International Journal of Agriculture, Environment & Biotechnology
Citation: IJAEB: 7 Special Issue : 459-465 July 2014

Molecular Biology

Molecular study of Pigeonpea [*Cajanuscajan* (L.) Mill sp.] Genotypes for *Fusarium*wilt using RAPD markers

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Abstract

The molecular study of seventeen genotypes of pigeonpea using 20 random amplified polymorphic DNA (RAPD) markers has been reported. A total of 179 loci were scored corresponding to an average of 8.95 loci per primer with 123 bands showing polymorphism (65.42%).Very low level of polymorphism in cultivated pigeonpeagermplasm had been earlier reported which was corroborated by many pigeonpea workers indicating the normal genetic base existing in this crop (Odeny*et al*. 2007). The average number of polymorphic loci obtained per primer (Assay Efficiency Index) was found to be 6.15. Jaccard's similarity coefficient ranged from 0.52 to 0.77 and the marker index value for pooled RAPD was found to be 11.65. A dendrogram constructed based on the UPGMA clustering method revealed two major clusters. Cluster-I comprised of 5 cultivars whichwas further differentiated into two sub-clusters. The cluster-II included remaining twelve cultivars. Genotypes that are susceptible to *fusarium*wilt of pigeonpea*viz*., GT-1, GT-100, GT-101, GT-102 and BANAS were closely related and they formed one cluster. It also revealed that genotypes *viz*., AGT-2 and AVPP-1 were closely related and formed one cluster whereas *viz*., T-15-15, LRG-41, C-11, BDN-2 and ICPL-87 were closely related and formed another cluster. The dendogram showed that genotypes that are resistant to *fusarium* wilt of pigeonpea*viz*., BSMR-853, WRGE-119, ICPL-87119, ICPL-84060 and ICP-8863 were related genotypes and they formed another cluster. The study reiterated that RAPD can be efficiently used for discriminating resistant and susceptible pigeonpea genotypes.

Highlights

- A total of 179 loci were scored corresponding to an average of 8.95 loci per primer.
- Jaccard's similarity coefficient ranged from 0.52 to 0.77 and the marker index value for pooled RAPD was found to be 11.65.
- Very low level of polymorphism (65.42%) in cultivated pigeonpea genotypes was reported.
- RAPD can be efficiently used for discriminating resistant and susceptible pigeonpea genotypes

Keywords: RAPD; Pigeon pea; *Fusarium* wilt; Dendrogram; Genotypes.

Pigeonpea or Red Gram [*Cajanuscajan* (L.) Millsp.] (2n = 22) is the second most important pulse crop and plays an important role in subsistence agriculture. The chromosome number of all *Cajanus* species is n=11, with a genome size of 808 Mbp (Greilhuber and Obermayer, 1998). India is considered as the primary center of origin for pigeonpea while Africa as the secondary center of origin (Van der Maesen, 1980). Its grains are highly nutritious and rich in protein content (21-23%), carbohydrates, fiber and minerals. During its life span, pigeonpea is attacked by more than 100 pathogens (Nene *et al.,* 1989). These include fungi, bacteria, viruses, nematodes and phytoplasma. In Gujarat, the *fusarium*wilt is one of the major diseases of concern followed by *Phytophthora* blight and sterility mosaic, affecting the productivity of this crop per unit area. *Fusarium* wilt of pigeonpea is a soil borne disease caused by fungus *Fusariumudum*. Therefore, to minimize yield losses due to *fusarium* wilt, it is necessary to tackle these problems at molecular level by developing cultivars which resist/

tolerate these biotic stresses and have greater recovery from damage. Genomic tools especially molecular markers have facilitated breeding in many cereal crops leading to development of several improved cultivars/ varieties with enhanced resistance / tolerance to biotic or abiotic stesses (Varshney *et al.,* 2006). Molecular markers, such as RAPD, SCAR, SSR, RFLP, AFLP etc., have been used to assess genetic variations at DNA level. RAPD, being a multi locus marker (Karp *et al*., 1997) with simplest and fastest detection technology, have been successfully employed for determination of genetic diversity in several grain legumes. This paper reports characterization of seventeenpigeonpea cultivars for *fusarium* wilt.

Materials and Methods

Plant material

Seventeen pigeonpea genotypes*viz*., GT-1, GT-100, GT-101, GT-102, BANAS, AVPP-1, AGT-2, T-15-15, LRG-41, C-11, BDN-2, ICPL-87, ICPL-87119, ICPL-84060, ICP-8863, BSMR-853 and WRGE-119 showing variable resistance / susceptible reaction against *fusarium* wilt were collected from Agricultural Research Station, Dahod and Pulse Research station, Vadodara, Anand Agricultural University, Anand and used in present study. Plants were grown in pots and leaf samples of all plants of each cultivar werecollected in labeled bags and stored in -40°C freezer prior to genomic DNA isolation.

