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Full Length Research Paper

In vitro mutagenesis and identification of new variants via RAPD markers for improving *Chrysanthemum morifolium*

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The objective of this study was to induce mutation in *Chrysanthemum morifolium* cv. 'Delistar White' through *in vitro* mutagenesis by treating the ray florets with two doses of gamma irradiation (0.5 and 1.0 gamma ray) and to apply RAPD analysis for the detection of genetic polymorphism among chrysanthemum mutants and their parent. Callus induction and shoot formation percentages were affected by gamma ray doses, whereas, the variation between medium protocols and the variations due to the interaction among medium protocols and doses were statistically insignificant. The shoot length was decreased with gamma ray treatment in comparison to the control. The results indicated that the irradiation dose 0.5 Gy was the most effective dose in inducing mutations in flower shape and number of florets per flower head, although no change in flower colour was observed, but conversion from tubular florets to spoon-shaped florets indicating no chimera formation, which concludes that flower colour/shape mutations can be developed through direct *in vitro* mutagenesis by avoiding chimeric phase. Five RAPD primers were used to amplify DNA segments from the genomic DNA of chrysanthemum and its 13 somaclones. The genetic similarity among the fourteen genotypes ranged from 0.43 to 0.95. The chrysanthemum cultivar and its 13 somaclones were classified into five clusters.

Key words: *Chrysanthemum morifolium*, *in vitro* mutagenesis, RAPD analysis, somaclones.

INTRODUCTION

Tissue culture studies in *chrysanthemum* are being done as a tool for mutation induction and as a means of micro propagation. However, the ability to regenerate plants from a single cell of florets is a useful approach to establish a mutant in pure form and facilitate the production of a wide range of new flower cultivars as stated by Mandal et al. (2000). Recently, the capability of nine *chrysanthemum* cultivars for plant regeneration has been determined (Barakat et al., 2010). Their results indicated that the cultivar "Delistar White" with the medium protocols A and B could be successfully utilized for further *in vitro* mutagenesis to select several unique traits relevant to chrysanthemum.

Radiation has been successfully used for the development of new flower colour/shape mutants in *Dendranthema*. Therefore, induced mutagenesis through irradiation or chemical treatment has become a very important method for plant breeding, including flower breeding. By 2005, 2335 varieties were released through mutagenesis in the world, in which ornamental crops and decorative crops are 552 varieties (IAEA, 2005). While, India has a particularly impressive share as it has commercially released 46 mutant cultivars in chrysanthemum alone in year 2004 (Chopra, 2005). Identification of varieties or breeding lines is very important in agricultural species, and is particularly interesting in *chrysanthemum* when in many cases the origin of varieties is unknown. Traditionally, identification has been based on morphological characters; however the development of new techniques has allowed basing these analyses on DNA information. One of them is the PCR based technique RAPD (Random Amplified Polymorphic DNA)

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(Williams et al., 1990) that has been widely used for plant germplasm characterization (Wilde et al., 1992; Wolff et al., 1994). The aims of the present investigation were to induce mutation in chrysanthemum cv. 'Delistar White' through *in vitro* mutagenesis by treating the ray florets with two doses of gamma radiation and to apply RAPD analysis for the detection of genetic polymorphism among *chrysanthemum* mutants and their parent.

MATERIALS AND METHODS

In vitro mutagenesis

Ray florets of the cultivar 'Delistar White' were irradiated in a gamma cell with a 60 Cobalt source at the National Center of Radiation Research and Technology, Nasr City, Cairo, Egypt, with the doses of 0.5 and 1 Gy (Walther and Sauer, 1986). The ray floret explants were cultured directly on the two selected culture media protocol A (MS medium supplemented with NAA 0.2 mg/L + BAP 0.5 mg/L) and protocol B (MS + NAA 0.5 mg/L + BAP 1.0 mg/L) then transferred to half strength 1/2 MS medium for rooting.

After four weeks of incubation, callus induction, and somatic embryogenesis response to irradiation was recorded. Calli with somatic embryogenesis, derived from ray floret explants were transferred to the two medium (protocols A and B) and the *in vitro* traits such as shoot formation, shoot length (cm) and the number of shoots per explants were observed. Shoots with 4 - 6 leaves were transferred to the rooting medium after six weeks. After shoots and roots had developed, regenerated plants were washed with tap water to remove agar from the roots and were transplanted to small pots filled with peat moss and perlite (1:1 v/v). The pots were incubated under moist conditions in the greenhouse for acclimatization, After 2 - 3 weeks of hardening; plants were transferred into the greenhouse. Variants that had shown different morphological characters from the parent were selected and PCR analysis was carried out by using the genomic DNA from the *Chrysanthemum morifolium* cultivar "Delistar White", and its mutants which were obtained by *in vitro* mutagenesis.

Statistical analysis

Data were, statistically, analyzed as a 2 - factor experiment (medium protocols and doses) in a Randomized Complete Block Design (RCBD) with ten replicates. Data with percentage was subjected to arcsine transformation prior to statistical analysis (Steel and Torrie, 1980).Comparisons among means were made using the Least Significant Differences Test (LSD).

