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EVALUATING AND VALIDATING THE PROTOCOL FOR GAMMA (γ) RADIATION INDUCED MUTATIONS IN FLORAL DISTINCT *ROSA* SPP.

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Abstract

Among the highly fragrant *Rosa* species, *Rosa gruss an teplitz* and *Rosa centifolia* have high value in terms of commercial importance and economical trade. However, the absence of floral diversification and diversity in color patterns in these species acts as limiting factors in floriculture trade of these species. In the present study, we aimed to induce mutations using gamma radiations up to 120 Gy to observe the rate and the correlated effects on the several plant traits in micropropagated shoot tips of *Rosa gruss an teplitz* and *Rosa centifolia.* Irradiated shoot tips were micropropagated for one culture cycle and were acclimatized in a green house after *in vitro* rooting. Plants of irradiated population at 60 and 30 Gy showed 78.12 and 38.50% less culture rooting % age as well as 23.82 and 7 % less flower size as compared to non irradiated population of *Rosa gruss an teplitz* and *Rosa centifolia* respectively. Moreover, flower color component *a** (+ redness – green, 36.16 and 27.16) and chroma (37.77 and 27.5) depicted minimum while *L** (Lightness, 45.12 and 76.64), b* (+ yellow – blue, 10.69 and -4.14) and hue angle (17.1 and -8.58) maximum value. Apart from these variations, mutants of *Rosa gruss an teplitz* also produced variegated, pink color and different shape flowers. Genetic variations observed among the putative mutants of *Rosa gruss an teplitz* and *Rosa centifolia* were evaluated using twelve decamer RAPD primers. Phylogenetic inferences showed large genetic diversity in putative mutants as compared to mother plant.

Key words: *In vitro* mutagenesis, Genetic variation, *Rosa gruss an teplitz, Rosa centifolia,* RAPD.

Introduction

Rose (*Rosa* species) has extremely high economic value in international market and its products are also very commonly used in cosmetic, perfume, and pharmaceutical industry (Hussain & Khan, 2004; Shinwari, 2010). Being an important ornamental plant, the breeding program is focused on identification and characterization of the traits linked to the flower color and size to boost and augment the ornamental and economical value. In addition, the availability of the improved varieties with new embodied traits can increase the economical importance and the sustainable demand of the rose in the flower market (Marchant *et al*., 1996). Conventional breeding approaches have been widely used for the effective trait integration but there are certain limitations (Rout *et al*., 2006). However, mutation breeding by radiation has been extensively used for the development of new cultivars with improved characteristics in vegetative propagated ornamental plants (Kumar *et al*., 2006). It is also most effective in producing number of new rose varieties (Datta & Chakrabarty, 2005). This technique can also improve existing cultivars by providing a limitable solution by mutating recessive gene (Schum & Preil, 1998). Previously, several studies have demonstrated the importance of tissue culture coupled with mutagenesis as a tool to enhance the crop variety in ornamental plants (Novak, 1990; Hussain *et al*., 2011). Among the radiation methods for mutational breeding, ultraviolet and ionizing radiation has been widely used for inducing mutation (Jain, 2010). Gamma rays used for this purpose are considered to be more suitable for obtaining mutants with less radiation damage (Yamaguchi *et al*., 2010) and have been successfully applied to study the role of the induced mutagenicity in miniature roses (Arnold *et al*., 1998). Gamma irradiation has also been utilized to develop the M1 lines in *Atopa bellanoda* (Hady *et al*., 2008). Moreover, application of the gamma irradiation in *Cicer aretinum* L. resulted in the development of the freezing tolerant mutagenic lines (Akhar *et al*., 2011).

Typically, flowers of *Rosa gruss an teplitz* and *Rosa centifolia* blooms in small clusters and have a strong aroma (Beales *et al*., 1998; Saeed, 2005) and are typically used as a raw material for the production of the rose oil. Despite, being unique in their floral characters, still the proper exploitation of the species to fulfill the recent needs of floriculture industry is in naive mainly due to the small flower size and absence of the color variation or variegation. The above-mentioned limitation strongly urge the need to outlook measures, which can be applied to develop mutant breeding approaches with species having large size and diversified floral color variation. We made an attempt to induce mutations and to understand the floral color elasticity relative to the gamma (γ) radiation induced mutations in *Rosa gruss an teplitz* and *Rosa centifolia*.

