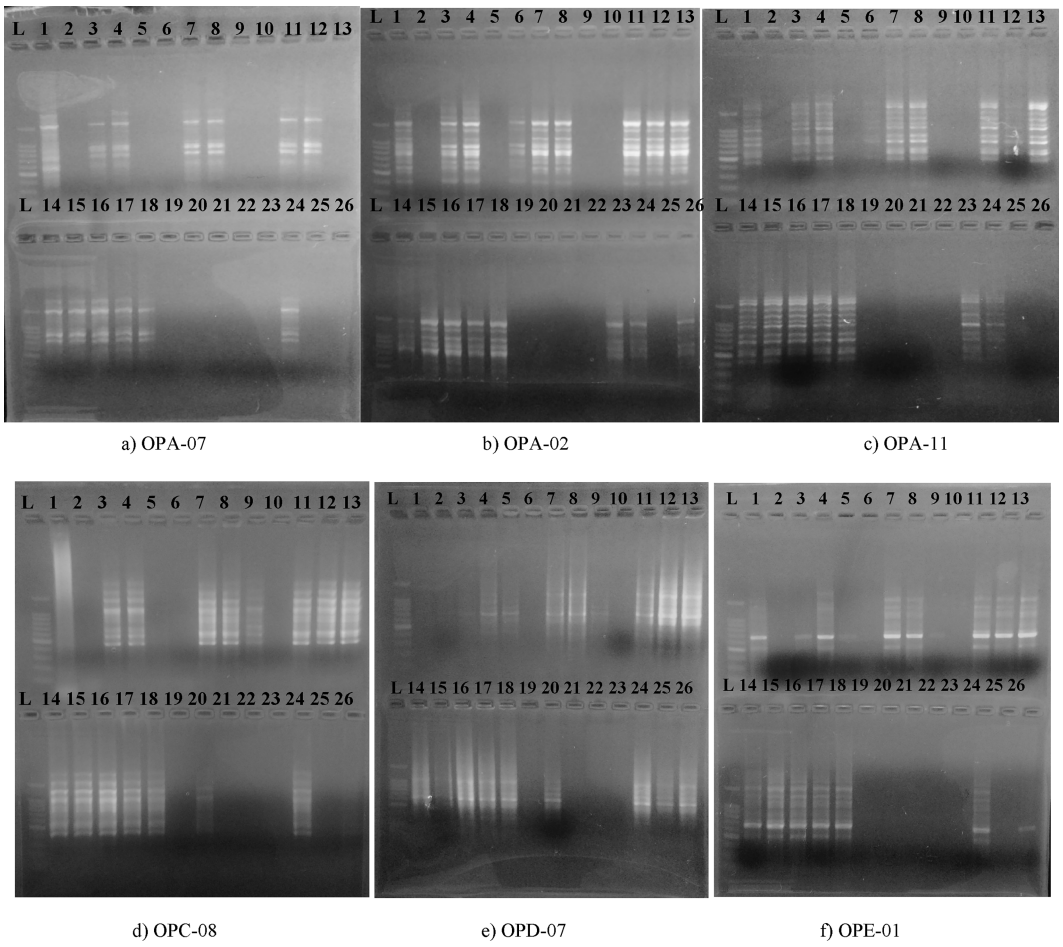

Figure 6. PCR banding profile of the RAPD primer. where *L* is the DNA concentration standard, 1−26 represents gel wells, and well 1 represents chrysanthemum cultivar *Candid* (parent), well 2 is blank, and the rest of the wells represent 24 mutants (physical and chemical): (1) candid (parent/control); (2) blank well; (3) 10 Gy 1; (4) 10 Gy 2; (5) 10 Gy 3; (6) 10 Gy 4; (7) 20 Gy 1; (8) 20 Gy 2; (9) 20 Gy 3; (10) 20 Gy 4; (11) 30 Gy 1; (12) 30 Gy 2; (13) 30 Gy 3; (14) 30 Gy 3; (15) 30 Gy 4; (15) 40 Gy 1; (16) 40 Gy 2; (17) 40 Gy 3; (18) 40 Gy 4; (19) 1 EMS 0.75%; (20) 2 EMS 0.75%; (21) 3 EMS 0.75%; (22) 4 EMS 0.75%; (23) 1 EMS 1.00%; (24) 2 EMS 1.00%; (25) 3 EMS 1.00%; (26) 4 EMS 1.00%. **Gy 1−4** represents sample number of gamma irradiation dose. **1−4 EMS** represents sample number of ethylmethanesulfonate dose.

