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DETERMINATION OF GENETIC MUTATIONS INDUCED BY CHEMICAL AND RADIATION TREATMENT IN *AEGLE MARMELOS,* USING INTER SIMPLE SEQUENCE REPEAT (ISSR) PRIMERS.

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ABSTRACT

Induced mutations in *in vitro* grown shoots of *Aegle marmelos* Corr. (Bael) due to the treatment with colchicines, Diethyl sulphate and gamma radiations were screened using fifteen ISSR and five RAPD primers were used for the testing of variability and reproducibility of the band. High level of genetic variation among the control and plantlets treated with three different mutagens was observed. Thirteen Out of 15 ISSR primers used for initial screening showed polymorphic profile which together produced 83 bands across four mutagenic variants of *A. marmelos*, of which 59 bands were polymorphic showing 71% polymorphism. The plants treated with colchicine grouped closer to control plant indicating lesser variation, DES treated plants in spite of major loss of genetic material showed better resemblance to control plants than gamma rays which has maximum level of mutations, grouping far away from the control plants

KEY WORDS : colchicines, gamma, DES, ISSR primers, genetic polymorphism

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INTRODUCTION

Aegle marmelos Corr. Family Rutaceae (Bael) is one of the most important medicinal plant as well as a fruit plant throughout the tropical countries. All parts of *Aegle marmelos*, such as the roots, bark, leaves, flowers, fruits and seeds are edible and possess medicinal properties. Leaves, stem, roots and fruits at all stages of maturity are used as ethano – medicines against various human ailments. The leaves of *Aegle marmelos* contain several bioactive compounds including essential oils, flavonoids, alkaloids, condensed tannins, anthocyanins and flavonoid glycosides. The leaves are astringent, laxative and an expectorant and are useful in treatment ofof inflammations, diarrhoea, dysentery, heart palpitation, and asthmatic complications. It is proved to have antibacterial activity[1], hypoglycemic activity[2], antialcer, anti-hyperlipidemic, antioxidant, anticancer, radioprotective, anti-inflammatory antipyretic, analgesic and antispermatogenic effects against various animal models[3]. *Aegle marmelos* is propagated through seeds, but has low viability and it leads to variability. Varietal purity and induction of mutations to improve the medicinal properties of this important ethanol – medicinal plant could be achieved through tissue culture of Bael. The plantlets grown *in vitro* were treated by mutagenic agents namely colchicine, Diethyl sulphate and Gamma radiations to induce mutations and to improve the medicinal properties of Bael. The plantlets obtained in M_3 generation were screened for genetic variations induced by mutagenic treatments and DNA finger printing of mutants using Inter Simple Sequence Repeat (ISSR) primers. ISSR is the molecular technique developed by Zietkiewicz *et al.,* (1994)[4] and it can screen 100 to 3,000 bp DNA fragments. ISSR is amplified by PCR using microsatellite core sequences as primers with a few selective nucleotides as anchors into the non-repeat adjacent regions (16-18bp). The main advantage of ISSR is that it is randomly distributed throughout the genome and results are reproducible

MATERIALS AND METHODS

Nodal explants of *Aegle marmelos* Corr. obtained from mature tree were grown *in vitro* using MS medium supplemented with 2 mg/lit of BAP and 0.5mg/lit NAA to obtain multiple shoots[5,6].The 60 days old shoots were used for the treatment with 0.8 % aqueous colchicine for 6 hours, 0.02% of Diethyl sulphate and 40G of Gamma radiations. The 90 days old M_3 plantlets obtained after each treatment were used for this genetic analysis using ISSR primers. The in vitro grown plantlets of *Aegle marmelos* Corr. without any treatment (T1-control), treated with colchicine (T2), Diethyl Sulphate (T3) and gamma rays (T4) was used to extract DNA. The DNA was amplified using 5 RAPD and 15 ISSR primers to detect the genetic variations induced by chemical and ionizing radiations.

a. Genomic DNA Isolation

Total genomic DNA was isolated from young leaves of plants growing *in vitro*. The leaf tissue was crushed in 4 ml of extraction buffer (100 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, 2% CTAB, 0.2% β-mercaptoethanol (v/v) and 1% PVP (polyvinylpyrrolidone, w/v, added immediately before use), mixed well to form a slurry and incubated at 65°C for 60 min with slow shaking for every 15 min. An equal volume of chloroform : isoamylalcohol (24:1 v/v) was added to the extract and centrifuged at 10,000 rpm for 20 min at room temperature (RT). The aqueous phase was aspired and placed in a new 2.0 ml tube. Equal volume of chilled isopropanol was added and incubated at –20°C for a minimum of 30 min followed by centrifugation at 10,000 rpm for 20 min at RT. The pellet was washed with 70% ethanol by centrifuging at 10,000 rpm for 8 min, dried and dissolved in 100 µl of autoclaved Milli Q water. The DNA was then treated with 1 µl of RNase A (10 mg/ml) incubated for 1 h at 37°C, followed by extraction twice with chloroform : Iso-amylalcohol (24:1) and precipitation with

