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RAPD-PCR EVALUATION OF *CARICA PAPAYA* PLANTS RAISED FROM THE SEEDS TREATED WITH ESTROGEN-A HUMAN SEX HORMONE

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RAPD-PCR evaluation of papaya (*Carica papaya*) plants raised from the seeds treated with Estrogen (Premarin) a human sex hormone was undertaken. Analysis of RAPD data of all the treatments showed significant genetic variation. Among 17 primers used, only two primers namely, OPF-12 and OPC-16 were properly amplified and had repeatable results. They were amplified template DNA to a total of 58 DNA fragments and showed polymorphism. The level of polymorphisms was different with different primers among different plants raised from 10 and 20 ppm premarin treated seeds. Cluster analysis based on UPGMA revealed two major clusters namely, A and B with primer OPF-12 at a similarity coefficient of 64%. The cluster A is the largest one with 10 genotypes and divided into two sub-clusters, namely, A_1 and A_2 at a similarity value of 76%. The sub-cluster A_1 again divides in two groups at similarity value 83%. In $A_{1(a)}$ only 10 ppm premarin treated plants for 12 h and in $A_{1(b)}$ control treated for 12 and 24 h were present. On the basis of present results, it is concluded that the plants raised from seeds treated with premarin human sex hormone have nearly same genotype.
Keywords: Estrogen (Premarin), sex hormone, polymorphism, UPGMA, OPF-12, OPC-16

Carica papaya (Caricaceae) is a widely cultivated popular and economically important fruit tree of tropical and subtropical countries. Three types of plants are recognized based on flower type: female, hermaphrodite, and male. If no male or hermaphrodite plants are nearby to provide pollen, female plants usually fail to set fruit. The ratio of male to female plants is sometimes very low and development of large number of male plants is not desirable. Several phytohormones (auxins, gibberellins, naphthalene acetic acid, maleic hydrazide and ethrel) have been used as foliar spray or for seed treatment to enhance the number of female flowers and fruits on monoecious plants, particularly in the cucurbits by a large number of investigators in India and abroad. Investigations have also been made to study the effect of phytohormones on sex expression in *Carica papaya* (Kumar and Jaiswal 1984, Mitra and Ghanta 1998, Begum *et al.* 1998). There are a few reports about the successful use of human sex hormones (estrogen) on increasing the number of female flowers in cucumber. However, the effect of human sex hormones has not been studied on other plants including *Carica papaya*. Present investigation proposes to study the effect of Estrogen-a female sex expression in this fruit tree. Random amplified polymorphic DNA (RAPD) analysis has been undertaken to differentiate between the sexual forms of plants raised from sex hormone treated seeds and control plants on molecular basis.

Brazil is currently the world's largest producer of papaya (*Carica papaya* L.), producing fruits for both the domestic market and export. According to Lemos *et al.* (2002) fruits only from hermaphrodite plants are marketed because they have the necessary commercial characteristics. Increased papaya yield has been limited mainly by the ratio of female to hermaphrodite (1: 2) plants normally occurring in orchards. This ratio causes great losses to papaya producers and the identification of the sex of seedlings during the nursery stage would be an important advance. Lemos *et al.* (2002) conducted random amplified polymorphic DNA (RAPD) analysis between the sexual forms of three commercial *C. papaya* cultivars. RAPD assays using the BC210 primer were able to detect hermaphrodites in all of the cultivars tested. The $BC210_{438}$molecular marker was much better at papaya sex differentiation than other

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markers described in the literature.

The occurrence of mammalian sex hormones e.g. 17B-estradiol, androsterone, testosterone or progesterone, have been reported in 60-80% of the plants (Anna and Andrzej 2005). Enzymes responsible for their biosynthesis and conversion were also found in plants. Treatment of the plants with sex hormones or their precursors influenced plant development: cell divisions, root and shoot growth, embryo growth, flowering, pollen tube growth and callus proliferation. Mammalian sex hormones - estrogens, were first detected in plants by Dohrn *et al.* (1926) and then in the 1930s, simultaneously by Butenandt and Jacobi (1933) and Skarzynski (1933). According to some reports, mammalian sex hormones can modulate sex expression in flowers of dioecious plants (which have masculine and feminine flowers on the same plant). For example, in *Ecballium elaterium* L., the application of estrogens considerably affected the total number of flowers as well as increased the ratio of female to male flowers. On the other hand, androgens increased the ratio of male flowers (Kopcewicz 1971). In cucumber, the number of female flowers increased after treating the plants with 17B-estradiol as well as with testosterone (Gawienowski *et al.* 1971). Moreover, cucumber plants treated with these hormones produced the first flower on the first node while control plants produced it on the fourth node. Other authors, however, do not confirm the results cited above (Jones and Roddick 1988). They tested the influence of 17B-estradiol, estrone and testosterone on sex expression in cucumber, pumpkin and spinach. No significant modification of sex expression tendency in the flowers of these plants was observed under the influence of the mentioned hormones. Estrogens and testosterone may play a role in the process of pollination and fertilization in plants. Estrogens and testosterone stimulated pollen tube growth of *Rumex tenuifolius* Waller. (Löve and Löve 1945).

