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DETERMINATION OF MUTAGENIC-SENSITIVITY AND INDUCED VARIABILITY IN THE MUTANT POPULATIONS OF 'BACARDI' CHRYSANTHEMUM CULTIVAR

Gulden HASPOLAT[1], Burak KUNTER[2] and Yaprak KANTOGLU[2]

[1]Aegean Agricultural Research Institute, Cumhuriyet Street, No:138, Mail Box: 9, 35661, Menemen, Izmir, Turkey
[2]Turkish Energy, Nuclear and Mineral Research Agency, Nuclear Energy Research Institute, Atom Street No: 27, 06983, Saray, Ankara, Turkey

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Mutation breeding is one of the most important breeding method for ornamental plants. Chrysanthemum is the genus that has the richest mutant varieties in ornamental plants. The objective of this study is to create variation by gamma irradiation and improving traits by mutation breeding. For this aim, *in vitro* bud explants of white Bacardi variety were irradiated by gamma radiation at 20 Gy (Gray). *In vitro* subcultures were continued until M_1V_4 period and observations were obtained in this period. Some changes were observed on heights and flowers of the plants such as; variable flowers, flowering time, differentiation on plant length, flower number per bunch and ray floret differentiations. The changes of the ray florets were determined as color changes to pink and yellow. Mutation frequency was calculated by 1.1% of the population. Approximately 0.9% of useful mutant lines determined from the selected mutants**.**

Keywords: Chrysanthemum, *in vitro* mutation, effective mutation dose (EMD-LD50), mutation frequency

INTRODUCTION

The basic aim of plant breeders is to create new genetic variability by using different techniques and select the individuals, which have the desired traits (SCHUM, 2003). Mutation breeding is one of the effective ways to generate new variability for ornamental plants. Spontaneous mutations can occur naturally by various kinds of radiations and cosmic rays received from the sun and emitted by several radioactive elements (OLADOSU *et al*., 2016). Other than this, the mutation can be artificially induced by several types of chemical agents belonging to few specified groups known as chemical mutagens and a number of physical agents like gamma rays or X-rays, ion beams (KHARKWALL, 2017; YAMAGUCHI, 2018). Physical mutagens especially gamma-rays have been most successfully used to generate new mutant varieties of ornamentals (VAN HARTEN, 1998;

Corresponding authors: Gulden Haspolat, Aegean Agricultural Research Institute, Cumhuriyet Street, No:138, Mail Box: 9, 35661, Menemen, Izmir, Turkey, guldenhaspolat@gmail.com

DATTA, 2009). The color, texture, shape of flowers and leaves changes, endurance of plants, plant growth type, and the difference of flowering time are the most seen features among new varieties, which were developed by mutation breeding in ornamental plants. According to IAEA's mutant variety database, the number of approved mutant varieties are 3377 in 2022 (http://mvgs.iaea.org/Search.aspx); the number of mutant ornamental plants are 728 and 285 of them are belong to the mutant Chrysanthemum varieties. Number of 400 mutants, which were released among the vegetatively propagated plants, are belong to the floricultural plants and fruit trees according to these records. These plants are including chrysanthemum, alstroemeria, dahlia, bougainvillea, rose, achimenes, begonia, carnation, streptocarpus, and azalea (AHLOOWALIA and MALUSZYNSKI, 2001).

The records of International Atomic Energy Agency (IAEA) mutant ornamental plants have 66 Genus. Superior properties obtained by mutation breeding are; changes in color and morphology of leaves, flower type and color, plant type, early flowering, nematode and sun tolerance, and flowering time. Alstromeria (35), Chrysanthemum (285), Dianthus (28), Euphorbia (1), Eustoma (3), Rosa (89) are officially announced commercial mutant cut flowers (IAEA, 2022).

