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Molecular Characterization of Chrysanthemum (*Dendranthema grandiflora* Tzvelev) Mutants using RAPD Analysis

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ABSTRACT

Chrysanthemum (*Dendranthema grandiflora* Tzvelev) is commercially cultivated as a cut flower, loose flower and pot plant. Physical and chemical mutagens were used in this study to induce variation in flower colour and form. Twenty five mutants from two chrysanthemum cultivars 'Ravi Kiran' (17) and 'Acc.116' (8) were characterized by RAPD to understand the extent of diversity and relatedness. Out of 28 random primers screened, 25 gave reproducible polymorphic bands. Cluster analysis of the mutants of both cultivars revealed that they fell into two major groups. In 'Ravi Kiran', of the two groups obtained, mutants caused by 0.5 kR gamma (γ) rays (3) obtained through *in vitro* mutation induction showed high genetic diversity with a maximum of 32 flowers per plant compared to the other mutants and parents. In 'Acc.116', of the two groups, mutants induced by 0.3% ethyl methane sulphonate (EMS) (14) and 0.5 kR γ rays + 0.1% EMS (*in vitro*) showed high genetic diversity with a maximum number of viable mutants (11) compared to the other mutants and parents. RAPD markers can be a useful tool to determine the genetic relationship among chrysanthemum mutants.

Keywords: mutagenesis, polymorphism
Abbreviations: RAPD, random amplified polymorphic DNA; **EMS**, ethyl methane sulphonate; **kR**, kilorad; **PCR**, polymerase chain reaction

INTRODUCTION

Flowers have been associated with mankind since time immemorial. Floriculture is fast emerging as a sunrise business activity and has taken a different perspective in the horticultural business domain. Enormous flower shops have materialized the markets in cities and towns across the country after westernization. Market led floriculture has assumed importance worldwide and demand for the flowers are being satisfied based on consumer preference.

The consumer preference has been changing from time to time for both traditional and cut flowers and there is demand for varieties based on colour, shape, size, shelf life, etc. Due to this phenomenal change, at present, India's floriculture industry has attained a status of a huge opportunity sector aiming to become an industry worth of Rs. 100,000 million by 2010 (Pawar 2007). Singh (2009) stressed the importance of research in developing high yielding varieties with year round production in chrysanthemum.

Chrysanthemum, *Dendranthema grandiflora* (Ramat.) Kitamura (Compositae), originates from the Greek *krus anthemon*, meaning gold flower, and has its roots in China and Japan (Fukai 1995). This culturally rich flower is also globally the second economically most important floricultural crop following rose, and one of the most important ornamental species, with the production value increasing exponentially as a result of the rapid improvement of living conditions and a greater enjoyment of life (Teixeira da Silva 2004). Chrysanthemum (*Dendranthema grandiflora* Tzvelev) is commercially cultivated as cut flower, loose flower and pot plant. The commonly grown chrysanthemums are hexaploid complex with average number of 54 chromosomes (Wolff 1996). It is propagated vegetatively as it has a strong sporophytic self-incompatibility as shown by the members of Asteraceae family. Since there exist a variety of tint and form in chrysanthemum, the ultimate challenge ahead the breeders is to develop a novel variety with really an outstanding bloom of ornamental value.

Mutation breeding has been widely applied in number of horticultural crops to obtain new varieties. By 2005, 2335 varieties were released through mutagenesis in the world, in which ornamental crops and decorative crops are 552 varieties (IAEA 2005). India has an impressive share as it has commercially released 46 mutant cultivars in chrysanthemum alone in year 2004 (Chopra 2005). This method of breeding is widely utilized to improve one or two traits of an outstanding cultivar. Induced mutations often lead to altered flower colour, shape, growth habit and other traits. Since the effect of mutation in ornamentals is clearly visible, selection is possible in M1 generation itself as most of the ornamental crops are vegetatively propagated. But the challenge associated with mutation breeding is that in the chimeric tissue, mutated cells are present along with the normal cells. During subsequent cell division, mutated cells compete with the surrounding normal cells for survival (diplontic selection). If these mutated cells survive in diplontic selection, they are expressed in plants (Datta *et al*. 2005). Hence *in vitro* mutagenesis serves as a rapid method for creation of solid mutants in chrysanthemum. The probability of plant regeneration from all the mutated tissues is also high.

