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Pathogenic and genetic diversity of *Fusarium oxysporum* f. sp. *ciceri* isolates causing wilt disease in chickpea

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ABSTRACT

An experiment was conducted to study pathogenic and genetic the variability of ten isolates of *F. oxysporum* f. sp. *ciceri* (FOC) using six chickpea genotypes and six RAPD markers. The *F. oxysporum* f. sp. *ciceri* isolates P-12, B-2 and BKN-1 proved to be most virulent to cause wilt disease in chickpea genotypes. These isolates were categorized based on mean wilt incidence into three groups: Group- I Highly virulent (P-12, B-2, BKN-1, FOC-1, S-13 and S-14), Group-II Moderately virulent (N-11 and J-7) and Group-III - Less virulent (D-3636 and U-549). Genetic diversity of *F. oxysporum* f. sp. *Ciceri* isolates examined for RAPD analysis revealed that the average number of polymorphic bands per primer were 7.83. The range of genetic similarity was found 0.79 (BKN-1 and FOC-1) to 0.10 (N-11 and B-2). The average genetic similarity coefficient observed was 0.31 ±.

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

Chickpea (*Cicer arietinum* L.) is the world's third most important food legume crop with India accounting for more than 60 per cent of world production (FAO 1993 and 2008). Chickpea wilt caused by *Fusarium oxysporum* f. sp. *ciceri* (Padwick) Snyd. and Hans. is one of the serious limiting factors of chickpea production world wide (Nene *et al.* 1989, Jalali and Chand 1992). The pathogen is soil-borne and difficult to eradicate as the fungal chlamydospores survive in soil up to 6 years even in the absence of host plant (Kaiser *et al.* 1994, Haware *et al.* 1996). The use of resistant cultivar is considered as one of the most practical and cost effective strategy for managing a disease like *Fusarium* wilt. However, the efficiency of resistant cultivar in disease management is seriously limited by appearance of virulent strains or races of pathogen. Hence, knowledge of pathogenic variability is essential in making strategies for developing disease resistant varieties and deployment of resistant genes.The conventional methods of grouping of isolates or races identification requires long time and subject to environmental variations. With the advent of molecular technologies one can overcome these limitations and generate useful information for fungal characterization (Ouellett and Seifert 1993). The molecular methods have also been used to distinguish closely related species with few morphological differences and to distinguish stains or isolates within a species. Genetic characterization of *F. oxysporum* f. sp. *ciceri* isolates is important for the efficient management of the disease through use of resistant cultivars.

Keeping in view of above facts and seriousness of this disease, the present work was undertaken to study the variability of different isolates of *Fusarium oxysporum* f. sp. *ciceri* using molecular marker and chickpea genotypes.

MATERIALS AND METHODS

Wilt infested chickpea plants were collected from different chickpea growing areas of Rajasthan, viz. Bikaner, Jaipur, Hanumangarh, Suratgarh, Sikar, Jhunjhunu, Churu, and Sriganganagar and adjoining states, i.e. Narnaul district of Haryana. These infested plants were gently washed in tap water to remove the soil and other extraneous materials adhering on root surface. The washed plant parts were cut into small pieces and surface sterilized in 0.1% mercuric chloride solution in Petri dishes for 1-2 minutes followed by washing in sterilized distilled water. These surface sterilized pieces were transferred on PDA medium in Petri dishes. The Petri dishes were incubated in BOD incubator for 7 days at 26°C for growth of the pathogen.

Pathogenicity of the isolated cultures of *Fusarium oxysporum* f. sp.*ciceri* was tested by growing chickpea plants in pots containing pathogen infested soil. For this purpose, the *F.oxysporum* f. sp. *ciceri* isolates were multiplied on sand maize meal (2:1) medium in Erlenmeyer flasks which were sterilized at 15 p.s.i. for 30 minutes. These

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flasks containing the sterilized media were inoculated with respective *F. oxysporum* f. sp. *ciceri* cultures and incubated at 26°C for 10 days. The individual *Fusarium* inocula were added to soil at 20 g/kg soil and mixed thoroughly. The inoculated soils were transferred to clean plastic pots (10 cm diameter), suitably moistened and allowed for 72 hr to stabilize the inocula before sowing of chickpea seeds (cv RSG 44). Five healthy seeds of chickpea were sown in each pot. In case of control, chickpea seeds were sown in pots containing uninoculated soil. These pots were irrigated usually on alternate days. The wilt symptoms developed in seedlings were recorded. Re-isolation of the pathogen was made from wilt infested seedlings and identified as chickpea wilt pathogen, i.e. *F. oxysporum* f. sp. *ciceri*. The selected virulent cultures were also got identified by Indian Type Culture Collections (ITCC), Indian Agricultural Research Institute, New Delhi (Table 1).

