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In vitro mutagenesis and detection of variability among radiomutants of chrysanthemum using RAPD

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Abstract: The present study was undertaken to induce mutations in *Dendranthema grandiflora* cv. Snow Ball through *in vitro* mutagenesis by exposing the *in vitro* shoots to 5, 10, 20 and 30 Gy gamma radiation. RAPD analysis was used to detect genetic polymorphism among the variants and the control. Morphological variations were not observed with the 5 Gy gamma dose during the first season. Shoot regeneration, rooting and survival of shoots were affected by gamma ray doses. 10 Gy of gamma irradiation was the most effective in inducing mutations in flower colour through direct *in vitro* mutagenesis. The shoots irradiated with 20 and 30 Gy gamma radiation did not root and died. Twenty RAPD primers were used to amplify DNA segments from the genomic DNA of the control and its 10 variants, and the genetic similarity among them ranged from 0.06 to 0.79 revealing high genetic diversity.

1. Introduction

Chrysanthemum is a major horticultural crop and it is the second largest in terms of cut flowers after rose, among the ornamental plants traded in the global flower market (Kumar *et al*., 2006). The common garden chrysanthemum is hexaploid with 54 chromosomes (Wolff, 1996). It is propagated vegetatively and has a strong self incompatibility system (Richard, 1986), hence new cultivars are difficult to obtain by crossing. Traditionally, new cultivars have been obtained from spontaneous mutations in vegetative reproduction, sports, being some variations more stable than others (Miñano *et al*., 2009). In the last few years, induced mutations and somaclonal variations derived from the tissue culture process have been employed as a new source of variability (Schum, 2003; Datta *et al*., 2005; Jain and Spencer, 2006; Zalewska *et al*., 2007; Jain, 2010; Barakat *et al*., 2010).

Although extensive work has been carried out to develop novelties in chrysanthemum through induced mutations using physical and chemical mutagens (Broetjes and Van Harten, 1978), there is always a need to explore the possibility of new variety for floriculture trade. Mutation breeding by radiation has been widely used to upgrade well-adapted plant varieties and also to develop new variations within improved agricultural characteristics. Since most cultivated chrysanthemum cultivars are polyploids with high genetic heterogeneity, mutants with allied flower colour, shape, floral size and shape are often recovered. Allied flower colours with chimeric tissue can be easily induced by radiation and can be isolated using *in vitro* tools (Kumar *et al*., 2006). Identification and characterization of cultivars is extremely important in horticultural crops in order to protect the plant breeder's rights. Traditionally, identification has been based on morphological characters; however the development of new technologies has made it possible to base this analysis on DNA information. One approach is a PCR-based technique - RAPD (William *et al*., 1990) - that has been widely used for plant germplasm characterization (Wolff *et al*., 1994; Huang *et al*., 2000; Martin *et al*., 2002; Martin and Gonzalez-Benito, 2005). The aim of the present study was to induce mutation in chrysanthemum cv. Snow Ball through *in vitro* mutagenesis by treating the *in vitro* shoots with gamma radiation and to apply RAPD analysis for the detection of genetic polymorphism among the mutants and control.

2. Materials and Methods

Plant material and in vitro mutagenesis

Nodal segments (2-3 cm) of *Chrysanthemum grandiflora* (Tzelev) cv. Snow Ball, collected from one-

