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Accessing genetic variability in chickpea (*Cicer arietinum* L.) varieties differing in susceptibility to *Fusarium oxysporum* f.sp. *ciceri* using ISSR markers

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Due to *Fusarium* wilt the production of chickpea has been affected in large. Molecular variability encompassed in chickpea offers an efficient means of exploiting disease resistance variety. The DNA-based molecular marker technique, inter simple sequence repeat (ISSR) was used to study the genetic variability for 5 chickpea varieties differing in their susceptibility to wilt disease using a different ISSR primers in present investigation. From the sixteen ISSR primer used, only two primers, primer 6956-022 (sequence of $(GA)_8$ CTC) and 6956-030 ($(AC)_8$ YG) gave three specific amplicon *viz.*,167,191, 272 and 196, 249, 408 base pair (bp) respectively, for resistant variety WR-315. Similarly, 6956-029 (146 bp), 6956-022 (311 bp), 6956-038 (1157 bp), 6956-031 (119, 139 bp) and 6956-033 (310 bp) markers associated with the chickpea variety JCP-27. For susceptible variety JG-62, three ISSR primer of a sequence $(GA)_8$ T, $(AG)_8$YT and $(AC)_8$CCTA generated unique band for DNA produced 1343, 534 and 231 bp markers, respectively.

Key words : Chickpea, *Fusarium oxysporum* f.sp. *ciceri*, ISSR

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INTRODUCTION

Chickpea (*Cicer arietinum* L., 2n=2x=16) is the most important pulse crop of India, which occupies an area of about 7.89 million ha. with an annual production of 7.06 million tones and productivity of 895 $kg.ha^{-1}$ (Gupta and Sahu, 2012). Wilt caused by *Fusarium oxysporum* f. sp. *ciceri* (Foc) is the major limiting factor of chickpea production (Nene *et al.,* 1996). Breeding for highly resistant variety is one of the best ways to control the disease. However, very little information is available regarding molecular and genetic variations and resistance to wilt in chickpea. Among the different techniques used to study the molecular and genetic variations, ISSR is a novel PCR technique that uses repeat anchored or non-anchored primers to amplify DNA sequences between two inverted SSR. Such amplification does not require genome sequence information and leads to multilocus and highly polymorphous patterns (Zietkiewicz *et al.,* 1994). The ISSR technique uses microsatellites, usually 16-25 bp long, as primers in a single primer PCR reaction targeting multiple genomic loci to amplify mainly the inter-SSR sequences of different sizes. Thus, genetic variability for 5 chickpea varieties differing in their susceptibility to wilt disease using a different ISSR primers were tested in present investigation.

RESEARCH METHODOLOGY

Chickpea (*Cicer arietinum* L.) seeds of five varieties (WR-315, JCP-27, GG-1, Saki and JG-62) differing in their susceptibility to wilt disease were obtained from Main Pulse Research Station, Junagadh Agricultural University, Junagadh, Gujarat, India.

DNA Isolation from chickpea :

Total genomic DNA was extracted from the leaves by a modified cetyltrimethyl ammonium bromide (CTAB) method (Keim *et al*., 1988). Fresh 10 days old chickpea leaves (1 g) from each varieties were powdered in liquid nitrogen using a pestle and mortar. The resulting powder was transferred to a 30 ml test tube and extracted with 5 ml of pre-warmed (65°C)

extraction buffer and then incubated it for 45 min at 65°C in water bath. After this, equal volume of chloroform: isoamyl alcohol (24:1) was added and transferred in centrifuge tube. The mixture was centrifuged at 5000 rpm for 10 min. This process was again repeated and the aqueous phase was transferred into another tube containing equal volume of chilled isopropanol and kept at –20°C for 1 hr to precipitate nucleic acid. Then tubes were centrifuged at 10000 rpm for 10 min. to collect precipitate. The pellet was washed with 70 per cent ethanol, air dried, dissolved in TE buffer, pH 8.0, containing RNAase (60 µg, 2µl from 30mg/ml RNase), and incubated for 1 hr at 37°C for RNA degradation. Degraded RNA was then removed with one volume of chloroform : isoamyl alcohol (24:1). DNA was precipitated with equal volume of chilled isopropanol and kept in –20°C for 30 min. and then centrifuged it at 10,000 rpm for 10 min. The pellet of DNA washed 2 times with 70% ethanol, dried at room temperature and re-suspended in 200 µl TE buffer.

