The Journal of Animal & Plant Sciences, 26(6): 2016, Page: 1819-1827
ISSN: 1018-7081

EFFECT OF RADIATION ON MORPHOLOGICAL CHARACTERS OF DIFFERENT CULTIVARS OF LILIUM AND GENETIC ANALYSIS OF MUTANTS THROUGH MOLECULAR MARKERS

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ABSTRACT

Morphological parameters and RAPD (Random amplified polymorphic DNA) markers have been used to evaluate the effect of gamma radiation on different varieties of Lilium plant. *In vivo* treated plants were studied to observe the alternations in growth parameters.Change in plant morphology in aspect of flower morphology (flower colour and shape), leaf length, leaf width, shoot length and number of leaves per plant was detected. Treated plants showed significant alterations in different morphological traits as contrast to control plants were further analyzed by using RAPD markers. Total no. of 101 amplified products were obtained by using 11 polymorphic RAPD primers. The genetic similarity among the 4 Lilium parent varieties and their mutant genotypes ranged from 0.14-0.34 in V4 (Samur La pink), 0.08-0.42 in V5 (Heaven White), 0.11-0.38 in V6 (Golden Tycon yellow) & 0.14-0.38 in V8 (orange colour Liliy). It is concluded that RAPD practice could be effectively applied to the newly Lily variants and can make a distinction among the mutants from their parents.

Key words: Lilium, Gamma radiation, Morphology, RAPD.

INTRODUCTION

Approximately Liliaceae contains 250 genera and 3500 species worldwide and in subtropical and temperate regions. The genus Lilium divides into seven sections (De Jong, 1974)with almost 100 species. This genus attained huge importance in the world flower market due to diversity and commercial availability of large number of hybrids and cultivars, mainly used as cut flower and also economic importance of the genus increased due to the medicinal and nutritive value. Plants are commonly used in floristry, especially for decoration purposes inside a house or building. Normally the cut flowers are decorated in a pot. Cut flowers used for different decoration purposes. Lilium belonging to the Liliaceae family is one of widely used ornamental flowers, after, roses, cloves, and chrysanths. This species is a valuable one from the perspective of horticulture, because of its aromatic prosperities, color spectrum, and adapt to different environmental conditions, and is commercially used as a cut or vase flower to enhance the beauty of gardens (Ko *et al*., 2002). In the Middle East and Turkey, Lilium has been cultivated for it uses in ornamental cut flower industry, obtaining different perfumes and also a number of secondary metabolic products from petals ranging from butanolic extracts to various types of saponins and pyroline derivatives from bulbs, which lead to improved its economic position to numerous times (Mucaji *et al*., 2002; Mimaki, 1999).

Ionizing radiations produce clear effects on plant cellular macromolecular components, such as membranes, cell walls and also at DNA level (McLennan, 1988). These effects are produced as a result of both the direct interactions between the macromolecular structures and ionizing radiation, and due to indirect action of AOS generated as product of water radiolysis.These both direct as well indirect actions of ionizing radiation create some other morphological and physiological changes in plants (Cho *et al*., 2000). Induced mutagenesis is a well-known technique for plant betterment, and the practice has been effectively exploited to generate some new and novel varieties in both horticulture and floriculture in general (Ahloowalia, 2004). Radiation induced genetic changes which cause stimulation in plant growth leads to production of new varieties in both horticulture and floriculture and it is the most vital role of atomic energy in the crop field. Mutagenesis has become an established method of creating changes within the plants.Technique of artificial mutagenesis by using ionizing radiation starts at the beginning of the 20th century. Such changes which produce variations at molecular level could be used in plant breeding have been proven after the experiments of over 30 years. Many new flower colour/shape mutants have been developed successfully through radiation technology. Therefore, artificial mutagenesis through irradiation or by use of different chemicals has become a very significant way for plant propagation in flower reproduction (Barakat & El-Sammak, 2011).Through induced mutation, a large number of plant varieties have been developed with improved traits such as high yield, early maturity, as well as high protein content, biotic and abiotic resistance. Also in ornamental plants improved

characters include compact growth, attractive variegated leaves and novel flower colour and shapes (Ahmad *et al*., 2011). RAPD practice produces high levels of polymorphisms and also shows detection of genotypes within given population. Genetic relationships among different *Lilium* species were studied by using RAPD markers (Ikinci & Oberprieler, 2010).The aim of the present study is to screen a variety of mutants showing changes in the morphological aspect of the plant. The best mutant variety will be selected to study its genetic diversity using random amplified polymorphic DNA.

