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Assessment of gamma radiation induced genetic variability in *Jatropha curcas* using RAPD and DAMD markers

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ABSTRACT

Gamma radiation induced variability among the *Jatropha* accessions was assessed using morphological and molecular markers (RAPD and DAMD) . Various treatments (0, 1, 3, 5, 10, 15, 20, 25, 50 Kr) of gamma radiation doses were given to *Jatropha curcas* L. genotype Urulikanchan and a total of 32 mutants and 1 control were included in the present study on the basis of various doses. Some variation in germination% was observed with different doses whereas characters like height, girth and canopy diameter showed non significant variation. The 15 RAPD and 4 DAMD primers were used which produced polymorphism percentage of 74.23% and 73.68%. PIC values of the markers ranged from 0.732 to 0.904 (RAPD) and 0.765 to 0.868 (DAMD) respectively. RAPD primers OPA 18, OPB 10, OPH 12, OPF 4 and OPI 5 produced bands which were specific to some mutants and hence helpful in identifying those mutants. Similarly, DAMD primers, HBV and HRV also produced specific banding profiles. Comparison studies showed very less correlation between the two markers as deduced from the r value (r=0.44). Both the markers proved efficient for polymorphism detection in gamma radiation treated *J.curcas* mutant.

Key words: DAMD, Genetic diversity, *Jatropha curcas,* RAPD

The genus *Jatropha* belongs to the Euphorbiaceae family and is morphologically diverse encompassing more than 200 species, which are distributed chiefly in dry tropical regions of America, and later introduced into Africa and Asia and are now cultivated worldwide. The approximate genome size of *Jatropha curcas* L. is 416 Mbp which is close to that of rice (430 Mbp) (Carvelho *et al.* 2008). *Jatropha curcas* is commonly known as Ratanjyot and its English synonyms include Physic nut, purging nut, Barbados nut, etc. *Jatropha* can grow well under adverse climatic conditions because of its low moisture demands, fertility requirements and tolerance to high temperatures. It is a small tree or a large shrub which can reach a height of up to six meters. *Jatropha curcas* is a multipurpose plant with many attributes and considerable potential. In today's world, it has attained an important position as an oil bearing crop which has contributed to its biodiesel properties. In spite of best nutritional composition, seed cake obtained from the toxic *J. curcas* remains unutilized as an animal feed due to its toxic nature and no successful attempts have been made till now for completely eliminating toxic principle (Makkar *et al.* 2008, Herrera *et al.* 2005, Ahmed and Salimon 2009). Both oleic acid (44.7%) and linoleic acid (32.8%) were detected as the dominant fatty acids while palmitic acid and stearic acid were the saturated fatty acids found in the *Jatropha* oil (Akbar *et al*. 2009).

Mutagenesis is a potential tool for creating new genotypes with useful character for crop improvement (Kahl *et al*. 2001, Lavi 2001). It is well known fact that the rate of spontaneous mutation in nature is too low for plant breeding. Therefore, physical and chemical mutagens can be used to induce mutation. The members of *Jatropha* complex predominantly outcross and are maintained by vegetative propagation, hence are highly heterozygous and display enormous plasticity in the phenotypic expression of traits. Estimation of genetic diversity is the first and foremost important step in genetic improvement of crop plants. Conventionally, variability is assessed using the morphological markers. However morphological traits are highly influenced by environment under which clones are grown or selected, limited in number, therefore do not provide correct estimation of diversity. On the contrary, DNA or molecular markers are not influenced by environmental changes and are highly abundant. Hence currently widely used for the estimation of genetic diversity and other plant

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breeding applications. In last two decades various PCR based marker techniques were developed and used in crop improvement which includes Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Direct Amplification of Minisatellite DNA (DAMD), Simple Sequence Repeats (SSR), etc. RAPD is an inexpensive and a rapid method not requiring any information regarding the genome of the plant and has been widely used to ascertain the genetic diversity in several plants (Deshwall *et al*. 2005). RAPD markers have been used for the assessment of genetic diversity in *Jatropha curcas* by several workers (Basha and Sujatha 2007, Ganesh Ram *et al.* 2008, Ranade *et al.* 2008)

The present study was carried out with the objective to assess genetic variability among the gamma radiation induced *Jatropha curcas* accessions using RAPD and DAMD markers.