PCR-ISSR :

The DNA concentrations were determined spectro photometrically at a wavelength of 260 nm. Depending on the concentration of DNA determined, a stock solution with a concentration of 1µg/µl was prepared for each isolate. These were subsequently diluted to 5 ng/µl and used in amplification. The genomic DNA fingerprinting of chickpea by ISSR was subjected to Anchored polymerase chain reaction using 12 primers obtained from Bangalore Genei, India. The Tm value of the primers varied between 42 and 62^0C. The PCR reactions for ISSR were carried out in 20 µl of reaction mixtures as described by Raina *et al.* (2001).

PCR reactions were carried out in 20µl of reaction containing 10X buffer (with 2.5mM $MgCl_2$), 2µl of 2mM dNTP mixture, 2 µl of 2µM primer, 5µl of Taq DNA polymerase 3U; 8ìl of H_2O, and 15 ng of template DNA samples were amplified on DNA thermocycler. (Bio-Rad Thermal Mycycler) using the PCR conditions 94^0C for 1 minutes, 42^0C- 62^0C (As per Tm value of ISSR primer) for 1 minutes, and 72^0C for 2 minutes. The total numbers of cycles were 36, with the final extension time of 10 minutes. A 10 µl of each reaction was electophorosed on 1.8 % agarose gel run at constant voltage (6v/cm) in 0.5 x TBE and stained with ethidium bromide (10mg/ml). The DNA marker used was 1kb ladder. The gel was viewed under UV Trans-illuminator for visualizing amplified DNA bands, and photographed by digital camera, and transferred to computer in JPEG format. Band positions for each isolates and primer combination were scored as either present (1) or absent (0) for phylogenetic analysis using NTSYS-pc (Numerical Taxonomy and Multivariate analysis) system version 2.2 by Exeter Software (Rohlf, 2004). The SIMQUALK programme was used to calculate Jaccard's similarity co-efficient and a graphical phenogram (dendrogram) of the genetic relatedness among the microbes was produced by means of the unweighted pair group method with arithmetic average (UPGMA) analysis (Sneath and Sokal, 1973). Size of specific bands of DNA or protein were determined using software *Alphaimager 2200* manufactured by Alpha Ease FC, USA.

Polymorphism information content (PIC) value calculation :

To measure the informativeness of the different markers, the polymorphism information content (PIC) of each marker was calculated according to following formula (Salem *et al.*, 2008) :

$PIC=1-(Pi^2 + Qi^2)$

where,

Pi=frequency of the ith allele in the set of 5 varieties.

Qi=n-Pi

n=total number of allele detected for a locus of a marker.