MATERIALS AND METHODS

Plant material: Bulbs of different cultivars of Lilium were procured from different seed nurseries of Lahore. Different doses of gamma rays i.e. (2.0-10.0) Gy were used for the plant treatment.

Plant growth: Treated bulbs were potted in green house containing sand + soil + vermicompost and having same environmental conditions. Conditions to grow plants were 22 °C with16 h of light and 8 h of dark periods. Spray of Hoagland solution and distilled water was given twice a day to the plants. Different parameters including, Shoot length, leaf length, leaf width, flower morphology and average number of leaves per treated plant were calculated.

DNA extraction method: CTAB (hexa decyltrimethyl ammonium-bromide) method (Murray and Thompson, 1980) with some modifications was used to isolate the genomic DNA. Young and healthy leaves were used as explants for the purpose. Fine powder of 2g leaves of Lilium plants for each variety was obtained by grinding in liquid nitrogen. Leaf powder was immediately transferred to 50-mL tube containing 3.5 mL of extraction buffer (Tris–HCl 100mM) pH 8.0, (EDTA 20mM), (NaCl 1.4 M), (CTAB, 2% w/v^{-1}) & 2% 2–mercaptoethanol and incubated the above at 65 °C for half hour. Chloroform – octanol (24:1) was used for DNA extraction and 70% ethanol was used for DNA washing. Dissolved the washed DNA in 100-400 µL of 0.1 X T.E buffer containing (Tris–HCl 10 mM) pH 8.0 (EDTA 1mM).

RAPD analysis: Diluted DNA (10 ng µL-1) was used for further RAPD-PCR reactions. Method used for RAPD analysis is described by (Naz *et al*., 2013) with some modifications (10 ng µL-1 of template DNA, 0.5 µM of single primer, Taq DNA polymerase 0.4 U & 0.1 µM of dNTPs). Initially 20 decamer primers were used for screening. After the initial screening, 11 primers were selected for further analysis. PCR products were separated by using 0.1% agarose gel electrophoresis technique and staining was made by using ethidium bromide. Visualization of bands was made under UV Tran's illuminator apparatus.

Statistical design

Study of morphological characters: For the experiments, a completely randomized design with three replicates was utilized. The data was subjected to ANOVA (analysis of variance) using the COSTAT V.63: statistical software (Cohort software, Berkely, California) for each parameter. The treatments mean values were compared with the least significant difference test following Duncan's new multiple range (DMR) test at 5 % level.

Study of genetic diversity: Polymorphism was calculated on the basis of presence and absence of clear and repeatable amplified products by using 1 for presence and 0 for absence. This data was further used to determine similarity using DNAMAN software and dendrogram was constructed by using a distance matrix with Nie and Lie's Coefficients to access the genetic similarity and dissimilarity among the different Lilium varieties and its mutants.

RESULTS AND DISCUSSION

Effect of radiation on morphological changes: From this study, it was noticed that in comparison with control plants, leaf length of treated plants showed different results in different varieties of Lilium. As shown in Table 1, Samur La pink showed increase in leaf length by increasing the dose of radiation (Figs. 1a, b, c, e & f) and the highest leaf length i.e. (9.5 cm) was observed at 10.0 Gy as compared to control plants having (6.9 cm) leaf length

Similarly increase in leaf length was also observed in treated plants of (Orange colour) Lilium cultivars at (2.0, 4.0 & 8.0) Gy of Gamma radiation. All other treated plants showed reduction in leaf length by increasing dose of radiation.