Based on the amount of the gamma (γ) radiation dosage, a decrease of 78.12 and 38.50% less culture rooting % age and 23.82 and 7% less floral size as compared to non-irradiated population of *Rosa gruss an teplitz* and *Rosa centifolia* respectively at 60 and 30 Gy. In addition, we observed morphological trait variations in the mutants of *Rosa gruss an teplitz*, which produced variegated, pink color and different shape flowers. Subsequently, molecular genotyping was used to access the levels of the induced genetic variations among the putative mutants of *Rosa gruss an teplitz* and *Rosa centifolia* using twelve decamer RAPD primers, which revealed large genetic diversity in putative mutants as compared to mother plant. The presented research, reports the evaluation of the gamma (γ) radiation as a potential

source for inducing mutational breeding and trait enhancement, which could potentially benefit the floriculture trade value of *Rosa* species.

Material and Methods

Induction of mutations using gamma (γ) radiation: In our experimental layout, micropropagated uniform sized shoot tips (1 cm) of *Rosa gruss an teplitz* and *Rosa centifolia* were directly irradiated with different doses (0, 10, 20, 30, 40, 50, 60, 70, 80, 90,100, 110 and 120 Gy) of gamma rays in a Co_{60} gamma radiation chamber for mutation induction (Hewawasam *et al*., 2004). Immediately followed by irradiation, shoot tip explants were cultured in already standardized MS medium for shoot proliferation (MS macro, micro elements and vitamins) supplemented with Indole-3-butyric acid (IBA, 0.01 mg L^{-1}), Gibberellic Acid (GA_3, 0.4 mg L^{-1}) and Benzylaminopurine (BAP, 1.0 mg L^{-1}), sucrose (30 g L^{-1}) and agar (7 g L^{-1}). The pH of the medium was adjusted at 5.8 before autoclaving. Explants were kept in growth room at 24°C under cool white light for 16 h photoperiod (Philips TL 40 W fluorescent tubes). After six weeks of shoot proliferation, single shoots were separated from proliferated shoot cluster and were transferred to *In vitro* rooting medium (1/2 MS macro, micro elements and vitamins) supplemented with 20 g L^{-1} sucrose and 0.50 mg L^{-1} IBA (Baig *et al*., 2011). Subsequently, six-week-old cultured rooted plantlets were transferred to pots and acclimatized under controlled glass house conditions.

Molecular genotyping: For the identification and validation of the putative mutants, Random Amplified Polymorphic DNA (RAPD) assay were conducted on the mutant and the control samples. For this purpose young tender leaves of putative rose mutants were taken and genomic DNA was extracted as per the protocols described by Dellaporta *et al*. (1983) with modifications as per Weising *et al*. (1995). In order to investigate the potential effect of the gamma radiation in inducing the genetic variation, we used two sets of decamer random amplified polymorphic DNA (RAPD) primers (OPS and OPV) from Operon Technologies Inc., Alameda, California, USA to determine genetic variation among the mutants and the mother plants. In brief, amplification reactions were carried out in 25 µL reaction volumes containing 2 µL primers, 3 µL genomic DNA, 0.5 µL Taq DNA polymerase, 4 µL dNTPs, 2.5µL Taq buffer, 5 µL $MgCl_2$ and 8 µL DEPC water. DNA amplification profile consists of an initial pre-denaturation at 94°C for 5 min followed by 40 repetitive cycles at 94°C (1 min), 38°C (1 min), 72°C (2 min) and a final incubation period at 72°C for 5 min in thermocycler (CreaCon M. 0005.400). All the amplified amplicons were gel-electrophoresed on 1% agarose gel in 0.5 x Tris-borate-EDTA (TBE) buffer and were subsequently stained with Ethidium Bromide (EtBr) stain as per Sambrook *et al*. (1989). Amplified amplicons were visualized using ultraviolet light and gel images were captured using the gel documentation system (AlphaImager HP).