In vitro culture in combination with induced mutations can speed up breeding programme for the generation of variability through selection, especially for flower colour and shape. This overcomes the problem of chimera formation. Currently, induced mutants also play an important role in plant molecular genetics. Progress in molecular studies relies to a greater extent on induction and identification of mutants which is used as a tool for study of developmental genetics and for further breeding programmes. Williams *et al*. (1990) developed DNA fingerprinting using RAPD markers. Since then, chrysanthemum cultivars and other mem-

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bers of Asteraceae have been characterized using RAPD technique (Wolff *et al*. 1995; Wolff 1996; and Martin *et al*. 2002). RAPD method was used earlier to study genetic variability in lady group of chrysanthemum (Ruminska *et al*. 2004).

Hence, molecular assisted breeding provides rapid and efficient methods for analyzing mutated generations by assessing the degree of polymorphism. With this in view, the present investigation was taken up – in chrysanthemum – to induce variability in flower colour and form.

MATERIALS AND METHODS

Two varieties, 'Ravi Kiran' (cut flower type) and 'Acc.116' (pot mum type) were selected for the study with the aim of inducing variation in flower colour and dwarfness. Mutation was induced by both *in vivo* and *in vitro* techniques. Gamma rays and ethyl methane sulphonate were the mutagens used individually and in combinations. The promising mutants of the varieties were isolated. The main objective of the study was to differentiate the promising mutants.

In vivo mutation

1. Gamma irradiation

The rooted cuttings were subjected to gamma irradiation at varying levels *viz*., 1.00, 1.50, 2.00 kR. Gamma chamber - 1200 established at the Center for Plant Breeding and Genetics, Tamil Nadu Agricultural University, Coimbatore, installed and maintained by the Board of Radiation and Isotope Technology (BRIT), DAE, Mumbai. Cobalt - 60 (^{60}Co) emitting 5000 rads per minute at the time of irradiation was used as the source of gamma rays. The formula suggested by Kodym and Afza (2003) was used for the calculation of duration of exposure.

The rooted cuttings were planted in pots immediately after irradiation. Untreated rooted cuttings were maintained as control.

2. Ethyl methane sulfonate (EMS)

Ethyl methane sulfonate ($CH_3SO_2OC_2H_5$) procured from M/s. Sigma-Aldrich Company, U.S.A was used as the chemical mutagen. Molecular weight of EMS is 24.60 g and the density is 1.17 g ml^{-1} respectively. EMS concentrations of 0.1, 0.2 and 0.3% were used in the present study. Rooted cuttings were soaked in different concentrations of aqueous solutions of EMS for five hours at room temperature with intermittent shaking. Then the treated cuttings were washed thoroughly and planted in pots. Untreated cuttings were maintained as control.

3. Combination of gamma rays and EMS

The rooted cuttings of Ravi Kiran and Acc.116 were first treated with gamma rays and then they were soaked in EMS solution for 5 hours and planted in pots. Untreated cuttings were maintained as control.

In vitro mutation

1. Gamma radiation

The explants after initial disinfection were subjected to gamma irradiation at 0.5 and 1.0 kR and then sterilized with ethanol and mercuric chloride and inoculated in the medium and incubated in the culture room at 25 ± 2ºC at a relative humidity of 60 to 70 per cent with a light intensity of 3000-Lux using white fluorescent lamps. A photoperiod of 16/8 h light and dark cycle was maintained.

2. Ethyl methane sulfonate (EMS)

The ray florets and nodal segments after surface sterilization were soaked in EMS solution (0.1, 0.2 and 0.3%) for 1 hour and washed thoroughly with sterile distilled water and inoculated in MS medium with growth regulators (as per the protocol standardized).