MATERIALS AND METHODS

Seeds of the genotype Urulikanchan from experimental plantations raised at the farm of Anand Agricultural University, Anand were exposed to various doses of gamma rays, i.e. 0, 1, 3, 5, 10, 15, 20, 25 and 50 Kr (Table 1). Plant population grown from treated seeds was selected for assessment of genetic variability. Morphological characters like plant height, girth and canopy diameter and seed germination percentage were analyzed and molecular markers, viz. RAPD and DAMD were used for genetic variability. The plants grown from seeds without treatment of gamma rays were taken as 'Control'. Four mutants for each dose were taken for the investigation.

Total genomic DNA was extracted by using the CTAB method (Doyle and Doyle 1980). The quality of the DNA was confirmed by running it on 0.8% agarose and staining with ethidium bromide. DNA concentration of each sample was determined using nanodrop

Amplification of RAPD fragments was performed according to standardized methods (Williams *et al*. 1990). Total 15 RAPD primers selected from initial screening of primers (Table 2) based on the resolution and those having more than five bands. The reaction was carried out in a 25 µl volume containing 1X *Taq* buffer with $MgCl_2$ (Bangalore Genei, India), 1.5 units *Taq* polymerase (Bangalore Genei India), 10mMdNTPs (2.5 mM each) (Fermentas,USA), 50ng template DNA, 15 pmoles primer (MWG biotech, Germany) and volume was finally made up with nuclease free water (Amresco,USA). Amplification was performed in a thermal cycler (Biometra, Germany) using following program : initial denaturation at 94° C for 4 min, 42 cycles of denaturation at 94° C for 1 min, annealing at 38° C for 1 min, extension at 72°C for 2 min and final extension at 72°C for 6 min. The amplicons generated were resolved on 1.8% agarose gel using 1X TBE. The gels were stained with ethidium bromide and documented using gel documentation system (Bio-Rad, California).

DAMD analysis was performed using the primers reported by Ranade *et al*. (2008) (Table 3). The reaction was carried out in a 25 µl reaction volume with all the reaction components similar to that in DAMD and also the reaction conditions remained the same except the annealing temperature which was adjusted according to Tm±3°C.

Clear and distinct bands amplified by RAPD and DAMD primers were scored as 1(for the presence of band)and 0 (for the absence of the band) for each genotypes. All the analysis were performed using NTSTSpc software version 2.02 (Rohlf 1998). Coefficients of similarity were calculated using Jaccard's similarity coefficient by SIMQUAL function and cluster analysis was performed by agglomerative technique using the UPGMA.

Table 1 Details of *J.curcas* mutants selected

Dose (Kr)	Dose (Kr)	Dose (Kr)	Dose(Kr)
0	5	15	25
1	5	15	25
1	5	15	50
1	5	20	50
1	10	20	50
3	10	20	50
3	10	20	
3	10	25	
3	15	25	

Table 2 Details of RAPD primers

Primer	Sequence (5' → 3')	GC content (%)
OPA09	GGGTAACGCC	70
OPA-18	AGGTGACCGT	60
OPB10	GTGACATGCC	60
OPB11	AAGACCCCTC	60
OPC18	CAGCTCACGA	60
OPD05	ACCAGGTTGG	60
OPF04	GGATGAGACC	60
OPF10	TGGACCGGTG	70
OPH12	ACGCGCATGT	60
OPH13	GACGCCACAC	70
OPI09	TGGAGAGCAG	60
OPI10	ACAA GCGAG	60
OPI13	CTGGGGCTGA	70
OPI15	TCATCCGAGG	60
OPK16	GAGCGTCGAA	60