Statistical inference: We collected and evaluated following data measures during induction of mutations using gamma (γ) radiation and *In vitro* culture conditions*:* percentage (%) of plants showing leaf abnormality, number of shoots, culture rooting percentage, number of roots per shoot, root length (cm), fresh and dry weight (mg) under *In vitro* condition while flower size, variation in flower color and variegation after 18 months of acclimatization in green house. To infer the effect of the gamma radiation on the genetic divergence, RAPD fragments were scored as presence (1) and absence (0) of each of the primer accession combinations. The experimental design consists of CRD having four replicates and 60 explants in each treatment. The data obtained from the experimental set-up was statistically evaluated using two-way analysis of variance (ANOVA) and the differences among means were compared using Duncan's Multiple Range Test at 5% probability level (Steel *et al*., 1997).

Results and Discussion

Gamma radiation induced mutagenic effects: The main aim of the present study is to demonstrate the effect of the mutability induced by gamma radiation in *Rosa* species. To observe the mutagenic effect, gamma radiations were used as a source to irradiate the *Rosa* species and to evaluate the patterns of mutagenic inhibition in floral color variation and size. In our results, we evaluate the efficiency of gamma radiations up to 120 Gy and observed that at LD_{50} of 54 and 33 Gy, *Rosa gruss an teplitz* and *Rosa centifolia* respectively, depicted a significant decrease in the numbers of shoots as shown in Fig. 1a. Moreover, the treated and untreated explants produce a single shoot during *in vitro* rooting stage. Previously, in *Crossandra infundibuliformis*, it has been demonstrated that the increase in gamma irradiation dosage subsequently reduced the production of lateral shoots, which is in line with the observed results in our experiment (Hewawasam *et al.,* 2004). The decrease in the number of the lateral shoots may be due to the decreased concentration of endogenous growth regulators, particularly the break down of cytokinins due to irradiation (Omar, 1988). After irradiation, the irradiated explants at the early stages of growth showed some morphological abnormalities in leaves (Fig. 1b). Initially, up to the level of 30 Gy, we found no abnormal leaves but with the further increase in the dosage of the gamma radiation, number of shoots showing leaf abnormalities significantly increased and maximum leaf abnormalities were observed at 60 Gy in *Rosa gruss an teplitz* (21.25%) and *Rosa centifolia* (31.75%) respectively. The development of abnormalities in the leaves of the irradiated mutant populations with subsequent increase in the dosage of the gamma radiation may be attributed to physiological disturbances and chromosomal aberrations.

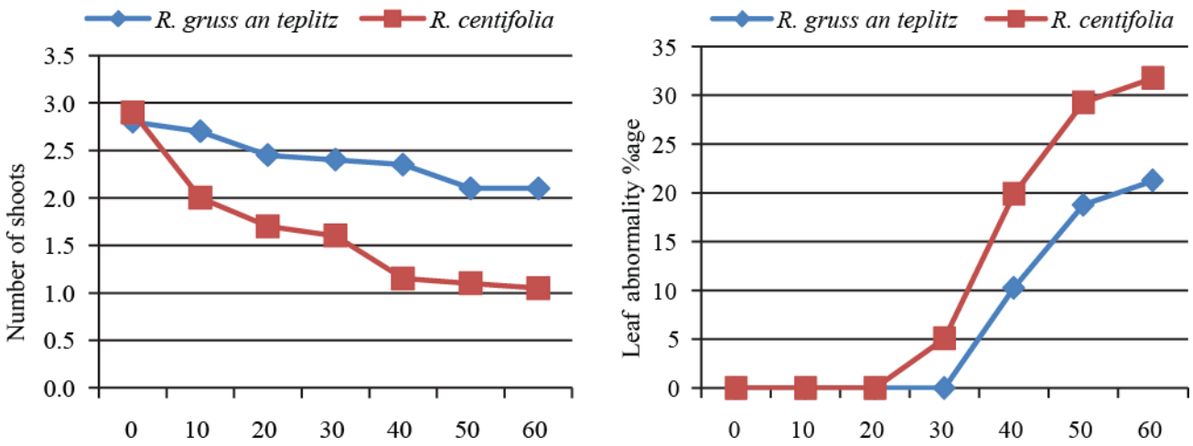

Fig. 1. Effect of gamma irradiation on number of shoots and percentage of plants showing leaf abnormalities in plants of *Rosa gruss an teplitz* and *Rosa centifolia*.

