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Research Article

Ray-Floret Based Rapid Propagation and Detection of Somatic Variation in Selected Mutant Chrysanthemum Individuals

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Abstract: Chrysanthemum (Dendranthema × grandiflora Tzvelev) is an attractive plant species that responds well to mutation breeding research conducted worldwide. The isolation and propagation of mutant individuals are very important for detecting mutations in *in vitro* cultures. Using *in vitro* cultures, it is easy to isolate solid mutants. In this study, the homogeneous reproductive capacity of selected mutant individuals after irradiation was examined using ray floret cultures at the M_1V_1 stage, based on various flower colors and architectures. The explant materials were obtained from selected mutant plants with yellow, dark red, orange, and spoon-shaped ray florets cultivated in full bloom. After the determination of an effective sterilization method, the ray florets were cultured in Murashige and Skoog's (MS) media, which contained 0.5 mg L^{-1} 1-Naphthalaneacetic acid (NAA) and 2.0 mg L^{-1} 6-Benzylaminopurine (BAP). The average rate of plantlet regeneration varied depending on the genotype; mutants with yellow-colored flowers generated 0.6 plantlets per explant, those with orange-colored flowers (1.11), those with dark red-colored flowers (1.16), and those with spoon-shaped flowers (2.71). After plant regeneration, plantlets were cultured in a hormone-free MS nutrient medium to ensure full-rooted plant development. The findings of this study showed that *in vitro* ray floret culture could be used to swiftly and successfully carry out vegetative reproduction of pot-type Brandevil mutants, which are more susceptible to in vivo cutting propagation than pot-type Chrysanthemum cultivars. In the propagating material, there were obtained four differently colored somaclonal plants, eleven partial somaclonal plants with incurved-type ray florets, eight somaclonal plants with spatulate-type florets, one somaclonal plant with semi-double-type flower heads, two somaclonal plants with pointed and reflexing ray florets.

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1. Introduction

Among ornamental plants, East Asian-originated chrysanthemum species have an important place. Chrysanthemum breeding has begun in China (Haspolat et al., 2022). This species is recognized as an indoor, outdoor, and potted decorative plant in this industry. Considering the varieties grown in