Dendranthema x grandiflora and Chrysanthemums have an important association with so many varieties. Consequently, flower breeders have developed numerous genotypes by breeding, which are enabled its establishment in the top ten cuts, potted flowering, and garden crops worldwide for years (ANDERSON, 2004). Chrysanthemum production area is 755 da in 2018 and increased 61.5% between 2011 and 2018 (KAZAZ *et al*., 2020) and it is one of the most consumed flowers in Turkey. Despite this increase, the vast majority of the varieties used in production are foreign varieties and it is of great importance to increase the share of local varieties in production rapidly. Appropriate breeding techniques for the creation of genetic diversity should consciously be integrated into breeding programs. Mutation breeding, which is one of these methods, is a method with very good results, especially for chrysanthemum. It is seen that *in vitro* mutation applications give very effective results in the studies carried out with the method of mutation breeding in chrysanthemum from past to present (KUMAR *et al*., 2012; DATTA, 2014; HASPOLAT *et al*., 2019, MELSEN *et al*., 2021).

In vitro techniques are regarded as a method that allows the propagation of plant material under controlled conditions and the selection of the propagated material in controlled environments. The use of *in vitro* methods for plant breeding is used as integrated into the classical breeding cycle in order to create genetic diversity in a short time and to evaluate the material rapidly in breeding studies based on disease, pest, salinity, and drought (VAN HARTEN, 2002). Since *in vitro* techniques allow for rapid clonal propagation and selection of mutants, good results are obtained in studies (DATTA, 2014). The mutation breeding technique, which has not been strategically ignored in the past, is now used in combination with *in vitro* techniques as a promising effective breeding method (KUNTER *et al*., 2016). *In vitro* mutation studies maintains more effective regeneration than *in vivo* conditions and increases the potential of obtaining mutant individuals as well as it allows for a considerable speed-up of all the stages of the plant breeding program. (ZALEWSKA *et al*., 2011).

Using proper strategies in mutation induction as an application of relevant and recurrent irradiation doses integrated with *in vitro* culture techniques may lead to rapid success also in homozygous, polyploid species (SCHUM, 2003). On the other hand, plant tissue culture techniques raise the efficiency of mutagen applications. It provides the generation of new genetic variation.

This method enables the handling, screening of large plant populations and mass cloning of selected plants (KUMAR *et al.*, 2012; SARSU *et al.*, 2018). Studies carried out on Chrysanthemums in *in vitro* conditions from past to present have shown that this species responds very well to *in vitro* propagation practices (ROUT and DAS, 1997; MANDAL *et al.*, 2000b; NENCHEVA, 2010; ZALEWSKA *et al.*, 2011; TEIXEIRA DA SILVA and KULUS, 2014; MILER and JĘDRZEJCZYK, 2018). The high regeneration capacity of the explants of the species appears to be an important factor that increases the applicability of mutation studies *in vitro* conditions. The aim of this study was to create *in vitro* variation in Chrysanthemum by gamma irradiation and to develop new traits by mutation breeding.

MATERIAL AND METHODS

Materials

Chrysanthemum morifolium (Ramat.) Bacardi is a cut flower which is spray type with white flowers, 7 weeks, green disc florets, high flower number and tolerance of diseases. Bacardi is one of the most preferred cultivars by the growers because of the strength of plants and the resistance to diseases. It was chosen in order to develop new varieties by using the positive features and adding new ones to them. *In vitro* bud explants of Bacardi were used as material. The study was carried out between 2016 and 2021 in the tissue culture laboratory in Aegean Agricultural Research Institute in Menemen. The hardened 36000 plants planted in the greenhouses at Bademler Village Agricultural Development Cooperative in Seferihisar, Izmir, Turkey and observed in November 2020 during full blooming.

Methods

Tissue Culture

All the explants were washed thoroughly in running tap water for 30 min. Explants were treated with 70% ethanol for 40 sec followed by surface sterilization with a H_2O_2 solution (25%) for 10 min and then washed thoroughly in sterile distilled water 5 times. Explants were cultured on MS medium (MURASHIGE and SKOOG, 1962) with 3% sucrose, 0.8% agar and 1 mg $L.^{-1}$ BA, Medium pH was adjusted to 5.8 before autoclaving at 121°C for 15 min. Cultures were incubated at 22±1°C under cool white light with a 16-h photoperiod (30 µmol m^{-2} s^{-1}) and 55–60% RH (relative humiditiy).