The molecular mechanism of mammalian sex hormones activity in plants is not explained and still requires investigations. Keeping this in view present investigation was undertaken to evaluate the molecular changes in *Carica papaya* plants raised from seeds treated with Estrogen used in the form of Premarin a female sex hormone.

MATERIAL AND METHODS

Seeds were collected from mature, ripe and large fruits of a local variety of *Carica papaya*. The fruits were cut and seeds were carefully taken out. These were washed and dried in shade and stored in plastic bags till used.

Seed treatments: Seeds were washed and soaked in distilled water for 4 h. After drying in shade the seeds were treated with different hormones solutions by the following procedure:

Estrogen: The tablets of premarin of Pfizer Limited were used. (Each tablet contains *0.625 mg* conjugated estrogens. It is water soluble and 100 **s**eeds were soaked in 10 and 20 ppm aqueous solutions for 12 and 24 h. For control 100 seeds were soaked in distilled water for 12 and 24 h.

Sowing of seeds: Variously treated seeds were sown at the depth of 1 cm in the soft black plastic bags of 6x16 cm with a drainage hole containing a mixture of sandy loam and sand (3:1) with adequate organic manure maintaining the pH at 6.5-7.0. They were irrigated with water containing 2% bavasten. The bags were covered with jute bags to maintain temperature and moisture till emergence of seedlings. Water was sprayed regularly to maintain the top soil moisture. Soon after germination, the jute bag cover was removed and the polythene bags were mildly irrigated to maintain constant moisture.

Following data was collected from chemically treated and control plants: Time taken for germination and germination percentage, plant height at the time of floral bud initiation, number of male, female and hermaphrodite plants, days taken for the initiation of male, female and hermaphrodite flowers, number of male flowers/plant, number of female flowers/plant, and number of hermaphrodite flowers/plant, size of different type of flowers,

Table 1: Types of samples used for RAPD-PCR analysis

S. No.	Abbreviation	Chemical	Concentration (ppm)	Treatment (h)
1.	P10(12)	Premarin	10	12
2.	P10(24)	Premarin	10	24
3.	P20(12)	Premarin	20	12
4.	P20(24)	Premarin	20	24
5.	C(12)	Control	--	12
6.	C924)	Control	--	24

Seeds were soaked in distilled water for 12 and 24 h to serve as control.

days taken for the formation of fruits on female and hermaphrodite plants, size and weight of fruits, and total yield/ plant. Data was statistically analyzed and results are published elsewhere (Chaurasia and Chauhan 2013).

Molecular Evaluation- The molecular changes in the young leaves of plants raised from the seed soaked in hormone solutions and control plants were compared by RAPD-PCR. RAPD analysis of the plants were used to differentiate between the sexual form raised from hormone treated seeds and control seeds.

DNA extraction - Total genomic DNA was isolated from different types of samples (Table 1) using the Hi Pura TM plant genomic DNA miniprep purification spin kit.

Quantity and purity of DNA: The yield of DNA per gram of leaf tissue was extracted and measured using a Thermo scientific nanodrop spectrophotometer at 260nm. The purity of DNA was determined by calculating the ratio of absorbance at 260nm to that of 280nm. DNA concentration and purity was also determined by running the samples on 0.8% agrose gel.