RCBD (Random Complete Block Design) with 2 replicates was used for morphological character analysis. Comparisons among means were made using the Least Significant Differences Test (LSD). The data were analyzed, using SAS program, version 6 (1985).

Molecular markers

DNA extraction

Frozen young leaves (500 mg) were ground to a powder with peastle and mortar in liquid nitrogen. The powder was poured into tubes, containing 9.0 ml of warm (65°C) CTAB extracting buffer. The tubes were incubated at 65°C for 60 - 90 min. An additive of 4.5 ml chloroform/octanol (24:1) was used and tubes were shaken in mixer for 10 min and centrifuged for 10 min at 3200 rpm. The supernatants were pipetted into new tubes and 6 ml isopropanol was added. After 60 min., the tubes were centrifuged for 10 min at 3200 rpm and the pellets obtained were put in sterile Eppendorf tubes, containing 400 µl of TE buffer of pH 8.0 (10 mM Tris - HCl, pH 8.0 + 1.0 mM EDTA, pH 8.0) (Sagahi - Maroof et al., 1984). The DNA of the genotypes were thus extracted and stored at -20°C until use.

Determination of DNA concentration by U. V. spectroscopy

Dilutions of DNA, by adding 20 µl of the refrigerated DNA solution to 0.98 ml of distilled water in a micro-centrifuge tube, were prepared and mixed well. The U. V. lamp in the spectrophotometer was warmed up for 20 min. and the wave length of the spectrophotometer was set to 260 nm. Distilled water was added to one cuvette as a blank and set the absorbance to zero. The absorbance of diluted DNA was measured. The concentration of DNA was calculated according to Sambrook et al. (1989).

PCR amplification

Five primers (Table 8), obtained from Pharmacia Biotech. (Amersham Pharmacia Biotech., UK Limited, HP79NA, England), were tested in this experiment to amplify the templated DNA. Amplification reaction volumes were 25 µl, each containing 1X PCR buffer with $MgCl_2$ [50 mM KCl, 10mM tris = HCl (pH = 9.0), 2mM $MgCl_2$ and 1% Trition X - 100], 200 µM of each of dATP, dGTP, dCTP and dTTP, 50 PM primer, 50 ng template DNA and 1.5 µl of Taq polymerase. Reaction mixtures were exposed to the following conditions: 94°C for 3 min., followed by 45 cycles of 1 min. at 94°C, 1 min. at 36°C, 2 min. at 72°C, and a final 7 min. extension at 72°C.

Amplification products were visualized with DNA marker on 1.6% agarose gel with 1X TBE buffer and were detected by staining with an ethidium bromide solution for 30 min. Gels were, then distained in deionized water for 10 min. and photographed on Polaroid films under U. V. light.

Data handling and cluster analysis

Data were scored for computer analysis on the basis of the presence of the amplified products for each primer. If a product was present in a cultivar, it was designated as "1", if absent, it was designated as "0", after excluding the unreproducible bands. Pair-wise comparisons of cultivars, based on the presence or absence of unique and shared polymorphic products, were used to determine similarity coefficients, according to Jaccard (1908). The similarity coefficients were then used to construct dendograms, using the Unweighted Pair Group Method with Arithmetic Averages (UPGMA) employing the SAHN (Sequential, Agglomerative, Hierarchical, and Nested clustering) from the NTSYS–PC (Numerical Taxonomy and Multivariate Analysis System), version 1.80 (Applied Biostatistics) program (Rohlf, 1993).

RESULTS AND DISCUSSION

In vitro mutagenesis

Induction of mutations based on ionizing radiations is one of the major breeding approaches for plant improvement. More than 2,300 mutant varieties have been released using irradiation mutagenesis (Jain, 2005) and among them 566 represent ornamental plants

Table 1. Analysis of variance of the effects of media, gamma radiation and their interaction on *Chrysanthemum morifolium* cv. "Delistar White" callus induction (%), shoot formation (%), shoot length and shoot number.

S.O.V.	D.F.	M.S.			
		Callus induction (%)[a]	Shoot formation (%)[a]	Shoot length (cm)	Number of shoots per explant
Blocks	9	121.26 $^{N.S.}$	299.37 $^{N.S.}$	0.18 $^{N.S.}$	5.27 $^{N.S.}$
Media (A)	1	3.22 $^{N.S.}$	558.31 $^{N.S.}$	4.79 **	25.35 **
Doses (B)	2	4631.17 **	3362.65 **	3.85 **	103.72 **
A x B	2	38.51 $^{N.S.}$	513.57 $^{N.S.}$	4.64 **	89.45 **
Error	45	244.49	196.08	0.36	3.66

[a]: Data were subjected to arcsine transformation. **: Highly significant at 0.01 probability level. $^{N.S.}$: Not significant at 0.05 probability level.

Table 2. Mean callus induction (%) and shoot formation (%) of *Chrysanthemum morifolium* cv. "Delistar White" as influenced by gamma ray doses.

Gamma ray dose (Gy)	Callus induction (%)	Shoot formation (%)
0.0	90.00	36.47
0.5	80.76	44.89
1.0	60.34	10.75

L. S. D. $_{(0.\ 05)}$ for doses means of callus induction = 9.96; L. S. D. $_{(0.\ 05)}$ for doses means of shoot formation = 8.92.