Table 1 Details of Ravi Kiran and its mutants used in RAPD analysis.

1.	Control
2.	1.00 kR γ rays + 0.1% EMS (*in vivo*)
3.	0.50 kR γ rays (*in vitro*)
4.	1.00 kR γ rays (*in vitro*)
5.	1.50 kR γ rays (*in vitro*)
6.	0.3% EMS (*in vitro*)
7.	0.50 kR γ rays + 0.1% EMS (*in vitro*)
8.	1.50 kR γ rays + 0.1% EMS (*in vitro*)

Table 2 Details of Acc. 116 and its mutants used in RAPD analysis.

1.	Control
2.	1.00 kR γ rays (*in vivo*)
3.	1.50 kR γ rays (*in vivo*)
4.	2.00 kR γ rays (*in vivo*)
5.	0.1% EMS (*in vivo*)
6.	0.2% EMS (*in vivo*)
7.	0.3% EMS (*in vivo*)
8.	1.00 kR γ rays + 0.1% EMS (*in vivo*)
9.	1.50 kR γ rays + 0.1% EMS (*in vivo*)
10.	2.00 kR γ rays + 0.1% EMS (*in vivo*)
11.	1.00 kR γ rays (*in vitro*)
12.	1.50 kR γ rays (*in vitro*)
13.	0.2% EMS (*in vitro*)
14.	0.3% EMS (*in vitro*)
15.	0.50 kR γ rays + 0.1% EMS (*in vitro*)
16.	1.00 kR γ rays + 0.1% EMS (*in vitro*)
17.	1.50 kR γ rays + 0.1% EMS (*in vitro*)

Table 3 List of random primers used in RAPD analysis in this study.

Primer	Sequence
OPA-01	CAGGCCCTTC
OPA-05	AGGGGTCTTG
OPA-07	GAAACGGGTG
OPA-08	GTGACGTAGG
OPA-13	CAGCACCCAC
OPA-15	TTCCGAACCC
OPB-04	GGACTGGAGT
OPB-07	GGTGACGCAG
OPF-01	ACGGATCCTG
OPF-02	GAGGATCCCT
OPF-03	CCTGATCACC
OPF-04	GGTGATCAGG
OPF-05	CCGAATTCCC
OPF-06	GGGAATTCGG
OPF-07	CCGATATCCC
OPF-08	GGGATATCGG
OPF-09	CCAAGCTTCC
OPF-10	GGAAGCTTGG
OPF-11	TTGGTACCCC
OPF-12	ACGGTACCAG
OPF-13	GGCTGCAGAA
OPF-14	TGCTGCAGGT
OPF-15	CCAGTACTCC
OPF-16	GGAGTACTGG
OPF-17	AACCCGGGAA
OPF-18	TTCCCGGGTT
OPF-19	CCTCTAGACC
OPF-20	GGTCTAGAGG

The untreated explants served as control. After inoculation, the cultures were incubated in culture room.

3. Combination of gamma rays and EMS

The explants were soaked in EMS solution after sterilization and then inoculated on MS medium and the cultures were incubated in the culture room until the initial establishment was noted. Then the culture bottles were exposed to gamma rays and the same were incubated in the culture room.

Plant material

Fresh leaves from 8 samples (1, control; 7, mutants) of 'Ravi Kiran' (**Table 1**) and 17 samples (1, control; 16, mutants) of 'Acc.116' (**Table 2**) were collected from the plants grown in pots.

DNA extraction

DNA was extracted from one gram of the fresh leaves ground in liquid nitrogen. Genomic DNA was extracted by the method recommended by Gawal and Jarret (1991) since the yield of DNA was high. The quality and quantity of the DNA was assessed on a 0.8% agarose (Bangalore Genei Ltd., India) gel. DNA concentration for PCR amplification was estimated by comparing the band intensity of a sample with the band intensities of known dilutions of 1 kb λ DNA marker (Fermentas, Germany). Based on the intensity the DNA was further fractionated to the required concentration (25-50 ng) using sterile double distilled water.