Calculating Effective Mutation Dose

The *in vitro* buds were irradiated with gamma rays at 7 doses which are 0, 5, 10, 15, 20, 25 and 30 Gy (Gray). Irradiation treatments were conducted in Turkish Energy, Nuclear and Mineral Research Agency, Nuclear Energy Research Institute using gamma rays of cobalt 60 (^{60}Co). Irradiation treatments were managed at *in vitro* conditions. Each treatment has 150 buds in 30 young plants and 1050 buds were used for the treatments totally. The gamma source rate was 4.72 kGy/h. After radiation applications, the explants were sub cultured at MS medium. The shoot length measured for calculating effective mutation dose (EMD 50). EMD was calculated as 20 Gy (HASPOLAT *et al.*, 2019).

Irradiation at EMD

After determination of EMD, 3000 explants were irradiated at 20 Gy and sub cultured after irradiation. Subcultures were continued during M_1V_1, M_1V_2, M_1V_3 and M_1V_4 growing periods. All the regenerated shoots (2–3 cm in length) were transferred to MS medium containing 3% sucrose and 0.8% agar for root induction. Rooted plantlets were transferred to plastic pots containing peat:perlite (3:1) and placed under high humidity for one week for hardening. Plantlets were planted in greenhouse one week after hardening. Irradiated plants kept under the same conditions in the greenhouse where the control plants were grown.

Selection of Mutant Plants

Mutation frequency was the rate of number different plants to irradiated-planted plants according to control group. Determination of mutant plants and selection of single plants were maintained during blooming. More flowering means, the consideration of flower number more than 10 flowers per bunch. Small florets were determined as the floret has diameter lower than 5 cm ($x \leq 5$). Big florets had the bigger diameter than 7 cm ($7 \leq x$). Single florets had only one flower per bunch. Different ray florets were unusual florets that had spinous or discrete ray flowers. Early blooming types bloomed one week earlier, late blooming genotypes bloomed one week later than all plants. Low and high plants had the length shorter and taller than the plants in the population. Higher plants were the plants longer than 110 cm and shorter ones were short than 70 cm. The color of the ray florets was determined and based on the Methuen Hand Book of Colour catalogue (KORNEUP *et al.*, 1978). The quantitative traits as plant height-width, stem height-width, number of shoots and flowers per plant, flower width and color, number of ray florets, leaf height-width and stem weight were used for the selection of useful mutants such as cut flower.

Statistical Analysis

NTSYS (Numerical Taxonomy and Multivariate Analysis System) 2.02 software package was used to determine the genetic similarity in terms of morphological characters. The analysis of the results was evaluated using the tree. The similarity matrix between the mutants was calculated using the core algorithm (ROHLF, 2000). The principal components analysis (PCA) of the original binary data matrix was also performed using NTSYS 2.02 software.

RESULTS

Changes of the Plants

Mutation frequency (MF) was calculated as 1.1% in the population and the useful mutant lines were considered with the percentage of 0.9. There was only one flower at some plants named as single with a ratio of 1.4%. Daisy (small florets) types were determined with a ratio of 6.3% in the mutant population. The plant height of some plants was higher or shorter than the control group and the ratio of long plant height was 5.6% and was 4.2% for short plants. The plants, which had different ray florets, had the ratio at 1.1%. Color changes of florets from white to yellow and pink. The ratio of yellow blooming types was 7% while the late and yellow blooming rate was 2.5%. The most common change of the selected population was color changing from white to pink. The rate was 30% for pink blooming types; it was 8.5% for pink and small florets; pink and late blooming

type's rate was 0.7%. Deformed plants had the ratio 23% including unusual florets, chimeric florets, and integrated florets (Figure 1).

