Ornamental Horticulture
ISSN 2447-536X | HTTPS://ORNAMENTALHORTICULTURE.EMNUVENS.COM.BR/RBHO
SBFPO Sociedade Brasileira de Floricultura e Plantas Ornamentais

Scientific Article

Induction of mutagenesis on Chrysanthemums

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Abstract

Crop genetic diversity has a significant role in improving new plants through breeding. The chrysanthemum contains the most mutant varieties, making mutation breeding one of the most widely utilized breeding procedures for ornamental plants. The goal of this research is to use gamma irradiation to induce genetic variation and mutation breeding to improve chrysanthemum features. *In vitro* bud explants of the white 'Bacardi' type were treated with gamma rays at 20 Gy on this scope. The explants were subcultured until M_1V_4 growing period occurred, and observations were made during blooming on this time. Variable flower head widths, distinction on plant heights and widths, numerous flower numbers, color and size variations of ray florets were among the mutagenic changes observed in plants and flowers. Ray florets varied in length, width, number of rows, and color. The mutation frequency of the population was estimated 1.1% and yellow-colored florets were developed whereas the control group remained white. The dendrogram was grouped into five groups with 1, 28, 31, and 41 mutants in each based on the plant height and width, plant stem height and width, number of shoots and flowers per plant, flower head width, ray florets' number- height- color, number of leaves, leaf length and width, and weight of flowering stems. The yellow-colored mutants were located in the first, second, and fourth groups. The advantageous mutations could result in improving new varieties. Gamma radiation is an effective mutagen for creating new chrysanthemum types when applied to *in vitro* bud explants.

Keywords: Chrysanthemum, *in vitro* mutant, mutation breeding, ornamental plants, variation.

Resumo

Indução de mutagênese em crisântemo

A diversidade genética das culturas tem papel significativo na melhoria de novas plantas por meio do melhoramento. O crisântemo contém a maioria das variedades mutantes, tornando a reprodução por mutação um dos procedimentos de reprodução mais amplamente utilizados para plantas ornamentais. O objetivo da pesquisa é usar a irradiação gama para induzir variação genética e criação de mutações para melhorar as características do crisântemo. Explantes de gemas in vitro do tipo 'Bacardi' branco foram tratados com raios gama a 20 Gy neste escopo. Os explantes foram subcultivados até que ocorresse o período de crescimento M1V4, e as observações foram feitas durante a floração neste momento. Larguras das inflorescências, distinção nas alturas e larguras das plantas, números de flores, variações de cor e tamanho dos raios das flores estavam entre as mudanças mutagênicas observadas em plantas e flores. Os raios das flores variavam em comprimento, largura, número de fileiras e cor. A frequência de mutação da população foi estimada em 1,1% e flores de cor amarela foram desenvolvidas, enquanto o grupo de controle permaneceu branco. O dendrograma foi agrupado em cinco grupos com 1, 28, 31 e 41 mutantes em cada um com base na altura e largura da planta, altura e largura do caule da planta, número de brotos e flores por planta, largura da inflorescência, número dos floretes nos raios. altura-cor, número de folhas, comprimento e largura das folhas e peso das hastes floridas. Os mutantes de cor amarela estavam localizados no primeiro, segundo e quarto grupos. As mutações vantajosas podem resultar na melhoria de novas variedades. A radiação gama é um mutagênico eficaz para a criação de novos tipos de crisântemo quando aplicawda a explantes de gemas in vitro.

Palavras-chave: crisântemo, melhoramento por mutação, mutante in vitro, plantas ornamentais, variação.

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https://doi.org/10.1590/2447-536X.v28i4.2523
Received June 08, 2022 | Accepted Oct 26, 2022 | Available online Dec 8, 2022

Area Editor: Bruno Trevenzoli Favero

Introduction

In the floriculture industry, developing a crop-wise database is an essential need (Datta, 2020). The basic goals of breeding are to create new genetic variability and to select individuals with desired features. (Suprasanna and Jain, 2022; Miler and Jędrzejczyk, 2018; Patil et al., 2017). Mutation breeding is one of the most effective breeding approaches for creating genetic variability in ornamental plants (Su et al., 2019; Suprasanna and Jain, 2022). Mutations can occur naturally or be created artificially by a range of physical agents like as gamma-rays or X-rays, as well as chemical mutagens (Kharkwall, 2017). The number of approved mutant varieties is 3377, the number of mutant ornamental plants is 728, and 285 of them are mutant Chrysanthemum varieties, according to the mutant variety database. (IAEA, 2022). These records show that 400 mutants were distributed among vegetatively produced plants. The majority of them are floricultural plants, with a few fruit trees (Kumari et al., 2019; Melsen et al., 2021).

According to records of the International Atomic Energy Agency, modifications on mutant ornamental plants were found in the color and morphology of the leaves, flower type and color, plant type, flowering period, nematode and sun tolerance (IAEA, 2022).