may result from chromosomal abnormalities produced by γ-rays or from the inactivation or reduction of the auxin concentration. Dilta et al.[15] found that when 10 chrysanthemum cultivars were exposed to 2.0 kR of gamma irradiation, their survival rates dropped significantly compared to the control group. The percentage of chrysanthemum cv. "Jaya and Lalima" plants that survived gamma irradiation decreased with increasing doses, as reported by Banerji and Datta.[40,41] According to research conducted by Singh et al.[42] using African marigold cv. "Pusa Narangi Gainda," plant viability drops significantly after being exposed to γ radiation at 100 Grays. The maximum dose was shown to reduce this characteristic (400 Grays). In addition, Rather et al.[43] found that when the radiation exposure to pot marigolds increased, the seedlings' chances of survival and growth decreased significantly (Calendula officinalis). Even after being exposed to γ rays, the survival rate of Dahlia cv. "Pinki" decreased, as found by Dwivedi and Banerji.[44] Inactivation and/or reduction in auxin content, which affects cell division and, in turn, results in poor establishment and survival after gamma irradiation[45,46,47] or the lethal effect of γ-rays caused by chromosomal aberration, are proposed explanations for the reduction in survival after gamma irradiation.[48] Using ethylmethanesulfonate (EMS) to induce mutation in immature floral pedicels, Latado et al.[49] developed numerous new chrysanthemum cultivars. They then induced and produced adventitious buds in vitro and finally revealed the sensitivity of pedicels to EMS at LD50, which was close to 0.82% (v/v), which is in close conformity with the present study. When exposed to chemical mutagens like ethylmethanesulfonate (EMS), diethyl sulfonate (DES), and methyl nitrosourea (MNH), Misra and Bajpai[50] found that the survival rates of all gladiolus cultivars were reduced by as much as 50% compared to the controls (*N*-methyl-*N*-nitrosourea). Toxic chemicals formed by some biochemical components may be to blame for the decreased plant survival.[51,52] Kapadiya et al.[53] found that chemical mutagens caused a greater decrease in percent plant survival compared to γ-rays. The leaf area increment is a result of the growth of cells mainly controlled by growth regulators (auxins). Higher exposure to gamma irradiation agitates synthesis of auxins, hence leading to decreased leaf area. Furthermore, Simard et al.[54] and Cassels et al.[55] recorded biological damage in carnation on increasing the dose of radiation. In addition, Gupta et al.[56] in tuberose; Misra et al.[50] in gladiolus; Gupta et al.[57] in costus; Acharya and Tiwari[58] in carnation; Siranut, et al.[59] in chrysanthemums; Srivastava, et al.[60] and Kahrizi, et al.[61] in rose; reported decrease in the number of leaves with the increase in dosage of gamma

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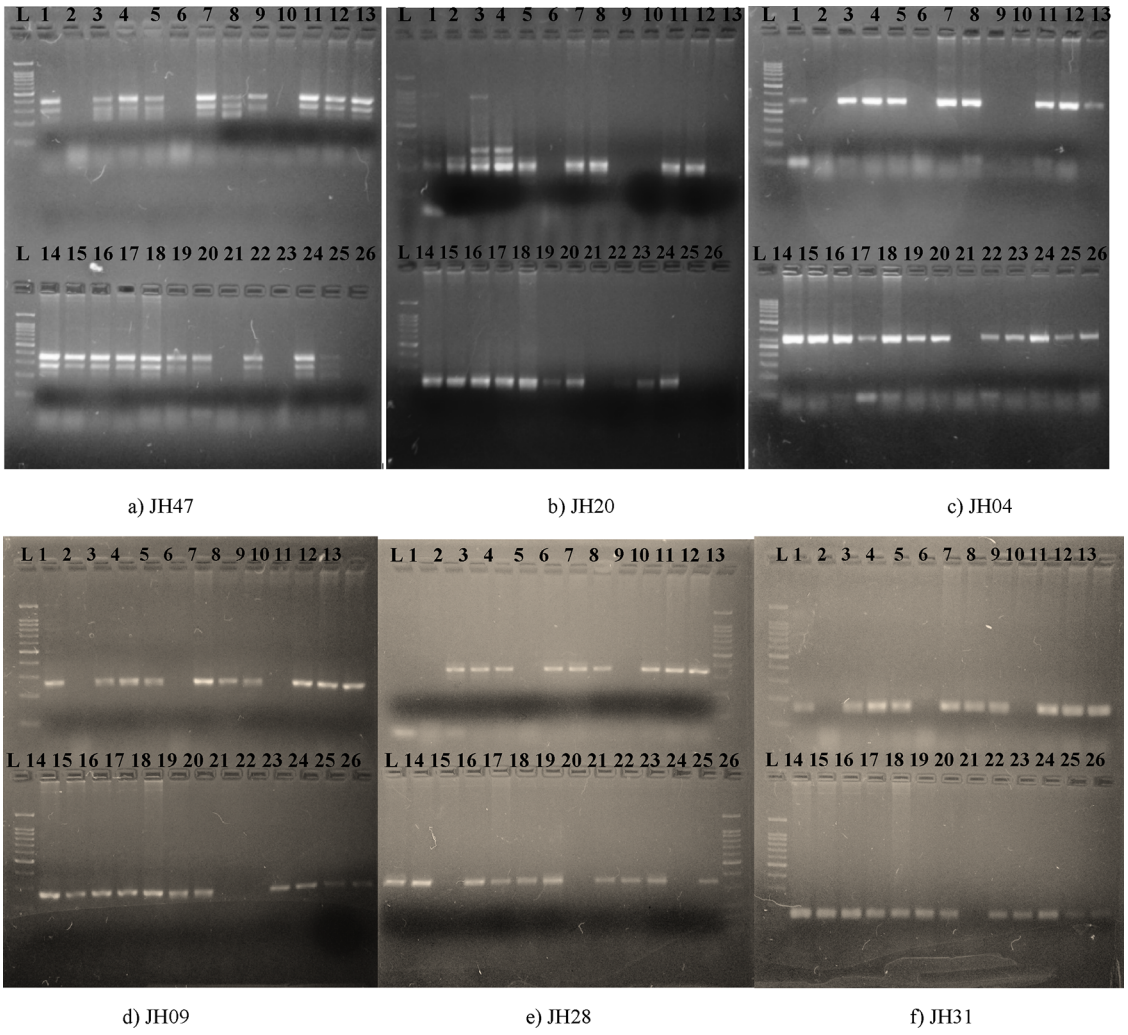

