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GENETIC ANALYSIS OF *RHODODENDRON* MUTANTS USING RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD)

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Abstract

The aim of our study was to consider the efficiency of randomly amplified polymorphic DNA (RAPD) analysis to show the differences between *Rhododendron* mutants and control plants. The mutant plantlets were selected due to their better *In vitro* shoot and root regeneration relative to control in both varieties. Six mutant plantlets from Alfred and seven mutant plantlets from Paars variety were selected from M_1V_2 generations. These mutants were selected from plantlets which were irradiated with doses 5 and 10 Gy. They were grown up *In vitro* conditions. The dendrogram shows one big cluster and the others to be distinguished. Genetic variability induced with gamma ray and RAPD methods were used to detect mutations at the *Rhododendron* shoot culture. Our results show that *Rhododendron* mutants were district from controls.

Introduction

Rhododendron species that belong to the Ericaceae, are always green and leafless in winter. Traditionally, most *Rhododendron* species are propagated by conventional methods like stem cutting. LineShould be removed *In vitro* micropropagation is becoming important in commercial production. *Rhododendron* shoot regeneration was obtained from flower, leaf explants and shoot tips (Hsia & Korban, 1997; Samyn & Bockstaele, 2000, Samyn *et al.,* 2002; Tomsone & Gertnere, 2003).

Induced mutation techniques can generate genetic variation and significantly increase. This is the first requirement for plant breeding. The desired genotype selection is the second step for new cultivar. Physical and chemical mutagens used from many researchers for increase of plant variability and the number of released mutant were found. NaCl (Hossain *et al*., 2006), heat (Das *et al*., 2000), herbicide tolerances (Atak *et al*., 2004) are the important characters to improve by mutation breeding. Many useful mutants were obtained by gamma irradiation at vegetative propagated plants (Ahloowalia & Maluszynski, 2001). In recent years, *In vitro* techniques were being used for plant breeding. Moreover, *In vitro* mutagenesis could give an opportunity to select the mutant seedlings. Induction of *In vitro* mutation for vegetative propagated *Musa* spp., produced some useful mutants (Roux, 2004).

Cytokinin and auxin are the important plant hormones that influence developmental processes. Interaction of auxin and cytokinin controls shoot and root development. Regulation of biosynthesis and degradation of these plant hormones plays an important role in organ growth (Kyozuka, 2007; Zhao, 2008; Bajguz & Piotrowska, 2009; Moubayini *et al*., 2009).

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RAPD analysis is suitable for genotyping, phylogenetic analysis and molecular selection (Williams *et al*., 1990; Göktürk *et al*., 2003; Atak *et al*., 2004; Akcicek *et al*., 2005; Yüzbasıoğlu *et al*., 2006). RAPD among other molecular marker methods has considerable advantages because it is fast, not expensive, applicability to any organism without prior information on the nucleotide sequence and in the potential detection of DNA damage and mutation (Ahloowalia & Maluszynski, 2001; Atienzar *et al*., 2002, 2006). RAPD markers have been widely used in the phylogenetic analysis of many plants and a general concordance was demonstrated among the results derived from RAPD and other techniques (Naugžemys *et al*., 2007).

RAPD method has been used to determine the genetic diversity and relationship of *Rhododendron* species (Lanying *et al*., 2008). Some workers have reported that RAPD marker detected a large genetic polymorphisms and detection mutation various plants which I*n vitro* induced mutagenesis using chemical mutagens (Hofmann *et al*., 2004; Khawale *et al*., 2007). Gamma radiation was widely used for changes in DNA and genetic variability was also created in various varieties. In these varieties RAPD analysis have been used to detect polymorphisms (Khan *et al*., 2007; Lu *et al*., 2007; Wang *et al*., 2007; Khan *et al*., 2009). Selvi *et al*., (2007) used RAPD analysis for identification of DNA polymorphism of gamma ray treated *Emblica officinalis* scions (V1M1 V2M). Jain *et al*., (2000) studied geographically isolated population of *Rhododendron arboreum* (Ericaceae). The level of genetic variability for these plants and the relationships within and among the disjunctions were evaluated by employing RAPD assay. RAPD revealed higher genetic variability among the individuals of temperate *Rhododendron* populations. Pillay *et al*., (2003) used RAPD to detect DNA variation among clones of banana cultivars. The RAPD primers produced identical banding patterns in all the samples. The differences among the atrazine mutants were examined by using RAPD and the differences between herbicide resistant mutants were shown (Atak *et al*., 2004; Sandhu *et al*., 2002). In this study we aimed to show the differences between the *Rhododendron* mutants and control plants with RAPD method.