Table 3 Details of DAMD primers

Primer name	Sequence (5'→3')	GC content (%)	Tm value (°C)
HBV	GGTGTAGAGAGGGGT,	60	50.6
HVR	CCTCCTCCCTCCT	69.2	44.0
33.6	GGAGGTTTTCA,	45.5	32.0
M13	GAGGGTGGCGGTTCT,	66.7	53.3

Un-weighted Pair Group Method with Arithmetic Mean method by SAHN clustering function of NTSYS-pc. Genetic relationships among the *Jatropha curcas* genotypes were expressed in the form of dendrograms and PCA (principal component analysis) plot.

The cophenetic correlation analysis was carried out using the COPH function of NTSYS-pc. In this method dendrogram and similarity matrix were correlated to find the goodness-of-fit of the dendrogram constructed based on similarity coefficients. The correspondence between RAPD and DAMD based on similarity coefficient matrices was tested using cophenetic correlation analysis and Mantel matrix correspondence test. The Mantel matrix correspondence test was carried out using the MXCOMP function in the NTSYSpc version 2.02.

Besides, we also calculated various parameters like total number of bands total number of polymorphic bands, Polymorphism Information Content (PIC), Effective Multiplex ratio (EMR), Polymorphism %. Polymorphism Information content (PIC) was calculated according to formula described by Shukla *et al*. (2011).

$PIC=1-\sum f^2$ where f is the frequency of i^{th} allele.

All the above mentioned variables were calculated individually for both the markers as well as the combined values for RAPD+DAMD were calculated for comparing ability of the markers for DNA polymorphism assessment.

RESULTS AND DISCUSSION

Morphological variability in gamma radiation mutants

Leaf abnormalities (light green patches, de-formed shaped leaves) (Fig 1 and 2) were observed in first few leaves (not the cotyledonary leaves) in treatments with 15, 20, 25 and 50 Krad radiation dose. However later sprouted leaves of these plants appeared to be normal.

In general, lower doses of radiation to the seeds appear to stimulate germination % and rate of germination. The germination percentage was higher in irradiated seeds than control (except in higher doses 25 and 50 Krad) which might be due to effect of mutagens on meristematic tissues of seeds. More than 50 % germination was observed on 6th day in the lower doses of radiation (1, 3, 5 and 15 Krad). More than 65 % germination was observed on 6th day in seeds irradiated with 1 Krad, while more than 65 % seed germination

Fig 1 Effect of radiation on leaves when treated with 15Kr

Fig 2 Leaf abnormalities observed with 20Kr dose

was observed on 7th day in seeds irradiated with 3 and 15 Krad dose. More than 80 % seed germination was observed in seeds treated with 1 and 15 Krad of radiation on 8th day after sowing.

No significant variation was observed when statistical analysis was carried out for characters like plant height, girth, and canopy diameter after 6 months and 1 year duration (Table 3). Dakshanamoorthy *et al*. (2011) also carried out similar studies in *Jatropha* and they found signicant variations in germination percentage.They found stimulatory effect (80.66%) with 10 Kr, whereas reduced germination (37%) with 15 Kr.

The results obtained through RAPD(Fig 3) and DAMD markers are presented in the Tables 4 and 5. The maximum no. of bands amplified by RAPD primers ranged from 6 (OPI 13) to 15(OPB 10), whereas those amplified by DAMD ranged from 5(33.6) to 9 (M13).The polymorphic bands for RAPD ranged from 1(OPF 10) to 13(OPB 10) and DAMD ranged from 3 (33.6) to 8 (M13). The lowest polymorphism percentage found in RAPD was 11.11 (OPF-10), whereas highest was 100% (OPI-10 and OPI-13). In case of DAMD, the lowest polymorphism observed was 60% (33.6) and highest was 88.88% (M13). The average polymorphism percentage for RAPD and DAMD was 74.23% and 73.68% respectively.