Figure 7. PCR banding profile of SSR primers. where *L* is the DNA concentration standard, 1−26 represents gel wells, and well 1 represents chrysanthemum cultivar *Candid* (parent), well 2 is blank, and the rest of the wells represent 24 mutants (physical and chemical): (1) candid (parent/control); (2) blank well; (3) 10 Gy 1; (4) 10 Gy 2; (5) 10 Gy 3; (6) 10 Gy 4; (7) 20 Gy 1; (8) 20 Gy 2; (9) 20 Gy 3; (10) 20 Gy 4; (11) 30 Gy 1; (12) 30 Gy 2; (13) 30 Gy 3; (14) 30 Gy 3; (15) 30 Gy 4; (15) 40 Gy 1; (16) 40 Gy 2; (17) 40 Gy 3; (18) 40 Gy 4; (19) 1 EMS 0.75%; (20) 2 EMS 0.75%; (21) 3 EMS 0.75%; (22) 4 EMS 0.75%; (23) 1 EMS 1.00%; (24) 2 EMS 1.00%; (25) 3 EMS 1.00%; (26) 4 EMS 1.00% . **Gy 1−4** represents sample number of gamma irradiation dose. **1−4 EMS** represents sample number of ethylmethanesulfonate dose.

Table 6. Monomorphic, Polymorphic Bands, and Calculated Parameters for RAPD and SSR Primers Used

primer		NB[a]	NPB	NMB	PPB	PIC
RAPD	OPA-02	13	13	0	100	0.34
	OPA-07	11	09	02	81.81	0.40
	OPA-11	18	18	0	100	0.28
	OPC-08	11	11	0	100	0.32
	OPD-07	05	05	0	100	0.08
	OPE-01	07	07	0	100	0.37
SSR	JH04	03	02	01	66.66	0.31
	JH09	01	01	0	100	0.27
	JH20	03	03	0	100	0.12
	JH28	01	01	0	100	0.32
	JH31	01	01	0	100	0.21
	JH47	06	06	0	100	0.37
average		6.66	6.41	0.25	95.70	0.27

[a]Where NB = total number of bands; NPB = number of polymorphic bands; NMB = number of monomorphic bands; PPB = percentage of polymorphic bands; PIC = polymorphic information.

irradiation. Meanwhile, Kumari, et al.[37] reported a reduction in leaf size in terms of length and width of plants treated with higher doses of γ-rays in variety "Otome Pink" and found that petiole length was shorter with increasing dose of mutagenic agents. Mahure et al.[47] recorded that lower doses, such as 10 and 20 Gy, increased the leaf area, but 30 Gy did so more than in the control. In yet another study by Dilta et al.,[39] reduction in the leaf number was reported in *Dendranthema grandiflorum* kitam cv. "Gulmohar" under gamma irradiation dose range of 1.0−3.0 kR. These unexpected shifts can be attributed to the alkylating chemicals, which are notoriously unstable. Moreover, as observed by Abdullah et al.,[62] there may be a difference between the control and experimental groups in morphological parameters because specific chemical mutagens elicit single base substitutions with various mutation spectra.

The findings of the current investigation can be attributed to natural alterations in plant physiology. Thus, this inhibitory action can cause flowering delays at greater concentrations. This is because mutagenesis did not lead to any chimeric growth in the shoot. According to a various reports,[63,64] chrysanthemums with nonchimeric shoots or tissue exhibit a

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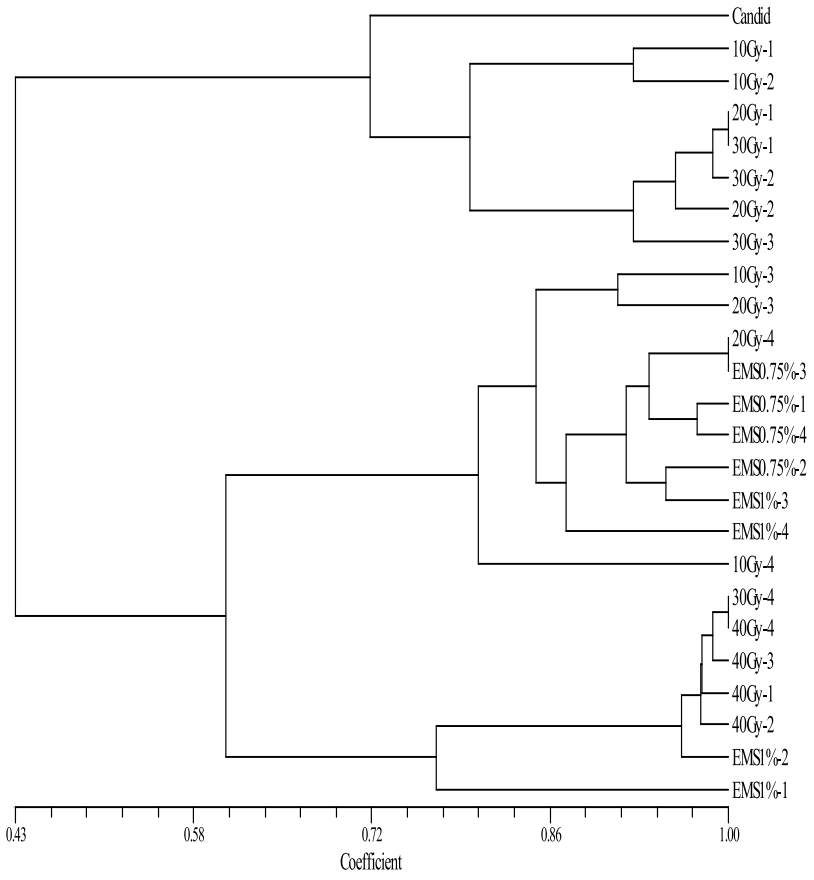

Figure 8. Dendrogram illustrating the genetic relationship among 24 mutants generated by UPGMA analysis of RAPD and SSR bands based on genetic distance obtained with 12 random and SSR primers.