RAPD analysis

A total of 28 primers synthesized at Sigma-Aldrich, Bangalore were used for the study (**Table 3**). Amplication reactions were in volumes of 25 µg containing 25 ng of genomic DNA, 2.50 µl of 10x PCR buffer, 0.50 µl dNTPs (2.5 mM) (Bangalore Genei Ltd., India), 1.00 µl of primer (Operon Technologies Inc., U.S.A)and 3 units/µl of *Taq* DNA polymerase (Bangalore Genei Ltd., India).

Amplifications were performed in thermal cycler (PTC-100^{TM} MJ Research Inc., U.S.A) programmed for an initial denaturation at 94^{o}C for 5 min, 35 cycles of 1 min denaturation at 94^{o}C, 1 min at a specific annealing temperature for each primer and 2 min extension at 72^{o}C and a final extension of 10 min at 72^{o}C and then at 4^{o}C until storage. Along with the PCR amplified products, 1 kb DNA ladder as standard marker were subjected to electrophoresis in 1.5% agarose gel in 0.5X TBE buffer at 8 v/cm for 4 to 5 hrs. The electronic image of the ethidium bromide stained gel was visualized and documented in gel documentation system (Model Alpha imager 1200, Alpha Innotech Corp., U.S.A).

Data scoring and analysis

Eight samples for 'Ravi Kiran' and 17 samples for 'Acc.116' were used for the study and were replicated thrice. Scoring was carried out only for the clear and unambiguous bands. The mutants and the parents were scored for the presence and absence of bands. The scores '0' and '1' were given respectively for the presence and absence of bands. RAPD bands within a genotype is scored as missing if they were poorly resolved on the gel or if the template DNA did not amplify well. Genetic distance was calculated on the basis of Jaccard's coefficient method. A dendrogram was constructed using the TREE procedure by the Numerical Taxonomy and Multivariate Analysis System (NTSYS) based on Jaccard's similarity coefficient using Unweighted Pair Group with Arithmetic Mean method (UPGMA).

RESULTS AND DISCUSSION

Among the 28 primers screened 25 gave clear and reproducible bands. Bands generated by RAPD fragments were of molecular weight ranging from 100 bp to 2500 bp (**Fig.**

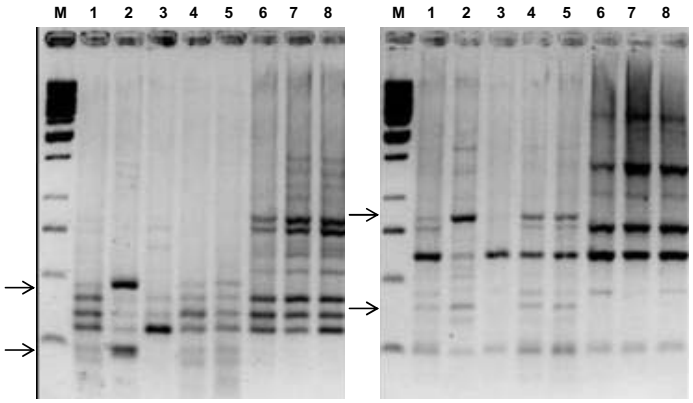

Fig. 1 RAPD analysis in Ravi Kiran and its mutants. Left: primer OPF-14; Right: primer OPF-15. Lanes 1-8 = treatments 1-8 in **Table 1**. Arrows represents bands which are different from the control indicating mutations.