Induction of plants with radiation showed no pronounced effect on the width of leaf in both treated and control varieties. As shown in Table 2, maximum leaf width i.e. (2.5) cm was found at 10.0 Gy in Samur La pink as compared to control plants in this variety while in all other varieties, positive results were obtained in non-treated ones and the highest width (4.1cm) was noticed in control plants of Montezuma O Red. Effects of various doses of radiation on average shoot length (cm) of different varieties of Lilium cultivars was also observed and shown in (Fig. 2a). Shoot length of treated plants shown significant change by gamma radiation exposure, decrease in shoot length occurred by increase in dose but in treated plants of Samur La pink dramatically increase in shoot length was observed at highest dose of gamma radiation i.e. 96 cm at 10.0 Gy. Cheng *et al*. (2010)

studied that with an increase in radiation dose, the percentage of plant growth, height and root length of minituber plants were drastically decreased at 40 and 50 Gy. Similarity to other parameters Samur La pink of Lilium cultivars showed significantly increase in number of leaves i.e. 80 leaves/ plant at 10.0 Gy in exposed plants in comparison with control plants while total number of leaves decreased significantly in gamma rays treated plants in all other cultivars (Fig. 2b.).

This study showed a remarkable change in flower morphology of Samur La pink. It was found that flower with smooth pattern, large flower which show brightness in the appearance was observed at 10.0 Gy in comparison with control plants (Figs. 1k & l). Similarly treated plants of Golden Tycon Yellow show a small change in flower petals morphology. It was noticed that treated plants at 6.0 Gy showed a variegated pattern at outer lining in compared to control plants (Figs 1m & n). Irradiation levels of 50-100 Gy could be used as a valuable parameter in the production of the *Torenia* flower color mutants by gamma-ray radiation because these levels produced inhibition of plant growth by inducing mutation is described by (Kasetsart, 2011). Effect of gamma rays on flower morphology was described by (Datta, 2009). 'Lilith' is a double Korean, white small-flowered chrysanthemum. Two and three plants showed flower color mutations in 1.5 and 2.0 krad respectively. In all cases the flower color mutation was yellow. Gamma-rays have been most successfully used and 76 new mutant varieties with changed flower color/shape, and chlorophyll variegation in leaves have been developed and released in different ornamentals. Nagatomi and Degi, (2009) obtained a total of 549 mutants (15%) from 3,688 plants in the field nursery; 79% of them fell into the category of light to dark pinks similar to the original variety and 21% were a different colour from the pinks. A wide spectrum of flower color appeared in individual regenerants derived from floral organ culture. Wide, continuous variations also appeared in the shape and size of the flowers and leaves in regenerants from petal and bud cultures. Xi *et al*., (2011) reported that 9 new varieties of Lilium were produced showing changed morphological characters of out of 50 mutant lines transferred to green housethan those of control Lilium plants.