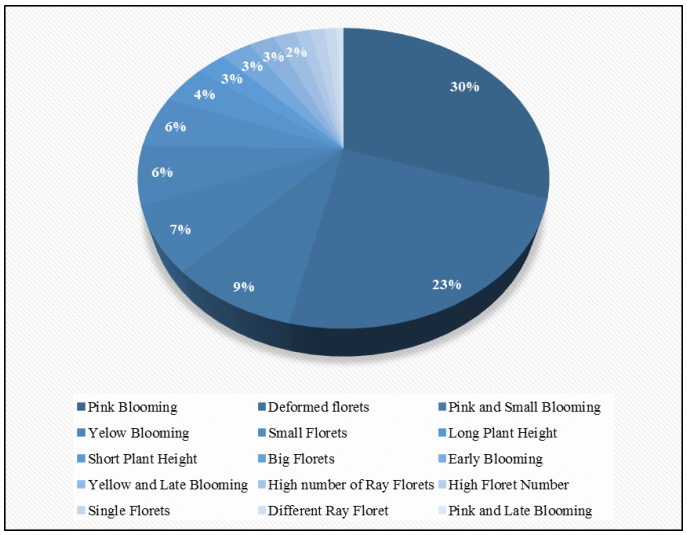

Figure 1. Changes of the plants

Plant heights differed from 44 to 121 cm. The mean plant height was 94.4 cm 16 mutants had a height between 110 - 121 cm. In selected population 3 mutants had the length between 44 -58 cm. Plant heights of 79 mutants were between 70-109 cm and 70 cm is the optimum length for cut flowers (Table 1).

Flower widths altered from 3.1 to 8.5 cm and 16 mutants among the 98 genotypes had the flower width from 7 to 8.5 cm. Small flowering 24 mutants had the flower width from 3.1 to 5 cm and 57 mutants had the diameter 5.5 to 6.8 cm (Table 1).

Flower numbers were between 1 to 52 per stem. The mean value was 16.5 and there were 10 genotypes whose flower number is higher than 30 ($30 \leq x$). It was observed that 69 genotypes had the flower number 10 and more ($10 \leq x$) and 29 genotypes had the flower number per stem between 1 to 9 (Table 1).

Flower colors were changed from white to pink and yellow. Pink colors had the codes 12/3A and 12/2A; yellow color code was 3/6A according to Methuen Hand Book of Colour catalogue (KORNEUP *et al.*, 1978).

Hierarchical cluster analysis was used to do an assessment of similarity between genotypes by quantitative characters like plant height and width, plant stem height and width, shoot number per plant, flower number and width, ray floret number and color, leaf length and width, weight of stem. According to the cluster analysis, varieties are divided into tree large groups with a 0.79 coefficient value. (Figure 2). The number of genotypes in a cluster was varied from 1 (Group 1) to 41 (Group 2) and 56 genotypes (Group 3).

Table 1. Plant height (P.H.), flower width (F.W.) and flower numbers (F.N.) of mutants

No	P.H. (cm)	F.W. (cm)	F.N.	No	P.H. (cm)	F.W. (cm)	F.N.	No	P.H. (cm)	F.W. (cm)	F.N.
B1	115	6.0	43	B34	110	6.3	26	B67	96	7.0	7
B2	120	5.0	31	B35	93	6.4	28	B68	82	5.0	3
B3	79	4.5	14	B36	99	5.8	28	B69	72	5.0	11
B4	97	5.5	18	B37	103	5.0	34	B70	81	6.2	7
B5	105	6.5	10	B38	104	5.5	30	B71	71	5.5	20
B6	110	6.0	16	B39	107	6.5	8	B72	95	4.5	10
B7	99	5.0	19	B40	98	6.5	11	B73	80	5.5	5
B8	103	6.2	23	B41	105	7.0	22	B74	91	5.5	20
B9	113	6.0	11	B42	101	6.8	30	B75	94	5.5	1
B10	109	6.0	7	B43	84	8.5	10	B76	85	5.5	15
B11	114	6.5	16	B44	99	7.0	6	B77	102	6.0	9
B12	100	6.0	16	B45	90	5.0	23	B78	110	6.3	14
B13	107	5.0	19	B46	100	5.5	5	B79	115	5.5	11
B14	101	5.5	31	B47	50	3.4	11	B80	58	5.0	7
B15	118	6.0	29	B48	90	6.5	11	B81	103	7.5	19
B16	103	7.6	19	B49	93	6.0	21	B82	104	6.5	10
B17	121	6.0	24	B50	103	6.5	22	B83	95	4.0	8
B18	100	4.0	9	B51	98	6.0	15	B84	85	5.0	5
B19	98	6.0	32	B52	87	6.5	11	B85	105	6.0	5
B20	78	4.5	14	B53	70	5.0	8	B86	44	4.0	7
B21	106	6.0	8	B54	73	5.5	22	B87	120	7.0	23
B22	86	6.0	18	B55	87	7.5	1	B88	98	5.5	18
B23	115	6.0	23	B56	85	7.5	9	B89	109	7.5	23
B24	116	7.0	19	B57	85	7.5	12	B90	100	7.2	23
B25	102	6.5	26	B58	80	5.0	17	B91	95	6.2	12
B26	96	6.5	16	B59	70	4.0	12	B92	98	4.5	12
B27	118	6.5	52	B60	90	5.5	9	B93	85	5.5	22
B28	97	6.5	34	B61	94	7.0	19	B94	74	5.6	8
B29	115	7.5	9	B62	76	6.5	6	B95	75	5.8	18
B30	93	5.5	49	B63	72	3.5	6	B96	95	7.0	7
B31	89	4.0	27	B64	84	5.5	20	B97	76	3.1	10
B32	118	7.0	12	B65	85	5.5	18	B98	83	4.0	20
B33	86	6.8	21	B66	80	6	5				