Ten selected pathogenic isolates of *F. oxysporum* f. sp. *ciceri* were used in the present test as given in Table 1. The isolates of FOC were tested in six chickpea genotypes, i.e. RSG-44, CSJ-195, RSG-895, RSG-902, RSG-963 and RSG-991 obtained from Chickpea Breeder, Agriculture Research Station, SKRAU, Durgapura, Jaipur in plastic pots (10 cm diameter) under green house conditions in year 2008-09. The *F. oxysporum* f. sp. *ciceri* isolates were mass multiplied on sand maize meal (2:1) medium. The sand maize meal inocula of respective FOC isolates were added to sterilized soil at 20 g/kg soil and mixed thoroughly. The plastic pots (10 cm diameter) were filled with sterilized soil containing FOC inocula and chickpea seeds were sown after 72 hr of soil inoculation. In each pot ten healthy seeds of each chickpea genotypes were sown with three replications. In case of control, healthy chickpea seeds of six corresponding genotypes were sown in uninoculated sterilized soil. The wilt symptoms developed in different chickpea genotypes were observed periodically up to 75 days of sowing. The per cent disease incidence in different genotype was calculated and analyzed statistically using completely randomized design (CRD).

Table 1 List of ten *F. oxysporum* f. sp. *ciceri* isolates used in present study

Fusarium isolates	Place of collection	ITCC Accession No.
B 2	Farmers field, Napasar, Bikaner, Rajasthan	6940-08
BKN-1	Deparment of Plant Pathology, College of Agriculture, Bikaner, Rajasthan	
FOC-1	Krishi Vigyan Kendra, Sardarshar, Churu, Rajasthan	
D-3636	Indian Agriculture Research Institute, Pusa,(ITCC, New Delhi)	
J-7	Agriculture Research Station Farm, S K RAU, Jaipur, Rajasthan	6943-08
N-11	Farmer, Narnaul, Haryana	6945-08
P-12	Punjab Agricultural University, Ludhiana, Punjab	
S-13	Farmers field, Sikar, Rajasthan	6946-08
S-14	State Farm, Suratgarh, Rajasthan	6947-08
U-549	Udaipur, Rajasthan (ITCC, New Delhi)	

The test isolates of *F. oxysporum* f. sp. *ciceri* were grown on potato dextrose broth for seven days at 26 ^{0}C in Erlenmeyer flasks and the harvested mycelia mats of the individual isolates were filtered through cheese cloth, followed by removal of excess moisture using tissue paper. Three grams of mycelial mat of the fungus were homogenized in liquid nitrogen (liq. N_2) using pestle and mortar till the mats were obtained in powder form. The homogenized material was used for isolation of DNA as per the method given by Doyle and Doyle (1990).

Eighteen primers belonging to OPB series were screened using one selected *F. oxysporum* f.sp. *ciceri* (FOC) isolate. Based on banding pattern, six Random Amplified Polymorphic DNA (RAPD) primers were used to detect the molecular variability among the above mentioned ten FOC isolates.Random amplification of polymorphic DNA(RAPD) was done by using six primers of OPB series obtained from Operon Technologies, Inc. Alameda, California. PCR Reactions were performed in final volume of 25 µl containing 10X Assay Buffer (Bangalore Genei), 1.0 unit of Taq DNA polymerase (Bangalore Genei), 200 µM each of dNTPs (Fermentas), 10 pmols/reaction of random primers (Operon Technologies) and 50 ng of template DNA. The PCR was performed in Biometra Thermocycler with an initial denaturation step at 94°C for 5 min; followed by 43 cycles of denaturation at 94°C for 1 min, annealing at 37°C for 1 min, and extension at 72°C for 2 min and a final extension step at 72°C for 7 min. The PCR products were resolved using a 1.2% agarose gels stained with ethidium bromide at 0.5 µg/ml, and photographed under UV lighting at gel documentation system (Bio-Rad).The presence of each band was scored as '1' and its absence as '0'. Faintly visible bands were not scored but a major band corresponding to faint band was considered for scoring. In order to confirm the presence of bands and determine reproducibility all the primers were replicated twice and if necessary thrice.