Material and Method

Plant tissue culture: In this study, we used mutant shoots of purple flowered *Rhododendron*. The shoot tip explants of *Rhododendron* varieties were irradiated with doses 0, 5, 10, 20, 30 and 50 Gy of gamma rays (Atak *et al*., 2003). The explants were regenerated in the growth chamber at 25^{o}C with 16 hours light /8 hours dark periods.

Mutant characteristics: The shoot induction decreased according to increased radiation doses. For this reason plantlets of "Alfred" and "Paars" *Rhododendron* varieties which irradiated with doses of 5 and 10 Gy of gamma ray, were grown on the medium described below (Atak *et al*., 2003). For this study, 6 mutant plantlets from Alfred (A2, A3, A4, A5, A6 and A9) and 7 mutant plantlets from Paars (B2, B3, B4, B5, B6, B7 and B9) variety were selected from M_1V_2 generations and they were grown up *In vitro* conditions. The characteristics of mutants were better *In vitro* root and shoot regeneration relative to control. Alfred mutants were selected from the explants, which were irradiated with doses of 10 Gy, except one mutant (A2) was selected from irradiation with 5 Gy. Paars mutants were selected from the explants, which were irradiated with doses of 5 Gy, except 2 mutants (B7, B9) were selected from irradiation with 10 Gy. These mutants were grown up in climate chamber which has light/dark (16/8) day period at 27^{o}C, under controlled conditions.

Shoot medium: Explants were placed in "Duchefa Steri Vent" container containing Anderson's macronutrients and micronutrients, B5 vitamins, 80 mg/L Adenin hemisulphate, 30 g/L sucrose, 8 g/L agar supplemented with 5mg/L 2iP. Twenty eight days later, shoots were transferred to the new medium and shoots were growing on medium which were 1/10 MS medium and vitamins plus 80 mg/L Adenin hemisulphate, 30 g/L sucrose, 8 g/L agar, 0.5 mg/L Zeatin, 0.5 mg/L TDZ and 0.0175 mg/L IAA (George *et al*., 1996; Hsia & Korban, 1997; Marks & Simpson, 1999; Tomsone & Gertnere, 2003).

Root medium: Long shoots were transferred to root medium (1/10 MS medium and vitamins, 600 mg/L charcoal activated, 10 g/L sucrose, 8 g/L agar, 5 mg/L IBA) (George *et al*., 1996; Almeida *et al*., 2005).

RAPD analysis: The differences between control and mutant plants were detected by the RAPD assay.

The genomic DNA isolation: The plant DNA extraction was carried out from the leaves described by Doyle & Doyle (1987). DNA extraction was carried out with minor modifications. Fresh leaves (0.05g) were ground using mortar. The grindate was added to 1 ml extraction buffer (2% (w/v) CTAB; 100 mM Tris-Cl buffer (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, (1% 8w/v) PVP-40) and incubated at 65^{o}C for 90 min. The homogenate was mixed with 500 ml 24:1 chloroform: isoamyl alcohol (v/v) and mixed well by gentle inversion. Following centrifugation at 10 000 rpm for 10 min, the upper aqueous layer was transferred to a fresh tube containing 600 ml of isoproponal, the mixture was allowed to sit at room temperature for 40 min. Following centrifugation at 5000 rpm for 3 min the samples were washed twice with 76% ethanol. The pellets were allowed to sit overnight at room temperature, and resuspended in TE buffer (10 mM Tris-Cl, pH 8.0, 1.0 mM EDTA pH 8.0).

Amplification conditions: The PCR amplifications were performed as described by Williams *et al*., (1990). Amplification was carried out in a Techne (UK) Progene Thermocycler. Fourty oligonucleotides from Operon Technologies were used for RAPD amplification. Of thirty, 8 primers (M13, B6, B18, OPU16, OPU06, A2, A7, B4) were chosen for further amplification of the DNA (Table 1). The amplification reactions were set in 100 µl final volume of reaction mixture containing 1 unit of Taq polymerase, 10 ml of 10X Taq DNA polymerase buffer, 10 pmol primer, 25 ng of genomic DNA and 1 mM $MgCl_2$. DNA amplification was performed in a Biometra thermocycler (T Personel) (This sentence should be removed). The thermocycler was programmed for 45 cycles, each of which had the following temperature profile: 30 s at 96ºC, 30 s at 35ºC and 30 s at 72ºC. 15 µl of amplification products were loaded in a 2% agarose gel that run 4 hours at 90 mV in 1xTAE buffer. A 100 base-pair DNA ladder was used as molecular weight markers. The gel was stained with Ethidium bromide and photographed under UV light (Maniatis *et al*., 1982).