Türkiye, it is clear that imported varieties are mainly used for breeding. Consequently, Turkey's significant production capability makes it very important. As a floral branch, the actual production in 2021 was 78 649 425, and in 2023 it was 78 763 020 (TUIK, 2024). This rise in output capacity demonstrates the significance of chrysanthemums in the Turkish ornamental plant industry as well as the importance of creating new native varieties (Kazaz et al., 2020). Numerous governmental research institutes and private companies have examined this topic using a cross-breeding approach. Breeding studies have been conducted on many plant species globally, utilizing a range of methodologies, from traditional methods to cutting-edge biotechnological technology. When these studies are evaluated from the perspective of the Green Revolution, it becomes evident that induced mutations are particularly valuable for molecular investigations, especially for the production and isolation of desirable genes (Pu et al., 2023). Mutation breeding has emerged as a critical breeding strategy for decorative plants and other plant groups. Therefore, breeders have evolved a number of strategies to make the induced mutation more favorable (Melsen et al., 2021; Yali and Mitiku, 2022; Datta, 2023). Because the Chrysanthemum species is genetically susceptible, numerous studies have already been conducted, and new ones are still being conducted to develop new cultivars through induced mutations (Lamsseejan et al., 2000; Mandal et al., 2000; Datta et al., 2005; Padmadevi and Jawaharlal 2011; Zalewska et al., 2011; Ahmad et al., 2012; Haspolat et al., 2019; Din et al., 2020). A total of 288 mutant Chrysanthemum cultivars were created using this technique and recorded in the International Atomic Energy Agency Mutant Plant Database (MVD, 2024). Türkiye has four new mutant cutting-type cultivars named Ozan, Kaan, Bademler Beyazı, and Ege Meltemi, which were registered in 2022 by the mutation breeding method of two government institutes (Anonymous, 2022). To accelerate breeding studies, which are typically carried out under field or greenhouse conditions, and to produce a much larger number of plants, *in vitro* mutations (Zalewska et al., 2011; Datta, 2023) and *in vitro* propagation techniques have also been used in addition to other propagation techniques (Barakat et al., 2010). Problems like self-sterility and incompatibility arise as limiting factors in plant breeding and propagation efforts. When these problems occur in ornamental plant species like chrysanthemums, vegetative propagation is the recommended technique (Misra and Datta, 2007; Nasri et al., 2018). Because of their genetic capacity, chrysanthemums are easily propagated using shoot tips, micro-cuttings, and ray florets using tissue culture techniques (Malaure et al., 1991; Datta et al., 2005; Barakat et al., 2010; Kumar et al., 2012; Teixeira da Silva 2014; Asoko et al., 2020; Prathusha et al., 2021). Particularly in mutation breeding research on chrysanthemums, it is straightforward to extract distinct mutants and sectoral chimeras (Mandal et al., 2000; Datta et al., 2005; Tymoszuk and Zalewska, 2014; Badigannavar et al., 2022; Datta, 2023). Ray florets were cultivated *in vitro*, which is thought to have shown their strong ability for regeneration in nutritional conditions containing different genotype-specific combinations of cytokinins and auxin (Mandal et al., 2000; Prathusha et al., 2021). Ray floret cultures can be used for clonal propagation of mutant material derived from in vivo or in vitro mutation breeding trials (Datta et al., 2005). The unique genetic material identified via breeding research may now be reproduced and preserved, according to Mandal et al. (2000) and Datta et al. (2005). In addition to propagating homogeneous clonal material with ray floret culture, important results were obtained in terms of revealing hidden somaclonal variations in the *in vitro* propagation process after mutation breeding (Datta et al., 2005; Datta, 2023). Point mutations were detected by the in vitro ray-floret method. Chrysanthemum is a plant that is prone to mutation breeding and somaclonal variation. *In vitro* propagation with ray floret culture and capture of different variants seems to be a very practical approach for this species (Sharmah et al., 2017). Similar to the mutations induced by different mutagens (physical or chemical), the rate of somaclonal variation is highly unpredictable. Hereditary differences resulting from genetic changes or, in some cases, non-hereditary differences can be revealed by different cultural methods applied *in vitro* (Chen and Henny, 2006; Eeckhaut et al., 2020). Pu et al. (2023) reported that auxin synthesis is an effective mechanism for controlling ray floret formation, particularly in chrysanthemum. Studies have shown that Auxin-related genes are effective in tubular-type ray florets. Therefore, these results are key for *in vitro* propagation studies of petal origins. The present study was carried out to propagate homogeneous mutant individuals through *in vitro* ray floret culture. The somaclonal variants and homogeneous clonal propagation rates of the parent plant were determined using this method.

2. Material and Methods

2.1. Material

The principal starting point was the pot-type Brandevil variety. The chosen M_1V_1 stage mutant individuals' ray florets were used *in vitro* plant propagation. Mutant individuals were created by ^{60}Co gamma ray treatment at a 27 Gray (Gy) effective mutation dose (Haspolat, 2022). A gamma irradiator was used at the Nuclear Energy Research Institute of the Turkish Energy Nuclear and Mineral Research Agency (dose rate 296 Gy/h). The explant materials were obtained from selected mutant plants with yellow, dark red, orange, and spoon-shaped ray florets cultivated in full bloom (Figure 1). As seen in Figure 1, ray florets belonging to single flowers that differ from other flowers on the main mutant plant were used for *in vitro* culture.

Figure 1. Different flower formations on the same plant and isolated flowers for ray floret culture (photos by B. Kunter).