The scores (0 or 1) for each band obtained from photograph were entered in the form of a rectangular data matrix (qualitative data matrix). The pair-wise association coefficients were calculated from qualitative data matrix using Jaccard's similarity coefficient. The equation for calculating Jaccard's similarity coefficients 'F' between two samples A and B is:

$$f = n_{xy} / (n_1 - n_z)$$

where, n_{xy}, Number of bands common to sample A and sample B; n_1, Total number of bands present in all samples; n_z, Number of bands not present in sample A or B but found in other samples.

Cluster analysis for the genetic distance was then carried out using UPGMA (Unweighted Pair Group Method with Arithmetic Mean) clustering method. The genetic distances obtained from cluster analysis through UPGMA

were used to construct the dendrogram, depicting the relationships of the clones using computer program NTSYS pc version 2.02 (Rohlf 1998).

To compare the efficiency of the markers in identification of fungal isolates, the discriminating power (D) of each primer was estimated. A single numerical index of discrimination (D) based on the probability that two unrelated strains sampled from the test population were placed into different typing groups and was calculated based on Simpson's index of diversity (Simpons 1949) as described by Hunter and Gaston (1988). This index can be derived from elementary probability theory and is given by the following equation:

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{S} n_j (n_j - 1)$$

where, N – is the total number of strains in the sample population, S – is the total number of types described, n_j – is the number of strains belonging to the j^{th} type.

This equation is derived as follows. The probability that a single strain sampled at random will belong to the j^{th} group is n/N. The probability that two strains sampled consecutively will belong to that group is $n_j (n_j - 1)/N (N-1)$. These probabilities can be summed for all the described types to give the probability that any two consecutively sampled strains were of the same type. This summation was subtracted from 1 to give the equation above.

RESULTS AND DISCUSSION

Pathogenic variability of F. oxysporum f. sp. ciceri

The disease development by 10 pathogenic isolates on six chickpea genotypes clearly revealed the existence of pathogenic variability among the *Fusarium* isolates. FOC isolates P-12, B-2 and BKN-1 proved to be most virulent on chickpea genotypes. The isolate P-12 was highly virulent to RSG-895 and CSJ-195 moderately virulent to RSG-44 and RSG-991. The chickpea genotype CSJ-195 and RSG-895 showed maximum susceptibility towards B-2 isolate. While, the isolate BKN-1 was highly virulent to chickpea genotype RSG-895. It was also observed that the mean disease incidence caused by FOC-1, S-13 and S-14 isolates to six chickpea genotypes was also quite high. The disease reaction recorded with respect to isolate J-7 and N-11 was variable. While, D-3636 and U-549 were comparatively less virulent to chickpea genotypes. The study also revealed that disease incidence recorded in individual chickpea genotypes in response to different *F. oxysporum* f. sp. *ciceri* isolates was variable.

The mean disease incidence recorded with respect to individual genotypes showed that RSG-963, RSG-895 and CSJ-195 were highly susceptible to the ten isolates of the fungus and rest three, i.e. RSG-44, RSG-902 and RSG-991 were comparatively less susceptible to *Fusarium* isolates tested. Based on these observations 10 *Fusarium* isolates can be categorized in to three groups (Table 2) and in Group-I isolates P-12, B-2, BKN-1, FOC-1, S-13 and S-14 were highly virulent (72.22 to 62.22% mean disease incidence), Group-II isolates N-11 and J-7 were moderately virulent (60.00 to 61.11% mean disease incidence) and Group-III isolates D-3636 and U-549 were less virulent (44.44 to 51.11% mean disease incidence). Iqbal *et al.* (2005) also recorded pathogenic variability in FOC isolates collected from major chickpea growing regions of Pakistan. The different isolates varied in their reaction to chickpea genotypes. Shrivastava and Agarwal (2006) categorized *F. oxysporum* f. sp. *ciceri* isolates into six groups based disease incidence on eight

Table 2 Pathogenicity of 10 *F. oxysporum* f. sp. *ciceri* isolates against six chickpea genotypes