(http://www.mvd.iaea.org). A combination of *in vitro* technique and radiation induced mutagenesis has been recommended to improve cultivars of vegetative propagated plants (Maluszynski et al., 2000). Some of the selected important agronomical traits of mutant ornamental plant were flower colour, flower morphology, flowering time and resistance to abiotic and biotic stress (Das et al., 2000; Misra et al., 2003).

Effect of gamma irradiation on *in vitro* culture

The basic requirement for an effective use of mutation induction in plant breeding programmes is the analysis of radio sensitivity of the explants material (Walther and Sauer, 1986). Predieri (2001) reported that one of the first steps in mutagenic treatments is the estimation of the most appropriate dose to apply. The aim of the present work was to determine the radio sensitivity of *in vitro* chrysanthemum culture, which was assessed by callus induction, shoot formation, shoot length and shoot number in order to select the suitable dose of gamma irradiation to conduct *in vitro* mutation for chrysanthemum improvement.

Data of the analysis of variance (Table 1) revealed that callus induction and shoot formation percentage were affected by gamma ray doses, whereas the variation between medium protocols and the variations due to the interaction between medium protocols and gamma ray doses were statistically insignificant. Data in Table 2 indicated clearly that decrease in callus induction and shoot formation occurred with increasing of irradiation dose. The callus induction was 80.76% at 0.5 Gy which decreased to 60.34% at 1.0 Gy (Table 2). Similar decreasing was observed with shoot formation which was 44.89 and 10.75% at 0.5 and 1.0 Gy, respectively, however, the shoot formation by 0.5 Gy was better than the control treatment (36.47). Statistical analysis of shoot length and number of shoots per explant showed highly significant influences of the medium protocols, gamma ray doses and their interaction (Table 1). Results in Table 3 showed that the medium protocol A produced longer shoots (2.11 cm) across the gamma ray doses which were significantly different from shoot length on medium protocol B (1.54 cm). The shoot length was decreased as a result of increasing gamma ray treatments in comparison to the control. Number of shoots varied among the gamma ray doses (Table 4). The dose 0.5 Gy gave significantly the highest mean number of shoots per explant (6.80 shoots / explant) across the medium protocols. On the other hand, the control gave the significantly lowest average mean of shoots number (2.25 shoots/ explant) across the medium protocols. The shoot number on the medium protocol A (5.23) was significantly higher than on the medium protocol B (3.93). The treatment of 0.5 Gy gave the highest number of shoots on the medium A (9.80). Several studies have been conducted on the radio sensitivity of *in vitro* cultures of several crops (Hell 1983; Walther and Sauer, 1986; Wang et al., 1988; Cheng et al., 1990; Shen et al., 1990; Charbaji and Nabuls 1999; Predieri and Gatti, 2000). They studied the effect of gamma irradiation on *in vitro* cultures in crop

Table 3. Mean shoot length of *Chrysanthemum morifolium* cv. “Delistar White” as influenced by medium protocols, gamma ray doses and their interaction.

Medium mean (cm)	Shoot length (cm)			Medium
	Gamma ray doses (Gy)			
	1.0	0.5	0.0	
2.11	1.82	1.38	3.12	A
1.54	1.37	1.72	1.54	B
	1.60	1.55	2.33	Doses mean (Gy)

L.S.D. $_{(0.05)}$ for medium mean = 0.31.
L.S.D. $_{(0.05)}$ for doses mean = 0.38.
L.S.D. $_{(0.05)}$ for cultivar x explant interaction = 0.53.

Table 4. Mean number of *Chrysanthemum morifolium* C.V. Delistar White shoots per explant as influenced by medium protocols, gamma ray doses and their interaction.

Medium mean	Number of shoots/explant			Medium
	Gamma ray doses (Gy)			
	1.0	0.5	0.0	
5.23	3.60	9.80	2.30	A
3.93	5.80	3.80	2.20	B
	4.70	6.80	2.25	Doses mean

L.S.D. $_{(0.05)}$ for medium mean = 0.99.
L.S.D. $_{(0.05)}$ for doses mean = 1.22.
L.S.D. $_{(0.05)}$ for cultivar × explant interaction = 1.68.

breeding application, with an objective of developing suitable *in vitro* mutagenic system for the induction and selection of desirable mutants.

Effect of gamma irradiation on morphological characters

At the flowering stage, plants in the greenhouse were screened for observing any variation in the morphological characters. Results revealed that there were 13 variants that had different morphological characters than the parent plant as shown in Table 5. Data of the analysis of variance (Table 6) revealed that morphological characters of *Chrysanthemum morifolium* cultivar "Delistar white" and its mutants was statistically highly significant affected by gamma ray doses.

Plant height

Statistical analysis of plant height was highly significantly influenced by differences between variants (Table 6). Plant height varied among the variants (Table 7). The variant v2 (medium protocol B with the 0.5 Gy dose) gave; significantly the, highest mean value of plant height (93.30 cm) compared with the control (85.0 cm) and 1.0 Gy dose. Comparison among means was made using the Least Significant Difference Test (LSD) as shown in Table 6.