2.2. Method

2.2.1. In vitro cultures

The sterilization phase is critical for the propagation of selected mutant ray floret explants. Therefore, the individual mutant flowers taken from the plants were first cleaned with tap water under controlled laboratory conditions. Each ray floret was removed from the flower bud and placed in sterile magenta boxes before being pre-washed with sterile distilled water inside a sterile cabinet. The ray florets were submerged in a 70% ethyl alcohol solution (EtOH) for 10, 12, 15, or 17 min. After the samples were cleaned three times with sterile distilled water for five minutes, 15% or 20% commercial sodium hypochlorite (NaClO) and two drops of Tween 20 were added. They were then shaken for 15, 12, or 10 minutes in 15% or 20% NaClO solutions. The samples were washed thrice in sterile distilled water for a total of five minutes. The water in the magenta box was poured well. The ray florets were then transferred onto sterile filter paper and preserved in sealed petri dishes to avoid contamination. Explants were placed on MS (Murashige and Skoog, 1962) baseline nutrient medium, which had a pH of 5.6 and contained 30 g L^{-1} sucrose, 3.5% Bacto-agar, 2.0 mg L^{-1} BAP, and 0.5 mg L^{-1} NAA (Datta et al., 2005). After starting direct plant regeneration from ray floret explants, the *in vitro* plantlets were moved to an MS medium without a growth regulator after 50 days (Figure 2). No additional rooting nutrient media was required for rooting the plantlets because the regeneration medium utilized promoted both root and shoot development. For subculturing, the regenerated plants were thus immediately transferred to a hormone-free MS medium. Four weeks after transfer, mutant individuals were reproduced by subculturing an average of three microcuttings per explant. *In vitro,* propagation of mutant explants was performed using three subcultures.

Figure 2. Ray floret cultures' of the 1st and 50th days (photos by K.Y. Kantoglu).

2.3 Incubation conditions, plantlet transfer to external circumstances, and observations

The *in vitro* ray floret cultures and regenerated plantlets were kept at a constant temperature of 24℃±1 and incubated for 16 hours of light and 8 hours of darkness in a growth chamber with an 8.000-lux lighting capability. After completing their *in vitro* development as whole plants, the plantlets were transferred to plastic pots containing sterile peat. The plants were then watered with a fungicide (Previcur, 20 ml 10 L^{-1}) solution to prevent fungal infection. The potted plantlets were kept humid and placed in sealed 50x40x40 cm clear plastic boxes over the course of two days, sprayed with water every four hours to maintain ambient humidity at 90%. The box lids were carefully opened gradually, and the plants were acclimatized to the climate room conditions. At the end of the fifth week, the plants were transferred to a fully controlled greenhouse. On the tenth day following the transfer, the plants were planted in larger pots, which included garden soil, peat, and burned dung (1:1:1 ratio), and the required maintenance operations were carried out. During the bloom period, observations were made to confirm true-to-true plant and flower formation and growth. The Royal Horticultural Society (RHS) Colour Chart Guide (sixth edition) (Anonymous, 2019) and Calibration Manual (Anonymous, 2020) were used to identify the ray floret type, flower head type, and flower color at full bloom.

2.4 Statistical analysis

Cultures were set up in a randomized plot design with 10 replicates and 30 explants in each replicate. The statistical software packages MINITAB and MSTATC were used to conduct analyses. The differences between the applications were determined using the DUNCAN test (at alpha 0.01).

3. Results

The aim of this study was to determine the capacity of *in vitro* ray floret culture for the homogeneous propagation of mutants of four distinct flower colors and morphologies (spoon-type, yellow, orange, and dark red-colored ray florets) and to detect somatic variation. To this end, the regenerative capacities of the explants and mutant individuals produced with the same structural features as those of the original mutant plants were evaluated. In addition, somatic variation was observed in the materials propagated by ray floret culture because ray floret culture is an important tool, especially in revealing somatic variations in mutant material. The best sterilization conditions were determined in the first step of this research. To accomplish this, ray florets were sterilized using various NaClO concentration ratios (10, 15, and 20 %) and treatment periods. The disinfection process in the high-dose application (20% NaClO) resulted in cultures blackening the ray florets. For this reason, ray florets were pre-treated with a 70% alcohol solution before using commercial NaClO and disinfecting the ray florets for 10, 12, and 15 minutes using a 15% sodium hypochlorite solution. Statistical analysis revealed that pre-cleaning with 70% alcohol for 10 minutes, rinsing the explants with sterile water, and then employing a 15% sodium hypochlorite solution with two drops of Tween 20 for 12 minutes produced the optimal treatment conditions (Table 1). The ray florets of the four different mutant flower types were sterilized using the A4 method and cultured on MS nutritional media containing 2.0 mg L^{-1} of BAP and 0.5 mg L^{-1} of NAA. The *in vitro* cultures were incubated for 50 days. The regeneration capacity of the ray floret explants was evaluated on the fiftieth day of culture incubation. According to the mean data acquired presented in Table 2, the spoon floret mutant of the ray floret cultures exhibited the highest regeneration rate, with 2.71 plantlets per explant (Table 2).