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year-old mother plants maintained in the glasshouse of the Department of Biotechnology, University of Horticulture and Forestry Solan (Himachal Pradesh), India, were used as explants. The explants were surface sterilized with 5% sodium hypochlorite ($NaOCl_2$) for 10 min, washed thoroughly three to four times with sterilized water and cultured on MS (Murashige and Skoog, 1962) medium supplemented with 2 mg/l benzyladenine (BA), 0.5 mg/l α-naphthalene acetic acid (NAA), 30 g/l sucrose (w/v) and 8 g/l agar (w/v; Sigma-Aldrich, Bangalore). The culture without growth regulators served as control. The pH of the medium was adjusted to 5.8 before autoclaving at 121ºC at a pressure of 1.1 kg cm^{-2} for 15 min. The cultures were maintained under 16-hr photoperiod with a light intensity of 50-60 μmol cm^{-2} s^{-1} provided by cool, white fluorescent lamps at 24±2ºC. After four weeks of culture, the shoots were subcultured on shoot multiplication medium consisting of MS salts supplemented with 0.5 mg/l BA, 0.1 mg/l indole acetic acid (IAA) and 1 mg/l gibberellic acid (GA_3). Shoots about 2.5-3.0 cm long were irradiated in a gamma cell (60 cobalt source) with 5, 10, 20 and 30 Gy doses. After four weeks, the shoots were multiplied and 3-4 cm long shoots were transferred to $^1/_2$ strength MS medium supplemented with 0.3 mg/l indolebutyric acid (IBA) and 0.2% activated charcoal for rooting. The shoots with well developed roots were removed from the culture vessel, washed with running tap water to remove the adhering agar and transferred to plastic pots (25 cm diameter) filled with soil:sand:FYM in 1:1:1 ratio and kept in the glasshouse with 80-90% relative humidity. After acclimatization for four weeks, the plants were observed for any variations in morphological characters from the control and PCR analysis was carried out using the genomic DNA.

Statistical analysis

The data recorded for different parameters were subjected to completely randomized design (Gomez and Gomez, 1984). The statistical analysis based on mean value per treatment was made using analysis of variance (ANOVA). The LSD multiple range test ($p \leq 0.05$) was used to determine differences between treatments.

Polymerase chain reaction

Genomic DNA was isolated from fresh, young green leaves of the control and gamma ray-irradiated plants following the method of Doyle and Doyle (1987) with some modifications. For amplification, reaction mixtures (21.8 μl) contained 1 μl oligonucleotide, 2.5 μl 10X Taq polymerase buffer, 1.5 nM $MgCl_2$, 2 μl each of dNTPs, 3 μl genomic DNA and 3 μl Taq polymerase (3 units/μl). Random oligonucleotide primers were used for RAPD amplification (Bangalore Genei, Bangalore, India). Amplification was performed in a thermocycler (MJ Research, USA) programmed for a first denaturation step of 3 min at 94ºC followed by 36 cycles of 94ºC for 30 s for denaturation, 50ºC for 30 s for annealing, and 72ºC for 2 min (extension). Twenty Operon random primers (Operon Technologies, Inc., USA), ten of the OPA series (1-10) and ten of the OPB series (1-10), were employed for amplification using the cycling condition mentioned above.

All samples were given 10 min at 73ºC for post-amplification. PCR products were separated on a 1.4% agarose gel using 1 x TAE buffer and were stained with ethidium bromide. In all amplification reactions, a reaction mixture with water instead of genomic DNA was used as negative control. 1 kb DNA ladder (Fermantas, Lithuania) was used as the size marker. The amplified products were visualized using a UV transilluminator and photographed using gel documentation system (Alpha Imager, USA). Amplified DNA was scored as either presence (1) or absence (0) of band. Pairwise comparison between the control and variants was performed to calculate similarity (J) between the samples (Jaccard, 1908) using SIMQUAL programme of NTSYS-PC (version 2). A dendrogram was produced from the resulting similarity using UPGMA method.

3. Results

Effect of gamma radiation on in vitro *cultures*

The data presented in Table 1 reveal that the survival of shoots was affected by gamma ray doses. A decrease in survival was observed with an increase of irradiation dose; a significantly higher survival percentage was observed in the control shoots. The variation in survival percentage between 5 and 10 Gy was statistically insignificant. A decrease was also observed on shoots forming roots. A lethal effect of higher doses (20 and 30 Gy) was noticed on rooting of shoots. The shoots did not root, hence they turned yellowish brown and died. No variation was observed in shoots forming roots with 5 and 10 Gy irradiation. A significant difference in percent root initiation was observed between the control and irradiated shoots. The rooted plants were acclimatized as explained above and allowed to grow in the glasshouse till flowering.