RAPD-PCR Reaction- DNA amplification was done using the primers OPE 01, OPAH 10, OPA1, OPH01, OPJO1, OPF12 and OPC16. PCR reactions were performed in a volume of 20 µl containing 2 µl PCR buffer 2 dNTPs, 0.5 µl taq polymerase,1 µl primer, 2 µl genomic DNA and rest amount of sterile de-ionized water (12.5 µl) to prepare 20 ml reaction mixture. PCR reactions were performed by denaturation at 95°C for 5 min on initial cycle and 30 seconds to 1 min on rest; annealing at 5°C for 30-45 seconds used as gradient and finally extension at 72°C for ~1 min/kb of expected product and only 5-10 min on last cycle with total ~30 cycles. The amplified PCR products were subjected to electrophoresis to separate the DNA fragments on 1.5 % agrose gel with 1µl ethidium bromide in 1X TAE buffer at a constant voltage of 70 volts for 2- 2.30 hrs. 1 µl loading dye was added in each PCR product, thereafter loaded into the wells. Markers were used for comparing the band size and visualized in a Transilluminator Gel Documentation system and images were photographed. The RAPD data of samples was extended for statistical analysis in order to measure the genetic distance among them. The bands were scored according to their presence (1) or absence (0) and were arranged as per the molecular size and NTYSIS PS version 20.2 software was used to calculate the frequency of polymorphic bands. Dendrograms based on similarity coefficient were generated by using the unweight pair group method arithmetic mean (UPGMA) to facilitate the grouping of genotypes into clusters based on their genetic relationship.

OBSERVATIONS AND DISCUSSION:

The germination of seeds treated with 10 and 20 ppp Estrogen (used in the form of Premarin) for 12 and 24 h was 64, 66, 70 and 74% as compared to 51, 53, 56 and 64% germination of seeds soaked in distilled water for same period to serve as control respectively.

Analysis of RAPD data of all the treatments showed significant genetic variation. Among 17 primers used only 7 primers namely, OPE 01, OPAH10, OPA1, OPH01, OPJ01, OPF-12 and OPC-16 properly amplified and only

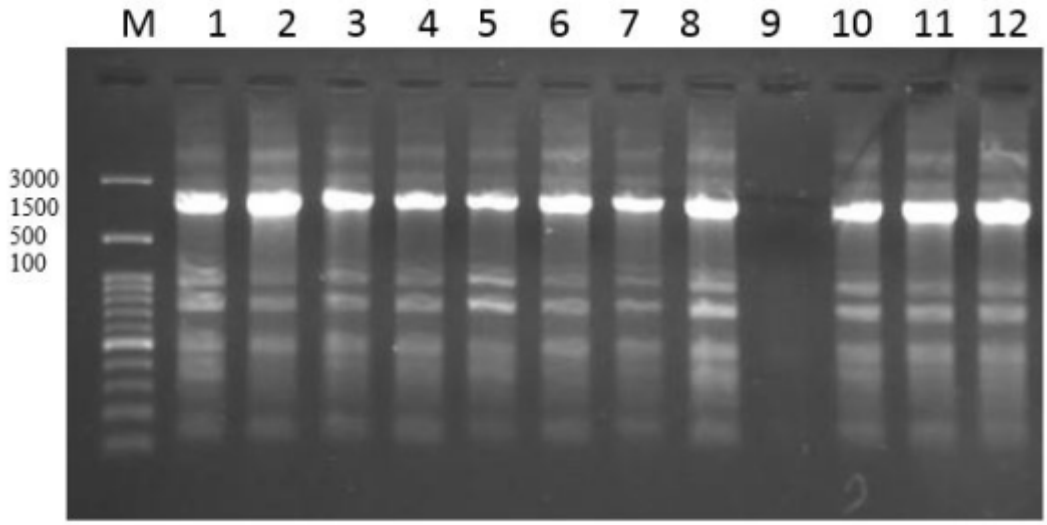

Figure 1 A: RAPD profiling with primer OPC-16 for 12 samples. M= 100bp DNA ladder.1. CD (12), 2. CD(24), 3.P10(12), 4. P10(24), 5. P20(12), 6. P20(24), 7. CD(12), 8.P10(12), 9. CD(24), 10.P10(24), 11. P20(24).