PIC values obtained by RAPD as well as DAMD varied greatly as observed in Tables 4 and 5 which indicate high genetic diversity among the mutant clones. Average PIC values for RAPD were 0.847 and DAMD 0.813.

Some of the RAPD and DAMD primers produced mutant specific banding patterns which may assist in identifying mutant. Primer OPH 12 showed the absence of bands with molecular weight 390.45 bp in the mutant 1Kr and 693 bp in the mutant 2Kr.Primer OPA 18 showed some specific bands in mutants 13 (10Kr), 15 (10Kr) and 23 (20Kr) with molecular weights 478.66, 655.35 and 149.69 base pairs. Primer OPB 10 showed the presence of a specific band of 1.3 Kb in the mutant 15 (10Kr), whereas mutant 23(20Kr) showed the absence of a band of molecular weight 227 bps which was present in all other mutants including control. Similarly, OPF 4 showed the absence of a product of 218 bp in the mutant 12 (5Kr) and OPI 5 showed the

Table 3 Mean table for 3 month, 6 month and 1 year data of different characters for different λ-irradiation Dose

λ-irradiation dose	Plant height (cm) (Average)			No. branches/ Plant (Average)			Stem girth (mm) (Average)			Canopy diameter (cm) (Average)		
	3 month	6 month	1 year	3 month	6 month	1 year	3 month	6 month	1 year	3 month	6 month	1 year
1Krad	45.91	105.97	179.08	2.29	3.11	13.69	28.33	31.65	50.70	41.50	71.20	182.24
3Krad	43.87	100.38	176.51	2.41	3.43	16.27	27.99	32.34	61.20	39.59	65.98	181.22
5Krad	42.23	101.75	177.70	2.05	3.07	12.17	26.85	30.15	53.22	37.58	62.88	185.24
10Krad	43.35	106.40	182.63	2.16	3.08	13.06	26.86	34.39	54.73	39.87	64.43	182.08
15Krad	44.91	99.34	176.06	2.21	3.15	13.19	27.91	30.20	50.08	41.10	66.10	187.43
20Krad	44.35	103.45	177.20	1.98	3.04	12.82	28.02	29.79	46.95	40.39	63.18	176.00
25Krad	41.87	103.68	174.41	1.97	3.37	12.70	28.23	31.54	51.42	40.15	62.55	177.26
50Krad	47.72	117.83	179.67	1.72	3.83	12.33	29.44	32.11	51.42	40.06	66.67	187.92
Control	42.84	103.20	182.53	2.11	3.87	12.81	26.48	29.62	46.72	38.03	66.52	184.80
Test	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
SEm	3.07	5.27	4.79	0.24	0.36	1.396	1.41	1.93	6.773	2.23	3.92	3.411
CD	9.21	15.80	14.36	0.73	1.08	4.185	4.21	5.77	20.304	6.68	11.75	10.226
CV%	12.07	8.72	4.65	20.02	18.83	18.28	8.76	10.66	6.42	9.70	10.36	11.40

absence of 469 bp product which was present in all other mutants. A specific amplicon of 386 bps was also observed in mutant 16 (10Kr) during amplification with the same primer.

DAMD marker also proved to be efficient in producing some specific banding profiles. Absence of 198 bp amplicon was observed in the mutant 3 (1Kr) while amplification with HBV (Fig 4), whereas 356 bp and 932 bp amplicons were found to be absent in the mutant 27 (25Kr) while amplifying with the primer HVR.