It has been previously demonstrated that chromosomal breakage, decrease in the auxin level, variation in the concentration of ascorbic acid and change in enzyme activity are few factors, which could potentially lead to abnormalities in leaves (Okamoto & Tatara, 1995). To observe the mutagenic effects of the gamma radiation on the rooting %, we tabulated culture-rooting percentage, number of roots per shoot, root length (cm) and observed a significant decrease in the culture rooting percentage with the relative increase in the dosage of gamma rays. A minimum culture rooting percentage i.e. 21.88% and 61.50% was recorded in *Rosa gruss an teplitz* and *Rosa centifolia* at 60 and 30 Gy gamma rays respectively (Fig. 2a). Subsequently, with the further increase in the dosage of gamma rays, no pattern of root formation was observed and an increased mortality rate was recorded in plantlets. Moreover, the observed number of roots and root length in plantlets also decreased with increase in gamma irradiation level. Increased level of irradiation at 60 and 30 Gy units of gamma rays showed least number of roots (5.40 and 6.02) and reduced root length (1.39 and 2.40 cm) in *Rosa gruss an teplitz* and *Rosa centifolia* respectively (Fig. 2b and 2c). The observed decrease in the number of roots and pattern of root development may be principally due to the down signaling and inactivation of the enzymes involved in auxin pathway balance by irradiation in gamma-irradiated mutant plants (Hewawasam *et al*., 2004).

We further extended the evaluation of the gamma irradiation to the last stage of *in vitro* rooting and observed significant variation in fresh and dry weight response of both rose species as a variable of different doses of gamma rays. A significant decrease in the fresh and dry weight in *Rosa gruss an teplitz* (15.00 and 13.12 mg) and *Rosa centifolia* (117.50 and 80.4 mg) at dosage of 60 and 30 Gy respectively was observed as compared to non-irradiated population. The observed decrease in the fresh and dry weight with increasing dosage of gamma irradiation, most plausibly may be due to slow cell division, abnormal transport of nutrients and metabolic activity disruption due to damage of apical meristem as a consequence of gamma irradiation (Okamoto & Tatara, 1995). Both species depicted same pattern of flower size with increase in the dosage of gamma rays. Non-significant reduction in the flower size of *Rosa centifolia* (4.91cm) (Fig. 3a) and *Rosa gruss an teplitz* (3.87cm) was observed at 30 and 60 Gy of gamma irradiation. This slight decrease in size might be due to the reason that the mutagenic application of gamma rays breaks the nuclear DNA during DNA repair mechanism and subsequently incorporates new mutations. Alternatively, the observed changes can happen also in cytoplasmic organelles, which might result in chromosomal or genomic mutations (Jain & Maluszynski, 2004). We evaluated several additional factors, which might contribute to color variance such as *L** (Lightness), a* (+ redness – green), b* (+ yellow – blue), chroma and hue angle. All the above-mentioned parameters were measured using Chromameter to record the variation in floral color of rose species. Highly significant results were recorded as shown in Fig. 3b, 3c, 3d, 3e and 3f. Maximum value of L* (45.12 and 76.64), b* (10.69 and -4.14) and hue angle (17.10 and -8.58) was observed in *Rosa gruss an teplitz* and *Rosa centifolia* at 60 and 30 Gy of gamma rays respectively. However, we observed that a* (36.16 and 27.16) and chroma value (37.77 and 27.50) decreased with increased level of gamma rays at 60 and 30 Gy dosage.