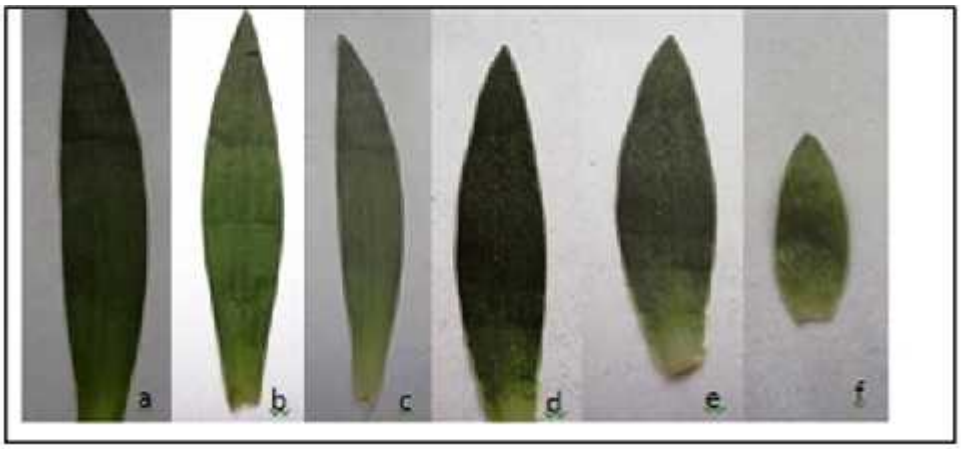


Fig. 1: Leaf length (cm) and width (cm) at different doses of gamma radiation in Samur La pink: (a) Leaf length and width at 10.0 Gy (1X). (b) Leaf length and width at 8.0 Gy (1X). (c) Leaf length and width at 6.0 Gy (1X). (d) Leaf length and width of control variety (1X). (e) Leaf length and width at 4.0 Gy (1X). (f) Leaf length and width at 2.0 Gy in Samur La Pink (1X). (g) Shoot length at 10 Gy (1X). (h) Shoot length of control variety (1X). (I & j) Flowering of radiated varieties of Samur La pink (1X). (k & l) Flowers of non - radiated varieties (1X). (m) Flower of radiated variety of Golden Tycon yellow (1X). (n) Flower of non - radiated variety of Golden Tycon yellow (1X).

Table 1. Leaf length at different doses of Gamma rays in different Lilium cultivars

Doses (Gy)	Different varieties showing leaf length (cm)								
	Star fighter	Courier La white	Advantage La salmon	Samur La pink	Heaven white	Golden tycon Yellow	Purple colour	Orange colour	Montezuma O red
2.0	4.5^{b}±0.056	5.3^{b}±0.063	5.3^{b}±0.063	3.8^{e}±0.109	8.4^{b}±0.109	6.6^{a}±0.089	6.0^{b}±0.04	7.2^{a}±0.028	4.0^{b}±0.0740.
4.0	4.0^{c}±0.074	4.8^{b}±0.063	4.5^{d}±0.056	4.0^{e}±0.074	5.5^{c}±0.074	5.4^{a}±0.07	5.0^{c}±0.08	6.5^{b}±0.116	3.1^{c}±0.063
6.0	3.3^{d}±0.04	2.3^{c}±0.093	5.0^{c}±0.089	7.7^{c}±0.056	4.8^{d}±0.063	2.1^{b}±0.12	3.8^{d}±0.10	4.0^{d}±0.0748	2.5^{d}±0.089
8.0	2.3^{e}±0.093	2.1^{c}±0.129	2.4^{e}±0.101	8.9^{b}±0.028	3.2^{e}±0.063	1.3^{b}±0.17	3.3^{e}±0.04	7.3^{a}±0.04	1.0^{e}±0.04
10.0	1.5^{f}±0.2	8.9^{c}±0.249	-	9.5^{a}±0.014	1.6^{f}±0.04	1.0^{b}±0.04	2.2^{f}±0.129	3.0^{e}±0.116	1.0^{e}±0.04
CONTROL	8.5^{a}±0.0632	10^{a}±0.241	5.7^{a}±0.048	6.9^{d}±0.089	9.4^{a}±0.014	8.5^{a}±0.06	7.1^{a}±0.052	5.4^{c}±0.0148	7.0^{a}±0.063

Each value is mean of five replicates with standard error (Mean ± S.E), Mean with same superscript are not significantly different by Duncan's new multiple range test ($p<0.05$).

Table 2. Leaf width at different doses of Gamma rays in different Lilium cultivars

Doses (Gy)	Different varieties showing leaf width (cm)								
	Star fighter	Courier La white	Advantage La salmon	Samur La pink	Heaven white	Golden Tycon Yellow	Purple colour	Orange colour	Montezuma O red
2.0	1.1^{abc}±0.05	0.8^{a}±0.04	0.8^{bc}±0.04	0.7^{d}±0.06	1.0^{b}±0.04	1.0^{a}±0.04	0.5^{cd}±0.04	0.9^{b}±0.05	0.9^{b}±0.05
4.0	0.9^{bc}±0.05	0.4^{b}±0.05	0.8^{bc}±0.04	0.9^{d}±0.05	0.6^{b}±0.06	0.6^{b}±0.06	0.6^{bc}±0.06	0.8^{bc}±0.04	2.5^{a}±0.08
6.0	0.8^{c}±0.04	0.3^{bc}±0.02	0.9^{b}±0.05	1.3^{c}±0.17	0.7^{b}±0.06	0.6^{b}±0.06	0.8^{a}±0.04	0.9^{b}±0.05	0.7^{bc}±0.06
8.0	0.9^{bc}±0.05	0.3^{bc}±0.02	0.7^{c}±0.06	1.8^{b}±0.04	0.8^{b}±0.04	1.2^{a}±0.04	0.6^{bc}±0.06	0.8^{bc}±0.04	1.0^{b}±0.04
10.0	1.3^{a}±0.17	0.2^{c}±0.04	-	2.5^{a}±0.08	1.6^{a}±0.04	1.2^{a}±0.04	0.7^{ab}±0.06	0.7^{c}±0.06	1.4^{c}±0.05
CONTROL	1.2^{ab}±0.04	0.7^{a}±0.06	1.1^{a}±0.05	1.0^{d}±0.04	0.7^{b}±0.06	1.1^{a}±0.05	0.4^{d}±0.05	1.1^{a}±0.05	4.1^{b}±0.05