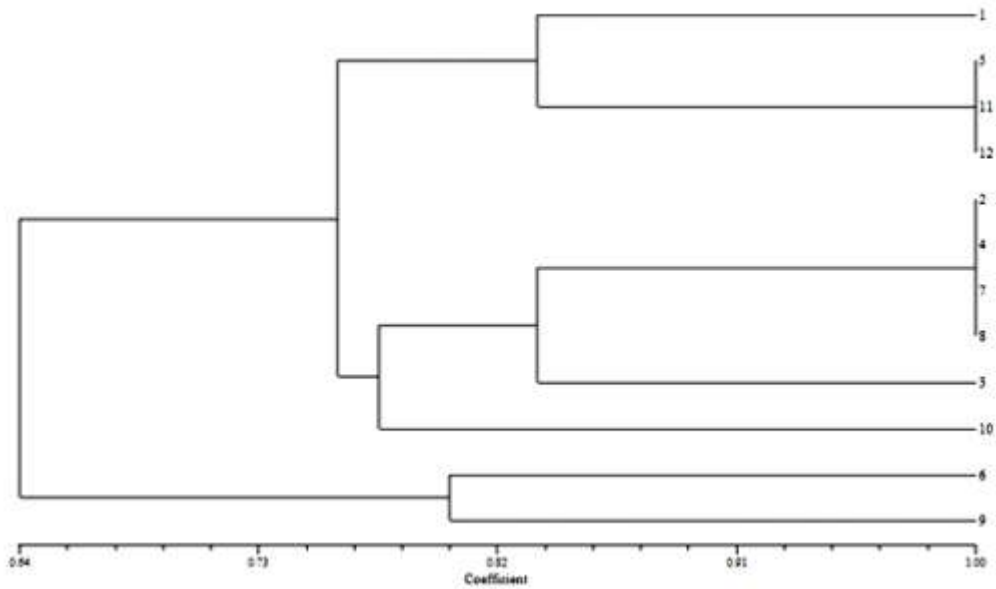

Figure1B: Dendrogram showing genetic distance of RAPD data by employing UPGMA method primer OPF-12 for 1(CD(12)=Control treated for 12 h; CD(24)=Control treated for 24 h; P10(12) 10ppm Premarin for 12 h; P10(24)=10 Premarin for 24 h; P20(12)= 20 Premarin for 12 h; P20(24)=20PPM Premarin for 24 h).

primers OPF-12 and OPC-16 produced repeatable results. They were amplified template DNA to a total of 58 DNA fragments and showed polymorphism. The level of polymorphisms was different with different primers among different samples. Electrophoretic pattern of different treatment with primer, OPF-12 showed 45 bands size ranging between 200-2300 base pair with 22.22% polymorphism. While with primer OPC-16 10 bands with band size 400-2000 base pair showed 30.77% polymorphism.

Cluster analysis based on UPGMA revealed two major clusters A and B with primer OPF-12 at a similarity coefficient of 64% (Fig.1A & B). The cluster A is the largest one with 10 genotypes could be divided into two subclusters, namely, A_1 and A_2 at a similarity

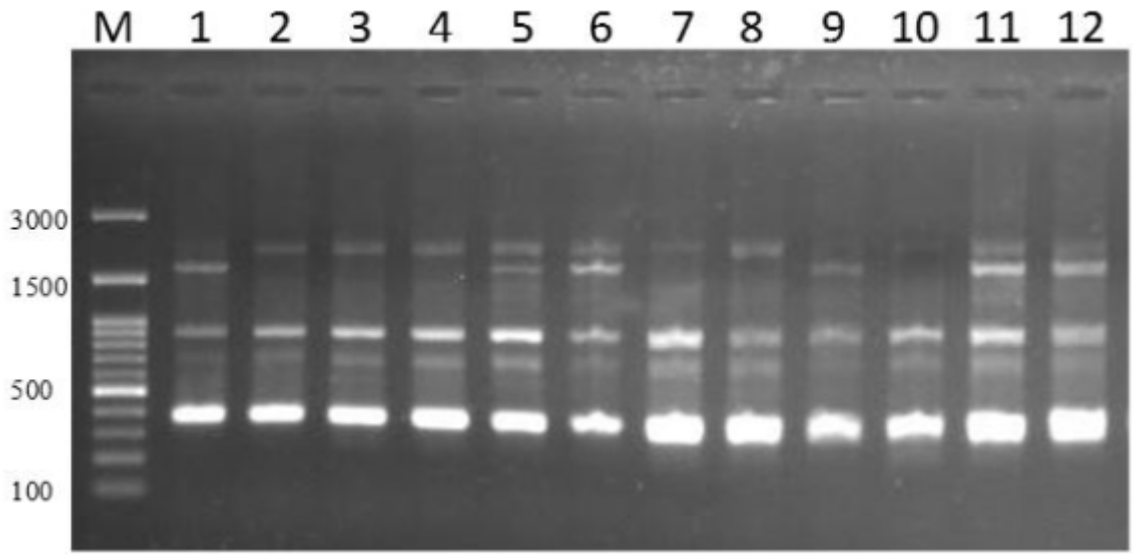

Figure 2A:. RAPD profiling of 12 samples with Primer OPF-12. [1. CD (12), 2. CD(24), 3.P10(12), 4. P10(24), 5. P20(12), 6. P20(24), 7. CD(12), 8.P10(12), 9. CD(24), 10.P10(24), 11. P20(24)].