Gamma rays are electromagnetic radiation, with a high energy level, have no particles, and penetrate profoundly. In order to develop new plant species, gamma irradiation breeding is highly useful. Although, after applying gamma-ray irradiation, plant growth can be impeded, the number of leaves can reduce, the flowering time can be delayed, and the number of flowers can fall, the variation rate of flower characteristics can also greatly have boosted at the same time (Wu et al., 2020). Lower gamma irradiation doses have favorable and effective effects on morphological and physicochemical traits in different types of ornamental plants, while greater levels of irradiation generally have a negative impact. The best gamma radiation dosages cannot be advised because they differ from plant to plant and affect different physical characteristics depending on their growth rate. Gamma irradiation plays a crucial role to develop successful and well-established plant breeding programs (Anne and Lim, 2020).

Chrysanthemums, *Dendranthema* x *grandiflora* Tzelev., have a long history of relationship with diverse cultures throughout the world. It has been bred for several years, resulting in a number of cultivars that are among the top 10 cuts, potted types, and garden plants in the world (Kishi-Kaboshi et al., 2017; Din et al., 2019; Shahrajabian et al., 2019). Chrysanthemums are one of Turkiye's most popular flowers with a production area of 755 da however, no indigenous varieties are available (Kazaz et al., 2020). The establishment of genetic diversity should be incorporated into breeding strategies for the development of new varieties (Patil et al., 2017; Datta 2020).

The rise of the chrysanthemum market every year should lead breeders and scientists to develop new varieties with fresh appearance, improved stress resistance and superior quality characteristics. The most popular methods for creating new Chrysanthemum varieties are crossbreeding and mutation breeding. Crossbreeding, along with sexual and asexual reproduction occurs naturally. Although this technique has been used as the foundation for numerous cultivars that have long since been used, Chrysanthemum is a self-incompatible cross-pollinated plant with difficulty matching parents and genetic factors are complicated for the selection of superior hybrid progenies (Anderson et al., 2017; Zhang et al., 2018; Kumari et al., 2019; Baghele, 2021).

Mutation breeding produces the desired effects for chrysanthemums (Kumari et al., 2019; Patil et al., 2017; Datta 2020). Some cutting-edge techniques have been crucial in the production of new and desirable features. A significant number of cultivars have been developed as a result of mutation breeding. Contrary to mutation breeding, which produces new, innovative traits quickly, conventional breeding requires more time and work. New types of chrysanthemums are being created for farmers to purchase commercially (Baghele, 2021).

On the other hand, tissue culture enhances mutagenesis and genetic transformation and creates somaclonal diversity for breeding by enabling regeneration from a single cell. Thus, *in vitro* somaclonal variation can optimize the variety attained naturally or artificially. Chrysanthemum plants with varying vegetative growth, ploidy levels, the flowering of ray florets and flower colors that developed from any part of plants like shoot tip callus, petals, capitula, stem culture and leaf explants (Eeckhaut et al., 2020).

The classic chrysanthemum breeding method of irradiation can be combined with tissue culture, and this method increases both the quantity and variety of color mutants. On another side, isolating mutants from nonmutated tissues encourages the development of novel varieties (Jain, 2010; Sarsu et al., 2018; Verma and Prasad, 2019; Eeckhaut et al., 2020). Mutations with *in vitro* culture techniques may be the sole way to improve an existing cultivar in some vegetatively propagated species (Kumari et al., 2019). The goal of this study is to use gamma irradiation to produce *in vitro* variation to improve identified features in order to save time by completing four development periods in the tissue culture laboratory rather than four seasons of growing plants at *in vivo* conditions.

Materials and Methods

Chrysanthemum morifolium (Ramat.) (*Dendranthema* x *grandiflora* Tzelev.), variety Bacardi is a white blooming spray cut flower with a 7-week response time, green disc florets, a high blossom number, and disease tolerance. Because of plant vigor and disease tolerance, it was chosen to enhance its beneficial properties and create new features by adding new ones to existing ones. The plant material was *in vitro* bud explants of 'Bacardi' variety. The study carried out between 2016 and 2021. Hardened 36,000 plants were planted in greenhouses and observed in November 2020 when they were fully bloomed. Among the 36,000 flowering plants 300 of useful homogenous mutants were selected and 100 of them evaluated in this study, while the remaining 98 were included in another manuscript (Haspolat et al, 2022).

Tissue culture of the explants

The bud explants were kept under running tap water for 30 minutes, then soaked in 70% ethanol for 40 seconds before being treated with a 25% H_2O_2 solution for 10 minutes and rinsed five times with sterile distilled water. MS (Murashige and Skoog, 1962) was the medium, which contained 3% sucrose, 0.8% agar, and 1 mg L^{-1} BAP (Benzylaminopurine). The pH was adjusted to 5.8 and autoclaved for 15 minutes at 121°C. Cultured bud explants were grown at 21°C under cold white light for 16 hours (30 mol m^{-2} sv^{1}) and 55%-60% relative humidity (Figure 1).