Cophenetic correlation between ultrametric similarities of tree and the similarity matrix was $r = 0.80$, $P < 0.01$. The three main groups were detected at 0.90. B1 was the only genotype in the Group 1 had the highest flower number, plant width and shoot number; with long plant - stem heights and stem width (Figure 2-3-4). According to the correlation matrix the similarity ratio between B1 and white colored genotype B2 was 84%; also B1 was similar to B59 (white color) with the ratio of 61. The ratios of B1with yellow colored genotypes B82 and B91 were 81% and

84% respectively. The similarity ratio of B1 with the pink colored genotypes were changed between 71% and 82%. Also the similarity of B1 to B72 and B84 was determined 82% while B73 and B80 was 77%.

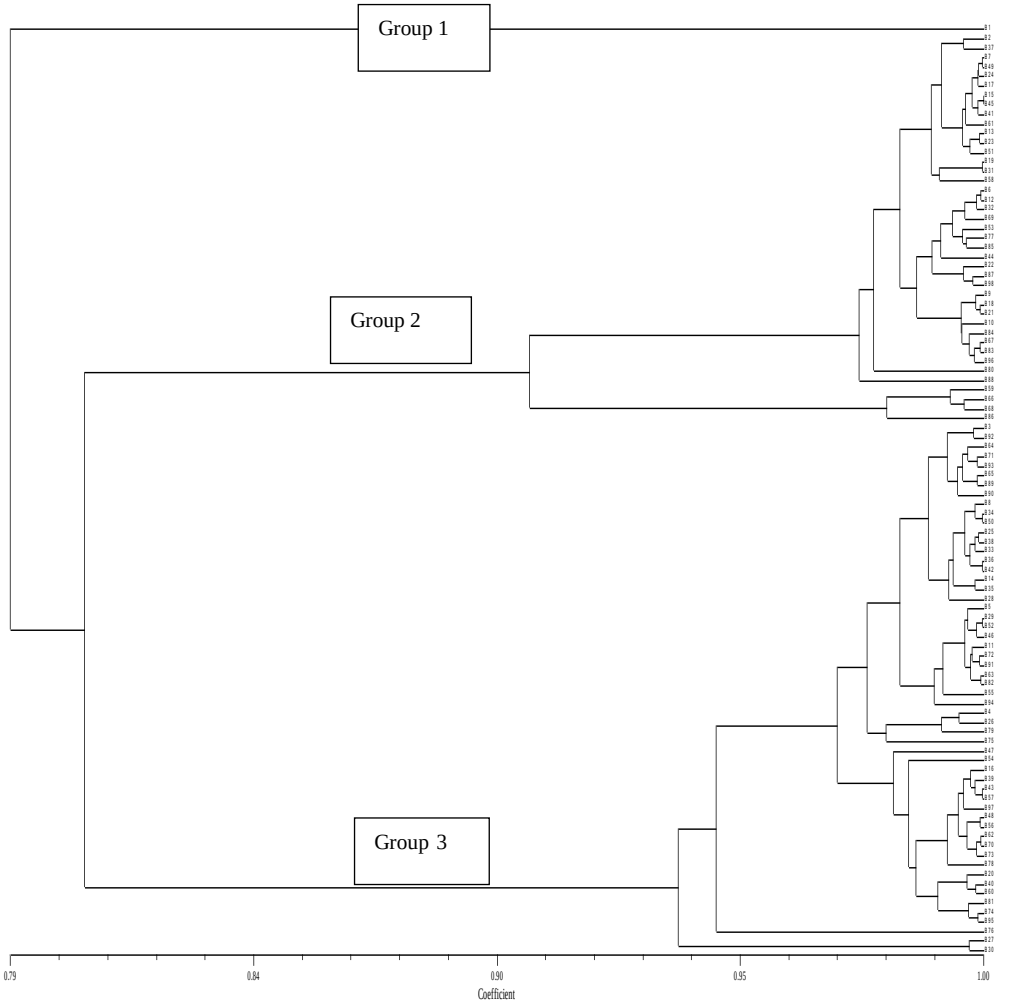

Figure 2. Diversity result of mutants UPGMA (unweighted pair group method arithmetic average)-based tree showing genetic similarities among mutants

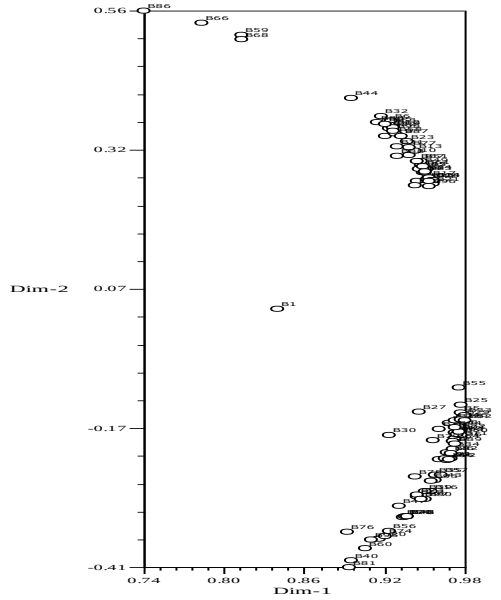

Figure 3. Two dimensional plot of the selected mutants in the principal components analysis

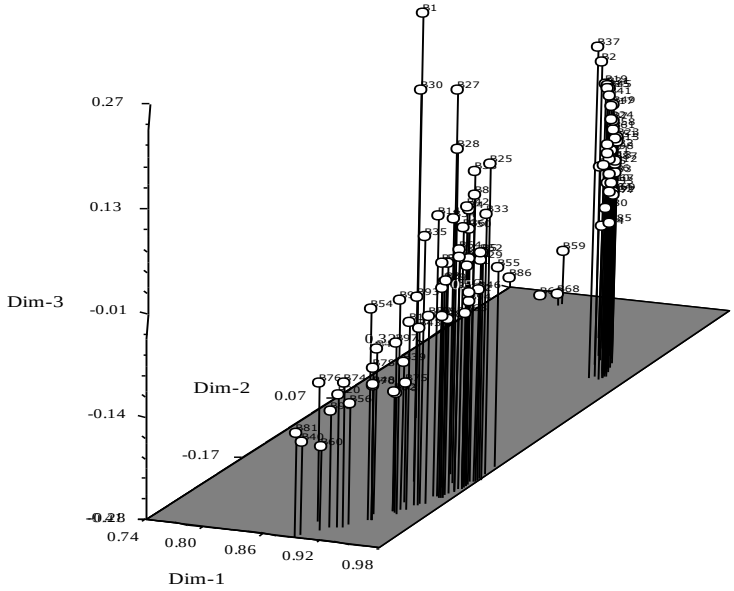

Figure 4. Three dimensional plot of the selected mutants in the principal components analysis