RESEARCH FINDINGS AND ANALYSIS

Inter-simple sequence repeat (ISSR) markers have been proposed as a new source of genetic markers that are inherited in Mendelian fashion and are scored as dominant markers (Ratnaparkhe *et al.*, 1998). Genomic DNA extracted from each chickpea variety was subjected to PCR using synthesized ISSR primers using 16 ISSR primers. These 16 primers were obtained from Bangalore Genei, India. Among the 16 ISSR primers one primer with the sequence $(AC)_8$CTC failed to give any amplified products of DNA. Possibly this may be due to absence of complementary sequence in the genomic DNA. Thus, 15 out of 16 primers were selected for evaluating molecular differences existing in 5 varieties of chickpea differing in response against fungal pathogen *Fusarium oxysporum* f.sp. *ciceri* for wilt disease. A total 110 bands were produced by 15 ISSR primers with an average frequency of 7 bands per primer. Total 77 polymorphic bands were generated out of which 56 were polymorphic and shared between at least two individuals, and 21 bands were polymorphic and unique while 33 were monomorphic. Except three primers (of a sequence $(AG)_8$YT, $(CA)_8$ AAGCT and $(AC)_8$YG) out of the 15 ISSR primers, produced monomorphic bands whereas these five primer produced only polymorphic banding patterns. ISSR primer of sequence $(GA)_8$ CTC generated the maximum 13 bands which was followed by primer with a sequence $(AC)_8$ YG and $(GA)_8$ T with 11 bands. Whereas primer with a sequence of $(CA)_8$ AAGCT generated the lowest number, only 1 bands. The per cent polymorphism furnished by each primer ranged between 28.6 and 100 (Table 1).

The calculated PIC values for ISSR markers were ranged from 0 to 0.897 (Table 1). The lowest PIC values obtained by 6956-021 and highest was with 6956-022 of a sequence of

$(CA)_8$ AAGCT and $(GA)_8$ CTC, respectively. Thus, on the bases of PIC value, primer 6956-022 of a sequence of $(GA)_8$ CTC (Plate 1) gave best results among the primer used for characterization of chickpea varieties studied in this experiment.

The ISSR profiles generated were further evaluated for studying the Jaccards similarity co-efficient (Table 2) and showed monomorphism between two varieties of chickpea. The variety JCP-27 showed the minimum similarity with the group of other chickpea varieties.

Dendrogram was constructed using UPGMA based on Jaccard's similarity co-efficient NTSYSpc-2.02i for five varieties of chickpea. The similarity co-efficient ranged from 0.143 to 0.943 (Table 2). The dendrogram obtained indicates that there was a major cluster A and B consisting of 4 chickpea varieties and one variety JCP-27 were found to be different from the rest of varieties. The major cluster A and cluster B consisted of 5 chickpea varieties and shared 36 per cent similarity (Fig. 1). Out of five varieties of chickpea, GG-1 and Saki showed more than 86 per cent similarity. Major cluster A was further divided in to sub-cluster A_1 and A_2 and consisted of 3(GG-1, Saki and JG-62)

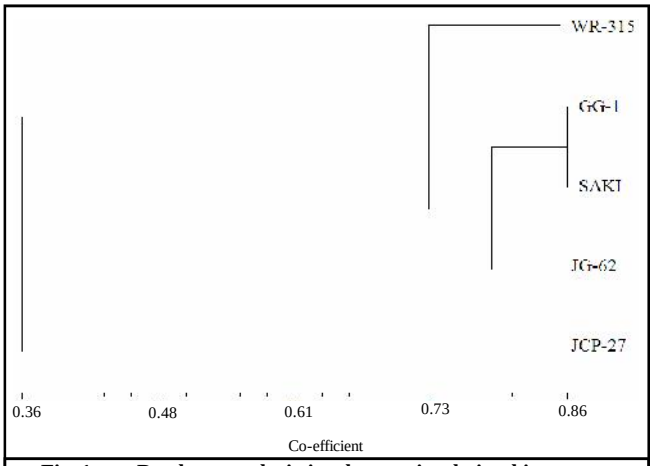


Fig. 1: Dendrogram depicting the genetic relationship among the chickpea varieties based on the ISSR data

Table 1: Polymorphism obtained with different ISSR primers generated from five varieties of chickpea