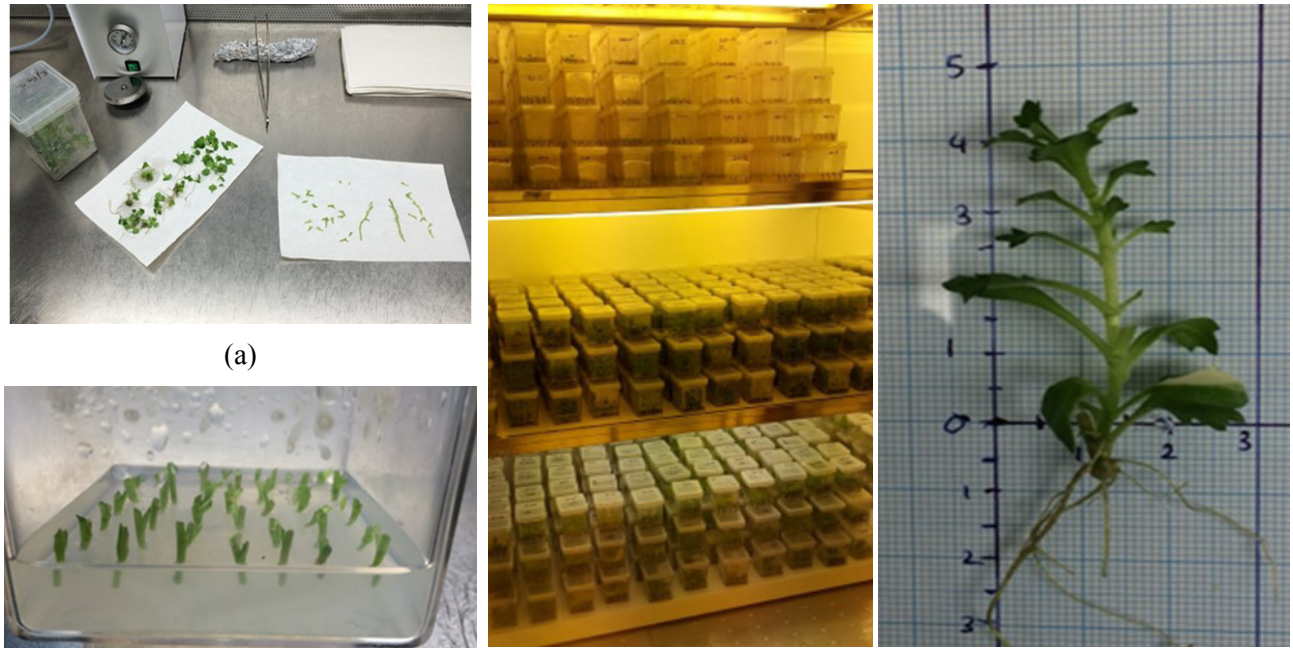

Figure 1. *In vitro* bud explants. (a) Subculturing, (b) bud explants, (c) growing plants, (d) measurements of shoots

Effective Mutation Dose Calculation

The *in vitro* bud explants were treated with gamma rays at seven doses: 0, 5, 10, 15, 20, 25, and 30 Gy (Gray). Irradiation treatments of gamma rays of cobalt 60 (Cobalt irradiator - ^{60}Co, Izotop, Ob-Servo Sanguis Co-60 Research Irradiator, Budapest, Hungary), were conducted in *in vitro* conditions. The effective mutagen dose (EMD 50) was estimated using linear regression analysis based on the shoot lengths of subcultured explants on MS media *in vitro* conditions and each treatment had a total of 1,050 buds. Explants were quickly subcultured after being exposed to radiation. To calculate the effective mutation dose, shoot lengths were measured in the 60th day of regeneration (Figure 1d) (Haspolat et al., 2019).

Irradiation and Hardening of plantlets

After the estimation of the effective mutagen dose (EMD 50), 600 *in vitro* plants with 5 buds were irradiated with 20 Gy effective mutagen dose and subcultured for four growth periods (M_1V_1 - M_1V_4). For root induction the explants were subcultured on MS media with 3% sucrose and 0.8% agar containing no plant growth regulators in M_1V_4. After regeneration of 2–3 cm long shoots of rooted plants, the plants were planted into plastic vials containing a 3:1 mix of peat and perlite, and the vials were kept for one week in a paper box covered with transparent wrapping paper at 22 ± 1 °C under cool white light with a 16-hour photoperiod (30 µmol m^{-2} s^{-1}) and 55%-60% RH. Small holes were drilled on the transparent wrapping paper day by day during hardening. Then hardened plantlets were planted into soil in the unheated polyethylene-covered greenhouse in July.

Selection of mutant plants

The ratio of variegated useful plants to irradiated plants was called the mutation frequency (MF). Single plant observations and selections were conducted while they were in bloom. The heights and widths of the plant, stem, leaf, and ray florets, as well as the weight of the blooming stem, flower head, and disc floret widths, were used to select the plants. On the other hand, the plant's shoot, leaf, and flower numbers were assumed. The Methuen Hand Book of Colour catalog was used to detect color variations in the ray florets (Kornerup et al., 1978).