Table 1. Sterilization experiments results for ray floret samples

Code	Sterilization Method	Non-contaminated explant rate
A1	70% EtOH (10 min)+%15 NaClO (10 min)	75% c
A2	70% EtOH (10 min)+%20 NaClO (10 min)	65% d
A3	70% EtOH (12 min)+15% NaClO 12 min)	80% b
A4	70% EtOH (10 min)+%15 NaClO (12 min)	95% a*
A5	70% EtOH (15 min)+%15 NaClO (15 min)	55% e
A6	70% EtOH (10 min)+%20 NaClO (15 min)	50% f

*Difference was found important and letters determined the statistical difference, P<0.01.

The mutant explants with orange flowers (1.11) and dark red flowers (1.16) had lower values. The mutant with yellow flowers (0.60) showed the lowest value. Statistical analysis showed that the ability to regenerate is strongly influenced by the genotype. At a 0.01 alpha level, there was a statistically significant difference in the regeneration rates of the mutant genotypes.

Table 2. Regeneration capacities of mutant ray floret explants

Mutant Genotype Code	Number of ray florets	Total number of regenerated plantlets	Mean plantlet regeneration per explant
Spoon Floret Mutant Clone	300	812	2.71 **a***
Orange-colored Mutant Clone	300	334	1.11 b
Yellow-colored Mutant Clone	300	181	0.60 c
Dark-red colored Mutant Clone	300	349	1.16 b

*Difference was found important and letters determined the statistical difference, P<0.01.

The spoon-floret mutant exhibited a plantlet regeneration rate of 48%, followed by genotypes with dark red (21%), orange (20%), and yellow-colored flowers (11%). After the regeneration phase, all *in vitro* plantlets were transferred to the greenhouse. A total of 450 plants per mutant clone were evaluated for morphological features during the two growing seasons (Figure 3).

Figure 3. Clones of the second-generation mutants that are true to type were propagated using ray floret culture (photos by B. Kunter).

The results showed that the ray floret culture method delivered the same homogeneity as the mutant clones for the selected mutant flower. Based on observations made during the flowering period of 1800 plants propagated by ray floret culture and transferred to external conditions, 26 plants were

found to differ from expected. Four of these plants had different flower colors (light yellow), eleven had incurved-type ray florets, eight had spatulate-type petals, two had pointed and reflexing-type ray florets and one had semi-double-type flower heads. Although 98.56% plant production with the desired properties was achieved from the ray florets of the selected mutant clones, somaclonal variants were obtained at a total rate of 1.44% (Figure 4). When the obtained somaclonal plant material was evaluated, the tendency to form incurved and spatulate petals was 0.61% and 0.44%, respectively, the rate of color change was 0.22%, the rate of pointed and reflecting ray florets was 0.11%, and the rate of semi-double flower head type was 0.056% (Figure 4 and Figure 5). When the emergence rates of these somaclonal variants, which emerged even at very low rates, were examined depending on the genotype, it was observed that the emergence rate of somaclonal variants from mutant ray florets with red flowers was high (75%). The remaining 25% of somaclonal variants were obtained from the ray florets of mutant flowers with orange flowers.