Number of flowers per plant

The analysis of variance, presented in Table 6, indicated that number of flowers per plant was, highly significantly, influenced to differences among variants. Results in Table 7 showed that the variant 1 (medium protocol A with dose 0. 5 Gy) produced the highest mean value of number of flowers per plant (11.85), While the parent showed the lowest mean value (5.2).These results agree with the findings of Misra et al. (2003) where number of flower heads were significantly reduced after 1 Gy.

Flower diameter (cm)

Data of the analysis of variance (Table 6) revealed that flower diameter was highly significantly affected by the differences between variants. The variant v6 (medium protocol B with 0.5 Gy dose) gave significantly the highest flower diameter (9.35 cm) compared to the other variants (Table 7).

Table 5. Conclusion for the morphological characters of *Chrysanthemum morifolium* cv. "Delistar White" somaclones as compared to the parent*.

Code variant	Source of the genotype (Protocol and irradiation)	Unique trait
V1	Protocol A (0.5 Gy)	Number of flowers per plant /Plant height
V2	Protocol B (0.5 Gy)	Response
V3	Protocol A (1.0 Gy)	Response
V4	Protocol B (1.0 Gy)	Response
V5	Protocol A (1.0 Gy)	Ray floret shape
V6	Protocol B (0.5 Gy)	Flower diameter
V7	Protocol A (1.0 Gy)	Ray floret shape
V8	Protocol B (1.0 Gy)	Ray floret shape
V9	Protocol B (0.5 Gy)	Ray floret shape
V10	Protocol B (0.5 Gy)	Ray floret shape
V11	Protocol A (0.5 Gy)	Vase- life
V12	Protocol A (1.0 Gy)	Ray floret shape
V13	Protocol B (0.5 Gy)	Ray floret shape

*The parent had white florets and spider form heads.

Table 6. Analysis of variance of the morphological characters of *Chrysanthemum morifolium* and its mutants.

S.O.V.	D.F.	M.S.				
		Plant height (cm)	Number of flowers/plant	Flower diameter (cm)	Vase life (Days)	Response (W)
Blocks	1	0.78 $^{n.s.}$	21.39 **	0.17 $^{n.s}$	0.78$^{n.s.}$	0.54 $^{n.s}$
Variants	13	27.22**	11.17 **	3.52 **	6.67**	23.26 **
Error	13	1.488	0.8530	0.2374	0.7368	0.0421

$^{*, **}$: Significant at 0. 05, 0.01 probability level, respectively.
$^{n.s.}$: Not significant at 0.05 probability level.

Table 7. Effect of γ-radiation on morphological characters of plants regenerated from ray florets of *Chrysanthemum morifolium* cv. Delistar white and its mutants.

Character variant	Plant height (cm)	Number of flowers per plant	Flower diameter (cm)	Vase life (day)	Response week
Parent	85.0 ^{ef}	5.2	7.5	12.5	7.0
V1	90.0^{abcd}	11.9	7.6	17	6.0
V2	93.3^{a}	5.8	8.0	15	6.5
V3	81.9^{fg}	5.8	8.1	15.4	8.2
V4	87.9 ^{bcde}	8.9	8	17.1	6.1
V5	92.9^{ab}	6.5	8.4	15.1	6.5
V6	91.3^{abcd}	7.2	9.35	14.65	7.5
V7	80.0^{g}	6.7	7.6	16.4	7.9
V8	82.6^{fg}	6.1	7.8	13.6	7.1
V9	89.9 ^{abcde}	9.2	8.6	13.8	7.4
V10	87.8^{cde}	8.2	9.4	15.3	7.5
V11	91.9^{abc}	11.7	7.3	17.3	6.0
V12	81.5^{g}	7.5	7.95	12.6	6.8
V13	86.5 ^{def}	10.4	8.5	14	7.4

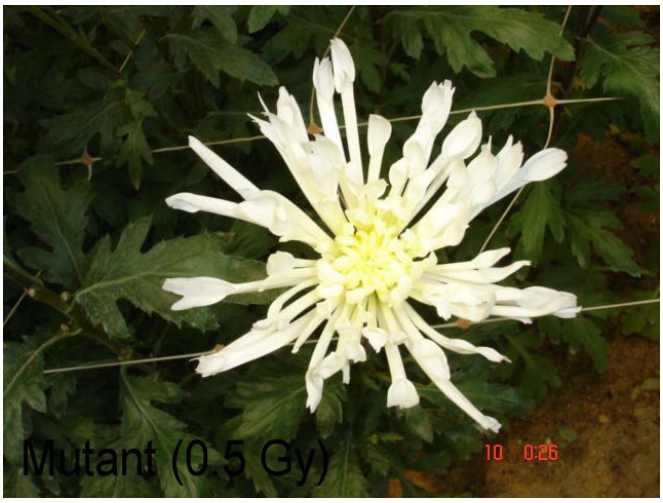

Figure 1. Mutants in *Chrysanthemum morifolium* cv. “Delistar White” ray floret shape after treating with 0.5 Gy.

Figure 2. Mutation in the number of florets per flower head in *Chrysanthemum morifolium* cv. "Delistar White" after treating with 0.5 Gy.