Isoporpanol, centrifuged at 12,000 rpm for 10 min followed by 80% ethanol washing twice, air dried and the DNA was dissolved in 100 µl of autoclaved Milli Q water.

b. Quantification of DNA

Quantity and quality of extracted DNA is checked and estimated by visual assessment of band intensities in comparison to Lambda DNA marker on 0.8% agarose gel stained using Ethidium bromide. DNA samples were diluted using sterile Milli Q water to 10 ng/µl and used as templates for PCR amplification.

c. ISSR and RAPD marker amplification

Fifteen ISSR Primer sequences from the Biotechnology Laboratory, University of British Colombia (UBC) were synthesized by Eurofins. PCR amplifications were performed in 15 µl volumes, using a VERITI 96 well thermal cycler (Applied Biosystems, USA). The reaction mixtures contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 10-20 ng genomic DNA, 10 pmol primer, 1.5 mM MgCl2, 200 µM of each dNTP (*In vitro*gen by Life technologies, USA), and 1U Taq DNA polymerase (Bangalore Genie). The temperature profile consisted of an initial denaturation step at 94°C for 4 min, followed by 40 cycles: 94°C for 1 min, Tm for 1min (Tm= 38°C for RAPD primers and 50°C for ISSR primers), and 72°C for 2 min. After the final cycle, samples were incubated at 72°C for 8 min to ensure complete extension and then stored at 4°C. The PCR products were separated on 1.5% agarose gel in 1X TBE buffer (Tris-borate-ethylene di-amine tetra acetic acid). The size of amplified DNA fragments was estimated with 100bp ladders (Life technologies, USA). The gels were visualized under UV using ALPHAIMAGER EC gel documentation system (Alpha Innotech Corporation, India). Fifteen ISSR and five RAPD primers were used for the initial testing of variability and reproducibility of the band. Eight ISSR primers were selected based on polymorphism level within the population and reproducibility was screened three times and band scored for diversity analysis

d. Data analysis

DNA profile with informative ISSR and RAPD markers which were reproducible in two independent amplifications were scored for the presence (1) or absence (0) of bands of various molecular weight sizes in the form of a binary matrix. The binary data was used to calculate Basic statistics using the genetic analysis package Power Marker ver. 3.23[7] for diversity measurements at each microsatellite locus, indicating the total number of alleles (NA), allele frequency, major allele (allele with the highest frequency), accession-specific alleles, gene diversity (GD), and polymorphism information content (PIC). (Table 2) Marker index was calculated to know the capacity of each primer to detect polymorphic loci among the genotypes. It is the sum total of the polymorphism information content (PIC) values of all the markers produced by a particular primer. PIC value was calculated using the formula PIC = 1-Σpi2, where pi is the frequency of the ith allele[8]. Pair-wise similarity matrices were generated by Jaccard's coefficient of similarity[9] using FreeTree program version 0.9.1.50. Jaccard's similarity coefficient is calculated with the formula: Sij = a/ a + b +c using suitable software. Where 'a' is the total number of bands shared between individuals i and j, 'b' is the total number of bands presents in individual i but not in individual j and 'c' is the total number of bands present in individual j but not in individual i. The Unweighted pair group method with arithmetic average (UPGMA)[10] cluster analysis of the Jaccard's similarity co-efficient generated a dendogram (Fig. 4) which illustrated the level of genetic similarity and differences among the mutants surveyed. The pair wise comparison of banding patterns was evaluated by Nei's[11] genetic distance using the Free-Tree-Free ware programme[12]. A dendogram was constructed with help of TreeView[13] software. Statistical stability of the branches in the dendrogram was established by bootstrap analysis with 100 replicates using FreeTree program version 0.9.1.50.

Table1
List of informative ISSR primers used to determine level of genetic mutation in Aegle marmelos (L) Corr..