Each value is mean of five replicates with standard error (Mean ± S.E), Mean with same superscript are not significantly different by Duncan's new multiple range test ($p<0.05$).

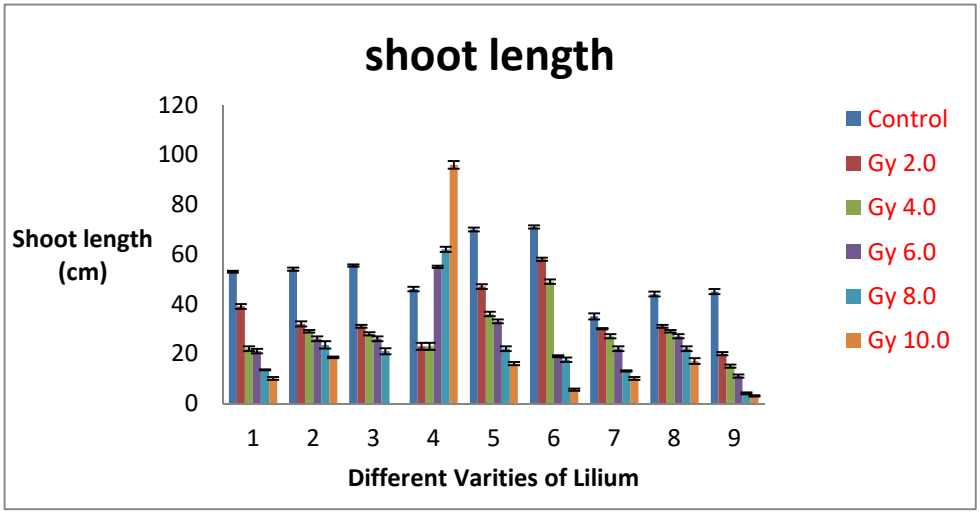


Fig. 2(a). Effect of various doses of radiation on average shoot length (cm) of different Lilium cultivars.

1 Star fighter
2 Courier La white
3 Advantage La salmon
4 Samur La pink
5 Heaven white
6 Golden tycon Yellow
7 Purple colour
8 Orange colour
9 Montezuma O red

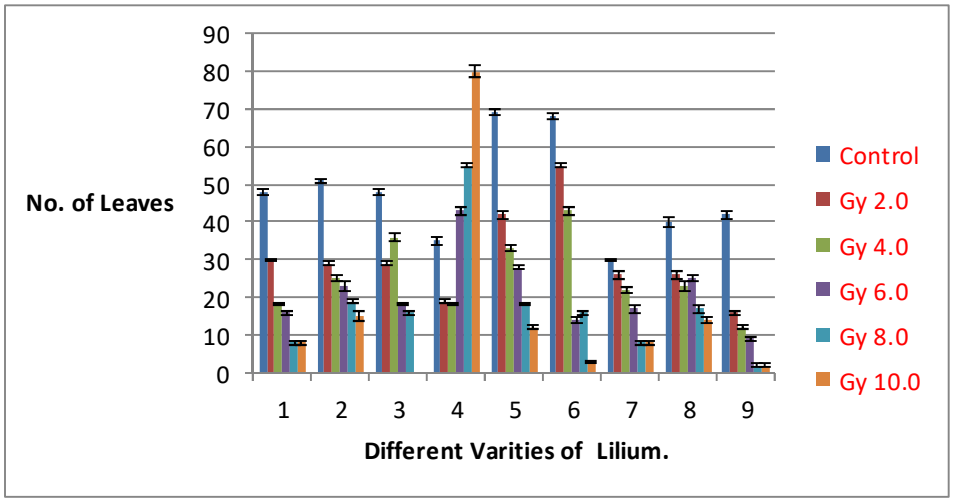


Fig. 2(b). Effect of various doses of radiation on average no of leaves formed in different Lilium cultivars.