Vase life (days)

Data of the analysis of variance (Table 6) revealed that vase life was highly significantly affected by variants. The significantly longest vase life (17.3 days) was given by the variant 11(medium A with 0.5 Gy dose). While the shortest vase life was given by the parent (12.5) (Table 7).

Response (week)

Data of the analysis of variance (Table 6) revealed that response week was highly significantly affected by variants. The 1.0 Gy dose gave the significantly delayed response week (8.2 weeks) as shown in variant 3 compared to the control (7.00 weeks) and the 0.5 Gy dose (6.8 weeks) as shown in Table 7. These results agree with the findings of Misra et al. (2003), where days to flower bud initiation, first colour showing and full bloom were delayed after treatment and with increase in exposure.

The results indicate that the irradiation dose 0.5 Gy was the most effective dose in inducing mutations in ray floret shape (Figure 1) and number of florets / flower head (Figure 2), although no change in flower colour was observed but conversion from tubular florets to spoon-shaped florets was recorded.

Molecular markers

RAPD analysis

Five primers were screened for their ability to amplify the genomic DNA of the *Chrysanthemum morifolium* cultivar "Delistar White", and its mutants. The number of DNA fragments amplified ranged from 6 to 14 depending on the primer and the DNA sample with a mean value of 9.6 bands per primer (Table 8).These results are almost similar to those reported in other chrysanthemum varieties and their somaclones (Martin et al., 2002). On the other side, these results are considered rather high for RAPD amplification, compared to the average numbers of amplified bands recorded in other crops; namely three fragments in *Triticum turgidium* L. (Joshi and Nguyen 1993), 4.3 fragment in *Solanum tuberosum* L. (Masuelli et al., 1995).

The size of fragments ranged from 300 to 1200 bp. A total of 48 fragments were produced by the five primers. Of these 48 amplified fragments, (47.9%) were not polymorphic; whereas, the remaining bands (52.1%) were polymorphic in one or other of the fourteen genotypes (one cultivar and its13 somaclones). However, Figure 3 shows the amplification profiles, generated by primers OPA-18 across the chrysanthemum cultivar and its somaclones. The 4 scorable bands of the primer were polymorphic across the chrysanthemum genotypes. RAPD analysis had been used in ornamental breeding to characterize genotypes (Yamagishi, 1995 and Scott et al., 1996), and for identification of genes controlling important traits. Also RAPD markers tightly linked to the locus controlling carnation (*Dianthus caryophyllus*) flower type were identified by Scovel et al. (1998).Genotype identification of ornamental species by RAPD had been reported by Benedetti et al. (2001). Likewise, RAPD analysis had been used also to identify DNA markers correlated to *Fusarium oxysporum* resistance in the greenhouse carnation (Scovel et al., 2001). Recently, RAPD analysis was used to set up marker assisted selection in chrysanthemum hybrid combinations including parents and offspring (Chanug et al., 2000).

Table 8. Number of amplification and polymorphic products, using five primers in *Chrysanthemum morifolium* cv. "Delistar White" and its mutants.

Polymorphism b/a (%)	Number of polymorphic[b]	Number of amplification[a]	Nucleotide sequence 5`to 3`	Primer number
35.7	5	14	GGACTGGAGT	OPB-04
50.0	6	12	GGGAATTCGG	OPB-18
83.3	5	6	AGGGGTCTTG	OPA-05
57.1	4	7	GAAACGGGTG	OPA-07
55.5	5	9	GGTGACGCAG	OPB-07