Statistical analysis

The genetic closeness in terms of morphological features was determined using the NTSYS 2.02 software

program (Numerical Taxonomy and Multivariate Analysis System). Hierarchical cluster dendrogram was created to analyse the similarity between mutant population. The core algorithm was used to calculate the similarity matrix between the mutants (Rohlf, 2000). Effective mutagen dose was calculated by linear regression analyse. Changing ratios of mutant genotypes according to the control group for some traits were calculated with the formula (1):

$$\%\ Change = \frac{Genotype - Control}{Control} x100$$

Results

The effective mutagen dose

Estimating the most appropriate mutagen dose is one of the first steps in mutation breeding. This requires determination of radio sensitivity and the dose that causes a 50% reduction in vegetative growth. Shoot lengths were measured on the 60th day following subculturing and 50% reduction in shoot length was used as an effective mutagen dose by calculating 20 Gy by linear regression based on shoot lengths (Figure 2).

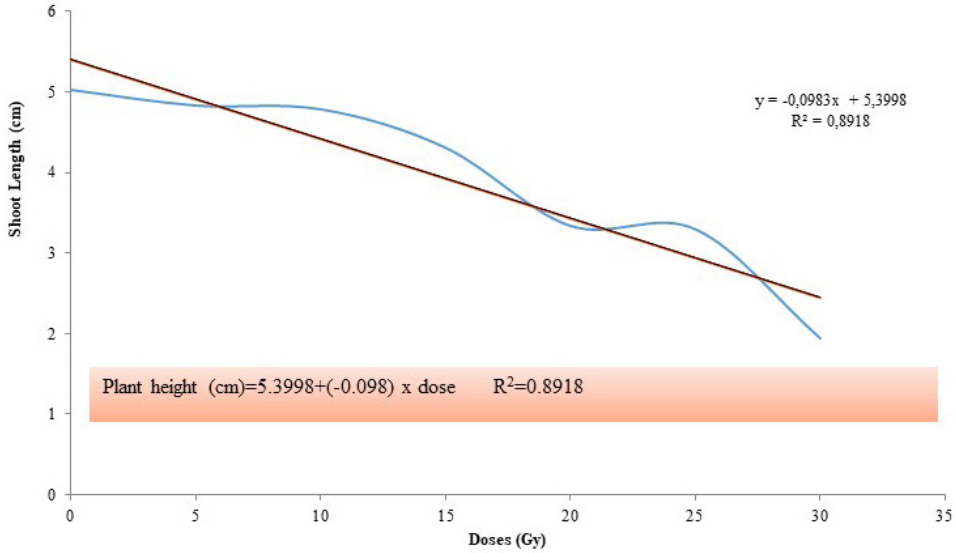

Figure 2. The shoot length decreasing with increasing doses

Changes of the plants

For the useful plants in the population, the mutation frequency was calculated to as 1.1%. The chimerism induction in population have not been considered in such useful mutants and the stable variegations were utilized.

Changes on plant height

In height the plants ranged from 44 to 125 cm. The average plant height was 92.2 cm while twenty-three mutants had a height between 110 - 125 cm. The plant lengths of eleven mutants ranged from 44 to 69 cm, whereas the heights of sixty-seven mutants ranged from 70 to 109 cm (70 cm is the ideal length for cut flowers). While the control group's plant height was 73 cm, the majority of the mutants had plant heights greater than 70 cm. When compared to the control group, the mutants' population decreased by 39.72 percent and their plant height increased by 64.38 percent (Figure 3 and Figure 6).

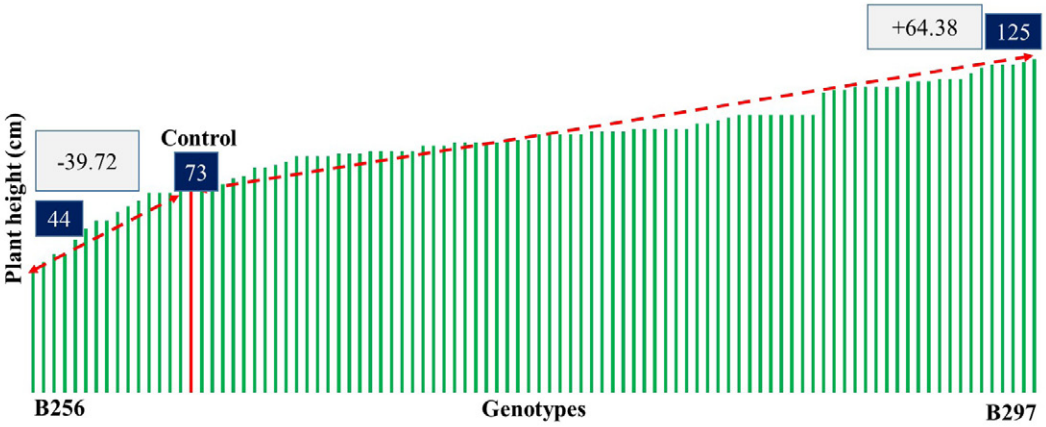

Figure 3. Changing ratios of the plant heights.

Changes on flower head width

Flower head widths ranged from 3.1 to 8 cm, with the two mutants having the largest flower head widths of 7 and 8 cm among the 100 mutants. The flower heads of forty small flowering mutants had a diameter of 3.1 to 5 cm, while fifty-nine mutants had a diameter of 5.1 to 6.8 cm. In the control group, the flower heads were 5 cm wide. The changing ratios were -48.33 and +33.33, respectively according to the control group (Figure 4 and Figure 7). Plants with a head diameter of 3.1 cm (two mutants) were considered to be 'Santini' forms. The 'Santini' flowers are significantly more compact than other Chrysanthemum variations and their popularity is raising.