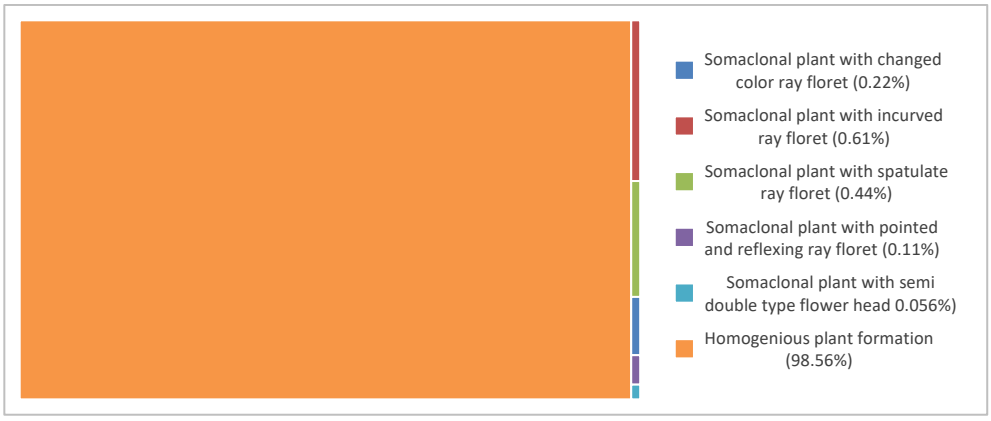

Figure 4. Homogeneous and somaclonal mutant plants obtain rates.

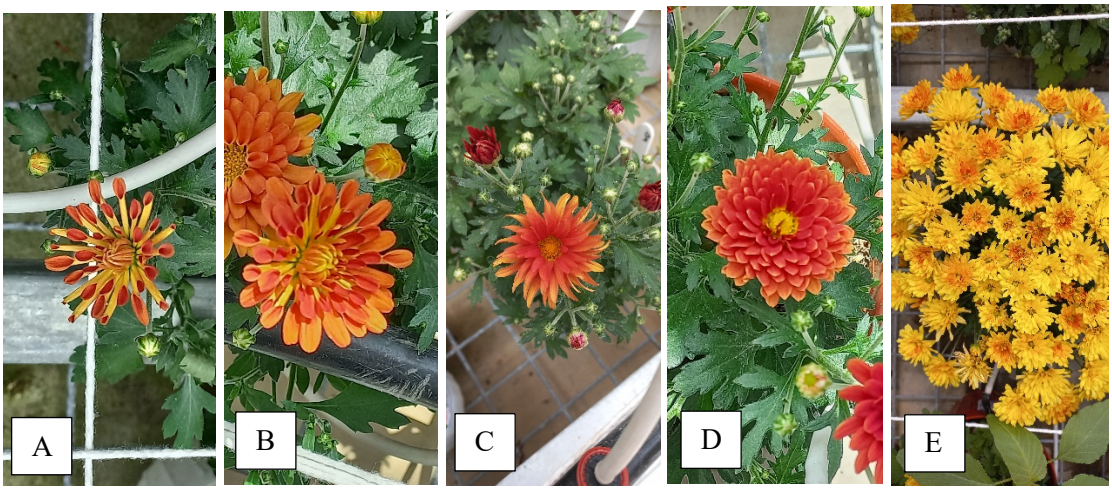

Figure 5. Somaclonal variation obtained in ray floret culture (A: spatulate type ray floret; B: incurved type ray floret; C: pointed and reflexing type ray floret; D: emi doube type flower head; E: changed color (photos by Burak Kunter).

4. Discussion

The objective of this study was to assess the possibility of uniform and quick *in vitro* mutant plant propagation and to detect somaclonal genotypes in the *in vitro*-propagated plants. The results showed that the method was practical for the rapid and uniform propagation of valuable mutant genetic material. Sterilization is essential for all *in vitro* plant propagations, especially chrysanthemums, as well as a small number of genetically varied plant materials, for optimal reproduction and preservation. Owing to the importance of this stage, several researchers have employed different sterilization