Table 1 - Effect of radiation doses on survival of *in vitro* shoots in *D. grandiflora*

Dose strength	Survival (%)	Number of shoots producing roots (%)
0	90.47 (79.93)	100.00 (90.90)
5	66.60 (59.25)	82.60 (65.48)
10	52.38 (46.39)	82.35 (65.40)
20	42.85 (40.83)	0(0)
30	19.05 (20.15)	0(0)
$LSD_{0.05}$	25.11 (21.89)	6.25 (4.88)

Figures within parentheses are arc sine transformed values.

Effect of gamma radiation on morphological characters

Morphological variations were not observed in the acclimatized plants exposed to 5 Gy gamma radiation. All the plants flowered true to the mother floret colour/shape. Therefore, selections were made among the plants exposed to 10 Gy gamma radiation for agronomic and molecular characterization of mutants.

At flowering stage, plants in the glasshouse were observed for any variations in morphological characters. Results revealed that there were 10 variants with different morphological characters compared to the control. The data presented in Table 2 reveal that morphological characters of *D. grandiflorum* cv. Snow Ball and its mutant were statistically, highly significantly affected by gamma ray doses.

Plant height

Plant height was significantly reduced in the variants compared to control (Table 2). A significant variation in plant height was also observed among the variants (Table 3). Variant V10 gave the significantly highest value for plant height (69.00 cm), compared with the control (62.10 cm). Variant V8 showed the lowest value for plant height, followed by variant V2. The plant height was significantly lower in most of the variants as compared to the control.

Leaf number and leaf area (cm^2)

The number of leaves was significantly reduced in the variants compared to control (Table 2). There was a significant increase in the number of leaves in variant V4. In general, the number of leaves in the variants was lower compared to the control. Variant V2 had the fewest number of leaves, followed by variants V7 and V8. Statistically leaf area did not differ between the variants and control (Table 2), however leaf area differed among the variants (Table 3). Variant V7 had significantly higher mean values of leaf area, followed by variants V10, V2 and V9 in comparison to the control.

Number of flower buds and flowers

Table 2 reports that the number of flower buds was significantly different between the variants, whereas the difference in the number of flowers was not significant. The number of flower buds and flowers varied among the variants (Table 3) with variants V3 and V8 having a significantly lower number of flower buds and flowers, followed by variant V7. It was observed that in most of the variants about 82-83% of buds opened into flowers while in variant V3 only 50% of the buds opened into flowers.

Flower diameter and flower colour

The variation in flower diameter between the control and the variants was statistically not significant (Tables 2 and 3). The original flower colour of the cv. Snow Ball was white with incurve ray florets (Fig. 1A), whereas one branch of variant V9 produced yellow-coloured flowers with incurve ray florets (Fig. 1B). The results indicate that the irradiation dose of 10 Gy was an effective dose in inducing mutations in flower colour, but no changes were observed in flower shape and size.

Table 2 - Analysis of variance of the morphological characters of control and variants of chrysanthemum

Treatment	Plant height (cm)	Number of leaves	Leaf area (cm^2)	Number of buds	Number of flower	Flowers diameter (cm^2)
Control	62.10	38.70	8.60	15.10	11.40	6.60
Variants	54.80	31.60	10.93	11.70	8.10	6.50
$LSD_{0.05}$	2.97	4.18	2.35	4.05	3.44	1.58

Table 3 - Effect of gamma radiation (10 Gy) on morphological characters in *D. grandiflora*

Treatment	Plant height (cm)	Number of leaves	Leaf area (cm^2)	Number of buds	Number of flower	Diameter flowers (cm^2)
Control	62.10	38.70	8.61	15.10	11.40	6.60
V1	56.00	23.00	7.39	15.00	10.00	5.50
V2	40.00	16.00	13.00	5.00	4.00	6.00
V3	60.00	40.00	8.48	14.00	7.00	8.00
V4	58.00	49.00	7.87	16.00	9.00	5.50
V5	60.00	38.00	10.85	14.00	11.00	6.60
V6	55.00	35.00	10.48	10.00	7.00	7.30
V7	58.00	19.00	15.16	7.00	5.00	6.00
V8	37.00	19.00	9.32	5.00	3.00	7.50
V9	55.00	40.00	12.56	13.00	10.00	6.80
V10	69.00	37.00	14.15	18.00	15.00	6.60