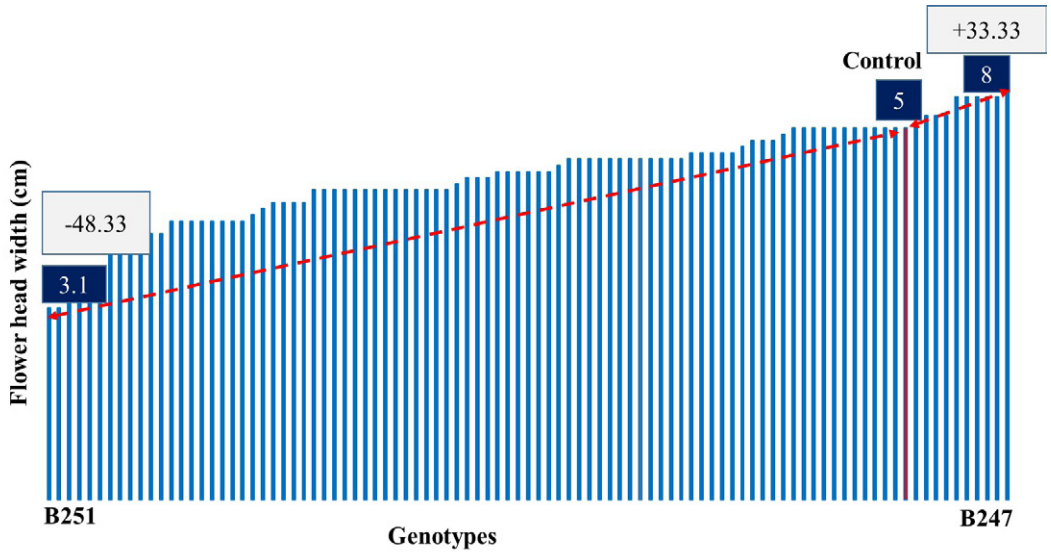

Figure 4. Changing ratios of the flower head widths.

Changes on flower numbers per stem

Flower numbers per stem were between 4 to 37. The mean flower number was 16.3 and there were thirteen mutants whose flower number is higher than 30 (30 ≤ x). It was observed that fifty-nine mutants had the flower numbers between 10 and 29 (10 ≤ x) and twenty-nine mutants had the flower number per stem from 4 to 9. The number of flowers was 11 in the control group and the changing ratios differentiated as -63.64 and +236.36 (Figure 5 and Figure 7). Although the flower numbers increased three times compared to the control plants, the branches with more flowers are not preferred global cut flower market as it is desired to have 5 stems in a spray cut flower chrysanthemum bouquet. The mutants with fewer florets, cannot be used in these normal spray-cut flower bouquets. It's important to pay attention to the number of florets in the candidate varieties that will be chosen for market use, which should be between 10 and 13. We had twenty-five mutants with flower numbers ranging from 10 to 13; of these selected mutants, five of them had 10 florets, three mutants had 11 florets, again five mutants had 12 florets and twelve mutants had 13 floret number per plant.

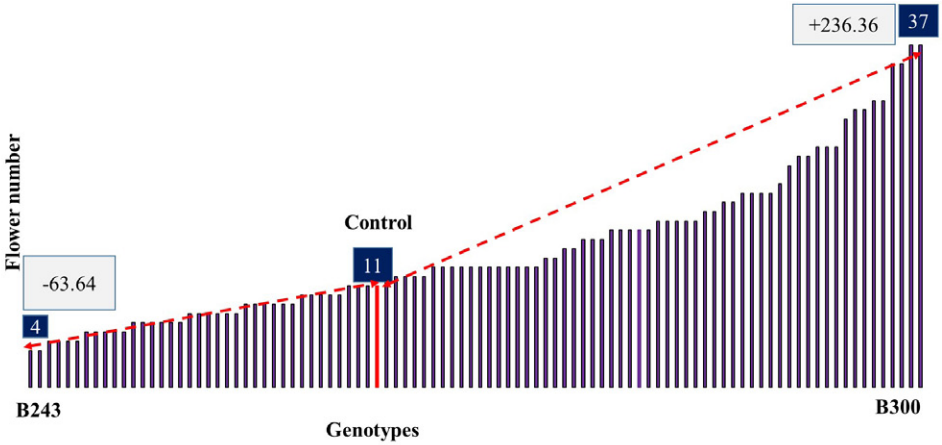

Figure 5. Changing ratios of the flower numbers.

Changes on plant widths

Plant widths were changed between 2.5 and 19 cm. The mean value was 10.5 cm. The changing ratios of the population were -66.67 and +26.67 according to the control group while the mean plant width of the control group was 15 cm (Figure 6 and Figure 7).

Increasing and decreasing ratios of the population

According to the control group, flower head widths and plant widths were reduced in the mutant population, whereas flower number per plant and plant heights were enhanced (Figure 7).