techniques for chrysanthemums (Verma et al., 2012; Teixeira da Silva et al., 2015; Hesami et al., 2019; Prathyusha et al., 2021). The best outcome of this study among EtOH+NaClO applications at different periods and rates was found to be 10% contamination and a 95% regeneration rate produced by 70% EtOH for 10 minutes and 20% NaClO for 12 minutes of application. Many experiments have been conducted with chrysanthemums to determine the most effective sterilization solvent. Different combinations of solvents used for ray floret sterilization, such as carbendazim+mancozeb+streptomycin+teepol, and utilizing artificial intelligence technologies (such as the MLP-NSGAII model), have demonstrated the importance of the link between the sterilizing agent and the application time (Verma et al., 2012; Hesami et al., 2019; Eisa et al., 2022). Another point is the effect of genotype on plantlet regeneration in the explants. In investigations to determine the effects of genotype and nutritional medium content on the potential of explants to regenerate, it was shown that mutant ray floret explants cultivated in the same nutrient medium had variable regeneration capacities depending on the genotype (Asoko et al., 2020). When the findings of this investigation were analyzed, the mutant donor with spoon-type ray florets had the highest regeneration rate of 48%, followed by the donor material with dark red, orange, and yellow flowers. Consequently, it has been found that in chrysanthemums, genotype greatly influences the *in vitro* regeneration rate of ray florets (Barakat et al., 2010; Prathyusha et al., 2021; Eisa et al., 2022). One of the possible factors contributing to the high mutation frequency is the flower color of the starting genotype used in the study. Studies have shown that as a result of mutagen application to genotypes with dark flower color, the rate of change in flower color, petal shape, and flower size is high in mutant variation (Haspolat, 2024). When the findings obtained from this research were evaluated, 75% of somaclonal variants obtained from mutant ray leaves with red flower color were obtained as a result of ray floret culture. Therefore, genotype has importance both in the formation of somaclonal variants and mutation frequency (Asoko et al., 2020; Eisa et al., 2022; Datta, 2023). The final stage of this study was to determine the effectiveness of this propagation method in obtaining the same plant formation as the main mutant individual. The results indicated that the mutant clones, which showed the same structure as the mutant floral structure, were 98.56% correct (true to true) after being reproduced as a whole plant and tested in greenhouse settings for two years. The findings of this study are in agreement with those of previous studies on chrysanthemum propagation by ray floret culture (Mandal et al., 2000; Nencheva, 2010; Nasri et al., 2018; Prathyusra et al., 2018). In contrast, *in vitro,* ray floret culture is an effective method for both identifying somaclonal mutants in selected mutant clones and propagation of the mutant clones. Using this method, a few new genetic materials with different characteristics (approximately 1.44%) that could not be predicted were introduced into the mutant gene pool. Malaure et al. (1991) and Sharmah et al. (2017) mentioned the same opinion regarding the detection of somaclonal variants in chrysanthemums.

Conclusion

In this study, it was aimed to propagate a sample of mutant plants using ray floret culture, which has the same characteristics as the flowers of the mutant material, and to detect somaclonal variants in the regenerated mutant material using ray floret culture. Chrysanthemum plants with different flower colors and structures were isolated for this purpose. Isolated ray florets were cultured in vitro and propagated as mutant clones. As a result of this study, the most suitable sterilization method for ray leaf explants was determined as a 70% EtOH (10 min)+%15 NaClO (12 min) sterilization cycle. 95% of explants survived without contamination. Therefore, optimum sterilization of a valuable and limited amount of genetic material was achieved, and losses due to infection were minimized. Second, it was determined that genotype-dependent differences were important for the in vitro regeneration capacities of ray florets and that the genotype was effective in regeneration success. Spoon floret mutant material regeneration rate was found to be the highest (2.71 plantlets per explant) compared with other mutant genotypes. In addition, the propagation of plant material from selected mutant flowers, which was the main goal of this study, was achieved at 98.56% with ray leaf culture. In vitro, ray floret culture provides another opportunity to detect somaclonal variants in mutant materials. In this research, 1.44% of the regenerated plants from ray florets showed differences according to the main selected mutant genotypes. The results showed that in vitro ray floret culture offers rapid and effective propagation. Additionally, this method hastens the breeding procedures for mutants. These findings must be strategically evaluated

for other attractive blooming plant propagation and to adapt the ray floret culture in breeding programs as well.

Ethical Statement

Ethical approval is not required for this study because no harm was done to nature and the environment.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

Author Contributions

Kadriye Yaprak Kantoğlu: Responsible *in vitro* propagation methods, statistical analyses and manuscript writing.

Burak Kunter: Responsible irradiation of plantlets, *ex vivo* plant propagation, and flower observations.

Ümran Şenel: Determination of *in vitro* ray floret culture conditions.

Gülden Haspolat: Responsible flower observations.

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