Isolate	Wilt incidence						
	Genotypes						
	RSG-44	CSJ-195	RSG-895	RSG-902	RSG-963	RSG-991	Mean
B 2	26.67 (31.00)*	93.33 (77.71)	93.33 (77.71)	60.00 (50.77)	80.00 (63.43)	73.33 (59.00)	71.11
D 3636	20.00 (26.57)	46.67 (43.08)	46.67 (43.08)	33.33 (35.22)	60.00 (50.77)	60.00 (50.77)	44.44
BKN-1	46.67 (43.08)	86.67 (68.86)	100.00 (90.00)	60.00 (50.77)	46.67 (43.08)	80.00 (63.43)	70.00
FOC 1	53.33 (46.92)	80.00 (63.43)	46.67 (43.08)	86.67 (68.86)	93.33 (77.71)	53.33 (46.92)	68.89
J 7	53.33 (46.92)	66.67 (68.86)	80.00 (63.43)	46.67 (43.08)	60.00 (50.77)	33.33 (35.22)	60.00
N 11	26.67 (31.00)	66.67 (54.78)	60.00 (50.77)	66.67 (54.78)	66.67 (54.78)	80.00 (63.43)	61.11
P 12	73.33 (59.00)	80.00 (63.43)	86.67 (68.86)	46.67 (43.08)	73.33 (59.00)	73.33 (59.00)	72.22
S 13	20.00 (26.57)	73.33 (59.00)	46.67 (43.08)	66.67 (54.78)	100.00 (90.00)	66.67 (54.78)	62.22
S 14	46.67 (43.08)	66.67 (54.78)	73.33 (59.00)	60.00 (50.77)	73.33 (59.00)	73.33 (59.00)	65.56
U 549	60.00 (50.77)	33.33 (35.22)	66.67 (54.78)	40.00 (39.23)	66.67 (54.78)	40.00 (39.23)	51.11
Mean	42.67	71.33	70.00	56.67	72.00	63.33	
		S Em	*CD*	*CV*			
Isolate		(3.32)	(9.28)	(6.67)			
Genotype		(0.65)	(1.82)				
Isolate × Genotype		(2.06)	(5.77)				

* Figures in parentheses are angular transformed values

chickpea differential cultivars. Haware *et al.* (1990) reported seven races of *F. oxysporum* f. sp. *ciceri* worldwide based on disease reaction to ten chickpea genotypes. The use of resistant cultivars is the most effective strategy for the management of the soil borne disease like chickpea wilt. In order to develop a resistant cultivar the knowledge of pathogenic variability is essential. The wilt pathogen *F. oxysporum* f. sp. *ciceri* can adopt a wide range of environmental conditions due to its physiological and genotypic variability (Jiménez-Gasco*et al*. 2001 and Sharma *et al*. 2009).

Genetic diversity

All the 10 isolates of *F. oxysporum* f. sp. *ciceri* were examined for Random Amplified Polymorphic DNA (RAPD) genetic marker with six decamer primers of OPB series (Operon Technology, USA). All the primers were repeated at least twice to confirm the polymorphism. This is because the stochastic nature of the band and banding pattern of DNA amplification with RAPD, reproducibility of the banding pattern has been found to change. Finally only those bands were considered as polymorphic, which did not amplify in certain isolates on repetition. The banding pattern generated by each primer was primer and isolate dependent and varied from 3 to 12 at 37^0C annealing temperature. Primer OPB 6 produced maximum number of bands (12) while OPB 11 produced least number of bands (3) (Table 3) (Fig 1,2). A total of 47 amplicons were obtained with 6 primers with an average of 7.83 bands per primer. All the 47 bands were found to be polymorphic and the level of polymorphism was 100 per cent. All six primers, viz. OPB 1, OPB 2, OPB 5, OPB 6, OPB 7 and OPB 11 yielded polymorphic bands (Table 3).

Discriminatory power (D)

The discriminatory power of different primers calculated according to Hunter and Gaston (1988) has been presented in Table 3. Primer OPB 6 has discriminatory power (D) as 1. This indicates that this primer generated unique banding pattern for all the isolates and the isolates can be identified by a single primer. Such primers discriminating all the isolates have been reported by Monga *et al.* (2004). These may be added to identification of isolates prevailing in the area and could prove important in formulating disease control strategies. This also indicates higher level of polymorphism available in the genome.