Genomic DNA isolation

Extraction of DNA from seedlings was done using modified Cetyl-Trimethyl Ammonium Bromide (CTAB) method (Murray and Thompson, 1980) with some minor modifications.

PCR amplification using RAPD primers

Twenty oligonucleotides primers were selected from available literature (Table 1). Out of which were synthesized from MWG biotech, Germany and the rest were Operon series primers. PCR was carried out in 25µl reaction volumes containing 2.5 µl of 10 x *Taq*assay buffer (Tris with15mM $MgCl_2$), 10 mM of each dATP, dCTP, dGTP and dTTP, 5U Taq polymerase (BioLabs, UK), 10picomole of primer and approx. 50ng of template DNA. Amplification were carried out in a thermo-cycler (Applied BiosystemVeriti, CA, USA) programmed for 40 cycles with an initial denaturation at 94°C for 4 min followed by cycling conditions of denaturation at 94°C for 1 min, annealing at 1min at 38°C and extension at 72°C for 2 min. After 40 cycles, there was a final extension step of 7 min at 72°C. The amplicons were analyzed on 1.6% agarose gels and detected by staining with ethidium bromide. UV trans- illuminated gels were photographed with gel documentation system (SYNGENE, USA).

Data collection and analysis

Clear and distinct bands amplified by RAPD and SSR primers were scored for the presence andabsence of the corresponding band among the genotypes. The scores 1 and 0 indicates the presence or absence of bands respectively. The softwares used for the analysis of the scored data were NTSYSpc version 2.02 (Rholf1994). The molecular weight of the PCR products were estimated by Alpha EaseFC4.0.0 software (Alpha Innotech Corporation, USA) for each primer to analyze allele range.Coefficients of similarity were calculated by using Jaccard's similarity coefficient by SIMQUAL function and cluster analysis was performed by agglomerative technique using the UPGMA (Un-weighted Pair Group Method with Arithmetic Mean) method by SAHN clustering function of NTSYSpc.

Results and Discussion

The data collected from random amplification of polymorphic DNA with 20 arbitrary primers produced 179 total loci and the total bands produced were 1942. Out of the 179 loci produced, 123 were polymorphic and hence the total polymorphism percentage was found to be 65.42 %, whereas Malviya*et al.,* (2010) and Choudhury *et al.,* (2008) obtained 80% and 74.7% polymorphism, respectively. Very low level of polymorphism in cultivated pigeonpeagermplasm had been earlier reported which was corroborated by many pigeonpea workers indicating the narrow genetic base existing in this crop (Odeny*et al*. 2007). The average PIC (Polymorphism Information content) values for RAPD was 0.84. Average number of loci per primer was found to be 8.95 and average number of polymorphic loci obtained per primer (Assay Efficiency Index) was found to be 6.15. The molecular size of the amplified PCR products ranged from 185 bp

Table 1: List of RAPD primers used for RAPD profiling.

Sr. No	Primer Series	Primer Sequence (5' 3')	GC content (%)	Sr.No	Primer Series	PrimerSequence (5' 3')	GC content (%)
1	13ES10C24	GGCTCGTACC	70	11	OPAC-11	CCTGGGTCAG	70
2	16ES10C27	CGCCACGTTC	70	12	OPAZ-18	CCGACGTTGA	60
3	17ES10C28	GCCTCCTACC	70	13	OPC-05	GATGACCGCC	70
4	22ES10G33	AGGCCCGATG	70	14	OPG-08	TCACGTCCAC	60
5	OPA-01	CAGGCCCTTC	70	15	OPN-04	GACCGACCCA	70
6	OPA-03	AGTCAGCCAC	60	16	OPN-09	TGCCGGCTTG	70
7	OPA-09	GGGTAACGCC	70	17	OPN-12	CACAGACACC	60
8	OPA-16	AGCCAGCGAA	60	18	OPP-01	GGGACGATGG	70
9	OPA-18	AGGTGACCGT	60	19	OPP-06	GTGGGCTGAC	70
10	OPA-20	GTTGCGATCC	60	20	OPP-08	ACATCGCCCA	60