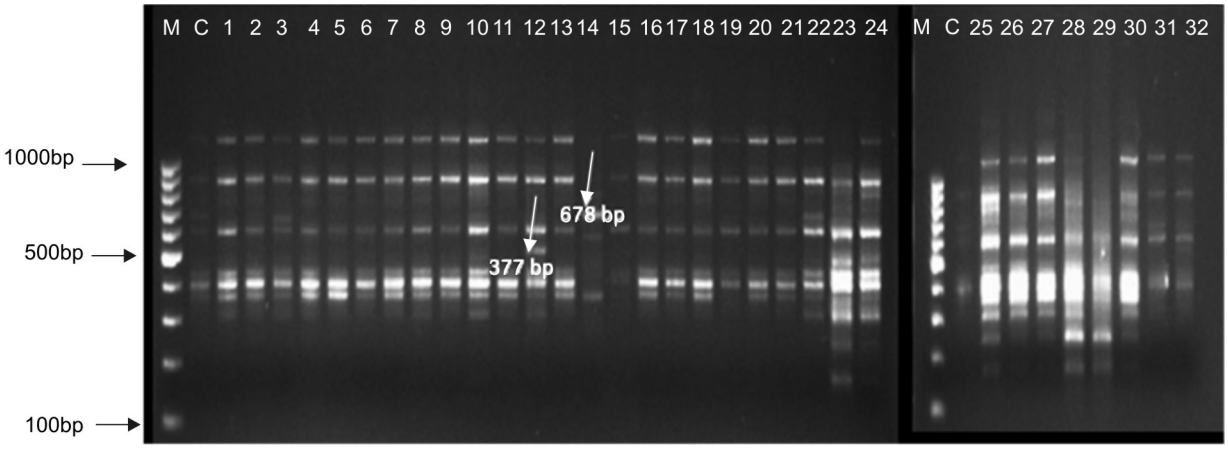

Fig 3 Amplification profile with RAPD primer OPA 18

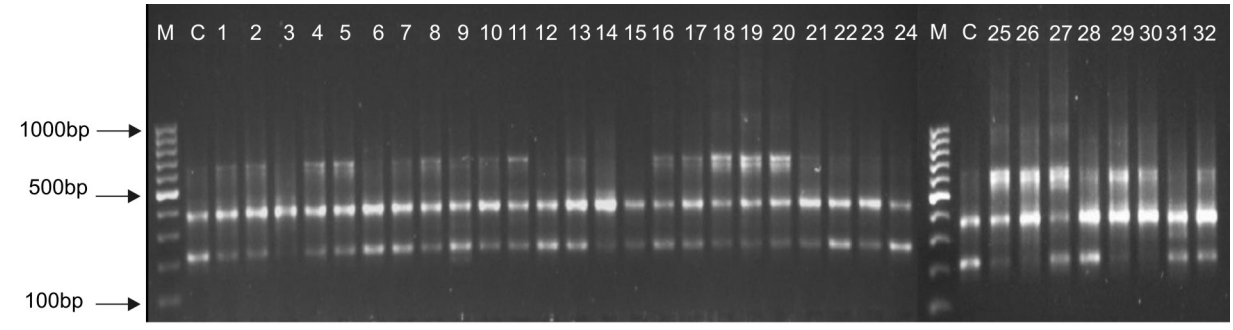

Fig 4 Amplification profile with DAMD primer HBV

Table 4 Results of RAPD analysis

Primer	Total no. of bands	No.of poly-morphic bands	Polymor-phism (%)	PIC	Range of molecular weight
OPA-9	12	10	83.33	0.904	171bp-1.3Kb
OPA-18	13	10	76.92	0.872	186bp-1.4Kb
OPB-10	15	13	86.66	0.893	105bp-2.0Kb
OPB-11	9	8	88.88	0.850	225bp-1.1Kb
OPC-18	9	5	55.55	0.873	181bp-1.1Kb
OPD-5	7	5	71.48	0.732	195bp-749bp
OPF-4	9	5	55.55	0.827	232bp-1.3Kb
OPF-10	8	1	11.11	0.879	169bp-2.0Kb
OPH-12	8	7	87.50	0.874	144bp-2.4Kb
OPH-13	8	6	75	0.863	346bp-1.3Kb
OPI-9	10	8	75	0.842	184bp-20Kb
OPI-10	10	10	100	0.863	209bp-2.2Kb
OPI-13	6	6	100	0.823	962bp-1.2Kb
OPI-15	7	5	71.42	0.751	448bp-1.2Kb
OPK-16	8	6	75	0.863	158bp-1.0Kb
Total	137	107			
Average	9.1	7.1	74.23	0.847	260.9bp-1.6Kb