Table 3 List of arbitery primers showing total and polymorphic amplicon generated for 10 *F. oxysporum* f. sp. *ciceri* isolates

Primer	Sequence [5' 3']	No. of bands	No. of Poly-morphic bands	Polymor-phism (%)	Discrimi-nation power [D]
OPB 1	GTTTCGCTCC	7	7	100	0.98
OPB 2	TGATCCCTGG	7	7	100	0.98
OPB 5	TGCGCCCTTC	8	8	100	0.91
OPB 6	TGCTCTGCCC	12	12	100	1.00
OPB 7	GGTGACGCAG	10	10	100	0.98
OPB 11	GTAGACCCGT	3	3	100	0.73
Total		47	47		
Mean		7.83	7.83	100	

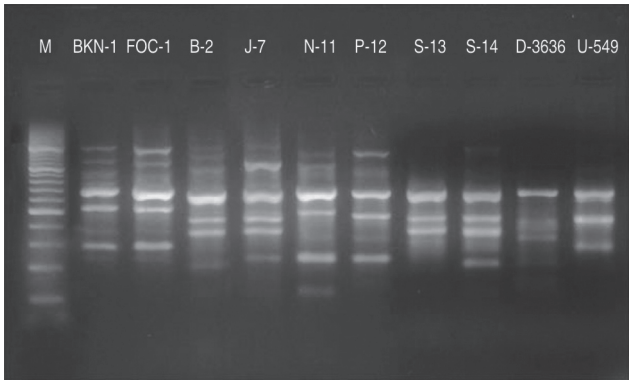

Fig 1 RAPD patterns generated by OPB-6 in ten isolates of *Fusarium oxysporum* f.sp. *ciceri.* M-Lambda uncut DNA marker, BKN-1-Bikaner, FOC-1-Churu, B-2-Bikaner, J-7-Jaipur, N-11-Narnaul, P-12-Ludhiana, S-13-Sikar, S-14-Suratgarh, D-3636-Delhi, U-549-Udaipur

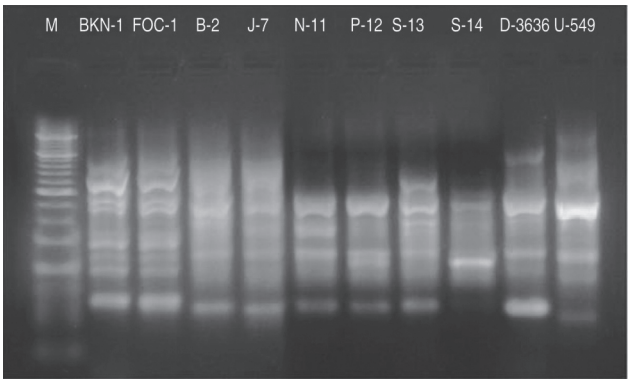

Fig 2 RAPD patterns generated by OPB-7 in ten isolates of *Fusarium oxysporum* f.sp. *ciceri.* M-Lambda uncut DNA marker, BKN-1-Bikaner, FOC-1-Churu, B-2-Bikaner, J-7-Jaipur, N-11-Narnaul, P-12-Ludhiana, S-13-Sikar, S-14-Suratgarh, D-3636-Delhi, U-549-Udaipur

Genetic relationship among the isolates and cluster analysis

Pair-wise genetic similarity estimates (Jaccard's coefficient) based on RAPD banding patterns were used for cluster analysis to present genetic relationships among various isolates in the form of Dendogram (Fig 3). The unweighted pair-group method with arithmetic average analysis (UPGMA) was used for cluster analysis. The Jaccard's pair wise similarity coefficient values for ten isolates of *F. oxysporum* f. sp. *ciceri* have been presented in the Table 4. The range of genetic similarity was found between 0.79 (BKN-1 and FOC-1) to 0.10 (N-11 and B-2). The average genetic similarity coefficient observed was 0.31 ±. The higher value of similarity coefficient close to