lack of petal production and a range of coloration. This cited remark fits quite well with our current research. An interruption in the metabolic pathway that aids in the synthesis of flower inducing chemicals could be the cause of delayed flowering. According to Ahirwar et al.,[65] flowering in *Microsperma lentil* var. HUL-57 was considerably delayed by 0.3% of EMS mutagens in both generations compared to control. Physiological alterations in plants at higher dosages due to an inhibitory impact were found to be the cause of chemical mutagens delaying flowering.[53] While irradiating chrysanthemum plants with a 10 Gy dose, the data showed that the mutation frequency in the blooms was 90%, which is exactly what was wanted. The frequency of mutations in flowers was measured in percentages under 20, 30, and 40 Gy dosages, although the resulting mutants were all undesirable. According to previous research, the frequency of mutations in plants increases after being exposed to ultraviolet light, which is consistent with the results of the current study.[66] Many new types of chrysanthemum were developed by Latado et al.[49] by injecting ethylmethanesulfonate (EMS) into the developing flower pedicels. In chrysanthemums, a dose of 1.00% EMS resulted in a highly favorable mutation frequency of up to 70% in flower color, as calculated by studying the plants' offspring. In comparison, at 0.75 and 0.50% EMS, the mutation frequency plummeted to 20% and 65%, respectively. Once EMS (at a concentration of 0.77%) was applied to the juvenile pedicels of the dark-pink chrysanthemum cv. "Ingrid," 48 mutants (5.2% of the total) with a wide range of petal colors developed (pink-salmon, light-pink, bronze, white, yellow, and salmon).

With differences in the DNA sequences contained on their chromosomes, polymorphism among individual genotypes is evident.[68] Greater polymorphic bands of primers indicate their efficacy for evaluating genetic diversity and genotype discrimination.[69] Six RAPD and six SSR primers were used to generate polymorphic products from 24 mutants (physical and chemical). The efficacy of the selected primers to resolve heterogeneity among the mutants and parent compounds varied considerably. In the case of RAPD primers, the percentage of polymorphism ranged from 81.81% (OPA-07) to 100% (OPC-08, OPD-07, OPE-01, OPA-11, and OPA-02), with an average of 96.96% per primer, and from 66.66% (JH04) to 100% (JH09, JH20, JH28, JH31, and JH47) with an average of 94.44% per primer in SSR. It is comparable to the findings of Kang et al.,[31] who utilized AFLP markers to identify *in vitro* explants generated from the standard type "Migok" from gamma irradiated mutants. The highest level of polymorphism (72.1%) was identified in plants irradiated with 30 Gy. Han et al.[70] analyzed the genetic diversity and relationship of 45 chrysanthemum varieties and observed a total of 486 unambiguous bands, of which 451 were polymorphic and produced at average total of 45.1 polymorphic bands per pair of primer and obtained the polymorphism frequency of 92.80%. Wu et al.[71] analyzed sixty-five chrysanthemum cultivars for genetic diversity based on AFLP. Six pairs of primer combinations generated a total of 244 bands, 178 of which were polymorphic. The median

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ACS Omega 2023, 8, 43836−43849

proportion of polymorphic bands was 72.95%. UPGMA cluster studies revealed close genetic links between cultivars of the same petal type. Their origin was associated with a considerable degree with the cluster analysis results, but the blossom color had no evident relevance to the cluster results. Some attributes like PIC[72] have been calculated to illustrate the discriminatory power of RAPD and SSR primers. It was determined that the polymorphic information content of each primer ranged from 0.08 (primer OPD-07) to 0.40 (primer OPA-07) and from 0.12 (JH20) to 0.37 (JH47), with an average of 0.27 for RAPD and SSR markers. The majority of primers exhibited good value. OPE-01 (0.37) and JH28 (0.40) had a greater PIC value (0.32). OPA-07 and JH47 were determined to be the optimal primers for genetic diversity analysis of physical and chemical mutants of chrysanthemum cv. "Candid", taking into account the aforementioned factors. OPD-07 and JH20 were found to be the least effective. Using RAPD, Chatterjee et al.[73] analyzed the similarity between cultivars and mutants, which ranges from 0.17 to 0.90, and revealed that a high genetic distance among the various chrysanthemum mutants (physical and chemical) have potential of introducing fresh and innovative genes from chrysanthemum gene pool was recommended.

A dendrogram was created by combining RAPD and SSR marker data, which split the population into two major clusters, which were subsequently subdivided into subclusters. 10Gy1 was discovered to be completely distinct from 3EMS 1%, 1EMS 0.75%, 2EMS 0.75%, and 4EMS 0.75%; however, 20Gy1 was discovered to be extremely similar to 30Gy1 with a similarity value of 1. Kaul et al.[75] used *in vitro* mutagenesis to generate mutations in *Dendranthema grandiflora* cv. "Snow Ball" by exposing the *in vitro* shoots to 5, 10, 20, and 30 Gy γ radiation and using RAPD analysis to identify genetic polymorphism among the variants and control and discovered that 10 Gy of gamma irradiation was efficient at inducing mutations in flower color. Chiu et al.[74] evaluated the genetic variation of 32 *Chrysanthemum morifolium* cultivars, 16 each for summer and autumn chrysanthemums, by using ISSR DNA markers and found 132 bands including 126 polymorphic bands in summer cultivars when analyzed with 14 ISSR primers. The average number of the bands was 9.4/primer. The genetic similarity among these 16 cultivars was 0.29 to 0.77, averaged at 0.43. Similar analysis revealed 127 bands, including 124 polymorphic bands, in autumn cultivars. The genetic similarity for autumn cultivars was 0.23 to 0.53, averaged at 0.32. Cluster analysis using unweighted pair group mean arithmetic revealed that there were 3 major groups in summer chrysanthemums. Two major groups were recognized in the autumn cultivars. The results of ISSR analysis suggested that dinucleotide poly(AC) *n* motifs were more abundant and they gave more polymorphic bands. Wu et al.[71] analyzed 65 chrysanthemum cultivars for genetic diversity based on AFLP. Due to the time and effort savings, molecular-based research has proliferated and are now widely used in breeding programs and germplasm collections. The molecular method is preferable to the agromorphological method because it generates a more overview of variety. Most desired features are the outcome of collaboration between expressed genes; therefore, molecular characterization can be thought of as balancing the standard approach. This is why morphological research is still necessary for identifying cultivars.