Data analysis: Bands on RAPD gels were scored as either present (1) or absent (0) for all subspecies studied. Common band analysis was conducted using the computer programme POPGEN (Dendrogram based Nei's 1978, genetic distance: method UPGMA modified from NEIGHBOR procedure of PHYLIP ver. 3) to determine the genetic distance between *Rhododendron* mutants. The values for genetic distance were then used in a cluster analysis to generate UPGMA-dendrograms (Nei, 1978).

Table 1. List of primers used in RAPD studies.

Primer no.	Sequence (5' --- 3')
M13	5'-GAGGGTGGCGGTTCT-3'
B6	5'-TGCTCTGCCC-3'
B18	5'-CCACAGCAGT-3'
OPU16	5'-CTGCGCTGGA-3'
OPU06	5'-CCACGGGAAG-3'
A2	5'-TGCCGAGCTG-3'
A7	5'-GAAACGGGTG-3'
B4	5'-GGACTGGAGT-3'

Results

Markers were selected based on their ability to generate a visible polymorphism between the samples. For RAPD analysis, of thirty primers, 8 primers were chosen for further amplification of the DNA. RAPD bands were ranging from 200 bp to 1500 bp in size. Some of the bands were monomorphic, while some of them showed at least one polymorphism (Fig. 1).

Samples from Paars and Alfred mutants were processed and analyzed separately using eight selected primers which gave clearly resolved band pattern. Table 2 shows genetic distance between mutants of Paars, Alfred and control individuals of these two *Rhodedendron* variety calculated based on RAPD data. Dendrogram was constructed using POPGENE computer program (Fig. 2).

The dendrogram shows one big cluster and the others to be distinguished. The lower cluster subdivided into two subcluster and the upper cluster divided into two subgroups. Control individuals of Alfred and A6 grouped together in upper subgroup and A3 and A5 are grouped in second subgroups, having genetic distance 12%. The upper subcluster contains KA1, KA3, KA2 and A6 having genetic distance 10%, 11% and 13% with each others respectively. As expected individuals from Alfred population are grouped together with one exception of A6 individual jumping to a control population.

B5 and B9 mutants from Paars are in the lower subgroup and B9 and B5 much closer to each other than the others with the genetic distance of 15%. The upper cluster contains individuals from Paars control and mutants and Alfred mutants. However, control individual of Paars seems distant than the rest of individuals, A6, KA2, KA1 and KA3.

The upper cluster contains the rest of Alfred mutants (A4, A2 and A9) and Paars mutants (B2, B3, B4, B6 and B7). Of these cluster members, A4 and A2 much closer to each other (20%) than A9 with the genetic distance 25%, 24%. The dendrogram shows Paars control sample having genetic distance between 24% and 33% with the Paars mutants.

Discussion

Induced mutation combining with I*n vitro* culture techniques and methods are used to make a research in plant breeding for improving varieties. Genetic variability occurred in all the mutagenic treatment and gamma radiation was used to induce new variation for traits.

Irradiation by this physical mutagenic agent leads DNA break formation via direct and indirect detrimental effects. In direct interactions, the radiation energy is transferred to the targets; in indirect interactions, energy is absorbed by the water present in the external medium. After hydrolysis of water, seconder messenger molecules (H_2O_2, $O_2^{\cdot}$-, $^{\cdot}OH$) affect the biomolecules (Esnault *et al*., 2010).