Sr. No.	Code	Sequence of ISSR primer	Tm	Polymorphic bands			Mono mor-phic. bands	Total bands	Poly-mor. (%)	PIC
				S	U	T				
1.	6956-029	$(GA)_8$ T	42.9	6	2	8	3	11	72.7	0.895
2.	6956-037	$(GA)_8$ C	43.3	2	0	2	2	04	50.0	0.716
3.	6956-022	$(GA)_8$ CTC	48.5	5	6	11	2	13	84.6	0.897
4.	6956-028	$(GA)_8$ G	48.8	2	0	2	5	07	28.6	0.856
5.	6956-036	$(CA)_8$A	52.7	1	1	5	2	07	71.4	0.846
6.	*6956-038	$(AC)_8$YA	49.8	5	2	7	3	10	71.4	0.887
7.	*6956-031	$(GA)_8$YC	48	6	2	8	1	09	88.9	0.872
8.	*6956-033	$(AG)_8$YT	45.4	2	2	4	0	04	100.0	0.694
9.	6956-020	$(CA)_8$ AAGG	61.3	3	0	3	2	05	60.0	0.793
10.	6956-021	$(CA)_8$ AAGCT	62.5	1	0	1	0	01	100.0	0
11.	*6956-030	$(AC)_8$ YG	53.7	7	4	11	0	11	100.0	0.888