CONCLUSIONS

At the conclusion of the second week after being exposed to γ-rays and EMS mutagenesis, chrysanthemum cv. "Candid" *in vitro* shoots had an estimated LD50 value of 23.19 Gy and 0.59% EMS, respectively. The highest survival rate was observed for shoots exposed to 10 Gy of γ radiation, indicating a negative relationship between increasing radiation doses and plant survival. In contrast, EMS treatments showed substantial variation among themselves, with a trend showing that survival percent increased with each successive dose of the mutagen, reaching a maximum under EMS 1.00%. During molecular characterization of the mutants using different molecular markers, the percentage of polymorphism was determined and an optimum primer for genetic diversity study of mutants was determined to be OPA-07 (RAPD marker) and JH47 (SSR marker) and observed distinction among physical mutation (dose of 10 Gy gamma irradiation) from chemical mutation (EMS dose of 1%, 0.75%). The finding of the present investigation indicates that Chrysanthemum cv. "Candid" mutants differ significantly in terms of their genetic makeup and has the potential to optimize chrysanthemum-breeding programmes. Furthermore, our results proved the effectiveness of SSR markers for the discrimination of gamma-irradiated and EMS-induced chrysanthemum mutants, allowing their earlier selection and reduction of the mutant population size.

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Conceptualization, methodology, investigation, A.D; supervision, Z.A.Q.; software, validation, S.Z.; writing − review and editing, M.A.W, S.I., S.A.M; project administration, N.B. and I.T.N.

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■ REFERENCES

(1) Wani, M. A.; Nazki, I. T.; Din, A.; Iqbal, S.; Wani, S. A.; Khan, F. U.; NeelofarFloriculture Sustainability Initiative: The Dawn of New Era. In: Lichtfouse, E. (eds) In *Sustainable Agriculture Reviews 27*. 2018 Springer International Publishing. DOI: 10.1007/978-3-319-75190-0_4.

(2) Wolff, K. RAPD analysis of sporting and chimerism in chrysanthemum. *Euphytica* **1996**, *89*, 159−164.

(3) Richards, A. J. *Plant Breeding Systems*; George Allen and Unwin: London, UK, 1986.

(4) Prudencio, A. S.; Devin, S. R.; Mahdavi, S. M. E.; Martínez-García, P. J.; Salazar, J. A.; Martínez-Gómez, P. Spontaneous, Artificial, and Genome Editing-Mediated Mutations in *Prunus*. *Int. J. Mol. Sci.* **2022**, *23*, 13273.

(5) Barakat, M. N.; Abdel, F. R. S.; Badr, M.; El-Torky, M. G. In-vitro culture and plant regeneration derived from ray florets of Chrysanthemum morifolium. *Afr. J. Biotechnol.* **2010**, *9*, 1151−1158.

(6) Din, A.; Qadri, Z. A.; Wani, M. A.; et al. Developing an efficient in vitro callusing and regeneration protocol in *Dendranthema* × *grandiflorum* Kitam. *J. Crop Sci. Biotechnol.* **2022**, *25*, 393−405.

(7) Miler, N.; Jedrzejczyk, I.; Jakubowski, S.; Winiecki, J. Ovaries of Chrysanthemum Irradiated with High-Energy Photons and High-Energy Electrons Can Regenerate Plants with Novel Traits. *Agronomy* **2021**, *11*, 1111.

(8) Nasri, F.; Zakizadeh, H.; Vafaee, Y.; et al. In vitro mutagenesis of *Chrysanthemum morifolium* cultivars using ethylmethanesulphonate (EMS) and mutation assessment by ISSR and IRAP markers. *Plant Cell Tiss Organ Cult* **2022**, *149*, 657−673.

(9) Zalewska, M.; Lema-Rumińska, J.; Miler, N. In vitro propagation using adventitious buds techniques as a source of new variability in Chrysanthemum. *Sci. Hortic.* **2007**, *113*, 70−73.

(10) Jin, C.; Dong, L.; Wei, C.; Wani, M. A.; Yang, C.; Li, S.; Li, F. Creating novel ornamentals via new strategies in the era of genome editing. *Front. Plant Sci.* **2023**, *14*, 1142866.

(11) Oladosu, Y.; Rafii, M. Y.; Abdullah, N.; Hussin, G.; Ramli, A.; Rahim, H. A.; Miah, G.; Usman, M. Principle and application of plant mutagenesis in crop improvement: A review. *Biotechnology and Biotechnological Equipment* **2016**, *30* (1), 1−16.

(12) Ma, L.; Kong, F.; Sun, K.; Wang, T.; Guo, T. From Classical Radiation to Modern Radiation: Past, Present, and Future of Radiation Mutation Breeding. *Front. Public Health* **2021**, *9*, No. 768071.

(13) Jain, S. M.; Spencer, M. M. Biotechnology and mutagenesis in improving ornamental plants. In *Floriculture, Ornamental and Plant Biotechnology: Advances and Topical Issues*; da Silva, J. A. T., Ed.; Global Science Books, Ltd: Isleworth, UK, 2006; Vol. *1*, pp 589−600.