1 Star fighter
2 Courier La white
3 Advantage La salmon
4 Samur La pink
5 Heaven white
6 Golden Tycon Yellow
7 Purple colour
8 Orange colour
9 Montezuma O red

RAPD Analysis: After screening of 20 primers for the amplification of the DNA isolated from the different cultivars of Lilium, 11 were selected for the production of amplicons ranged from 4-16 (Table 3).Total number no of 105 amplified loci were produced with an average of 9.54±0.05 bands out of which 61.4% were polymorphic in nature. Phylogenetic tree was also generated by using this software. Maximum bands (16) were generated by primer LP9 and LP11 while the minimum bands were produced by primer LP1.Effect of gamma radiation in screening the best mutants in different plants was also reported by other literature. Gamma- ray irradiation is a conventional technique to produce mutants in plant breeding.Genetic difference between different species of Lilium was investigated by Ikinci, (2010) using random amplified polymorphic DNA profiles. These markers were also used for the genetic analysis, identification and calculating the genetic diversity in different Lilium plants (Persson *et al*., 1998).

Figure 3 shows the pattern of number of bands generated by different primers. Primer number 6, 8 & 9 produced total of 32 bands in Golden Tycon Yellow. Primer number 2 generated 10 bands in V5 (Heaven white). Primer number 9 generated highest number i.e. 16 fragments in V8 (Orange colour Lily) while primer no. 4, 7 & 10 produced total of 29 fragments in V4 Samur La pink (Figs. 3a, 3b &3c).RAPD analysis used for genotype identification has been reported by (Benedetti *et al*., 2001; Scovel *et al*., 2001).

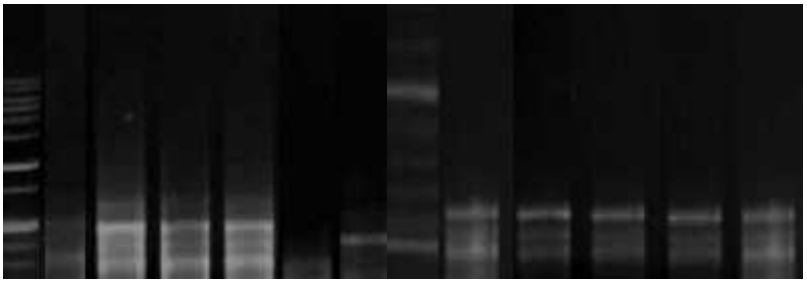
Genetic polymorphism among control and treated Lilium cultivars: RAPD markers produced by the selected 11 primers were further used to generate a similarity index which ranged from 0.14-0.34 in V4 (Samur La pink), 0.08-0.42 in V5 (Heaven White), 0.11-0.38 in V6 (Golden Tycon yellow) & 0.14-0.38 in V8 (orange colour Liliy). RAPD analysis becomes a rapid method to detect changes and variations between different genotypes. It has been noticed that selection of primers is an important factor in obtaining the genetic variations (Wolff, 1996).

Clustering of Lilium parental varieties and their mutant varieties is described in the present study (Fig. 4). Data was analyzed on the basis of genetic distances of mutants from their respective controls. Mutants of Samur La pink were divided into two main clusters: first group included mutants irradiated with 10.0, 8.0, 6.0 and 4.0 Gy doses of gamma irradiation while control plants of Samur La pink and mutants at 2.0 irradiation dose were clustered in one group. Dendrogram of mutants and control plants of Heaven white were also clustered into two groups as well: first group included mutants treated with 10.0, 8.0 and 6.0 doses of gamma rays whereas second group had control and mutants irradiated at 2.0 and 4.0 Gy dose. Mutants of Golden tycon yellow irradiated at 4.0 to 10.0 Gy were combined in one group while control plants and treated plants at 2.0 Gy were separated in the second group. In case of Orange pixie, mutants irradiated at 2.0, 4.0, 6.0, 8.0 and 10.0 Gy gamma irradiation were grouped in one group while control plants were clustered in another group.