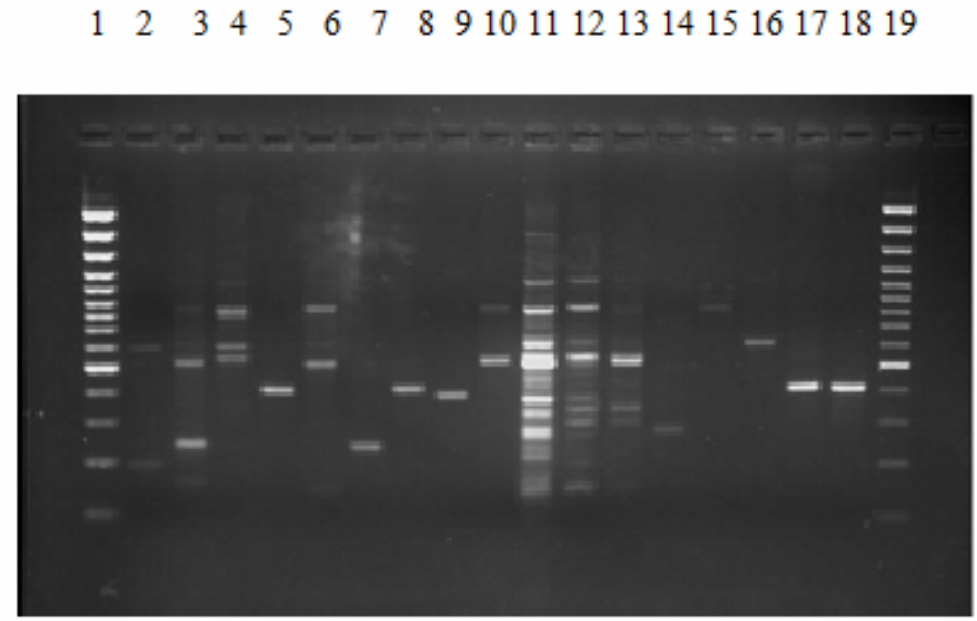

Fig. 1. RAPD profiles of genomic DNA of Alfred, Paars and control samples of *Rhododendron*. Lane 1 and 19. Molecular size marker (100 bp molecular size ladder), Lane 2-Lane 18: KA1, KA2, KA3, A2, A3, A4, A5, A6, A9, KB, B2, B3, B4, B5, B6, B7, B9.

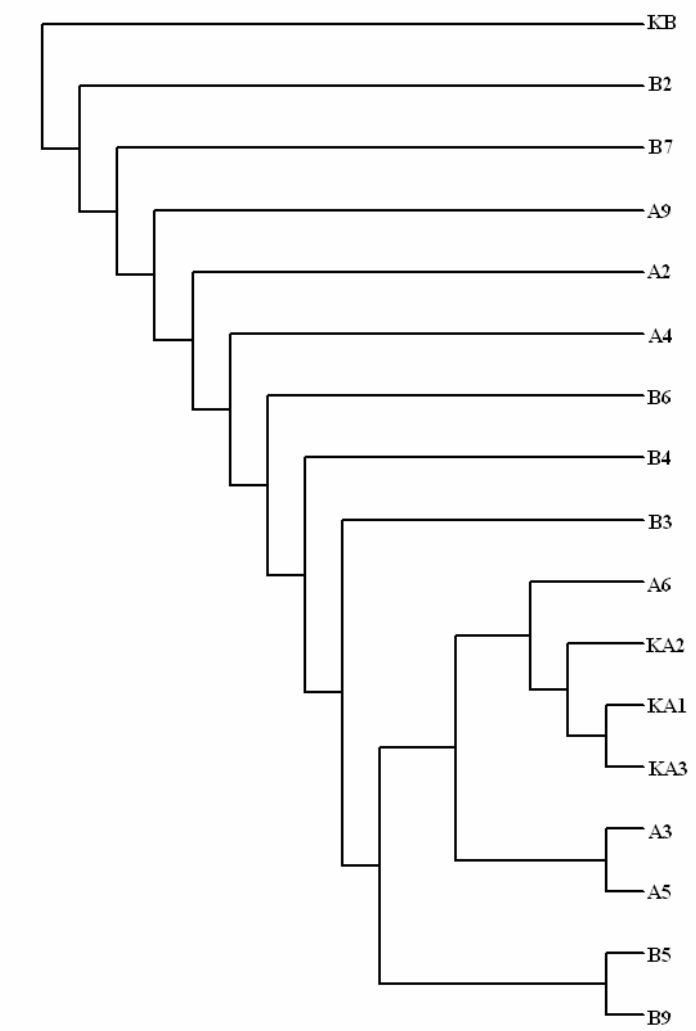

Fig. 2. UPGMA Dendrogram showing the relationships between individuals from Alfred, Paars and control sample of *Rhododendron*.

Table 2. Nei's genetic distance (below diagonal) among Paars, Alfred and control sample.

pop ID	KA1	KA2	KA3	A2	A3	A4	A5	A6	A9	KB	B2	B3	B4	B5	B6	B7	B9
KA1	**																
KA2	11	**															
KA3	10	11	**														
A2	17	20	20	**													
A3	14	13	15	18	**												
A4	13	14	18	20	20	**											
A5	14	15	15	18	12	20	**										
A6	11	11	13	20	14	14	14	**									
A9	18	21	18	24	14	25	20	19	**								
KB	23	24	24	29	26	28	30	25	31	**							
B2	20	21	21	24	20	27	23	20	25	28	**						
B3	16	16	18	20	16	18	19	16	21	24	20	**					
B4	14	18	18	22	16	20	18	17	18	29	20	18	**				
B5	14	15	18	18	15	18	16	13	18	27	20	16	18	**			
B6	15	18	17	20	18	19	18	18	23	31	25	17	20	16	**		
B7	16	21	20	23	21	22	23	16	25	33	25	23	21	21	22	**	
B9	14	13	16	20	16	18	18	14	21	27	21	16	16	15	16	17	**