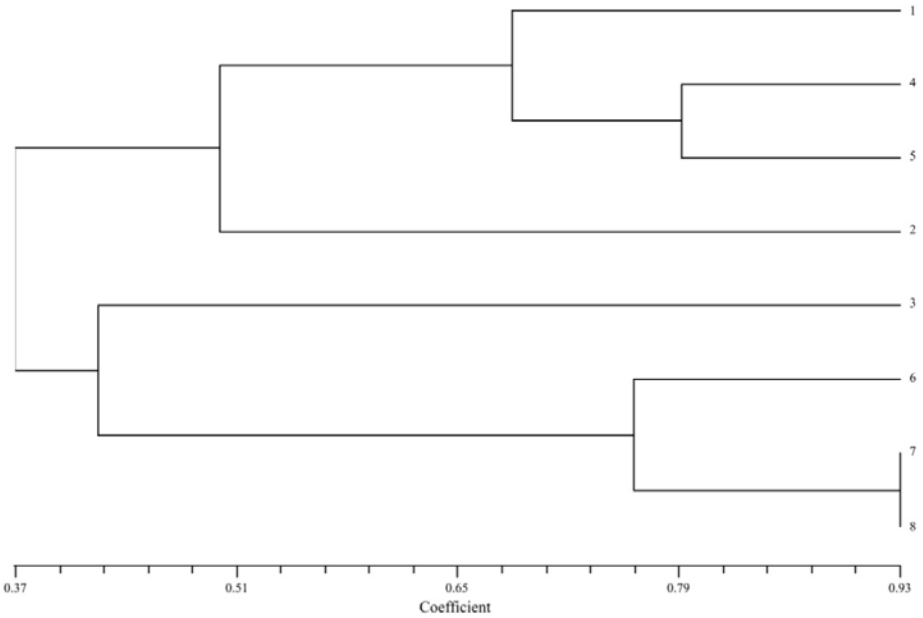

Fig. 2 Dendrogram for Ravi Kiran and its mutants. Numbers 1-8 represent lanes 1-8 in **Fig. 1**.

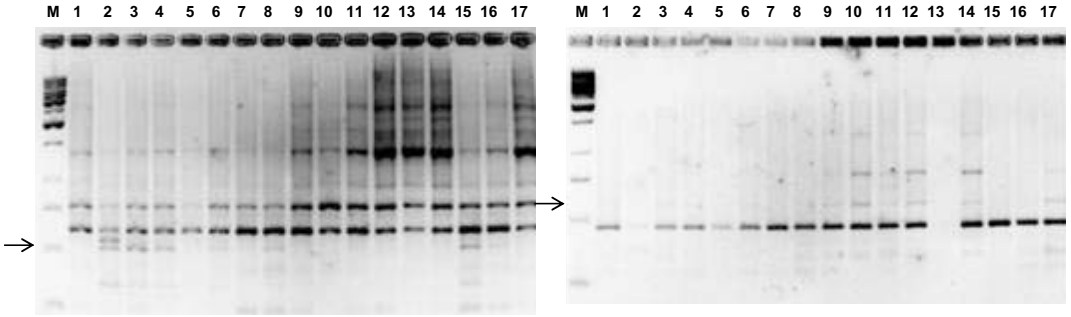

Fig. 3 RAPD analysis in Acc. 116 and its mutants. Left: primer OPF-14; Right: primer OPF-15. Lanes 1-8 = treatments 1-17 in **Table 2**. Arrows represents bands which are different from the control indicating mutations.