DNAMAN Software was used to generate the multiple sequence alignment showed a sequence similarity index of 81.61% in V4, 82.41% in V5 & 80.81% in V6 and 81.79 % in V8 of Lilium parental varieties and their variants. Genes related with flower types in *Dianthus caryophyllus* were also identified by using the RAPD markers by Scovel *et al*. (1998). To characterize the *in vitro* grown plants of some ornamental plants, RAPD analysis has been carried out by Rady (2006) successfully in which changes occurred at DNA level during *in vitro* growth of the plants. Recently this technique has been used to check out the genetic polymorphism between parental chrysanthemum varieties and their mutants (Barkat *et al*., 2010). Williams *et al*. (1990) reported that nucleotide changes induce mutations which alter the genetic makeup so that the amplified product generated would be in different sizes.

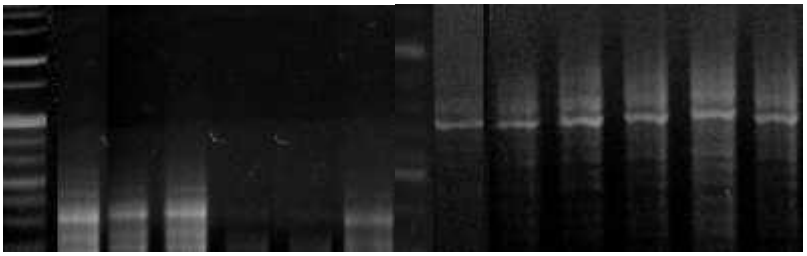
Table 3. Number of amplification and polymorphic products, after screening of eleven primers in different Lilium cultivars.

Primer number	Primer Sequence	Number of amplification	Polymorphic bands	Polymorphism (%)
LP1	TCA TCC GAG G	4	3	75
LP2	GGA AAC CCC T	10	8	80
LP3	GTG CGC AATG	5	3	60
LP4	AAG TGC GAC C	11	9	81.2
LP5	CCC GGA TGGT	9	4	44.5
LP6	CCG CAT CTA C	11	5	45.5
LP7	TGT CTG GGT G	10	5	50
LP8	AAA GCT GCGG	5	2	40
LP9	TGC GTG CTT G	16	8	50
LP10	CAC ACT CCA G	8	7	87.2
LP11	ACT TCG CCA C	16	10	62.5
	Mean	9.54±0.05	5.81±0.07	61.44



L C 2.0 4.0 6.0 8.0 10.0L C 2.0 4.0 6.0 8.0 10.0

Fig. 3(a): RAPD polymorphism inV6 (Lilium Golden Tycon Yellow) and its variants using primer 6 & 8.