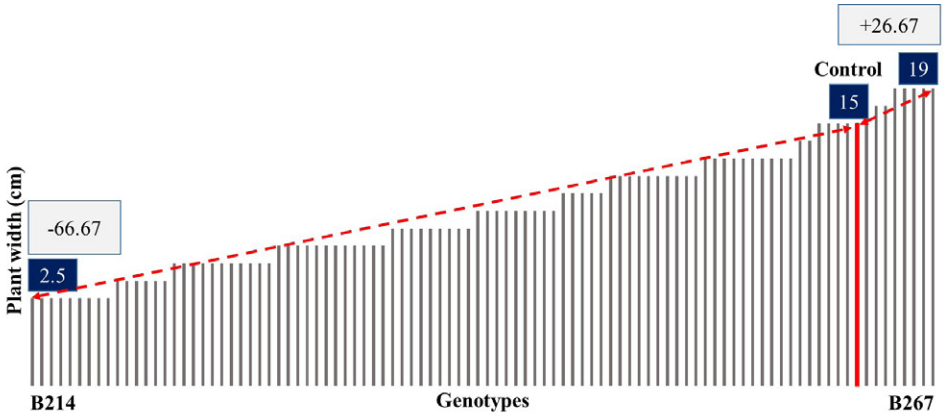

Figure 6. Changing ratios of the plant widths.

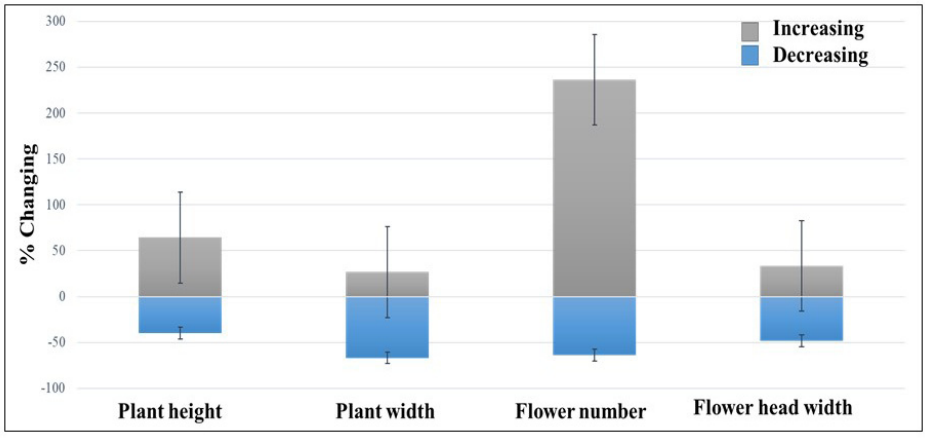

Figure 7. Increasing and decreasing ratios of the population for the plant height, plant width, flower number and flower head width per plant.

Hierarchical clustering

In the present study, hierarchical cluster analysis was used to assess similarity among mutants by quantitative characters like: Plant height and width, plant stem height and width, shoot and flower number per plant, flower head width; numbers, height, width and color of ray florets; leaf length and width, the weight of the flowering stems. We obtained the dendrogram including five clusters (Figure 8). The number of mutants in a cluster was varied from 31 (Group 1), 41 (Group 2), 1 (Group 3 and 5) and 28 (Group 4) mutants. The cophenetic correlation between ultrametric similarities of the tree and the similarity matrix was $r = 0.62$, $p \leq 0.01$.

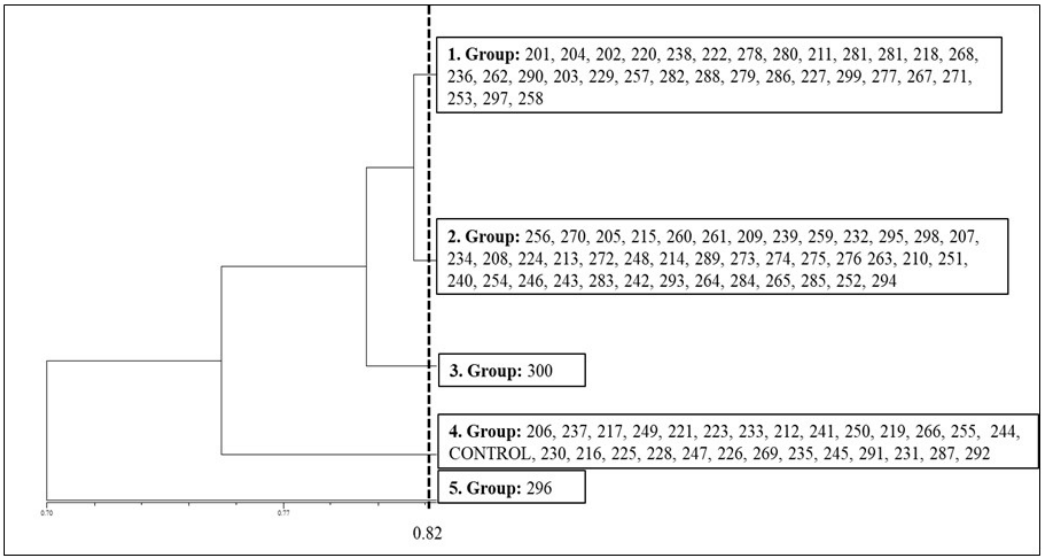

Figure 8. Dendrogram of the selected mutants.