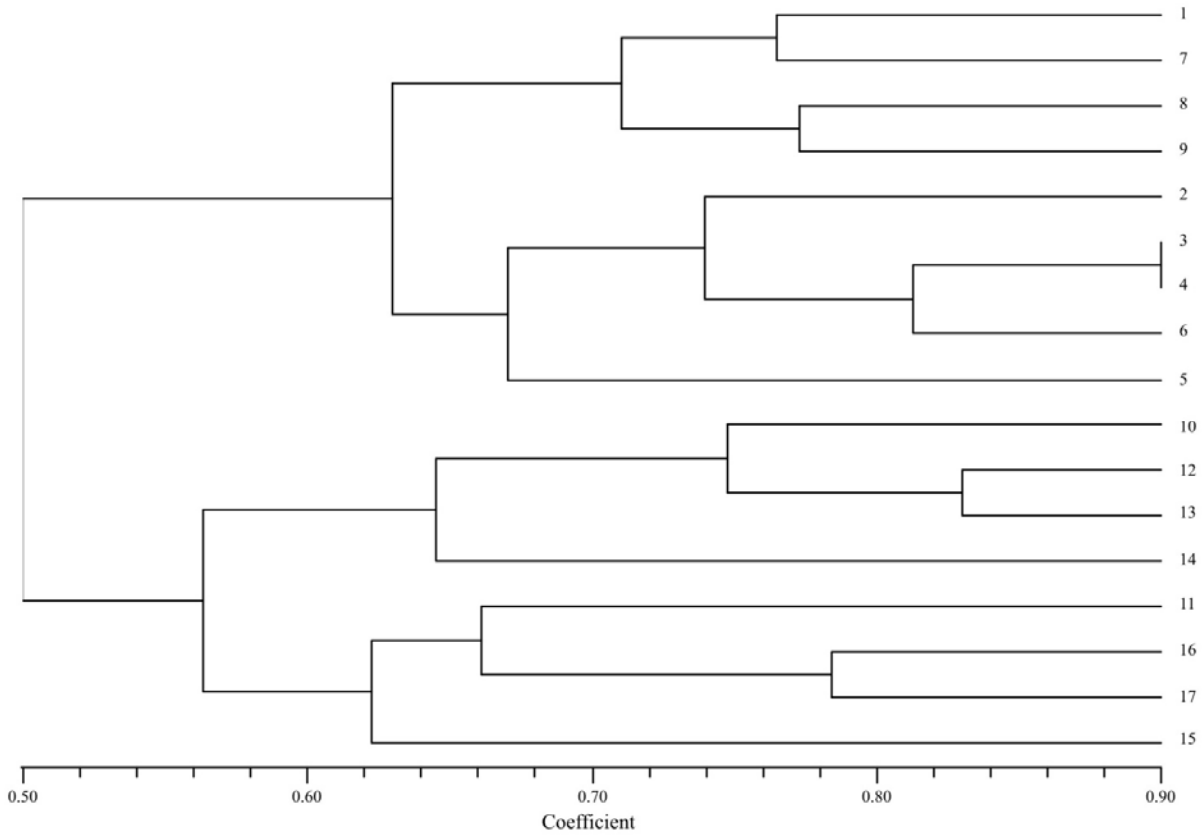

Fig. 4 Dendrogram for Acc. 116 and its mutants. Numbers 1-17 represent lanes 1-17 in **Fig. 3**.

1). Mutant obtained using 1.00 kR gamma rays + 0.1% EMS (2) in *in vivo* treatment in Ravi Kiran produced an additional band at 2000 bp using the primer OPA 15. Bands of 400 bp were present in untreated plants but not in mutants. The mutants from the treatments 0.50 kR gamma rays + 0.1% EMS (7) and 1.50 kR gamma rays + 0.1% EMS (8) had low genetic distance for most of the primers screened (**Fig. 2**). Each primer produced an average of 10 bands per individual.

The Jaccard's similarity values showed that the highest genetic similarity to the original cultivar was observed in mutants 0.50 kR gamma rays + 0.1% EMS (7) and 1.50 kR gamma rays + 0.1% EMS (8) and the lowest was in 1.00 kR gamma rays + 0.1% EMS (2) and 0.50 kR gamma rays (3). The cluster analysis separated the mutants into two main groups. Both the groups are divided into two sub groups. Group one includes the parent in a sub group and mutants of 1.00 kR gamma rays (4) and 1.50 kR gamma rays (5) obtained by *in vitro* mutagenesis in the other sub group. Group two includes mutants of 0.50 kR gamma rays (3) in the first sub group while mutants of 0.3% EMS (6), 0.50 kR gamma rays + 0.1% EMS (7), and 1.50 kR gamma rays + 0.1% EMS (8) treatments in the second sub group. Mutants of 0.50 kR gamma rays + 0.1% EMS (7), and 1.50 kR gamma rays + 0.1% (8) were closely related with the similarity co-efficient of 0.93.