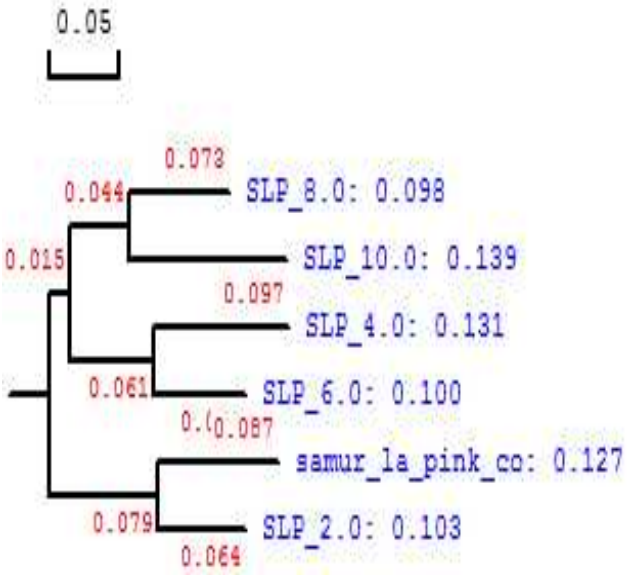


L C 2.0 4.0 6.0 8.0 10.0L C 2.0 4.0 6.0 8.0 10.0

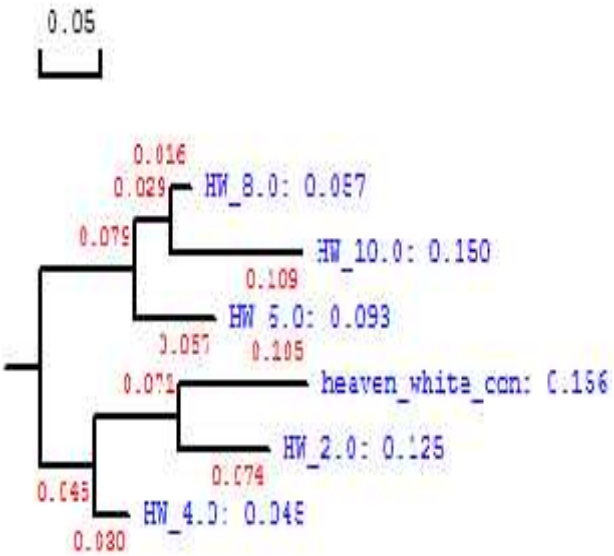
Fig. 3(b): RAPD polymorphism inLiliumV5 (Heaven White) & V8 (Lilium Orange colour) varieties and its variants using primer 2 & 9.

L C 2.0 4.0 6.0 8.0 10.0L C 2.0 4.0 6.0 8.0 10.0 L C 2.0 4.0 6.0 8.0 10.0

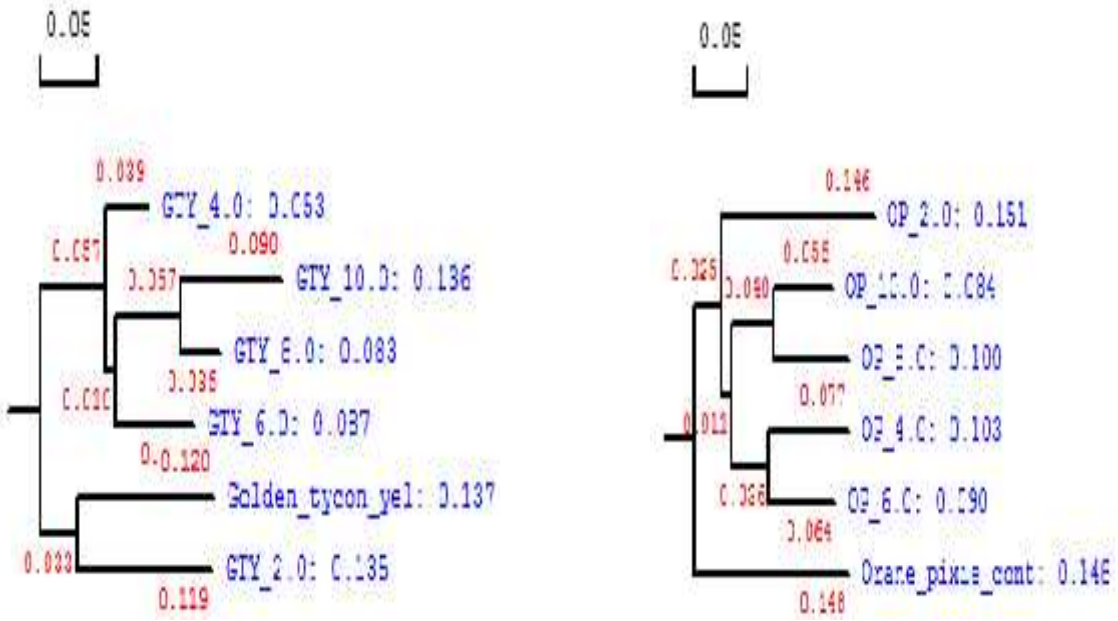
Fig. 3(c): RAPD polymorphism inV4 (Lilium Samur La pink) and its variants using primer 4, 7 & 10.



V4 (Samur La pink)



V5 (Heaven White)



V6 (Golden tycon Yellow) V8(Orange colour Liliy)

Fig. 4. Phylogenic tree showing the relationship among the parental varieties of Lilium cultivars and its variants using RAPD data and theUPGMA method of clustering to show DNA similarity.

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