In the present study the mutants of Alfred and Paars were better *In vitro* shoot and root regeneration relative to control. They may be modified in their shoot and root forming capacity in tissue culture, because of altered in endogenous auxin and cytokinin levels in the hormones transport or in the hormones signaling pathway. *In vitro* growth of cultured tissue or organs of plants and regeneration are affected by the genotype. The shoot forming phenotype and rooting capacity are linked to altered cytokinin and auxin content and signaling (George *et al*., 2008; Moubayini *et al*., 2009; Perilli *et al*., 2010).

RAPD analysis is used for detection of DNA alterations (DNA adducts, DNA breakage, point mutations, large rearrangements). The present or absent RAPD bands are used to estimate diversity and similarity measurement (Upadhyay *et al*., 2004; Gostimsky *et al*., 2005; Atienzar & Jha, 2006; Choi *et al*., 2006; Wang *et al*., 2009).

Mutant plantlets from Alfred and Paars varieties showed polymorphic bands. We observed that dendrograms show Paars and Alfred mutants, which were distinct from control. Cluster analysis separated mutants into different groups. Genetic distance of 33% between Paars *Rhododendron* mutants and control showed maximum distinct. Although mutant of Alfred was better *In vitro* root and shoot regeneration relative to control, the genetic distance observed between control and mutant of Alfred (A6) was low and control individuals and A6 are grouped together. At the *Rhododendron* shoot culture, Paars were more radiosensitive according to Alfred, the upper cluster of the dendrogram shows Alfred and Paars mutants.

The detection of the differences with the mother and mutant plants were performed with RAPD method in different species. Li & Nelson, (2002) studied wild (*G. soja*) and cultivated soybean (*G. max*) and they had large morphological differences but they showed that the genetic distance within the *G. soja* group was larger than within *G. max*

group. Sandhu *et al*., (2002) irradiated seeds of 21 rice lines with 200, 250 and 300 Krad/h with ^{60}Co source. The differences between resistant and susceptible lines for glufosate were analysed with RAPD method. Lema-Rumińska *et al*., (2004) differed the genetic variations of *Dendranthema grandiflora* Tzvelev irradiated with X or 15 Gy gamma radiation dose. They obtained polymorphic bands from the mutant plants with 8 RAPD primers. Soniya *et al*., (2001) used RAPD method to detect the genetic variation among callus regenerated plants of tomato. Puchooa, (2005) observed no differences of RAPD profiles between mother *Anthurium* plant and mutant plants irradiated with 5 Gy gamma radiation dose which showed phenotypic differences. Kumar *et al*., (2006) detected genetic variability among chrysanthemum radiomutants (flower shape, floret shape and flower color) with RAPD. The cluster analysis of chrysanthemum radiomutants separated into different groups but the genetic distance, which was observed between them, was low, except two mutants. Khan *et al*., (2007) studied genetic variability in sugarcane induced somatic mutations using gamma ray and their RAPD data showed that the similarity between mutants and parents decreased while the irradiation doses increased.

The level genetic variability and relationships within and among the populations of temperate and tropical *Rhododendron* have studied by Sakakibara, (1997) and Jain *et al*., (2000). They used RAPD assay for revealed genetic variability among individuals of *Rhododendron* populations. There are many studies for genetic relationship between *Rhododendron* species and hybrids (Iqbal *et al*., 1995; Scheiber *et al*., 2000; Zha *et al.,* 2008; Caser *et al.,* 2010). Also 2 mutant varieties for *Rhododendron simsii* for flower color and 13 mutant varieties for *Rhododendron* sp., were development for flower color and dwarfness improved by mutation breeding (Maluszynski *et al*., 2000).

In vitro mutation studies by Alfred and Paars varieties began in this study. Mutant plants have better root and shoot growth than the mother plants i*n vitro* culture conditions. Application of gamma radiation to *Rhododendron* explants possibly caused changes in expression profiles of the genes. We could have selected these *Rhododendron* mutants which their genes have roles in cytokinin-auxin crosstalk. RAPD methods showed the genetic difference between *Rhododendron* varieties and mutants, which exposed to the gamma radiation doses 5 and 10 Gy.

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