DISCUSSION

The results, which obtained from this study, showed that the *in vitro* mutation breeding method gives an opportunity in chrysanthemums to generate new varieties in a short time. Our results also have similarities with ZALEWSKA *et al*. (2011). By *in vitro* mutation techniques, ZALEWSKA *et al*. (2011) created new chrysanthemum cultivars as a result of the conducted study by *in vitro* mutagenesis on different explant types. They reported flower color changes among the observed individuals. As a result of this study, the researchers could have generated different colored variants from white-colored material by *in vitro* induced mutation techniques. In our study, we also obtained yellow and pink florets from white cultivar similarly (Figure 5).

Figure 5. a.Yellow ray florets, b. High number of ray florets c. Bacardi flower, d. Pink ray florets, e. Short ray florets, f. Yellow flowering plant, g. Pink and short ray florets (bars = 1 cm).

BRAKAT *et al.* (2010), were conducted a research on *Chrysanthemum morifolium* cv. Delistar White by *in vitro* mutagen treatment on ray florets with gamma irradiation (0, 0.5, and 1.0 Gy). According to their results, the shoot length was decreased with gamma ray treatment in comparison to the control. 0.5 Gy gamma irradiation treatment was found as the most effective dose to obtain different types of flower shapes and the number of florets per flower head. Whereas there was no difference obtained on flower color, they did not indicate any chimera formation. They reported that the flower color and shape mutations could be developed through direct *in vitro* mutagenesis by avoiding the chimeric phase. We have similar results like decreasing shoot length and color changes can occur without chimera formation. Besides, the formation of sectorial chimeras was considered as negative mutants got the place in-group of deformed florets in the presented study.

DATTA *et al.* (2005), reported that after mutagen treatment in chrysanthemum cuttings, generally abnormal leaves/flower heads were seen in the first generation, we have observed in this study deformed flower heads with the rate of 23.2%. MANDAL *et al*., (2000a and 2000b) used rooted seedlings of 'Purnima' chrysanthemum varieties with flower color 'White Colchi Bahar' and red flower color. They irradiated them at gamma source, ray leaves taken from plants were cultured *in vitro* conditions. Sectoral somatic mutations that affect flower color were observed in mutant plants. The flower color was determined as yellow in some of the mutant plants and it was found that this feature was preserved in plants that were reproduced vegetatively. According the findings of this presented data have a similar correlation with the MANDAL *et al*. (2000a and 2000b). These data showed that the *in vitro* mutation induction has a great effect on chrysanthemum to change flower color and other plant characteristics.

SUSILA *et al.* (2019), indicated that increasing the dose of gamma irradiation inhibited plant growth. Irradiation at 10 Gy and 20 Gy produced the color changes in flowers compared to other doses. They have obtained dark purple and deep red flower colors irradiating at 10 Gy and 20 Gy, while the flower color was purple at control group. The treatment of gamma ray irradiation significantly affected the leaf length, leaf width, stem diameter, stem length and diameter of flowers. In our study we had the flower changes and differentiation of flower diameters similarly.

KAUL *et al.* (2011), reported that *in vitro* mutation provided to chance in flower color in one branch of the same plant by 10 Gy irradiation. By this research original floral color of Snow Ball was changed as yellow with flat and incurving florets. All ray florets were observed as the same color and shape. In presented data you can follow, similar results were achieved like getting pink and yellow flowers from white ones with same shape and size. Homogeny was one of most important aims in this study.

Main target of that mutation research on Chrysanthemum was to change flower color by mutation (IAEA, 2022). 'ARTIpurple' and 'ARTIqueen' named varieties which were developed from spray-type Chrysanthemum. There are different varieties as 'Argus Joy Prelude Afu' and 'Joy Prelude Coe', which were developed *in vitro* mutation studies (IAEA, 2022). Similarly, flower color changes were also obtained at *in vitro* plants after hardening in this research.