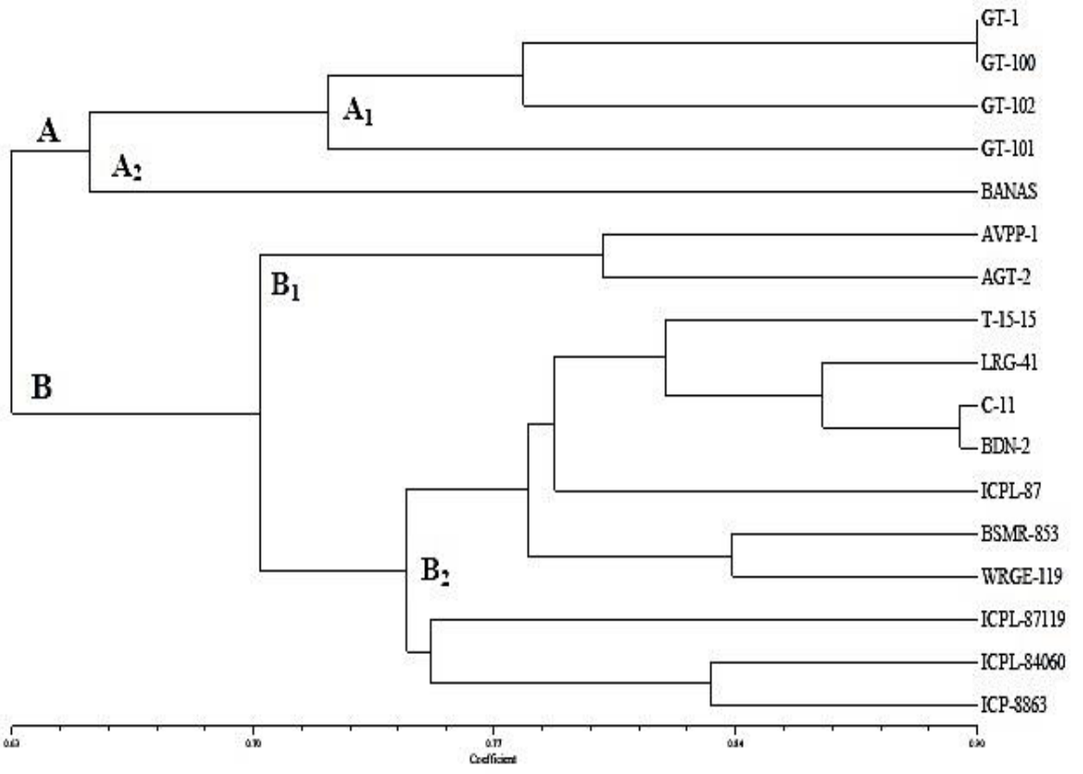

Figure 1: Dendrogram constructed using UPGMA cluster analysis

Table 2: The number of loci detected in different cultivars of pigeon pea using random primers

Sr. No	Locus Name	Molecular size range (bp)	Total no. of bands	No. of Loci	No. of polymorphic loci	Percent Polymorphism (%)	PIC value	Unique loci	
								Cultivers	Size(bp)
1	13ES10C24	225bp-2890bp	138	16	14	87.5	0.91	ICPL-87	1243
								ICPL-87119 C-11 ICP-8863	225
2	16ES10C27	342bp-2095bp	150	13	10	76.92	0.90	ICP-8863	445
3	17ES10C28	735bp-3294bp	68	8	6	75	0.80	LRG-41	3098
4	22ES10G33	450bp-1845bp	88	10	9	90	0.86	BANAS	1128
5	OPA-01	252bp-2387bp	108	8	3	37.5	0.85	ICPL-87 ICPL-84060	252
6	OPA-03	273bp-2378bp	83	7	5	71.42	0.83	BSMR-853 WRGE-119	273
7	OPA-09	461bp-3428bp	85	10	8	80	0.84	T-15-15	628
								GT-101 AVPP-1	657
8	OPA-16	298bp-2050bp	96	7	4	57.14	0.84	ICPL-87119	298
9	OPA-18	340bp-2316bp	68	5	2	40	0.77		
10	OPA-20	365bp-2386bp	87	7	4	57.14	0.83		
11	OPAC-11	361bp-3190bp	118	11	10	90.9	0.88	T-15-15	361 1265
12	OPAZ-18	320bp-2545bp	60	9	8	88.8	0.82		
13	OPC-05	225bp-1960bp	121	9	3	33.3	0.87		
14	OPG-08	306bp-2375bp	94	8	4	50	0.84	ICP-8863 WRGE-119	924
								ICPL-87119	908
15	OPN-04	270bp-2885bp	70	7	6	85.71	0.81		
16	OPN-09	346bp-3210bp	121	8	2	25	0.86		
17	OPN-12	185bp-2182bp	118	8	3	37.5	0.86		
18	0PP-01	430bp-1676bp	78	5	3	60	0.79		
19	OPP-06	380bp-3249bp	104	10	8	80	0.88		
20	OPP-08	136bp-2840bp	87	13	11	84.61	0.86	GT-101	136
								ICPL-87119	154
Total	-	-	**1942**	**179**	**123**	-	-		
Average	-	**335bp-2559bp**	**97.1**	**8.95**	**6.15**	**65.42**	**0.84**		