In addition to the above-mentioned results, we found some other mutants in *Rosa gruss an teplitz* species as shown in Fig. 4. Two plants in *Rosa gruss an teplitz* with variegated flowers having pink and red petals in the same flower were observed in the form of chimeras as a result of 60 Gy dosage of gamma irradiation. Moreover, two plants having pink flowers were also found in *Rosa gruss an teplitz* as compared to red color flower of untreated population. Apart from these mutants, a single plant having different flower shape was also noticed in this species. The observed changes may be due to ionizing irradiation, which may results in a complete or partial inactivation of genes, involved in the biosynthesis of color pigments (Lema-Rumińska & Zalewska, 2005). Previous studies in *Gerbera* resulted in the formation of several flower mutants by gamma irradiation in form of chimera (Laneri & Altavista, 1990). Similarly, variegated flower mutant plants were isolated in *Petunia hybrida* by irradiation of *Surfinia* (Miyazaki *et al*., 2002).

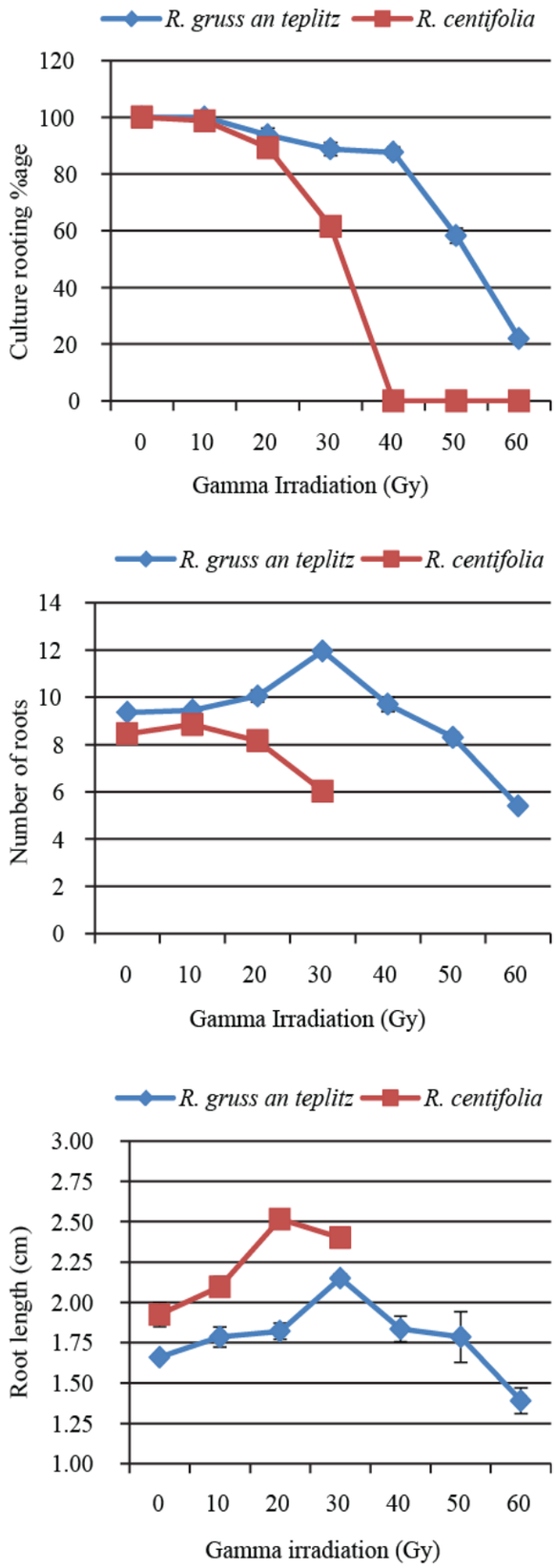

Fig. 2. Effect of gamma irradiation on culture rooting % age, number of roots and root length of *Rosa gruss an teplitz* and *Rosa centifolia*.

Observance of genetic variance among the mutants: Identification of the induced genetic variation by gamma irradiation can play an important role in understanding the behavior of the mutant plants towards the treatment of the ionizing radiation. RAPD is a useful technique for the rapid and easy assessment of genetic variation of mutants and may become a potential tool for the quick selection of mutants with great genetic variation during early growth stages (Teng *et al*., 2008). We evaluated genetic variation among the mutants using a set of 40 decamer RAPD primers from S and V series (Operon Technologies Inc. Almeda CA, USA). All the 40 RAPD primers were screened for amplification and 12 primers were found to be amplifying across mutant rose genotypes of *Rosa gruss an teplitz* and *Rosa centifolia* species induced by gamma irradiation (Table 1). Amplification profiles revealed a high number of dominant amplified loci (11) in mutant GM1, which is a putative mutant of *Rosa gruss an teplitz* species. In case of *Rosa centifolia*, maximum amplicons (10) were recorded in control and mutant CM2. Total number of bands amplified by these primers across mutants of *Rosa gruss an teplitz* and *Rosa centifolia* were recorded as 204 and 241 respectively.