The mutants in the groups had the similarity ratio between 0.19 and 0.99. The five main groups were detected at 0.82. The third and fifth groups had only one mutant, the 300 was only mutant in the 3rd group and 296 were located in the 5th group. These two mutants had white color and plant height was longer than 70 cm. Flower color variations of yellow-colored mutants were observed on twenty plants. The yellow-colored mutants were located in the first, second and fourth groups (Figure 8). The yellow color code was 3/6A according to the Methuen Hand Book of Colour catalog (Kornerup et al, 1978) (Figure 9). According to the correlation matrix, the similarity ratio was 99.6% between the closest white-colored mutants 275 and 276. The furthest ones were Control and 256 with a ratio of 19.9%.

Discussion

It is possible to say that gamma radiation treatments influenced the size and shape of flowers changed with color distinction, as also the size and shape of ray florets. These mutants can be selected and grown to produce more commonly accepted cut flowers. It would be advantageous for the flower industry and its customers to breed for unusual colors.

Brakat et al. (2010), investigated *in vitro* mutagenesis by treating chrysanthemum ray flowers with two doses of gamma irradiation (0.5 and 1.0 Gy). Shoot length decreased with gamma ray treatments compared to control. We got the similar results according to a prior article we published the increasing gamma treatment dosages caused the decrease in shoot lengths (Haspolat et al., 2019). We detected smaller flower head diameters and color shifts to pink (8.5%) in our other evaluation of the study's initial selected group of mutants (Haspolat et al., 2022).

Color modifications of eight variants with yellow decorative inflorescences from the white cultivar 'Albugo' were seen after *in vitro* mutagenesis of distinct explant types (Zalewska et al., 2011). We observed flower colors changed from white to yellow similarly (Figure 9). According to the mutant variety database (MVD), the mutant varieties 'Amason' and 'Hae-no-Yuugure' were created by gamma ray irradiation of *in vitro* culture and the flower colors were the most improved features of these mutant types (IAEA, 2022). According to Shafiei et al. (2019), the purple cultivar of chrysanthemums produced the largest change in petal color and mutation frequency when 25 Gy gamma-ray irradiation was applied to leaf explants. Color changes might be assessed in order to improve new cultivars. *In vitro* mutagenesis can be utilized to develop differences in flower color or flower form that can be used immediately as new cultivars. It is important to create a new floral type that will be popular among consumers, as flower color has a great impact on consumer demand and expresses aesthetic satisfaction in the marketing sector.

On the 'Delistar White' cultivar, irradiation of the ray florets with two doses of gamma (0.5 and 1.0 Gy) resulted in the most effective dose, causing differences in floral shape and number of ray florets, but no change in flower color (Brakat et al., 2010). We observed declines in plant widths and variations in the number and shape of ray florets, along with many other features (Figure 9). In contrast, we saw color changes from the white-colored control group to yellow-colored mutants, and most mutants exceeded the control group in terms of plant length.

Mandal et al. (2000a and 2000b) determined yellow-colored flowers among the mutant population of gamma-ray irradiated white-colored cultivar. Our mutant population had 20% yellow-colored mutants similarly. Suchlike the original floral color was white with flat and incurving florets, the mutant floret color was yellow with flat and incurving florets as published by Kaul et al. (2011) with a similar side of present data. They detected that *in vitro* mutation is in flower color in one branch of the same plant with 10 Gy irradiation. We got similar white-colored plants that turned yellow color at one branch in the same plant. Due to their inhomogeneous nature, these inductions were not considered in the selected useful mutants (Figure 10). Despite the fact that these plants can be popular among customers, they are not preferred by growers as they are not uniform.