Table 5 Results of DAMD analysis

Primer	Total number of bands	Total number of polymorphic bands	Poly-morphism (%)	PIC	Size of bands
HBV	6	5	83.33	0.765	207bp-1.1Kb
HVR	8	5	62.50	0.868	283bp-1.2Kb
M13	9	8	88.88	0.840	199bp-2.0Kb
33.6	5	3	60.00	0.780	225bp-1.5Kb
Total	28	21		3.255	
Average	7	5.75	73.68	0.813	

Genetic relationships

Similarity matrix values constructed on the basis of combined results of RAPD+DAMD varied greatly from 0.50 (5KR2 and 5KR4) to 0.89 (15KR1 and 15KR2) with mean value 0.74 which indicated remarkable variation among the mutants.

Cluster and principal component analysis

The cluster diagram based on combined RAPD+DAMD results generated through NTSYSpc version 2.02 using Jaccard's similarity coefficient and UPGMA (Unweighted Pair Group Mathematical Average) method revealed two major clusters at similarity value 0.76. and mutant 10Kr2 was uniquely identified. (Fig 5) .The second cluster was further subdivided into 2 sub clusters at coefficient of 0.78 where 50KR1 was distinguished from all others. New PCR amplification products may reveal a change in some oligonucleotide priming sites due to mutations [new annealing event(s)], large deletions (bringing to preexisting annealing site closer), and/or homologous recombinations (juxtaposing two sequences that match the sequences of primer) (Atienzar *et al*.1999).The new bands could be attributed to mutation, while the disappearance of bands could be attributed to DNA damage (Atienzar and Jha 2006)

Results of seed germination showed some correlation with that of clustering pattern observed. For example, the mutants with the doses of 1, 3,5 and 15 Krad which showed more than 50% germination on 6th day were separated into same cluster upto some extent. Other mutants (10, 15, 20, 25 and 50 Krad) which didn't show significant response to radiation with respect to germination % were resolved into almost separate groups in the dendrogram generated. Also, within the same treatment (replications) also, variation was observed among the mutants which could be ascertained from the dendrogram results.

The Principal component analysis results for RAPD+DAMD also coincided with the results of cluster analysis and the first three components calculated through EIGEN module of NTSYS 2.02, revealed the maximum variation of 75% in RAPD.

Studies pertaining to diversity in *Jatropha curcas* mutants using RAPD were performed by Dhakshanamoorthy and Selvaraj (2010). PIC values obtained in their study varied from 0.00 to 0.40 which was very less as compared to present study. Average polymorphism obtained was 67% whereas in the present study it was 74.23%.Similar studies have also been conducted in mutants of other crops like amla (Selvi *et al*. 2007), chrysanthemum (Barakat *et al*. 2009), Sugarcane (Sajida bibi *et al*. 2010) and paparika (Kumar and Ponnuswamy 2010).

Comparison studies were done to analyze the efficiency of both the markers for diversity studies in mutated clones of *J.curcas*. Polymorphism percentage for both the markers did

Table 6 Comparison of RAPD and DAMD markers

Marker characteristics	RAPD	DAMD	RAPD+DAMD
Number of Primers used in analysis	15	4	19
Total number of bands	137	28	165
Total No. of polymorphic bands	107	21	128
Polymorphism %	74.23	73.68	73.95
Size of bands	260bp–1.6Kb	228bp–1.4Kb	228 bp–1.6Kb
Average Polymorphism Information Content (PIC value)	0.847	0.813	0.83