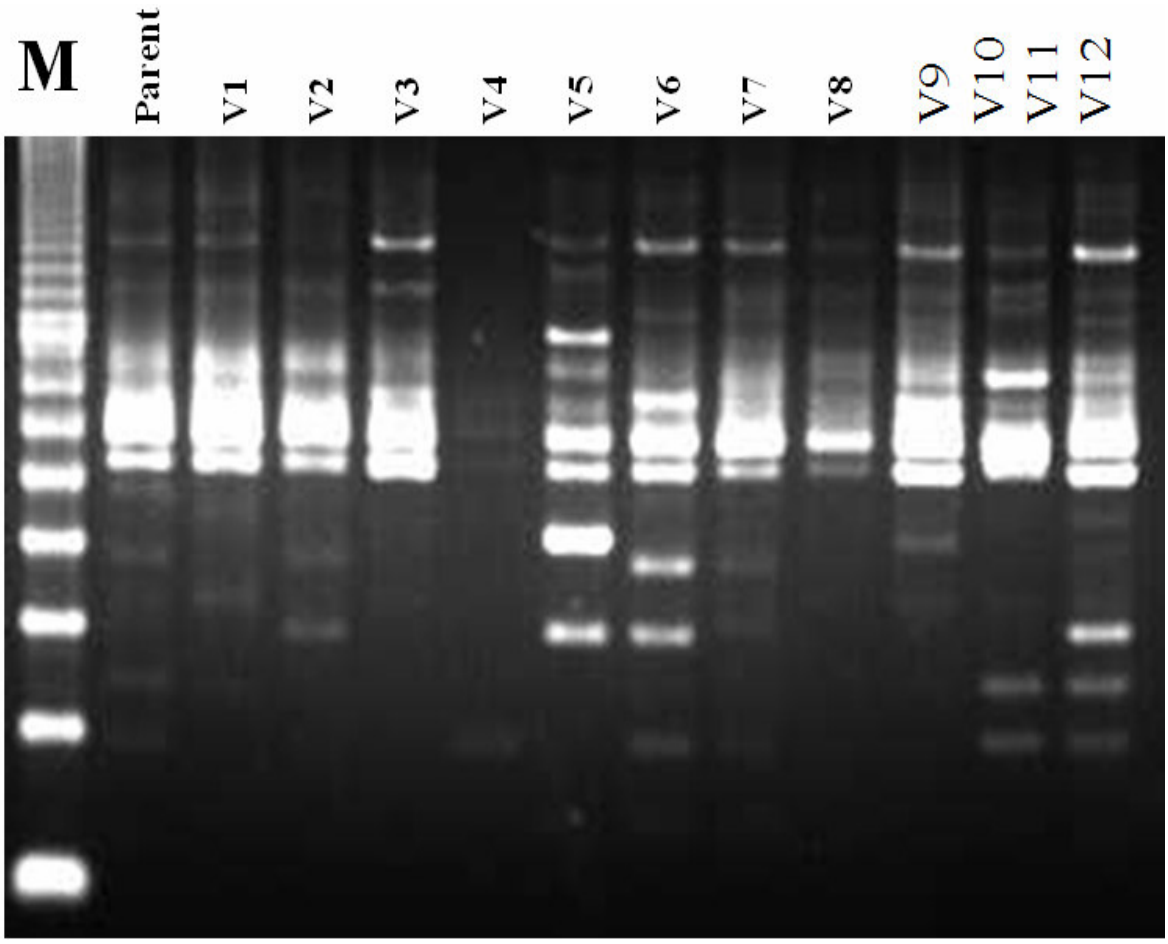

Figure 3. RAPD polymorphism in *Chrysanthemum morifolium* CV. "Delistar White" and its variants.

They reported that forty-five random primers were screened, of which twenty-two primers were selected to detect the molecular marker in three hybrid combinations of chrysanthemum. Martin et al. (2002) used RAPD analysis with ten primers to characterize fifteen commercial varieties of chrysanthemum. More recently, Kumar et al. (2006) also noticed a high degree of polymorphism in chrysanthemum cultivars using RAPD markers. Their results indicated that out of forty primers screened, twenty-one were selected on the basis of robustness of amplification, reproducibility, scorability of banding patterns and were employed for diversity analysis. Williams et al. (1990) reported that polymorphism among individuals could arise through nucleotide change that prevented amplification by introducing either a mismatch at one priming site, deletion of a priming site, insertions that rendered priming sites to distant to support amplification and insertions or deletions that change the size of the amplified product. RAPD has proved to be a very useful and rapid method to detect variation between different chrysanthemum varieties, even when not a very high number of markers have been scored. Wolff (1996)

Table 9. Simple Matching Coefficient of Similarity matrix for *Chrysanthemum morifolium* cv. "Delistar White" and its somaclones determined from RAPD analysis using 5 different primers and analyzed by UPGMA program.

	Parent	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10	V11	V12	V13
Parent	1.00													
V1	0.72	1.00												
V2	0.60	0.60	1.00											
V3	0.67	0.67	0.60	1.00										
V4	0.42	0.42	0.42	0.42	1.00									
V5	0.86	0.72	0.60	0.67	0.42	1.00								
V6	0.75	0.72	0.60	0.67	0.42	0.75	1.00							
V7	0.81	0.72	0.60	0.67	0.42	0.81	0.75	1.00						
V8	0.72	0.87	0.60	0.67	0.42	0.72	0.72	0.72	1.00					
V9	0.81	0.72	0.60	0.67	0.42	0.81	0.75	0.84	0.72	1.00				
V10	0.77	0.72	0.60	0.67	0.42	0.77	0.75	0.77	0.72	0.77	1.00			
V11	0.75	0.72	0.60	0.67	0.42	0.75	0.78	0.75	0.72	0.75	0.75	1.00		
V12	0.81	0.72	0.60	0.67	0.42	0.81	0.75	0.84	0.72	0.87	0.77	0.75	1.00	
V13	0.81	0.72	0.60	0.67	0.42	0.81	0.75	0.84	0.72	0.87	0.77	0.75	0.95	1.00

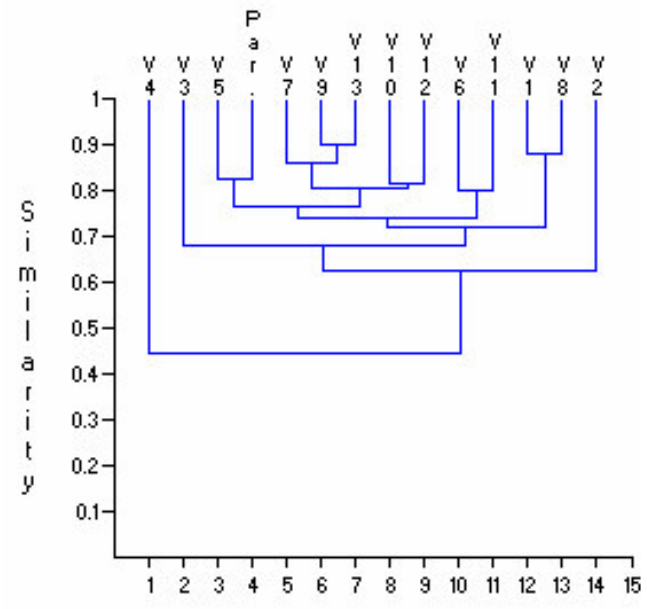

Figure 4. Phylogenic tree illustrating the relationship among the parent and its 13 somaclones/ variants (v1-v13) using RAPD data and the UPGMA method of clustering to show DNA similarity.

reported that the choice of the primers may be an important factor in obtaining a rapid discrimination between samples.