Among the V series primer, OPV-10, OPV-15, OPV-17 and OPV-18 showed promising amplification across maximum genotypes (5) as compared to OPV-6 and OPV 8, which amplified minimum genotypes (1) in *Rosa gruss an teplitz* (Fig. 5). Among OPS series OPS-3 and OPS-5 amplified DNA of five genotypes while OPS-18 amplified DNA of one genotype of *Rosa gruss an teplitz*. In OPS series, OPS-11 amplified maximum number of genotypes (5) as compared to other primers of same series. In case of mutants of *Rosa centifolia*, a uniform amplification rate was observed. In total, OPV-10 and OPV-15 primers showed amplification in 5 genotypes as compared to OPV-6 and OPV-8.

On the basis of the computed genetic similarity (Nei and Li's genetic coefficient), dendrogram was constructed to infer the genetic relationship among different genotypes of *Rosa gruss an teplitz* (Fig. 6). We observed that mutant GM4 was found to be more diverse to control RG (32.7%) and also relatively different from other genotypes. It was observed that mutant GM2 and GM3 were also diverse from other genotypes but showed high similarity (77.5%) among them. Phylogenetic dendrogram of genotypes of *Rosa centifolia* revealed (Fig. 7) that mutant CM1and CM3 were dissimilar as compared to control RC. Mutant CM2 and CM4 showed 73.7% similarity to each other. Earlier RAPD has been successfully applied for the identification of the genetic relationship among the mutants in *Chrysanthemum* (Teng *et al*., 2008).

To conclude, we induce mutation in *Rose* species through the successful application of the gamma radiation and effectively demonstrated the effect of the various dosage of gamma radiation to develop mutant rose cultivars for floral size and variation.