Table 3.Genetic similarity matrix of pooled RAPD data based on Jacard's similarity coefficient

	GT-1	GT-100	GT-101	GT-102	BANAS	AVPP-1	AGT-2	T-15-15	LRG-41	C-11	BDN-2	ICPL-87	ICPL-87119	ICPL-84060	ICP-8863	BSMR-853	WRGE-119
GT-1	1.00																
GT-100	0.90	1.00															
GT-101	0.74	0.71	1.00														
GT-102	0.76	0.78	0.70	1.00													
BANAS	0.70	0.66	0.60	0.62	1.00												
AVPP-1	0.60	0.58	0.59	0.64	0.60	1.00											
AGT-2	0.61	0.60	0.56	0.72	0.60	0.79	1.00										
T-15-15	0.62	0.63	0.60	0.71	0.64	0.64	0.73	1.00									
LRG-41	0.62	0.62	0.59	0.73	0.63	0.64	0.76	0.85	1.00								
C-11	0.62	0.61	0.56	0.75	0.61	0.70	0.81	0.80	0.87	1.00							
BDN-2	0.65	0.65	0.57	0.76	0.62	0.70	0.76	0.78	0.84	0.89	1.00						
ICPL-87	0.62	0.62	0.60	0.70	0.56	0.66	0.74	0.70	0.79	0.82	0.81	1.00					
ICPL-87119	0.62	0.60	0.58	0.64	0.59	0.59	0.64	0.69	0.75	0.73	0.76	0.74	1.00				
ICPL-84060	0.64	0.63	0.56	0.69	0.61	0.62	0.69	0.70	0.73	0.74	0.76	0.75	0.79	1.00			
ICP-8863	0.64	0.63	0.52	0.70	0.59	0.64	0.72	0.66	0.71	0.75	0.76	0.75	0.70	0.82	1.00		
BSMR-853	0.66	0.65	0.54	0.71	0.62	0.68	0.73	0.69	0.73	0.81	0.84	0.76	0.75	0.73	0.79	1.00	
WRGE-119	0.64	0.64	0.58	0.70	0.58	0.69	0.76	0.71	0.77	0.82	0.81	0.77	0.75	0.75	0.75	0.83	1.00

