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ASSESSMENT OF GENETIC DIVERSITY OF *FUSARIUM OXYSPORUM* F. SP. *CICERI* USING SSR MARKERS

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Abstract- *Fusarium* wilt of chickpea caused by *Fusarium oxysporum* f.sp. *ciceri* (Foc) is a major constraint to chickpea production world-wide. A total seven different isolates of *F. oxysporum* f. sp. *Ciceri* (FOC) were obtained from wilt-infected chickpea plants from different agro climatic regions of central India. The isolates were identified according to the identification key of F. *oxysporum*. All the isolates were single spored and was stored in tubes containing PDA at 4 °C. All isolates were characterized using four Simple Sequence Repeat Marker (SSR). Cluster analysis revealed that races of FO under study fell in to two groups, major group A and minor group B.Major Group A divided into two sub groups, first subgroup containing two isolates namely I-19 and I-4. Second sub group had also two isolates I-28 and I-1 respectively. Group B divided into two sub group, first sub group containing two isolates I-20 and I-13. Second sub group having only one isolate, i.e. I-80. These diverged from other accessions were placed at end of the cluster.

INTRODUCTION

Fusarium wilt of chickpea caused by *Fusarium oxysporum* f. sp. *Ciceri* (Foc) is a major constraint to chickpea production world-wide. The disease is widespread in the chickpea growing areas of the world and is reported from at least 33 countries, causing 10 - 15% annual losses. It is more important between the latitudes 30°N and 30°S where the chickpea-growing season is dry and warm (Sharma *et al.,* 2009). India is the largest producer of chickpea and accounts for 68.47% of the total area and 67.02% of total production globally. Chickpea represents 35.16% of total pulse area and 50.34% of total pulse production in India (AGRISTAT, 2011).

Chickpea wilt is gradually prevailing in India as a result of the increased drought condition since last few years. Therefore, the issue needs great attention to enhance the yield (Lines *et al*., 2008). The disease is soil or seed born (Jamil *et al*., 2010), which is difficult to control by the use of chemicals or fungicides. The pathogen enters host through roots and causes systemic infection by progressive vascular damage, wilting and finally death of the plant. The cultivation of resistant varieties is one of the most durable and economical practice for the management of FW. However, performance of varieties differs from place to place owing to existence of physiological races among the Foc isolates. Eight races of Foc (0, 1A, 1B/C, 2, 3, 4, 5 and 6) have been reported worldwide Haware and Nene (1982); Races 1A (also known as race 1), 2, 3 and 4have been reported from India, whereas races 0, 1B/C, 5and 6 were found mainly in the Mediterranean region andin the United States (California). The identification of pathogenic races of Focis important for disease resistance breeding and for the efficient use of available *Fusarium* wilt resistant cultivars in chickpea. Monitoring pathogenic variability of fungus based on DNA markers will greatly help in understanding pathogen diversity and their pathogenicity. The recent years, several types of molecular marker systems such as RAPD (random amplified polymorphic DNA) Honnareddy and Dubey (2006), RFLP (restriction fragment length polymorphism) SSR (simple sequence repeat) Dubey and Singh, (2008) and amplified fragment length polymorphism (AFLP) have been increasingly used to study the variability in pathogenic populations of Foc. The present study therefore was undertaken to assess the extent of genetic diversity in Focisolates

collected from the different chickpea growing regions of India using SSR markers.

MATERIALS AND METHODS

Fungal Isolates

A total seven different isolates of *F. oxysporum* f. sp. *Ciceri* (FOC) were obtained from wilt-infected chickpea plants from different agroclimatic regions of central India. Isolations were made from the wilted plants on potato dextrose agar (PDA) medium. The isolates were identified according to the identification key of *F. oxysporum* (Nelson *et al*., 1983). All the isolates were single spored and was stored in tubes containing PDA at 4 °C.