Sr. No	Marker	Primer sequence (5'-- 3')	Total bands	Polymorphic bands	P(%)	PIC	Gene diversity
1	UBC 807	AGAGAGAGAGAGAGAGT	12	9	75.00	0.70	0.75
2	UBC 808	AGAGAGAGAGAGAGAGC	5	1	20.00	0.38	0.50
3	UBC 810	GAGAGAGAGAGAGAGAT	8	6	75.00	0.70	0.75
4	UBC 811	GAG AGA GAG AGA GAG AC	6	5	83.33	0.70	0.75
5	UBC 814	CTC TCT CTC TCT CTC TA	1	1	100.00	0.38	0.50
6	UBC 818	CACACACACACACACAG	6	6	100.00	0.70	0.75
7	UBC 834	AGAGAGAGAGAGAGAGYT	6	6	100.00	0.55	0.63
8	UBC 835	AGAGAGAGAGAGAGAGYC	5	3	60.00	0.55	0.63
9	UBC 840	GAG AGA GAG AGA GAG AYT	11	8	72.73	0.70	0.75
10	UBC 841	GAG AGA GAG AGA GAG AYC	5	2	40.00	0.55	0.63
11	UBC 844	CTC TCT CTC TCT CTC TRC	4	3	75.00	0.30	0.38
12	UBC 859	TGTGTGTGTGTGTGTGRC	9	6	66.67	0.70	0.75
13	UBC 888	BDBCACACACACACACA	5	3	60.00	0.55	0.63
	Total		**83**	**59**	**71.08**		
	Mean		**6.38**	**4.54**	**71.08**	**0.58**	**0.64**

- Y= Pyrimidine's: C or T, B = Non A nucleotide; D = Non C nucleotide; P% : percent polymorphism; PIC: Polymorphic Information Content

Figure1
Estimation of DNA Quality and quantity on 0.8% Agarose gel.

T1 T3 T2 T4

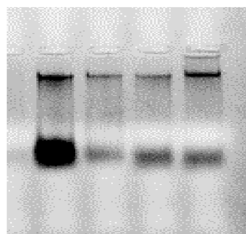
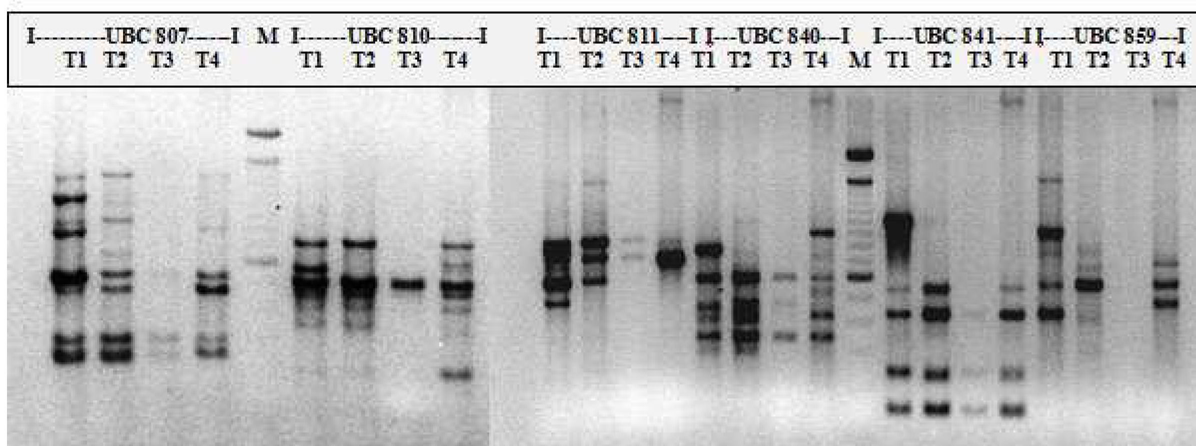


Figure 2
Amplification profile of four mutants of Aegle marmelos genotypes on agarose gel produced using ISSR primers (lane M is a 100 base pair ladder). T1: control, T2: colchicine treated plant, T3: DES treated plant and T4: gamma ray treated plant.



M - marker

Figure 3
Tree drawn by Tree View softwareFig 3: Tree drawn by Tree View software

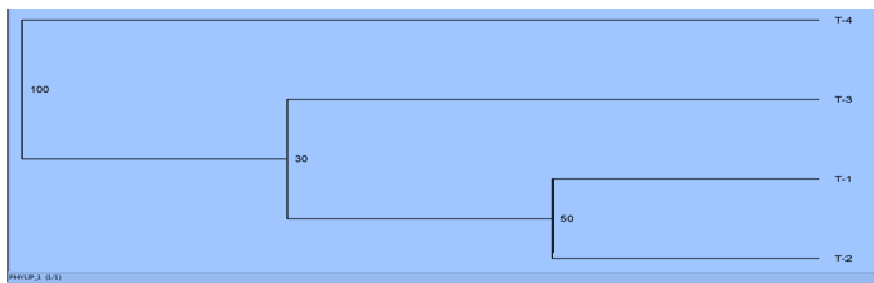


Table 2
Jaccard's similarity coefficient Matrix of four mutants of Aegle marmelos

	T-1	T-2	T-3	T-4
T-1				
T-2	0.24561			
T-3	0.48315	0.39241		
T-4	0.35714	0.27451	0.37662	

RESULTS AND DISCUSSION

Quality and quantity of DNA isolated using CTAB method was checked and estimated on 0.8% agarose gel stained using Ethidium bromide (Figure 1). The DNA samples were diluted to10 ng/µl and used as templates for PCR amplification.