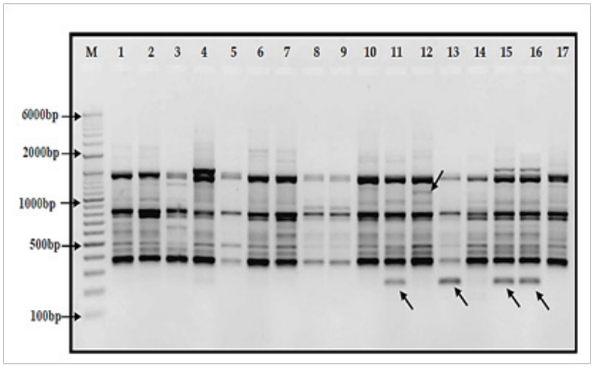

Figure 2: RAPD profile of primer 13ES10C24

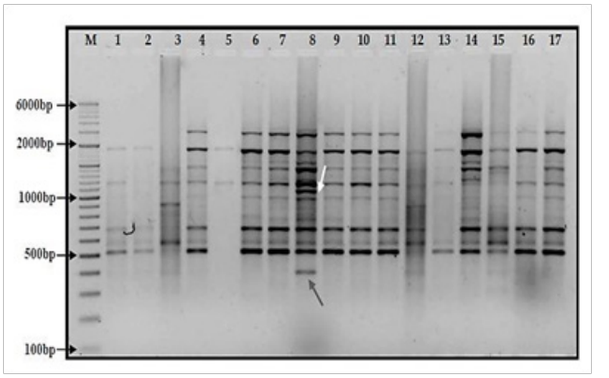

Figure 3: RAPD profile of primer OPAC-11

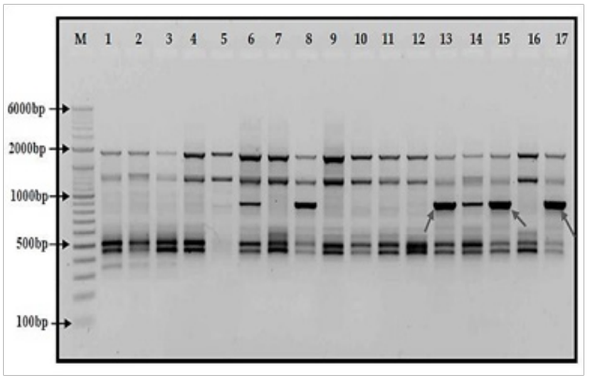

Figure 4: RAPD profile of primer OPG-08

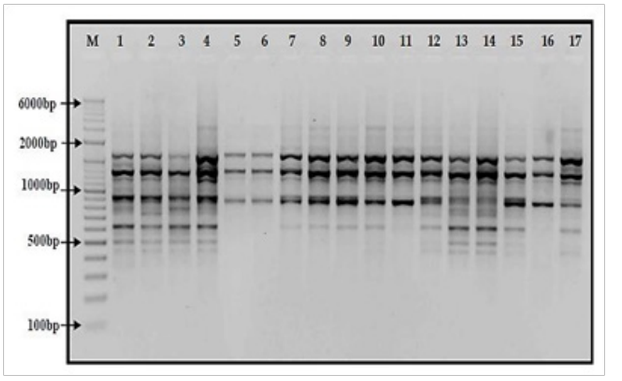

Figure 5: RAPD profile of primer OPP-06

(OPN-12) to 3428 bp (OPA-09) (Table 2). The highest similarity index value of 0.90 was found between GT-1 and GT–100, while the least similarity index value of 0.52 was found between GT-101 and ICP-8863. The average similarity coefficient among genotypes was 0.77. The RAPD marker 16ES10C27 produced maximum number of 150 bands, while OPAZ-18 produced the minimum number of 60 bands. OPAC-11, 22ES10G33 and OPAZ-18 produced highest polymorphism of 90, 90 and 88.8 % respectively. The RAPD marker OPN-09 gave the lowest polymorphism *i.e.* 25%. The highest PIC value obtained was 0.91 for 13ES10C24 marker and lowest PIC value was 0.77 for OPA-18. Marker index value for pooled RAPD data was found to be 11.65. A dendrogram was generated showing the grouping of genotypes according to their resistance and susceptibility reaction to *fusarium*wilt.

The RAPD cluster pattern is presented Figure 1. It showed two major clusters namely A and B formed at a similarity coefficient of 0.63 (Fig. 1). Cluster A was divided into two sub-clusters A1 and A2. Grouping of four genotypes that are susceptible to *fusarium* wilt were in one majorcluster 'A1'. Sub-cluster A1 included two minor clusters in which one consisted GT-101 alone and another minor cluster included genotypes *viz*., GT-1, GT-100 and GT-102. Sub-cluster A2 consisted of BANAS genotype alone which is susceptible to *fusarium*wilt. Cluster B was divided into two sub-clusters B1 and B2. Sub-cluster B1 included two genotypes, AVPP-1 and AGT-2. Sub-cluster B2 included three minor clusters in which one consisted of *viz.,* T-15-15, LRG-41, C-11, BDN-2 and ICPL-87. It contained both resistant and susceptible genotypes. Since resistance and susceptibility

reactions are usually monogenic or oligogenic in nature the molecular differences may be associated with even differences in a single nucleotide. Higher resolution in such clusters may be possible if large number of markers or SNPs are used for such type of study. The second minor cluster of B2 included resistant genotypes *viz*., BSMR-853 and WRGE-119. The third sub-cluster B2 consist genotypes *viz.*, ICPL-87119, ICPL-84060 and ICP-8863.

Conclusion

RAPD analysis of seventeen pigeonpea genotypes with 20 random primers revealed that this dominant marker can efficiently distinguish susceptible genotypes from resistant ones. RAPD markers can very well serve the purpose of identifying resistancy from susceptibility. Moreover, conversion of distinct molecular bands to more efficient SCAR markers should to be the next logic step to characterize resistant genotypes. The study also revealed that from the tendency of resistant and susceptible genotypes to cluster together, it can be inferred that these genotypes share a common phylogenetic pathway and the resistancy and susceptibility to *fusarium* wilt may be due to mono or oligogenes which can in turn to be targeted using more efficient marker (molecular) systems.

Acknowledgements

We thank Retd. Head and Professor Dr. J. C. Jadeja, for the helpand encouragement during these study and also thank to Plant Biotechnology Laboratory, Department of Genetics and Plant Breeding, B. A. College of Agriculture.

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