12.	6956-032	$(AC)_8$G	54.9	4	0	4	1	05	80.0	0.782
13.	6956-024	$(AC)_8$CCTA	55.3	3	1	4	6	10	40.0	0.891
14.	6956-025	$(AC)_8$CCTG	58.9	2	0	2	4	06	33.3	0.831
15.	6956-023	$(AC)_8$CTC	51.4	0	0	0	0	00	00.0	0
16.	6956-026	$(AG)_8$ CTG	52.5	4	1	5	2	07	71.4	0.835
		Total		56	21	77	33	110		

Tm=Melting Temp.; S= Shared; U= Unique; T= Total Polymorphic Bands; PIC = Polymorphism Information Content,
* Anchored primer, Y= Pyrimidines (C/T)

Table 2: Jaccard's similarity coefficient between the chickpea varieties based on the ISSR data

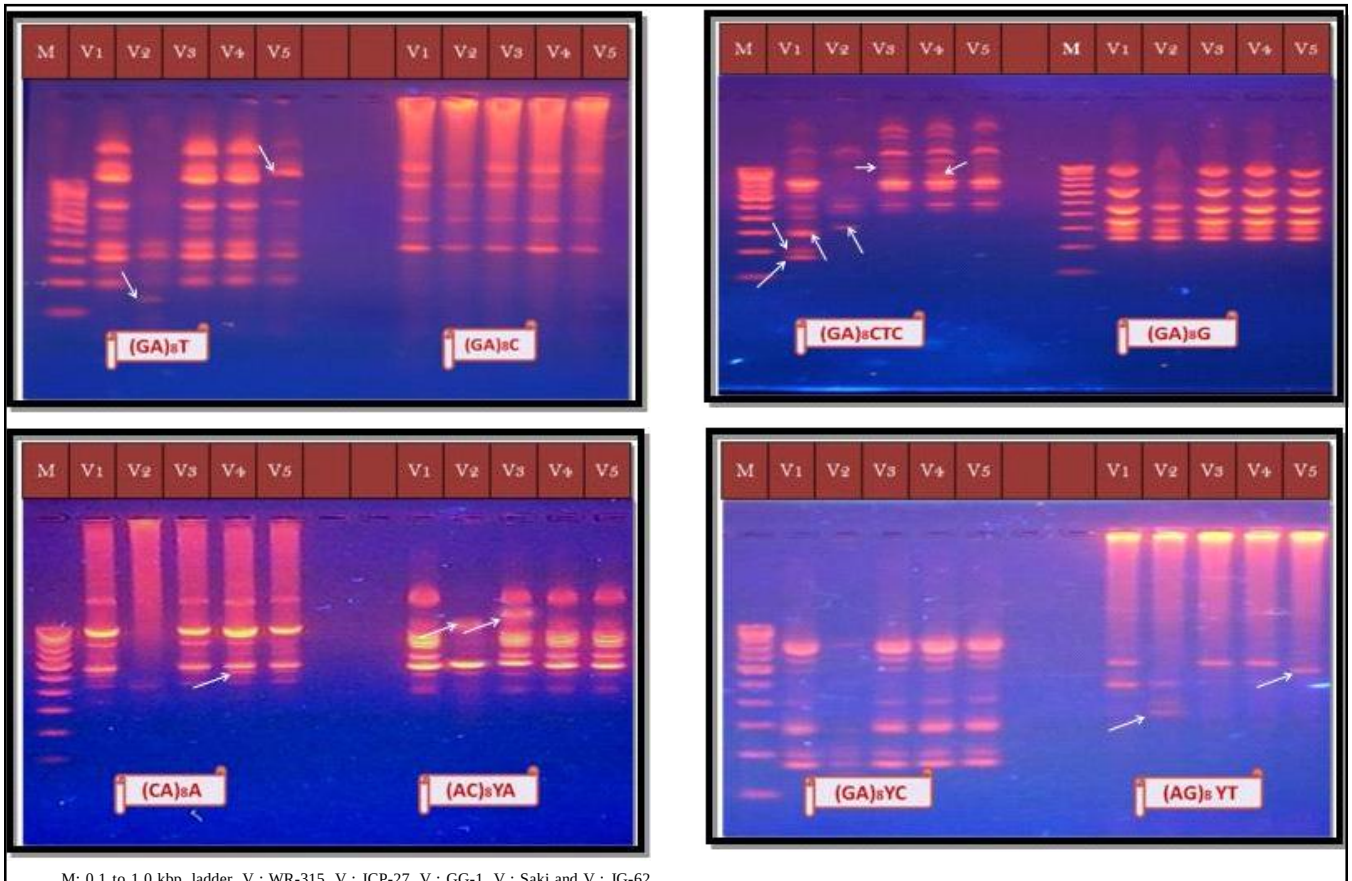
	WR-315	JCP-27	GG-1	SAKI	JG-62
WR-315	1.000				
JCP-27	0.367	1.000			
GG-1	0.767	0.340	1.000		
SAKI	0.717	0.344	0.860	1.000	
JG-62	0.711	0.376	0.798	0.783	1.000