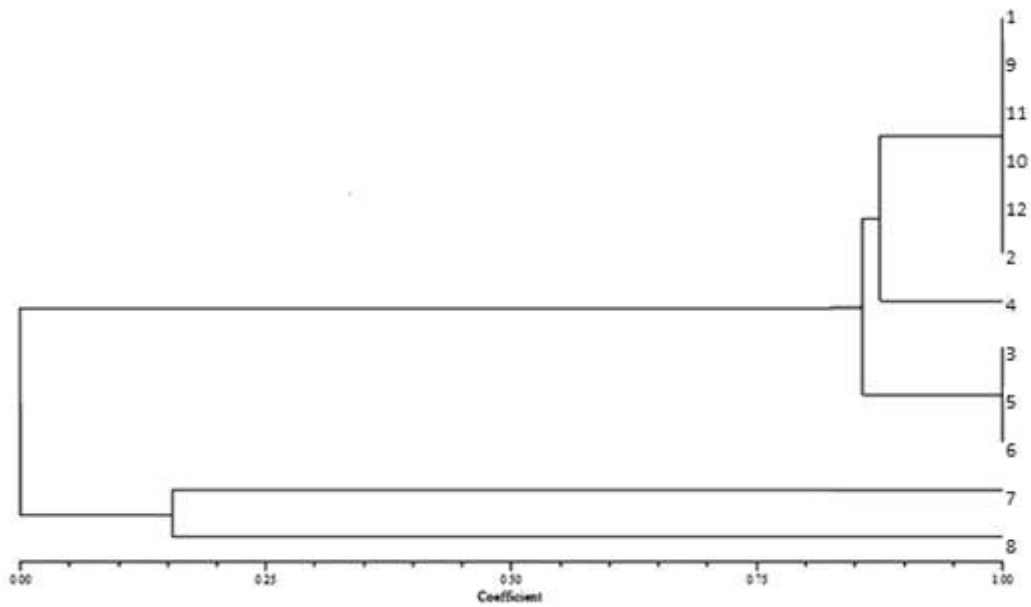

Figure 2B. Dendrogram showing genetic distance of RAPD data by employing UPGMA method primer OPC-16 for 12 Samples. (CD(12) = Control treated for 12 h, CD(24) = Control treated for 24 h, P10(12) = 10 ppm premarin for 12 h, P10(24) = 10 ppm premarin for 24 h, P20(12) = 20 ppm premarin for 12 h, P20(24) = 20 ppm premarin for 24 h.

value of 76%. The subcluster A_1 again divided in two groups at similarity value 83%. In $A_{1(a)}$ only 10 ppm premarin treated plants for 12 h and in $A_{1(b)}$ control treated for 12 and 24 h were present. Thus it is concluded that they have nearly same genotype. The sub cluster A_2 again subdivided into 3 groups $A_{2(a)}$ & $A_{2(b)}$ at a similarity value 83% $A_{2(a)}$ in plants raised from 10 ppm premarin treatments with 12 and 24 h and 20 ppm premarin treatment for 12 h. While in $A_{2(b)}$ it has only plants raised from 20 ppm premarine treatment for 12 h. However, in $A_{2(a)}$ has only, control plants (24 h).

The cluster B is divided into two groups at similarity value 80.2% and both have only 20 ppm premarin treated plants (24 h). Cluster

analysis with primer OPC-16 (Figs. 2A & B) showed two major clusters A and B. The cluster A is subdivided into 3 sub-groups, namely, A_1, A_2 & A_3 at similarity coefficient 82%. The cluster A_1 has control plants treated for 12 and 24 h and 10 ppm premarin treated plants for 12 h. The genotype of these plants was nearly similar. On the other hand, cluster A_2 has only 10 ppm premarin (24 h) treated plants. In A_3 group, the plants rose by treatment with 10ppm premarin for 24 h are present with 20 ppm premarin (12 h) treated plants. However, the cluster B divided in two groups has only 20 ppm premarin treated (24 h) plants.

Thus, cluster analysis clearly showed that the plants rose by 20 ppm premarin treatment for 24 h is genotypically different from other treated and control plants.

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