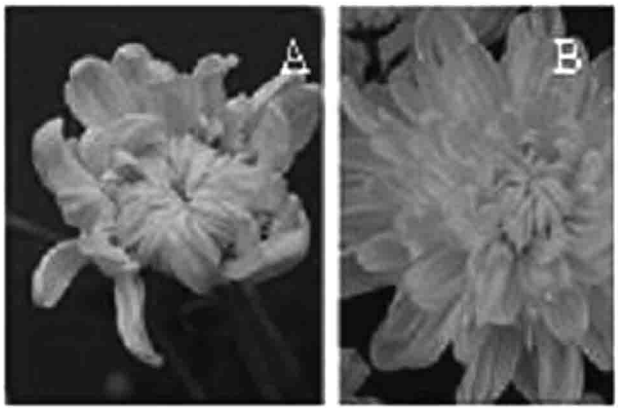

Fig. 1 - Induced somatic mutations in chrysanthemum cv. Snow Ball after 10 Gy gamma radiation treatment. (A) control, white-coloured flowers; (B) mutated, yellow-coloured flower.

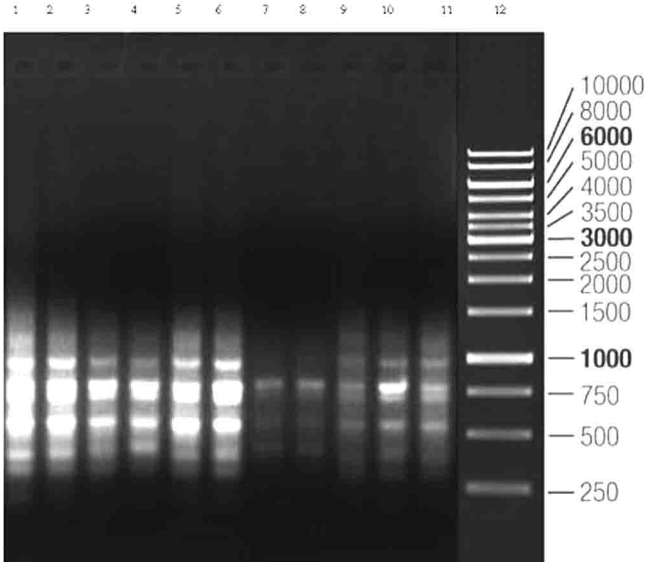

Fig. 2 - RAPD profile with primer OPB-4 showing polymorphism in control and its variants in chrysanthemum cv. Snow Ball. Lanes 1- 10: variants (V1 to V10); Lane 11: control; Lane 12: DNA ladder (1kb).

Characterization

Twenty decamer primers (Table 4) were used for the amplification of genomic DNA of control and mutants of *Dendranthema grandiflora* Snow Ball. The number of DNA fragments amplified ranged from one to five depending upon the primer and the DNA sample with a mean value of 2.90 bands per primer (Table 4). The amplification product ranged from 250 to 3000 bp. A total number of 58 markers were produced by the 20 primers. A total of 100% of the 58 scored bands were polymorphic in 11 genotypes (one control and its ten variants). Figure 2 shows the amplification profiles, generated by primer OPB-4 across the chrysanthemum genotypes. The RAPD markers produced by the 20 primers were used to construct a similarity matrix (Table 5). Five clusters can be observed. The first cluster includes only

Table 4 - Nucleotide sequences and RAPD amplification results of the primer used in the PCR amplification