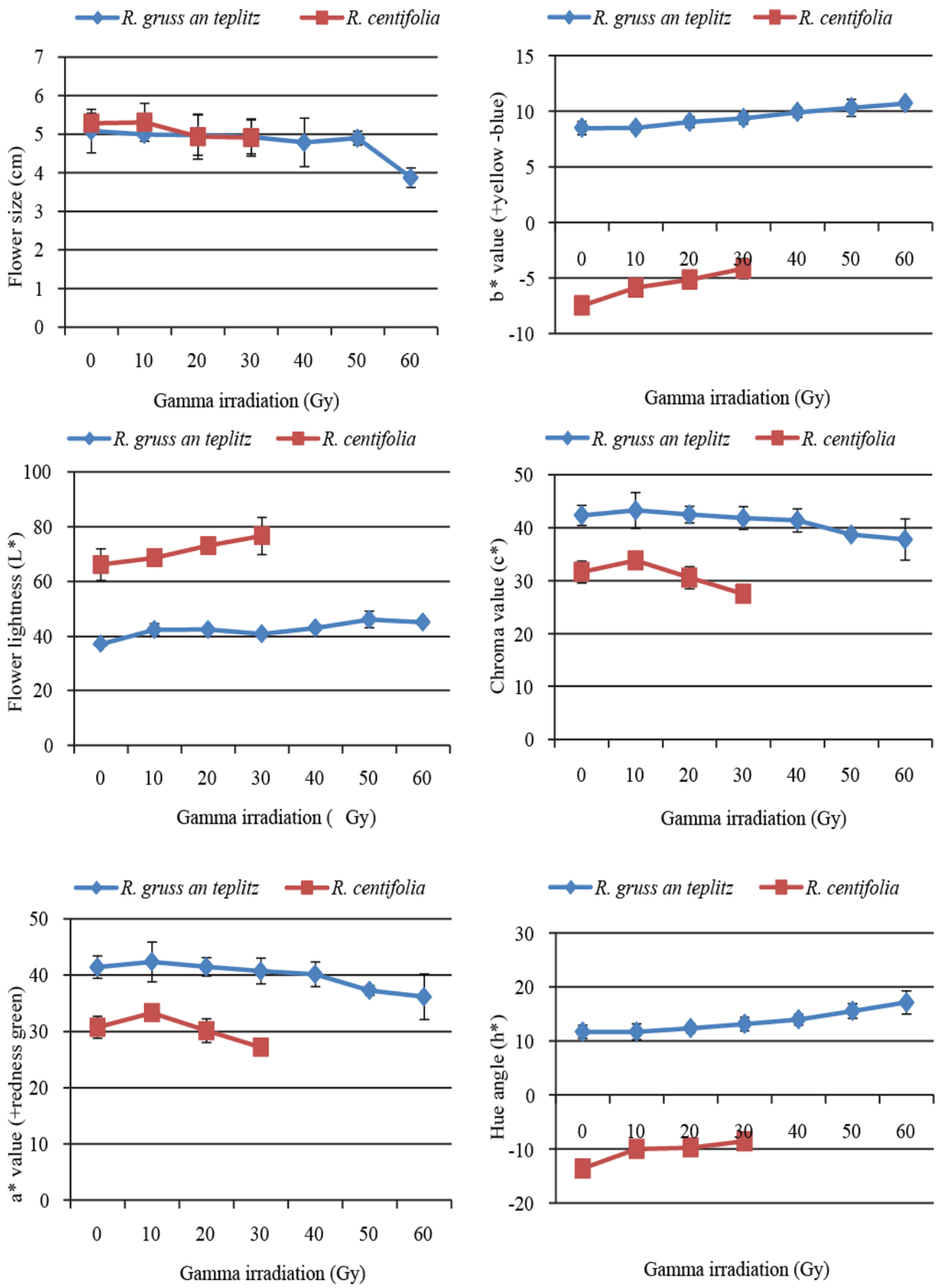

Fig. 3. Effect of gamma irradiation on flower size, *L**, *a**, *b**, chroma and hue angle in flowers of *Rosa gruss an teplitz* and *Rosa centifolia*.

Fig. 4. Mutants (b, c, d, e, f, g and h) obtained after gamma (γ) irradiation as compared to control (a) in *Rosa gruss an teplitz*.

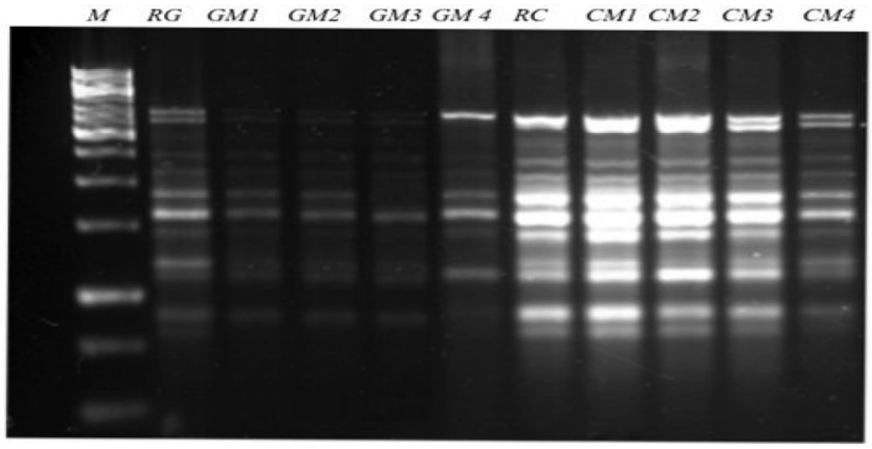

Fig. 5. Agarose gel showing RAPD banding pattern produced with OPV-18. * RG (*Rosa gruss an teplitz*), GM1 GM2 GM3 GM4 (Mutants obtained from *Rosa gruss an teplitz*), RC (*Rosa centifolia*), CM1 CM2 CM3 CM4 (Mutants obtained from *Rosa centifolia*).