a. ISSR band pattern

Fifteen ISSR oligonucleotides were used for amplification of treated and untreated four lines of *Aegle marmelos.* High level of genetic variation among the control and plantlets treated with three different mutagens was observed. Thirteen Out of 15 ISSR primers used for initial screening showed a polymorphic profile (Table 1). Figure 2 shows the observed polymorphism among four variants using the UBC 807, UBC 810, UBC 811, UBC 840, UBC 841, UBC 859 and UBC 888 primer. ISSR primers produced different numbers of DNA fragments, depending on their simple sequence repeat motifs. PCR amplification of DNA, using 13 informative ISSR primers for genetic analysis, produced 83 DNA fragments that could be scored in all genotypes. The number of amplified fragment varied from twelve (UBC 807) to one (UBC 814) with an average 6.38 bands per primer, which varied in size from 150 bp to 1700 bp. Of the 83 amplified bands, 59 were polymorphic, with an average of 4.54 polymorphic bands per primer. Percent polymorphism ranged from 20 (UBC 808) to 100 (UBC 814, UBC 818, UBC 834), with an average percentage polymorphism of 71. The PIC values varied from 0.30 (UBC844) to 0.70 (UBC 807, UBC 810, UBC 811, UBC 814, UBC 835, UBC 840 and UBC 859) with an average of 0.57, while average expected gene diversity ranged from 0.37 (UBC 844) to 0.75 (UBC 807, UBC 810, UBC 811, UBC 814, UBC 835, UBC 840 and UBC 859) across four variant's of *Aegle marmelos* (Table 2). There had been considerable reduction in number of amplified bands in all three mutants as compared control plants. Out of 83 amplified bands, 62 bands

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amplified in control (untreated) *Aegle marmelos* plant, colchicine treated plant showed amplification of 52 bands, gamma treated plants showed amplification of 50 bands and DES treated plant showed lowest number of bands (27 bands) . Only 24 of total 83 bands were common in all four lines. Band UBC 818500 was unique to the plant treated with DES (T3), whereas UBC 811600, UBC859720 and UBC859620 were unique to Colchicine treated (T2) plants. Comparatively major polymorphism with 9 unique bands (UBC 810600, UBC 818520 &400, UBC 834450,400&380, UBC 840900 & 550 and UBC 859650) were recorded in Gamma rays treated (T4) plants showing highest mutagenic effect at DNA level.

b. Analysis of Bands

Thirteen ISSR primers together produced 83 bands across four mutagenic variants of *A. marmelos*, of which 59 bands were polymorphic showing 71% polymorphism. Over 99% of the ISSR fragments were reproducible when repeated three different times using same DNA and primers in the present experiment. Thirteen out of 15 ISSR primers used were informative may be due to the use of selective primers reported to be informative in one or more plant species in previous reports. To determine the level and distribution of genetic variation in the lines tested, gene diversity statistics was calculated using Powermarker v. 3.23 [7]. The results indicate loss of genetic material / primer binding sights in all three treatments. The neighbour joining tree shows plants treated with colchicine grouped closer to control plant indicating lesser variation as compared to other treatments. DES treated plants in spite of major loss of genetic material showed better resemblance to control plants than gamma rays treatment. This clearly indicates higher level of deletion or loss of primer binding sight and comparatively limited transition and transversion at chromosomal level in DES treated plants. The same has also been proved in dendrogram by grouping DES treated plants in same cluster but were more diverse than colchicine treated plant. The neighbour joining tree shows that plant treated with gamma rays has a maximum level of mutations, grouping far away from the control plants (Figure3).

CONCLUSION

Aegle marmelos (L) Corr. is a very important medicinal plant in Indian subcontinent, with varied uses. An attempt is made to induce variability in the properties of this plant by inducing mutations with the help of colchicine, an agent that induces polyploidy, Diethyl Sulphate a chemical mutagen and gamma radiations, the physical mutagenic agent. The effects of these mutagenic treatments was studied at the molecular level using five RAPD and fifteen ISSR primers. Thirteen out of 15 ISSR primers used for initial screening showed polymorphic profile which together produced 83 bands across four mutagenic variants of *A. marmelos*, of which 59 bands were polymorphic showing 71% polymorphism. The plants treated with colchicine, which induces polyploidy were found to be closer to control plant indicating lesser variation, DES, an alkylating agent causes point mutations showed a major loss of genetic material in treated plants, in spite of which, showed better resemblance to control plants than ionising gamma rays which has maximum level of mutations, grouping far away from the control plants.

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Conflict of Interest

Conflict of interest declared none.

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