Primer	Sequence (5'-3')	Scored bands	Polymorphic bands	Polymorphism (%)
OPA-1	GTTTCGCTCC	3	3	100
OPA-2	TGATCCCTGG	4	4	100
OPA-3	CATCCCCCTG	3	3	100
OPA-4	GGACTGGAGT	1	1	100
OPA-5	TGCGCCCTTC	4	4	100
OPA-6	TGCTCTGCCC	5	5	100
OPA-7	GGTGACGCAG	4	4	100
OPA-8	GTCCACACGG	4	4	100
OPA-9	TGGGGGACTC	0	0	0
OPA-10	CTGCTGGGAC	2	2	100
OPB-1	CAGGCCCTTC	4	4	100
OPB-2	TGCCGAGCTG	1	1	100
OPB-3	AGTCAGCCAC	1	1	100
OPB-4	AATCGGGCTG	3	3	100
OPB-5	AGGGGTCTTG	2	2	100
OPB-6	GGTCCCTGAC	2	2	100
OPB-7	GAAACGGGTG	4	4	100
OPB-8	GTGACGTAGG	3	3	100
OPB-9	GGGTAACGCC	3	3	100
OPB-10	GTGATCGCAG	5	5	100
Total		58		58

Table 5 - Jaccard's similarity matrix in control (C) and mutated (V) plants based on RAPD analysis

	C	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10
C	1.00										
V1	0.32	1.00									
V2	0.31	0.79	1.00								
V3	0.31	0.67	0.54	1.00							
V4	0.28	0.76	0.65	0.67	1.00						
V5	0.37	0.37	0.37	0.37	0.42	1.00					
V6	0.20	0.30	0.26	0.32	0.32	0.37	1.00				
V7	0.29	0.65	0.68	0.53	0.72	0.40	0.45	1.00			
V8	0.20	0.50	0.54	0.37	0.50	0.26	0.37	0.62	1.00		
V9	0.06	0.27	0.26	0.28	0.29	0.24	0.26	0.33	0.25	1.00	
V10	0.28	0.62	0.61	0.44	0.51	0.31	0.32	0.62	0.59	0.35	1.00

variant 9 (V9); the second includes the control (C); the third includes variants 5 and 6 (V5 and V6); the fourth includes variants 8 and 10 (V8 and V10), and the last cluster includes variants 3, 4, 7, 2 and 1 (V3, V4, V7, V2, V1). It can be seen from figure 3 that the shortest genetic distance was found between variant 1 (V1) and variant 2 (V2), whereas the greatest distance was observed between the control and variant 9 (V9).

4. Discussion and Conclusions

Analysis of explant sensitivity is one of the basic requirements for an effective use of mutation induction in plant breeding programmes (Walther and Sauer, 1986). In the present study, the radio sensitivity of shoots was assessed by survival and rooting of shoots after irradiation in order to select a suitable dose of gamma irradiation. Predieri (2001) also reported the necessity to identify an appropriate dose to apply in mutagenic treatments. The effect of gamma radiation on *in vitro* cultures in crop breeding has been studied by many workers (Shen *et al*., 1990; Charbaj and Nabul, 1999; Predieri and Gatti, 2000; Datta *et al*., 2005; Barakat *et al*., 2010).

Reductions in survival, plant height, number of leaves and flowers were recorded after gamma irradiation. Mutation in flower colour was detected as chimera in one branch of the plant, which produced yellow-coloured flowers (Group 5C, Fan 1) (British Colour Council, 1938). In order to check the stability of flower colour, the mutated branch was propagated vegetatively. The plants were grown in a glasshouse, where they expressed the same colour/shape.