(14) Riviello-Flores, M.d.l.L.; Cadena-Iñiguez, J.; Ruiz-Posadas, L.d.M.; Arévalo-Galarza, M.d.L.; Castillo-Juárez, I.; Soto Hernández, M.; Castillo-Martínez, C. R. Use of Gamma Radiation for the Genetic Improvement of Underutilized Plant Varieties. *Plants* **2022**, *11*, 1161.

(15) Dilta, B. S.; Sharma, Y. D.; Gupta, Y. C.; Bhalla, R.; Sharma, B. P. Effect of gamma rays on vegetative and flowering parameters of chrysanthemum. *J. Ornamental Hortic.* **2003**, *6*, 328−334.

(16) Din, A.; Qadri, Z. A.; Wani, M. A.; Rather, Z. A.; Iqbal, S.; Malik, S. A.; Hussain, P. R.; Rafiq, S.; Nazki, I. T. Congenial In Vitro γ-ray-Induced Mutagenesis Underlying the Diverse Array of Petal Colours in Chrysanthemum (*Dendranthema grandiflorum* kitam) cv. "Candid". *Biol. Life Sci. Forum* **2021**, *4*, 21 DOI: 10.3390/IECPS2020-08780.

(17) Shirasawa, K.; Hirakawa, H.; Nunome, T.; Tabata, S.; Isobe, S. Genome-wide survey of artificial mutations induced by ethyl methanesulfonate and gamma rays in tomato. *Plant Biotechnol J.* **2016**, *14* (1), 51−60.

(18) Ibrahim, R; Ahmad, Z.; Salleh, S; Hassan, A. A.; Ariffin, S. Mutation breeding in ornamentals. In: *Ornamental Crops* 2018. Springer International Publishing, pp 175−211 DOI: 10.1007/978-3-319-90698-0_8.

(19) Begum, T.; Dasgupta, T. A comparison of the effects of physical and chemical mutagens in sesame (*Sesamum indicum* L.). *Genet Mol. Biol.* **2010**, *33* (4), 761−766.

(20) Jankowicz-Cieslak, J.; Huynh, O. A.; Brozynska, M.; Nakitandwe, J.; Till, B. J. Induction, rapid fixation and retention of mutations in vegetatively propagated banana. *Plant Biotechnol J.* **2012**, *10* (9), 1056−1066.

(21) Luan, Y.-S.; Zhang, J.; Gao, X.-R.; An, L.-J. 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* (1), 77−81.

(22) Hong, Y.; Bai, X.; Sun, W.; Jia, F.; Dai, S. The numerical classification of chrysanthemum flower color phenotype. *Hortic. Sci.* **2012**, *39*, 1330−1340.

(23) Zhang, Y.; Zhu, M.; Dai, S. Analysis of karyotype diversity of 40 Chinese chrysanthemum cultivars. *Journal of Systematics Evaluation* **2013**, *51*, 335−352.

(24) Lin, L. Z.; Harnly, J. M. Identification of the phenolic components of chrysanthemum flower (*Chrysanthemum morifolium* Ramat.). *Food Chem.* **2010**, *120*, 319−326.

(25) Chen, S.; Li, C.; Zhu, X.; Deng, Y.; Sun, W.; Wang, L.; Chen, F.; Zhang, Z. The identification of flavonoids and the expression of genes of anthocyanin biosynthesis in the chrysanthemum flowers. *Biological Plant* **2012**, *56*, 458−464.

(26) Gu, C.; Chen, S.; Liu, Z.; Shan, H.; Luo, H.; Guan, Z.; Chen, F. Reference gene selection for quantitative real-time PCR in chrysanthemum subjected to biotic and abiotic stress. *Molecular Biotechnology* **2011**, *49*, 192−197.

(27) Kalia, R. K.; Rai, M. K.; Kalia, S.; Singh, R.; Dhawan, A. K. Microsatellite markers: An overview of the recent progress in plants. *Euphytica* **2011**, *177*, 309−334.

(28) Esselink, G. D.; Nybom, H.; Vosman, B. Assignment of allelic configuration in polyploids using the MAC-PR (microsatellite DNA allele counting—peak ratios)method. *Theor. Appl. Genet.* **2004**, *109*, 402−408.

(29) Zane, L.; Bargelloni, L.; Patarnello, T. Strategies for microsatellite isolation: a review. *Mol. Ecol.* **2002**, *11*, 1−16.

(30) Billotte, N.; Risterucci, A. M.; Barcelos, E.; Noyer, J. L.; Amblard, P.; Baurens, F. C. Development, characterization and across-taxa utility of oil palm (*Elaeis guineensis* Jacq.) microsatellite markers. *Genome* **2001**, *44*, 413−425.

(31) Kang, E. J.; Lee, Y. M.; Sung, S. Y.; Ha, B. K.; Kim, S. H.; Kim, D. S.; Kim, J. B.; Kang, S. Y. Analysis of the genetic relationship of gamma irradiated *in vitro* mutants derived from standard type chrysanthemum cv. Migok. *Horticulture Environment and Biotechnology* **2013**, *54* (1), 76−81.

(32) Rohlf, F. J. 1998. *NTSYSpc Numerical Taxonomy and Multivariate Analysis System Version 2. 0 User Guide*. Applied Biostatistics Inc.: Setauket, NY, *37* 17.

(33) Gray, L. H. Some characteristics of biological damage induced by ionizing radiations. *Radiat. Res.* **1954**, *1*, 189−213.

(34) Evans, H. J. Effects of radiation on meristematic cells. *Radiation Botany* **1965**, *5*, 171−182.