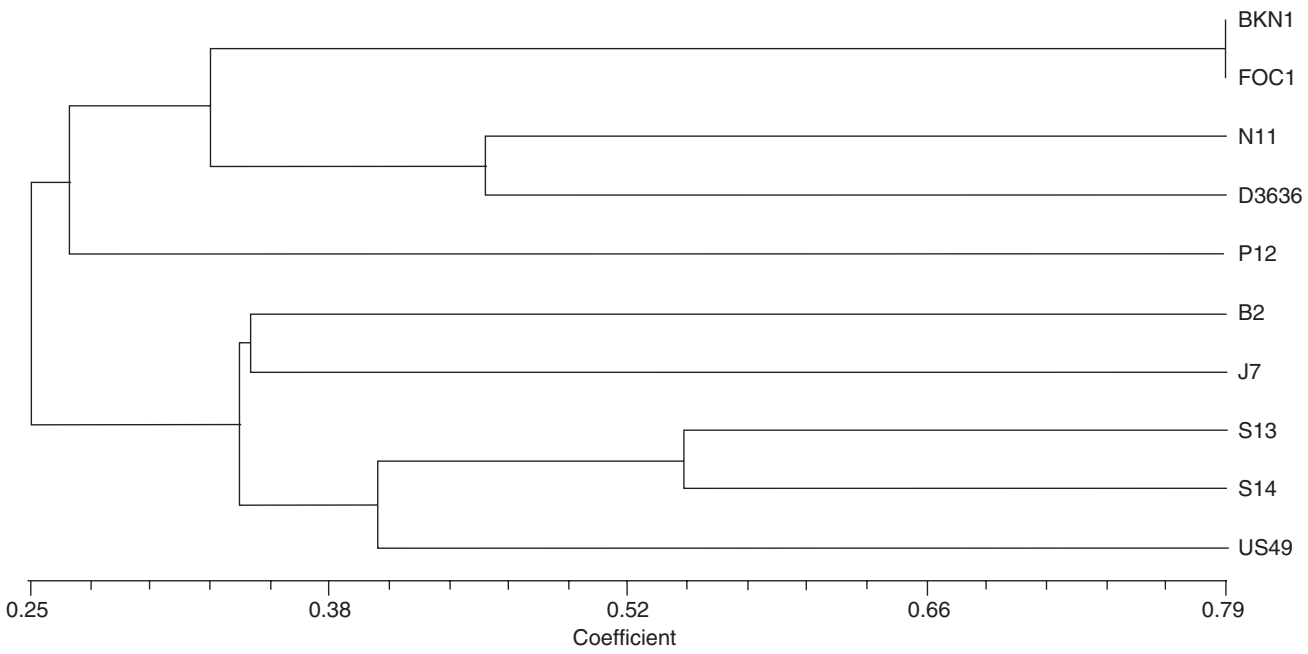

Fig 3 Dendogram generated by UPGMA analysis based on RAPD data showing relationship among ten isolates of *F. oxysporum* f. sp. *ciceri*

one was indicative of close resemblance between the different isolates of *F. oxysporum* f. sp.*ciceri*. Isolates S-13 - S-14, N-11- D-3636 and S-13 - U-549 had higher similarity coefficient, whereas, isolates N-11 - U-549 and B-2 - D-3636 had lower similarity coefficient.

The clustering analysis separated all the ten isolates in two major groups. Group A included Bikaner (BKN-1), Churu (FOC-1), Narnaul (N-11), Delhi (D-3636) and Ludhiana (P-12) isolates, whereas, Group B consisted of isolates from Bikaner (B-2), Jaipur (J-7), Sikar (S-13), Suratgarh (S-14) and Udaipur (U-549). Within group diversity for group A was 64 per cent and for group B was 62 per cent. Both the groups were separated from each other at higher level of diversity, i.e. 75 per cent (Fig 3).

RAPD analysis of *Fusarium oxysporum* in different hosts was studied and correlation between pathogenicity and polymorphism was established (Jimenez-Gasco *et al*. 2002, Jimenez- Gasco *et al*. 2004). Sivaramakrishnan *et al*. (2002) also recorded high level of DNA polymorphism using molecular markers. They also suggested the rapid evolutions of new recombinants of the pathogen in chickpea growing regions. Singh *et al.* (2006) studied the genomic variation existing in 30 isolates of *F. oxysporum* f. sp. *ciceri* employing PCR amplifications with a set of 40 RAPD primers and two IGS primers. Genetic similarity between the isolates indicated that very little genetic variability existed among the FOC isolates collected from north India.

However, there may not be a clear correlation of genetic molecular markers (RAPD) with the virulence, but highly virulent isolates from Bikaner (BKN-1), and Churu (FOC-1) clustered as most related isolates. The present study generated useful information in terms of pathogenic and molecular variability in *F. oxysporum* f. sp. *ciceri* isolates existing in chickpea growing regions of Rajasthan and adjoining areas. It is suggested that after further verification with more number of isolates and primers it would be possible to develop a rapid and reliable method of race identification *F. oxysporum* f. sp. *ciceri* through use of RAPD markers and other molecular methods.

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