Cluster analysis

The RAPD markers, produced by five primers, were used to construct a similarity matrix (Table 9). Simple matching coefficient, ranging from 0.425 to 0.95, suggested a broad genetic base for chrysanthemum genotypes. Table 9 indicates the genetic similarity estimates of the 91 pair wise comparisons among the Delistar White cultivar and its variants, based on 48 polymorphic bands. Figure 4 represents the clustering of chrysanthemum cultivar generated by UPGMA analysis of the parent; namely, "Delistar White and its 13 somaclones. The results of characterization analysis revealed a high diversity between the cultivar and its somaclones. Five clusters could be observed. The first cluster included only the

variant 4 (V4), while the second one included the variant 3 (V3). The third cluster included the variant 1 and the variant 8 (V1 and V8). The fourth cluster included only variant 2 (V2). The last cluster included the parent Delistar White as well as the other variants (Figure 4). It can be seen from this figure that the shortest genetic distances (the highest similarity value) was found between variant 12 and variant 13 (V12 and V13), whereas the highest distance (the low similarity value) was observed between the parent and the variant 4 (Delistar White cultivar and V4). These results indicated that RAPD technique could be successfully applied to ornamental crops.

RAPD analysis in the characterization of chrysanthemum varieties and their somaclones allows the detection of changes produced during culture and also, with samples taken subsequently through the entire process, to detect at which time the alteration has been produced (Martin et al., 2002).The phenotypic changes that these variations could produce must be checked subsequently in order to select those favorable phenotypic modifications that can be applied for commercial propagation. Bhattacharya et al. (2006) reported that the similarity among the chrysanthemum cultivars and mutants varied from 0.17 to 0.90 using RAPD analysis. Mutant with different flower colour could be identified at the molecular level using RAPD technique holding promise to identify unique genes as SCAR markers. The results of Badr et al. (2006), El-Nashar (2006) and Kumar et al. (2006) indicated that RAPD markers could be effectively used for genetic diversity studies among radio mutants. Their results suggested that by using RAPD markers, the newly evolved chrysanthemum cultivars can be easily differentiated from their parents and can be a useful tool to supplement the distinctness uniformity and stability analysis for plant variety protection in the future.

In conclusion, these results must be regarded as preliminary studies because of the small size analyzed, the low number of used primers and the low number of generated RAPD bands. Nevertheless, they are encouraging. Moreover, it gives information on the level of genetic polymorphism existing among these variants (somaclones) and brings new prospective for the use of such markers in a breeding program for improving ornamental characters of chrysanthemum cultivars.

REFERENCES

Badr M, El-Torky MG, Abbas R, El-Mezawy A, Gaber G (2006). Breeding studies on *Salvia* sp. II. Biochemical and Biotechnological identification of some *Salvia* genotypes. Alex J. Agric. Res. 51(2): 169-176.

Barakat MN, Abdel FRS, Badr M, El-Torky MG (2010). *In vitro* culture and plant regeneration derived from ray florets of *Chrysanthemum morifolium*. Afr. J. Biotechnol. Vol. 9.

Benedetti LD, Mercuri A, Bruna S, Burchi G, Schiva T (2001). Genotype identification of ornamental species by RAPD analysis. Acta Hort. 546: 391-394.

Bhattacharya A, Teixeira da Silva JA (2006). Molecular systematics in *Chrysanthemum grandiflorum* (Ramat.) Kitamura Sci. Hort. 109: 379-384.

Chanug HS, Tsai CC, Sheu CS (2000). Genetic analysis of *Chrysanthemum* hybrids based on RAPD molecular markers. Bot. Bull. Acad. Sin. 41: 257-262.

Charbaj I, Nabul S (1999). Mutational analysis of racial differentiation in *Oryza sativa*. Curr. Sci. 32: 451-453.

Cheng KT, Wang CL, Lu YM, Shen M, Afza R, Duren MV, Brunner H (1990). Anther culture in connection with induced mutations for rice improvement. Euphytica 120: 401-408.

Chopra VL (2005). Mutagenesis: Investigating the process and processing the outcome for crop improvement. Curr. Sci. 89(2): 353-359.

Das A, Gosal SS, Sidhu TS, Dhaliwal HS (2000). Induction of mutations forheat tolerance in potato by using *in vitro* culture and radiation. Euphytica 114: 205-209.

El Nashar Y (2006). Effect of chemical mutagens (Sodium Azide and Diethyl Sulphate) on Growth, Flowering and induced variability in *Amaranthus caudatus L. and A. hypochondriacus L.* Ph.D Thesis. Floricult. Ornamental Hortic. Dept., Fac. Agric., Alexandria University.