In Acc.116, each primer produced an average of 10 to 12 bands per individual. Mutants from the treatment 1.00 kR gamma rays (2) produced a distinct band at 600 bp using the primer OPF 14 (**Fig. 3**) and 500 bp using OPA 8 only in this mutant which shows its diversity from others. Mutants of 1.50 kR gamma rays (3) and 0.2% EMS (6) produced a distinct band at 2000 bp using OPB 7. The cluster analysis separated the mutants into two main groups each being divided into different sub groups (**Fig. 4**). In the first group, mutants of 1.50 kR gamma rays (3) and 2.00 kR gamma rays (4) had high similarity. In group one, mutants of 0.1% EMS were distinct from others. In group two, mutants of 2.00 kR gamma rays + 0.1% EMS (10), 1.50 kR gamma rays (3), 0.2% EMS (6) and 0.3% EMS (7) showed similarity for duration of flowering which were placed in a separate sub group. In the sub group two, 1.00 kR gamma rays (2) and 1.50 kR gamma rays (3) were closely related while mutant of 0.50 kR gamma rays + 0.1% EMS (15) was distinct.

The characterization of mutants occupies an important step in the improvement of chrysanthemum. It is often difficult to distinguish mutants having a common progenitor.

Since then, chrysanthemum cultivars and other closely related families of Asteraceae have been characterized by RAPD. Wolff and Van Rijn (1993) noticed a high degree of polymorphism in chrysanthemum cultivars using RAPD markers. Kumar *et al*. (2006) also noticed a high degree of polymorphism in chrysanthemum cultivars using RAPD markers, as did Bhattacharya and Teixeira da Silva (2006).

RAPDs have been widely used for determining the genetic relationships between different related species for identification of cultivars and for estimating the genetic relationships among the crop germplasm (Teixeira da Silva *et al*. 2005). Genetic variations between genetically diverse tissues and three chimeral types were detected earlier using RAPD (Wolff 1996). The main advantage of RAPD method lies in its rapidity, simplicity, and requirement of only small quantity of DNA, applicability to any organism without prior knowledge of nucleotide sequence, ability to generate numerous polymorphisms and to detect mutations (Cheng *et al*. 1997). Kumar *et al*. (2006) detected variability among the chrysanthemum radiomutants using RAPD markers.

Non-related cultivars were distinguished from each other though the level of similarity between them seemed high. However Wolff *et al*. (1995) suggested that there is no possibility at the DNA level to distinguish mutant cultivars derived vegetatively from one original cultivar.

For finger printing closely related genotypes of chrysanthemum, primers which could amplify many closely adjacent complementary sites would be more useful (Ruminska *et al*. 2004).

In the present study, the induced mutants particularly those which resulted from 1.00 kR gamma rays + 0.1% EMS in Ravi Kiran and 1.00 kR gamma rays in Acc.116 showed polymorphic bands which distinguished them from others and suggested greater changes in the genome. This might have been due to point mutations in genes as well as greater chromosomal aberrations, as reported earlier by Wolff (1996) and Shibata *et al*. (1998).

The primers used could not discriminate some of the mutants those derived using 1.50 kR gamma rays + 0.1 % EMS, 1.00 kR gamma rays and 1.50 kR gamma rays in both the cultivars, though they were phenotypically different. The possible causes for the lack of distinct difference of RAPD patterns between these mutants might be that polymorphic RAPD markers might have resulted from the amplification of both coding and non-coding genome regions. Bhattacharya *et al*. (2006) observed low genetic similarity among the mutants and their parents as the mutants did not vary greatly in their morphology.

A large similarity index indicates genetically similar backgrounds while a small similarity index indicates distantly related backgrounds. Although the mutants varied in their morphology especially in flower colour and plant height, bands specific for flower colour or plant height could not be distinguished. Some bands may code for flower colour or plant height, the traits of our concern and it can be verified using SCAR markers and cloning cDNA in the future generations.

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