Matrix correlation: r = 0.996 (Normalized Mantel test statistic)

Table 3 : ISSR markers associated with five varieties of chickpea on the basis of unique band

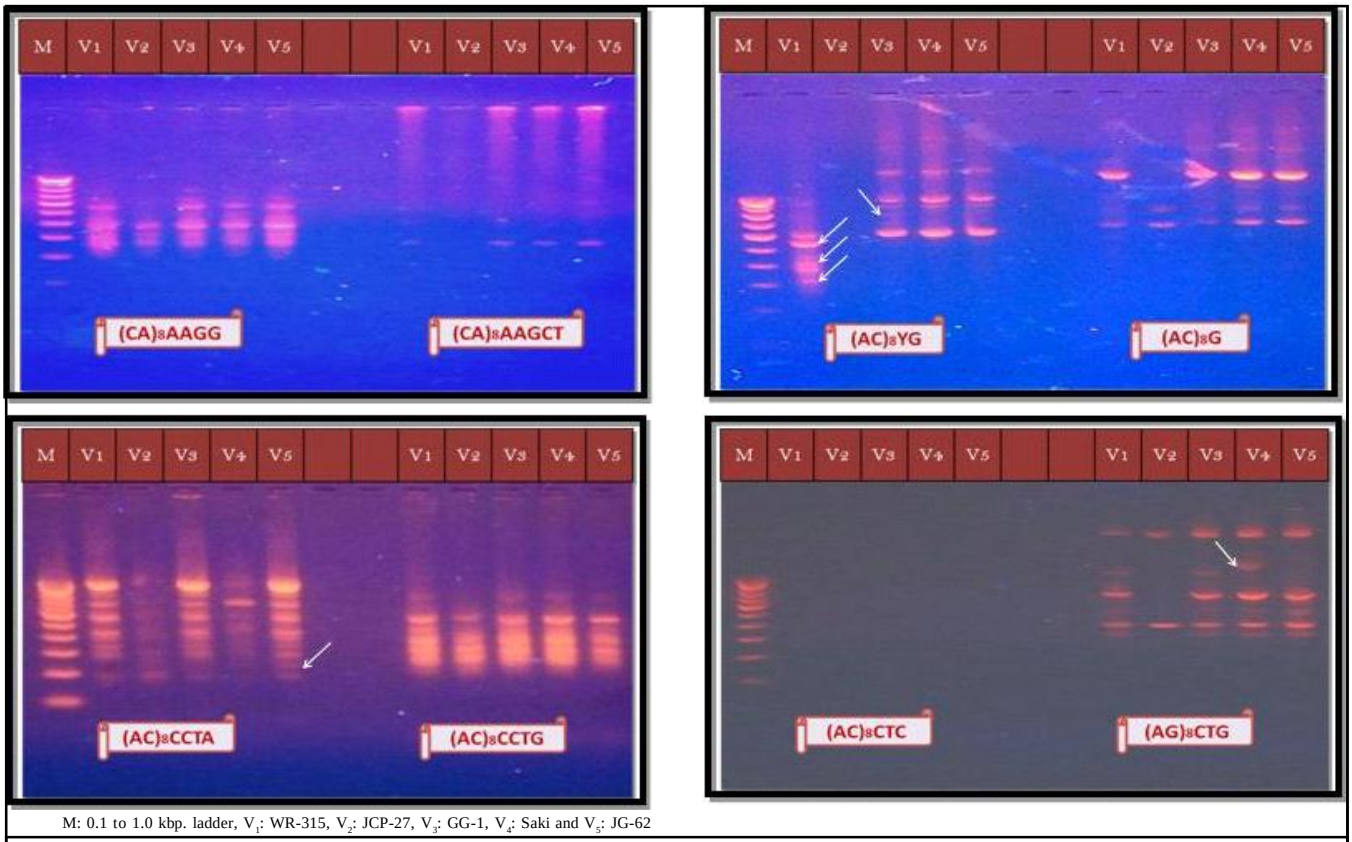
Sr. No.	Code	Sequence of ISSR primer	WR-315 (V_1)	JCP-27 (V_2)	GG-1 (V_3)	SAKI (V_4)	JG-62 (V_5)
1.	6956-029	$(GA)_8$ T	-	146	-	-	1343
2	6956-037	$(GA)_8$ C	-	-	-	-	-
3	6956-022	$(GA)_8$ CTC	167,191,272	311	1071	936	-
4	6956-028	$(GA)_8$ G	-	-	-	-	-
5	6956-036	$(CA)_8$A	-	-	-	462	-
6	*6956-038	$(AC)_8$YA	-	1157	1357	-	-
7	*6956-031	$(GA)_8$YC	-	119,139	-	-	-
8	6956-033	$(AG)_8$YT	-	310	-	-	534
9	6956-020	$(CA)_8$ AAGG	-	-	-	-	-
10	6956-021	$(CA)_8$ AAGCT	-	-	-	-	-
11	*6956-030	$(AC)_8$ YG	196,249,408	-	715	-	-
12	6956-032	$(AC)_8$G	-	-	-	-	-
13	6956-024	$(AC)_8$CCTA	-	-	-	-	231
14	6956-025	$(AC)_8$CCTG	-	-	-	-	-
15	6956-023	$(AC)_8$CTC	-	-	-	-	-
16	6956-026	$(AG)_8$ CTG	-	-	-	1539	-
		Total	6	6	3	3	3

* Anchored primer, Y= Pyrimidines (C/T)



M: 0.1 to 1.0 kbp. ladder, V_1: WR-315, V_2: JCP-27, V_3: GG-1, V_4: Saki and V_5: JG-62

Plate 1: Banding pattern obtained from five varieties of chickpea generated with different ISSR primers



M: 0.1 to 1.0 kbp. ladder, V_1: WR-315, V_2: JCP-27, V_3: GG-1, V_4: Saki and V_5: JG-62

Plate 2 : Banding pattern obtained from five varieties of chickpea generated with different ISSR primers

and 1 variety (*i.e.* WR-315) of chickpea, respectively, sharing 73 per cent similarity. Sub cluster A_1 consisted of 3 isolates, and shared 78 per cent similarity. Thus, the cluster analysis from the ISSR data clearly revealed a difference between the genotypes.