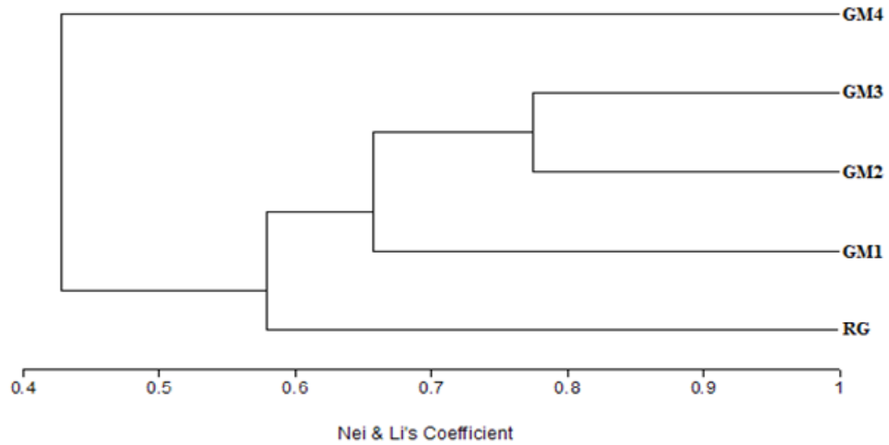

Fig. 6. RAPD based genetic relationship between mutants of *Rosa gruss an teplitz* obtained through gamma irradiation. [RG (*Rosa gruss an teplitz*), GM1 GM2 GM3 GM4 (Mutants obtained from *Rosa gruss an teplitz*)].

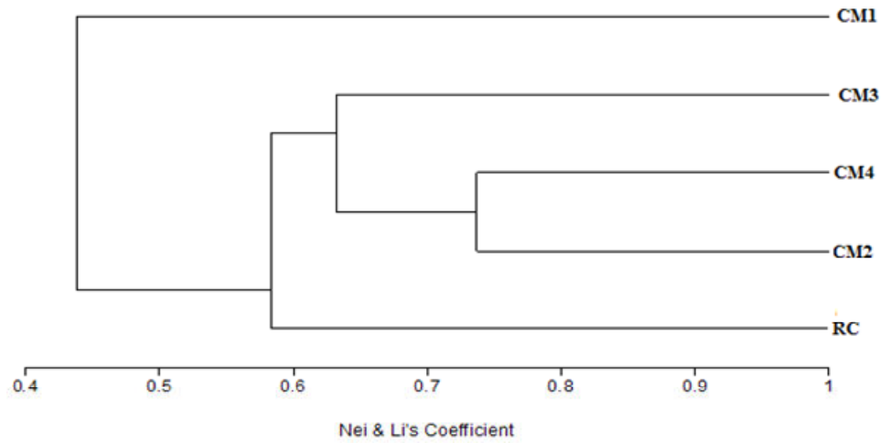

Fig. 7. RAPD based genetic relationship between mutants of *Rosa centifolia* obtained through gamma irradiation. [RC (*Rosa centifolia*), CM1 CM2 CM3 CM4 (Mutants obtained from *Rosa centifolia*)].

Table 1. List of the amplifiable primers for mutant detection in *Rose* species.

Sr. No.	Primer name	Sequence (5'-3')	Amplified bands in mutants of *Rosa gruss an teplitz*	Amplified bands in mutants of *Rosa centifolia*
1.	OPS-03	CAGAGGTCCC	22	24
2.	OPS-05	TTTGGGGCCT	16	16
3.	OPS-11	AGTCGGGTGG	9	11
4.	OPS-18	CTGGCGAACT	7	19
5.	OPV-06	ACGCCCAGGT	4	3
6.	OPV-08	GGACGGCGTT	8	24
7.	OPV-10	GGACCTGCTG	29	29
8.	OPV-12	ACCCCCCACT	20	25
9.	OPV-15	CAGTGCCGGT	26	26
10.	OPV-17	ACCGGCTTGT	37	14
11.	OPV-18	TGGTGGCGTT	19	19
12.	OPV-20	CAGCATGGTC	7	31

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