(35) Walther, F.; Sauer, A. Development of families of cultivars in *Gerbera jamesonii*. *Dtsch. Gartenbau* **1985**, *39* (45), 2097−2098.

(36) Dubec-Lebreux, M. A.; Vieth, J. The effects of ^{60}Co gamma rays on *Gerbera jamesonii* plantlets irradiated *in vitro*. *Can. J. Bot.* **1987**, *65* (2), 261−267.

(37) Kumari, K.; Dhatt, K.; Kapoor, M. Induced mutagenesis in *Chrysanthemum morifolium* variety 'Otome Pink' through gamma irradiation. *Bioscan* **2013**, *8*, 1489−1492.

(38) Wang, H. Y.; He, X. D.; Huang, Y. G.; Wang, H. Y.; He, X. D.; Huang, Y. G. Chrysanthemum tissue culture for breeding studies. *J. Jiangsu For. Sci. Technol.* **2003**, *30* (4), 25−26.

(39) Dilta, B. S.; Sharma, Y. D.; Dhiman, S. R.; Verma, V. K. Induction of somatic mutations in chrysanthemum by gamma irradiation. *Int. J. Agric. Sci.* **2006**, *2*, 77−81.
(40) Banerji, B. K.; Datta, S. K. Gamma ray induced flower shape mutation in chrysanthemum cv 'Jaya'. *J. Nucl. Agric. Biol.* **1992**, *21* (3), 73−79.
(41) Banerji, B. K.; Datta, S. K. Induction and analysis of gamma ray-induced flower head shape mutation in 'Lalima' chrysanthemum (*Chrysanthemum morifolium*). *Indian J. Agric. Sci.* **2002**, *72*, 6−10.
(42) Singh, V.; Banerji, B. K.; Dwivedi, A. K.; Verma, A. K. Effect of gamma irradiation on African marigold (*Tagetes erecta* L.) cv. Pusa Narangi Gainda. *Journal of Horticultural Science* **2009**, *4* (1), 36−40.
(43) Rather, Z. A.; Dar, K. R.; Peer, F. A.; Nelofar. Gamma irradiation studies in pot marigold (*Calendula officinalis*). *J. Ornamental Hortic.* **2011**, *14* (1−2), 66−69.
(44) Dwivedi, A. K.; Banerji, B. K. Effect of gamma radiation on dahlia cv. Pinki, with particular reference to induction of somatic mutation. *J. Ornamental Hortic.* **2008**, *11* (2), 148−151.
(45) Gordon, S. A. Studies on the mechanism of phytohormone damage by ionizing radiation. *Proc. Int. Conf. Peaceful Uses At. Energy* **1956**, *2*, 283−292.
(46) Gordon, S. A. The effect of ionizing radiation on plants: biochemical and physiological aspects. *Quart. Review of Biological* **1957**, *32*, 46−56.
(47) Mahure, H. R.; Choudhary, M. L.; Prasad, K. V.; Singh, S. K. Mutation in chrysanthemum through gamma irradiation. *Indian J. Hortic.* **2010**, *67*, 356−358.
(48) Zafar, S. A.; Aslam, M.; Albaqami, M.; Ashraf, A.; Hassan, A.; Iqbal, J.; Maqbool, A.; Naeem, M.; Al-Yahyai, R.; Zuan, A. T. K. Z. Gamma rays induced genetic variability in tomato *(Solanum lycopersicum* L.) germplasm. *Saudi J. Biol. Sci.* **2022**, *29*, 3300 DOI: 10.1016/j.sjbs.2022.02.008.
(49) Latado, R. R.; Adames, A. H.; Tulmann, N. A. *In vitro* mutation of chrysanthemum (*Dendranthema grandiflora* Tzvelev) with ethyl methane sulphonate (EMS) in immature floral pedicels. *Plant Cell, Tissue Organ Culture* **2004**, *77* (1), 103−106.
(50) Misra, R. L.; Bajpai, P. N. Mutational studies in gladioli (gladiolus) I. Effect of physical and chemical mutagens on sprouting and survival of corms. *Haryana J. Hortic. Sci.* **1983**, *12* (1/2), 1−6.
(51) Sax, K. The effect of ionizing radiation on plant growth. *American Journal of Botany* **1955**, *42*, 360−364.
(52) D'Amato, F.; Hoffmann-Ostenhof, O. Metabolism and spontaneous mutation in plants. *Adv. Genet.* **1956**, *8*, 1−22.
(53) Kapadiya, D. B.; Chawala, S. L.; Patel, A. I.; Ahlawat, T. R. Exploitation of variability through mutagenesis in Chrysanthemum (*Chrysanthemum morifolium* Ramat.) var. Maghi. *Bioscan* **2014**, *9* (4), 1799−1804.
(54) Simard, M. H.; Michaux-Ferriere, N.; Silvy, A. Variants of carnation (Dianthus caryophyllus L.) obtained by organogenesis from irradiated petals. *Plant Cell Tissue Organ Cult.* **1992**, *29*, 37−42.
(55) Cassels, A. C.; Walsh, C.; Periappuram, C. Diplontic selection as a positive factor in determining the fitness of mutants of Dianthus 'Mystère' derived from x-irradiation of nodes in in vitro culture. *Euphytica* **1993**, *70*, 167−174.
(56) Gupta, M. N.; Sumiran, R.; Shukla, R. 1975. Mutation breeding of tuberose Polianthus tuberose L. In *Use of Radiations and Radioisotopes in Studies of Plant Productivity, Proceedings of a Symposium Held at GB Pant University of Agriculture and Technology, Pantnagar,12−14 April 1974*; Department of Atomic Energy, Food and Agriculture Committee: Bombay, India, pp 169−179.