Hell BP (1983). Frequency and types of mutations induced in bread wheat by some physical and chemical mutagens. Genet. 31: 449-480.

IAEA (2005). Strategies of the Joint FAO/IAEA Programme for the Use of Induced Mutations for Achieving Sustainable Crop Production in Member States.

Jaccard P (1908). Nouvelles recherches sur la distribution lorale. Bull. Soc. Vaud. Sci. Nat. 44: 223-270.

Jain SM (2005). Major mutation-assisted plant breeding programs supported by FAO/IAEA. Plant Cell Tiss. Org. Cult. 82:113-123.

Joshi CP, Nguyen HT (1993). RAPD analysis based intervarietal genetic relationships among hexaploid wheats. Plant Sci. Lim. 93(1-2): 95-103.

Kumar SK, Prasad V, Choudhary ML (2006). Detection of genetic variability among chrysanthemum radio mutants using RAPD markers. Current Sci. 90(8): 1108-1113.

Maluszynski M, Nichterlein K, van Zanten L, Ahloowalia BS (2000). Official released mutant varieties- the FAO/IAEA database. Mut. Breed 12: 1-88.

Mandal AKA, Chakrabarty D, Datta S (2000). *In vitro* isolation of solid novel flower colour mutants from induced chimeric ray florets of chrysanthemum. Euphytica 114: 9-12.

Martin C, Uberhuaga E, Pèrez C (2002). Application of RAPD markers in the characterization of *Chrysanthemum* varieties and the assessment of somaclonal variation. Euphytica 127: 247-253.

Masuelli RW, Tanimoto EY, Brown CR, Comai L (1995). Irregular meiosis in a somatic hybrid between *Solanum bulbocastanum* and *S. tuberosum* detected by species-specific PCR markers and cytological analysis. Theor. Appl. Genet. 91:401-408.

Misra P, Datta SK, Chakrabarty D (2003). Mutation in flower colour and shape of *Chrysanthemum morifolium* induced by γ-radiation. Biol. Plantarum 47(1): 153-156.

Predieri S, Gatti J (2000). Pear mutagensis: *In vitro* treatment with gamma- rays and field selection for vegetative form triats. Euphytica 114: 79-90.

Predieri S (2001). *In vitro* propagation and breeding of ornamental plants: Advantages and disadvantages of variability. Euphytica 115: 12-14.

Rohlf FG (1993). Numerical Taxonomy and Multivirate Analysis System. Exeter Software, New York.

Sagahi-Maroof M, Soliman K, Jorgenson RA (1984). Ribosomal DNA spacer length polymorphisms in barely: Mendelian inheritance, chromosome location and population dynamics. Proc. Natl. Acad. Sci. 81: 8014-8018.

Sambrook J, Fritsch EF, Maniatis J (1989). Molecular cloning, a laboratory manual. 2nd ed. Cold Spring Harbor Laboratory Press (Ed). New York.

SAS Institute Inc (1985). SAS/STAT. Guide for personal computers. version 6, 4th ed. vol. 2 Cary. NC, USA.

Scott MC, Caetano-Anolles G, Trigiano RN (1996). DNA amplification fingerprinting identifies closely related chrysanthemum cultivars. J.

Am. Soc. Hort. Sci. 121(6):1043-1048.
Scovel G, Ben-Meir H, Ovadis M (1998). RAPD and RFLP markers tightly linked to the locus controlling carnation (*Dianthus caryophyllus*) flower type. Theor. Appl. Genet. 96(1): 117-122.
Scovel G, Ben-Yephet Y, Ovadis M, Reuven M, Vainstein M (2001). Markers assisted selection for resistance to *Fusarium oxysporum* in the greenhouse carnation. Acta Hort. 552:151-156.
Shen T, Li W, Dai C, Chen JY (1990). The induction of sports in chrysanthemum by gamma radiation. Theor. Appl. Genet. 88(3): 129-136.
Steel RGD, Torrie JH (1980). Principles and Procedures of Statistics. A Biometrical Approach. (2nd ed.). McGraw Hill Book.
Walther F, Sauer A (1986). *In vitro* mutagenesis in roses. Acta Hort. 189: 37-46.
Wang K, Mao Y. Zhang X (1988). Induced mutation for crop improvement in China. Zhong Yao Cai. 6: 109-115.
Wilde J, Waugh R, Powell W (1992). Genetic fingerprinting of *Theobroma* clones using randomly amplified polymorphic DNA markers. Theor. Appl. Genet. 83: 871-877.
Williams GK, Kubelik AR, Livak KL, Rafalski JA, Tingey SV (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 18: 6531-6535.
Wolff K (1996). RAPD analysis of sporting and chimerism in chrysanthemum. Euphytica 89: 159-164.
Wolff K, Peters-Van RJ, Hofstra H (1994). RFLP analysis in *Chrysanthemum*. I. Probe and primer development. Theor. Appl. Genet. 88: 472-478.
Yamagishi M (1995). Detection of section-specific random amplified polymorphic DNA (RAPD) markers in *Lilium*. Theor. Appl. Genet. 91: 830-835.