A new late-blooming chrysanthemum type (as in case of its parent from which it is derived after mutagenesis) that blooms till late January is developed and named 'Kesar' in India. The novelty of the new variety lies in its 'Yellow-Pink' bi-colored florets and bigger capitulum size (~10% bigger) (IAEA, 2022). Capitulum sizes differentiated in this study as well.

According to mutant variety database the mutant variety 'Ion-no-Kouki' was developed in 2006 by irradiation of *in vitro* culture (petal culture) with 5 Gy C ion beams. Main improved attribute of mutant variety is complex with light pink and bright orange yellow. The mutant variety 'Ion-no- Hatsune' was was developed by irradiation of *in vitro* culture (petal culture) with 20 Gy C ion beams. This mutant variety has also color changes such as complex with light yellow and pink. The mutant variety 'White Lineker OW-1' was developed by irradiation of *in vitro* culture with x-rays. Main improved attribute of mutant variety is white flower color. (IAEA, 2022). In this experiment we got the color changes similarly. NAGATOMI and DEGGI (2009), indicated that flower color mutation could be more easily induced in plants which were regenerated from buds and petals, than from leaves. It was conjectural that the gene loci fully expressed on floral organs may be unstable for mutation by mutagenesis or culture, but could perhaps induce mutation in a desired direction (NAGATOMI and DEGGI, 2009). We have observed color changes from bud explants and our results confirm the findings of the researchers.

The flower head of the 'Yellow sun' mutant is very small, approximately half the size of its control. The mutant was obtained via *in vitro* culture using ion beams with 0.5 Gy. In the early stage of flowering, only its bright yellow disc florets were clearly visible whilst its ray florets were almost non-existence. These ray florets were only visible during full bloom (IAEA, 2022). Similar results were seen that we have gained small flowers even with color changing and very short ray florets comparing the control plants.

CONCLUSIONS

Mutation induced by related biotechnology is an effective method to create genetic variability, especially in ornamental plants, and there are many studies on this subject. In our study, *in vitro* mutation breeding methods are very efficient and suitable for chrysanthemum breeding. To develop new Chrysanthemum varieties, the application of 20 Gy gamma radiation to bud explants *in vitro* is a useful mutagen. Variations were observed for the plant size, flower length, pigmentation and shape, which changed the ray blooms with color differentiation. The mutants were selected and propagated to produce cut flowers that will be further tested for market acceptance. The results showed that *in vitro* mutation studies are very effective for generating new variation and this method is very promising and rapid for developing varieties released in Chrysanthemum.

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ODREĐIVANJE MUTAGENE OSETLJIVOSTI I INDUKOVANE VARIJABILNOSTI U MUTANTSKIM POPULACIJAMA KULTIVARA HRIZANTEME „BACARDI"

Gulden HASPOLAT[1], Burak KUNTER[2,] Yaprak KANTOGLU[2]

[1] Egejski institut za poljoprivredna istraživanja, 35661, Menemen, Izmir, Turska
[2]Uprava za istraživanje energetike, nuklearne energije i rudarstva Turske, Institut za istraživanje nuklearne energije, 06983, Sarai, Ankara, Turska

Izvod

Oplemenjivanje mutacijom je jedan od najvažnijih metoda oplemenjivanja ukrasnih biljaka. Hrizantema je rod koji ima najbogatije mutantne sorte u ukrasnim biljkama. Cilj ove studije je stvaranje varijacija gama zračenjem i poboljšanje osobina korišćenjem mutacija u oplemenjivanju. Za ovaj cilj, *in vitro* eksplanti pupoljaka bele sorte Bacardi su ozračeni gama zračenjem na 20 Gi (Grai). *In vitro* subkulture su nastavljene do perioda M1V4 I u ovom periodu su ocenjene. Neke promene su primećene na visinama i cvetovima biljaka kao što su: varijabilni cvetovi, vreme cvetanja, diferencijacija po dužini biljke, broj cvetova po grozdu i diferencijacije cvetova. Promene cvetova utvrđene su kao promena boje u ružičastu i žutu. Učestalost mutacija je izračunata na 1,1% populacije. Približno 0,9% korisnih mutantnih linija je određeno iz odabranih mutanata.

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