(57) Gupta, M. N.; Laxmi, V.; Dixit, B. S.; Srivastava, S. N. Gamma rays induced variability in Costus Speciosus. *Prog. Hortic.* **1982**, *14*, 193−197.
(58) Acharya, N. N.; Tiwari, D. S. Effect of MMS and gamma rays on seed germination, survival and pollen fertility of Hamatocactus setispinus in M1 generation. *Mysore J. Agric. Sci.* **1996**, *3*, 10−13.
(59) Siranut, L.; Peeranuch, J.; Arunee, W.; Surin, D.; Prapanpongse, K. Gamma-rays induced morphological changes in Chrysanthemum. (Chrysanthemum morifolium). *Agric. Nat. Sci.* **2000**, *34*, 417−422.
(60) Srivastava, P.; Singh, R. P.; Tripathi, V. K. Response of gamma radiation (60Co) on vegetative and floral characters of gladiolus. *J. Ornamental Hortic.* **2007**, *10*, 135−136.
(61) Kahrizi, Z. A.; Kermani, M. J.; Amiri, M. E.; Vedadi, S. 2010. Identifying the correct dose of gamma-rays for in vitro mutation of rose cultivars. In *Acta Horticulturae, Proceedings of the XXVIIIth International Horticultural Congress on Science and Horticulture International Symposium on Micro and Macro Technologies for Plant Propagation and Breeding in Horticulture*; Fabbri, A.; Rugini, E.,Eds.; International Society for Horticultural Science: Leuven, Belgium, Vol. *923*, pp 121−127 DOI: 10.17660/ActaHortic.2011.923.17.
(62) Abdullah, T. L.; Endan, J.; Nazir, B. M. Changes in flower development, chlorophyll mutation and alteration in plant morphology of *Curcuma alismatifolia* by gamma irradiation. *Am. J. Appl. Sci.* **2009**, *6* (7), 1436−1439.
(63) Langton, F. A. Chimerical structure and carotenoid inheritance in Chrysanthemum morifolium (Ramat.). *Euphytica* **1980**, *29*, 807−812.
(64) Ohmiya, A. Molecular mechanisms underlying the diverse array of petal colors in chrysanthemum flowers. *Breed. Sci.* **2018**, *68* (1), 119−127.
(65) Ahirwar, R. N.; Lal, J. P.; Singh, P. Gamma-rays and ethylmethane sulphonate induced mutation in microsperma lentil (*Lens culinaris* L. *Medikus*). *Bioscan.* **2014**, *9* (2), 691−695.
(66) Du, Y.; Feng, Z.; Wang, J.; Jin, W.; Wang, Z.; Guo, T.; Chen, Y.; Feng, H.; Yu, L.; Li, W.; Zhou, L. Frequency and Spectrum of Mutations Induced by Gamma Rays Revealed by Phenotype Screening and Whole-Genome Re-Sequencing in *Arabidopsis thaliana*. *Int. J. Mol. Sci.* **2022**, *23* (2), 654.
(67) Doyle, J. J.; Doyle, J. L.; Brown, A. H. D. Isolation of plant DNA from fresh tissue. *Focus* **1990**, *12*, 13−15.
(68) Miler, N.; Kulus, D.; Sliwinska, E. Nuclear DNA content as an indicator of inflorescence colour stability of in vitro propagated solid and chimera mutants of chrysanthemum. *Plant Cell Tiss Organ Cult* **2020**, *143*, 421−430.
(69) Serrote et al., 2020 Serrote, C.; Reiniger, L.; Silva, K.B.; Rabaiolli, S.; Stefanel, C.M. Determining the polymorphism information content of a molecular marker Gene, *726* (2020), 144175, DOI: 10.1016/j.gene.2019.144175.
(70) Han, J.; Hu, N.; Li, Y. G.; Shang, F. D. Genetic diversity of chrysanthemum cultivars revealed by AFLP analysis. *Acta Hortic. Sin.* **2007**, *34* (4), 1041−1046.
(71) Wu, Z. S.; Li, H. L.; Liu, J. H.; Zuo, Z. R.; Tian, R. C. Analyses of genetic diversity among 65 chrysanthemum cultivars based on AFLP. *J. Nanjing For. Univ. Nat. Sci.* **2007**, *31* (5), 67−70.
(72) Cornea-Cipcigan, M.; Pamfil, D.; Sisea, C. R.; Margaoan, R. Characterization of *Cyclamen* genotypes using morphological descriptors and DNA molecular markers in a multivariate analysis. *Front. Plant Sci.* **2023**, *14*, 1100099.
(73) Chatterjee, J.; Mandal, A. K. A.; Ranade, S. A.; Teixeira-da-Silva, J. A.; Datta, S. K. Molecular systematics in Chrysanthemum grandiflorum (Ramat) Kitamura. *Sci. Hortic.* **2006**, *110*, 373−378.
(74) Chiu, Y. S.; Ong, P. L.; Tsai, C. T. Evaluation of genetic variation of chrysanthemum (*Dendranthema x morifolium* Ramat.) varieties with inter simple sequence repeat DNA markers. *J. Taiwan Soc. Hortic. Sci.* **2008**, *54* (1), 1−9.
(75) Kaul, A.; Kumar, S.; Ghani, M. *In-vitro* mutagenesis and detection of variability among radiomutants of chrysanthemum using RAPD. *Adv. Hortic. Sci.* **2011**, *25* (2), 106−111.
(76) Jaccard, P. Nouvelles recherches sur la distribuitionflorale. *Bull. Soc. Vaud. Sci. Natl.* **1908**, *44*, 223−270.

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