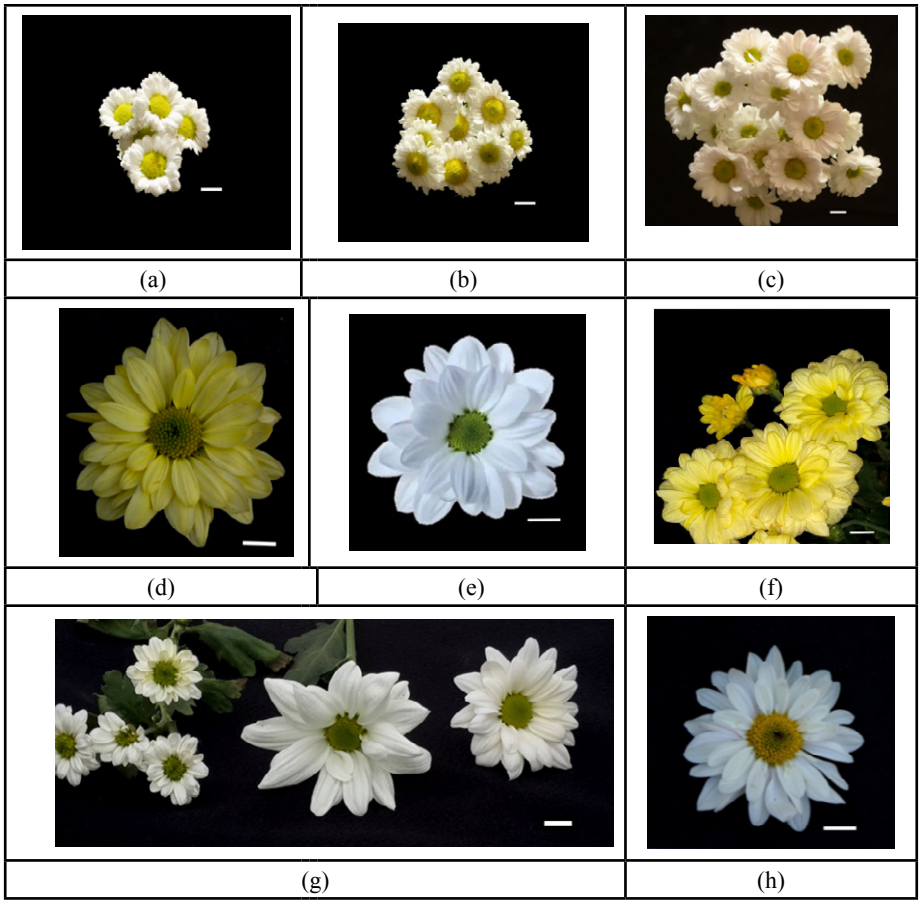

Figure 9. Different plant widths, shapes and colors of the mutants. (a) - (b) - (c) small florets, (d) - (f) yellow florets, (e) Bacardi flower, (g) different flower and ray floret widths, (h) variated flower shape (bars = 1 cm).

Figure 10. Yellow and white colored flowers in same plant (bar = 1 cm).

The mutant ‚Hhae-no-Kirameki' had yellowish-orange flowers after being irradiated with gamma rays on tissue culture. Another mutant variety, ‚Hae-no-Hatsu-yuki,' was created using gamma ray irradiation of tissue culture and possessed yellowish-white flowers. Irradiation of tissue culture explants with gamma rays (10 Gy) improved ‚Golden Cremon,' which featured golden-yellow ray flowers with anemone disc florets (IAEA, 2022). Combination of gamma irradiation with *in vitro* plants is expected to play a preeminent role with the new mutants having a distinctive color. The mutant variety 'Yellow Prism' was improved by irradiation of *in vitro* culture with gamma-rays with erected flower petal and bright yellow petal color. (IAEA, 2022). In this experiment, we got the color changes similarly. Nagatomi and Deggi (2009) indicated that flower color mutations could be more induced in regenerated plants from petals and buds, than from leaves. We have discovered color changes from bud explants and our results confirm the researchers. On the other side, Puripunyavanich et al. (2019) irradiated *in vitro* leaf explants with 20 Gy gamma-rays. Originally pink parents modified some color mutants varied from pink-orange to yellow shades with numerous ray florets. In addition, compared to the control group, our research produced color alterations in flowers as well as smaller and larger flower heads with more ray florets. These modifications of new mutants may be acceptable to flower traits and consumers with various physical traits.

In vitro explants of single-node fragments, leaves, and leaf-originating callus were used by Zalewska et al. (2010), that irradiated the explants with gamma irradiation at a dose of 15 Gy. They suggest that using induced mutagenesis in combination with the *in vitro* adventitious bud method is a very effective method for chrysanthemum breeding. Their mutations could result in new and appealing original chrysanthemum varieties. It is also expected to obtain new chrysanthemum cultivars in this research.

The variety 'Cream Marble' was developed through irradiation of nodal cultures with acute gamma rays (35 Gy). The variety had orangish-red flowers, yellowish cream leaves and medium height plants (IAEA, 2022). When comparing the control plants to the mutants, we found comparable results in flower color, plant heights, and flower head size variations that can be utilised as new varieties. Chrysanthemums are available in a huge variety of colors and traits, but despite this, there is a market that always demands the creation of new types on both growers' and consumers' sides.

Conclusions

Mutation breeding is a particularly gentle method of developing new ornamental plants with immediately visible color, form, and size features. Irradiation of Chrysanthemums at an effective mutagen dose, resulted in plant height variation and significant alterations in floral parts and leaves. The beneficial alterations may result in improved new varieties. In addition, applying gamma radiation to *in vitro* bud explants is an effective mutagen for producing new chrysanthemum types. *In vitro* bud explants were irradiated with EMD50 as 20 Gy gamma rays. After hardening various florets were observed, including discernible variations in ray floret sizes and colors that might be utilized as new cut flower varieties. As the result of *in vitro* treatments of mutagenic gamma radiation, it can be mentioned that induced homogenous mutations method is an effective method for chrysanthemum breeding. The mutants can be selected and vegetatively propagated to generate cut flowers that will be accepted.

Acknowledgements

This study was a part of project of Ministry of Agriculture and Forestry, General Directorate of Agricultural Research and Policy with the collaborations and larger efforts led by the Turkish Energy, Nuclear and Mineral Research Agency, Nuclear Energy Research Institute and Bademler Village Agricultural Development Cooperative. Special thanks to Dr. Yaprak Kantoglu and Dr. Burak Kunter for their contributions to this paper, as well as Dr. Veysel Aras for his assistance with NTSYS.

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