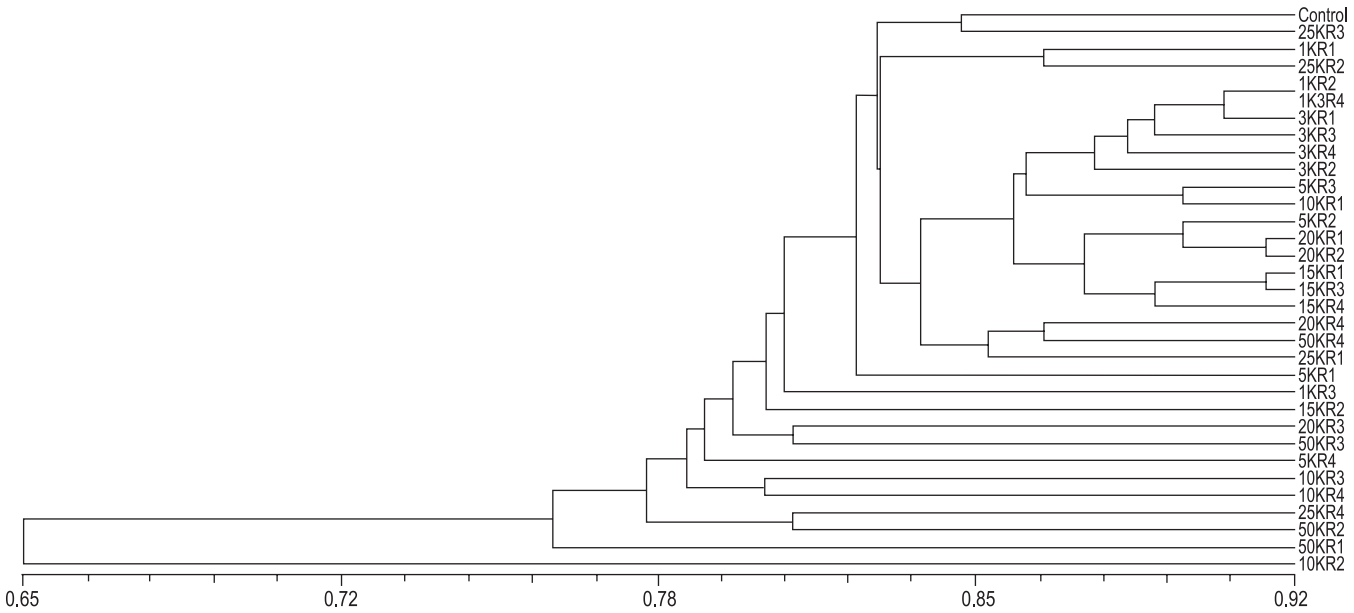

Fig 5 Cluster diagram of RAPD+DAMD results using Jaccard's coefficient

not vary so much. However PIC value was high for in RAPD markers (Table 6). Matrix correlation studies were also performed to estimate the correlation between the two markers using the the Mantel's test and it was found that overall correlation was very low between the two (r=0.44). One of the reasons for this low correlation could be due to the different genome target sites of both the markers. RAPD markers target the whole genome whereas DAMD are specific to minisatellite regions. Also the difference in the no. of primers can also be attributed as one of the reasons as only 4 DAMD primers were included with comparison to 15 RAPD primers.

CONCLUSIONS

The results obtained from the present study suggest that gamma ray radiation as a physical mutagen could be used for mutation induction and the present study based on RAPD and DAMD markers showed that these DNA markers are useful tools for identification of polymorphism in gamma radiation treated *Jatropha curcas*. Still better conclusions can be derived by evaluating more no. of morphological traits like root length, shoot length, vigour index, fruit morphology etc. which may prove to be useful in order to establish the correlations between the markers and the morphological/quantitative traits. Also, chemical mutagens along with physical mutagens may also assist in deriving better conclusions about the best mutagens for mutation breeding purpose.

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