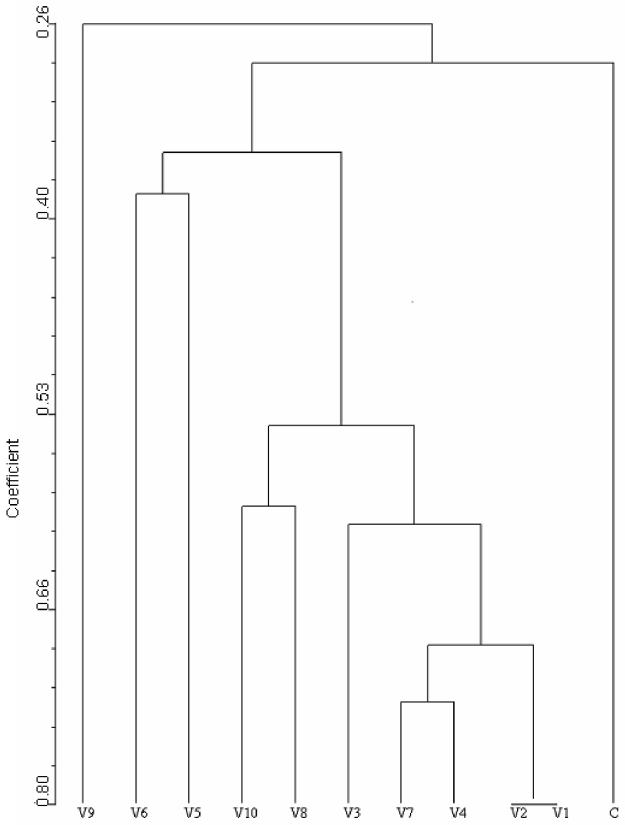

Fig. 3 - Dendrogram of control and mutants of chrysanthemum cv. Snow Ball

Over recent years, RAPD analysis has been used in ornamental breeding to characterize genotypes and to identify genes controlling important traits (Huang *et al*., 2000; Rumińska *et al*., 2004; Kumar *et al*., 2006; Miñano *et al*., 2009). In the present study, a high level of polymorphism was observed in 10 radiomutants of chrysanthemum. Wolff and Van Rijn (1993) also noticed a high degree of polymorphism in chrysanthemum cultivars using RAPD markers. This high level of polymorphism may be due to the strict out-crossing, resulting in a higher level of heterozygosity in chrysanthemum (Wolff *et al*., 1994). RAPD markers were used to construct a similarity matrix and the results of characterization analysis revealed a high diversity between the control and its somaclones. The greatest genetic distance between control and variant 9 (V9) may be due to the fact that V9 is a somaclone where one branch was mutated and produced yellow-coloured flower heads. Although the plants differed in flower colour, bands specific for flower colour could not be distinguished due to the resolution capacity of the tested primers. Wolff (1996) reported that the choice of the primers may be an important factor in obtaining a rapid discrimination between samples. Barakat *et al*. (2010) reported that mutants with different flower colour could be identified at the molecular level using RAPD technique, holding promise to identify unique genes as SCAR markers. Bhattacharya and Teixeira da Silva (2006) attempted to understand the molecular systematic and genetic difference between 10 original chrysanthemum cultivars and 11 mutants and reported that similarity ranged from 0.17 to 0.90 using rapid analysis. Kumar *et al*. (2006) reported genetic distances from 0.43 to 0.96 between 13 chrysanthemum cultivars. A great genetic distance among the different cultivars showed the existence of introgressing new and novel genes from the chrysanthemum gene pool. It may be suggested, with regard to the present study, that by using RAPD markers, it is possible to differentiate newly evolved chrysanthemum cultivars from their parents which can be a useful tool to supplement the distinctness, uniformity and stability analysis for plant variety protection in future. Our results are in accordance with those of other workers who reported RAPD as a powerful tool in the assessment of genetic variability as well as for genetic characterization, allowing differentiation of chrysanthemum mutants/cultivars (Huang *et al*., 2000; Rumińska *et al*., 2004; Kumar *et al*., 2006; Chatterjee *et al*., 2006; Miñano *et. al*., 2009).

Therefore, it may be concluded that 10 Gy gamma irradiation was most effective in inducing variations in flower colour and other morphological characters. Morphological variations were not observed with the 5

Gy gamma dose and a lethal effect of 20 and 30 Gy doses was observed on rooting and survival of *in vitro* shoots. One hundred percent polymorphism was observed among the radiomutants using PCR technique. The present results also indicate the applicability of gamma radiation in crop breeding, and the assessment of genetic variability and characterization of radiomutants at genomic level by RAPD can be a useful tool in breeding programs aimed to improve ornamental characters of chrysanthemum cultivars.

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