ISSR markers linked to different variability in chickpea varieties related to tolerance against wilt disease is tabulated in (Table 3). Only two primer, out of sixteen, primer 6956-022 (sequence of $(GA)_8$ CTC) and 6956-030 ($(AC)_8$ YG) gave three specific amplicon (167,191, 272and 196, 249, 408 bp, respectively) for resistant variety WR-315. Similarly, 6956-029 (146 bp), 6956-022 (311 bp), 6956-038 (1157 bp), 6956-031 (119, 139 bp) and 6956-033 (310 bp) markers associated with the chickpea variety JCP-27. However, 6956-022 generated ISSR markers for maximum number of chickpea varieties *i.e.* WR-315(167,191, 272 bp), JCP-27 (311 bp), GG-1(1071 bp.) and Saki (936.bp). Thus, the maximum (6) numbers of unique marker bands having varying base pairs were generated by this primer. Three ISSR primer of a sequence $(GA)_8$ CTC, $(AC)_8$YA and $(AC)_8$ YG generated unique band for DNA for GG-1 variety and produced 1071, 1071 and 715 bp markers, respectively. For susceptible variety JG-62, three ISSR primer of a sequence $(GA)_8$ T, $(AG)_8$YT and $(AC)_8$CCTA generated unique band for DNA produced 1343, 534 and 231 bp markers respectively (Table 3).

To test the goodness of fit of the clustering of ISSR data, matrix of cophenetic values were also computed using the program COPH in same way as in RAPD analysis. In the present study also the mental test statistics Z was normalized and degree of goodness of fit for a cluster analysis (Matrix correlation r = 0.996) as categorized by Rohlf (1994) was found to fall under the category of "very good fit".

Literature showed efficiency of ISS.56R to identify various chickpea varieties differing in response against fungal pathogen through molecular characterization. Bhagyawant and Srivastava, (2008) reported from their study in chickpea crop that an amplification of genomic DNA of the 12 genotypes using ISSR analysis yielded 492 fragments that could be scored. The total number of bands amplified by 3' anchored primers varied from 36 to 96. The maximum band also was produced by primer having poly AC sequence. Brinda and Ravikumar (2005) also found polymorphism for JG-62 and WR-315 using the ASAP (allele-specific associated primer) marker in a chickpea. Rao *et al.* (2007) also noted from their study that the wild species shared 20.3 per cent common bands and 79.6 per cent polymorphic bands with ISSR markers. 43.75 per cent common bands and 56.25 per cent polymorphic bands were found among cultivated chickpea varieties and

34.37 per cent common bands and 65.63 per cent polymorphic bands were found among wild accessions.

Thus, Based on an ISSR marker, many new markers have been identified in present study linked with different variability in chickpea varieties related to tolerance against wilt disease. Only two primers, primer 6956-022 (sequence of $(GA)_8$ CTC) and 6956-030 ($(AC)_8$ YG) gave three specific amplicon (167,191, 272and 196, 249, 408 bp, respectively) for resistant variety WR-315. Similarly, 6956-029 (146 bp), 6956-022 (311 bp), 6956-038 (1157 bp), 6956-031 (119, 139 bp) and 6956-033 (310 bp) markers associated with the chickpea variety JCP-27. For susceptible variety JG-62, three ISSR primer of a sequence $(GA)_8$ T, $(AG)_8$YT and $(AC)_8$CCTA generated unique band for DNA produced 1343, 534 and 231 bp markers.

Conclusion :

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