Genomic DNA extraction

Fresh fungal mycelium (~200 mg) was transferred to a sterilized 1.5 ml eppendrofmicro centrifuge tube with the help of sterilized scalpel and 800 µL of extraction buffer (0.1 M Tris-HCl pH 8, 10 mM EDTA pH 8, 2.5 M NaCl, 3.5% CTAB, 150 µL of 20 mg/mL proteinase K) was added with mixed sterilized 0.5-1 mm glass beads. The mixture was vortexes at high speed on a homogenizer (Spinix, Tarsons, India) for 5 min. The samples were placed in a water bath at 65 ºC for 30 min. The samples were then centrifuged at 10,000 rpm for 10 min at room temperature. Supernatant was collected and equal volume of phenol-chloroform-isoamylalcohol (25:24:1) was mixed. The samples were again centrifuged at 10,000 rpm for 10 min at room temperature. Supernatant was again collected and equal volume of chloroform isoamylalcohol (24:1) was mixed. Samples were again centrifuged under the conditions mentioned above. Supernatant was collected and equal volume of ice-cold isopropanol was added. Samples were incubated at "20 ºC for 1-2 h. The samples were centrifuged for 15 min at 13,000 rpm to pellet the DNA. Supernatant was decanted and DNA pellet was washed with 800 µl of 70% ethanol. DNA pellet was air-dried and dissolved in 200 µl TE buffer (10 mMTris-HCl pH 8, 1 mM EDTA). 5 µl RNase A (20 mg/ml) was added to DNA samples, mixed and incubated at 37 ºC for 1 h. DNA was recovered and air-dried as described above. DNA was reconstituted in TE buffer for further use for PCR amplification.

Primer screening

Fifteen SSR primers were initially screened using one individual clone to determinate the suitability of each primer for the study. After preliminary testing on a few samples, eight primers were selected for further analysis based on their ability to detect distinct clearly resolved amplified products within the population. To ensure reproducibility the primers generating no, weak, or complex patterns were discarded.

PCR amplification

PCR amplification was performed in 25 µL reaction mixture containing: 5 U Taq DNA polymerase (Promega) along with 10× manufacturer's buffer (Promega), 200 µM each deoxynucleoside triphosphate (dNTPs), 20 pM SSR primers and 50 ng genomic DNA. The reaction conditions were as follows: initial denaturation step at 94 °C for 4 min, 35 amplification cycle of denaturation at 94°C for 1 min, annealing at 55 °C for 30 min and primer extension at 72 °C for 1 min; followed by a final extension at 72 °C for 10 min. PCR amplifications were carried out using a Thermo-Hybaid PCR thermal cycler (Thermo Fisher Scientific, USA). Aliquots of the PCR products (5 µl) were analyzed in 1% (w/v) agarose gels (Sigma, USA) by horizontal gel electrophoresis. DNAs were visualized by UV excitation after staining with Ethidium bromide (0.5 mg/l).

Data Analysis

SSR bands were scored using binary matrix '1' for presence and '0' for absence. Data was analyzed using NT-SYS-pc version 2.1. Pair-wise genetic similarities between accessions were estimated using the similarity coefficient. A dendrogram was constructed based on similarity coefficient values by adopting the sequential hierarchical agglomerative non-overlapping (SHAN) clustering technique of unweighted pair group method of arithmetic mean (UPGMA) which is a variant of the average linkage clustering algorithm.

RESULTS AND DISSCUSSION

SSR analysis: SSR analysis is simple technique and can be performed even in a moderately equipped laboratory. Initially 15 primers were screened and a total of 8 primers were selected on the basis of sharp and clear banding pattern for final SSR PCR analysis. The PCR reaction was carried out using a single SSR primer at a time. The sequences of these primers are presented in Table 1.

15 SSR primers amplified 24 loci. The size of

Table 1. The location and race distribution of *Fusarium oxysporum* f. sp. *ciceri* isolates used in this study

Isolates	Race/Variants	Locatation/State representing Race/Variants
I- 19	Race 2	Ghootna, Jabalpur, MP
I-28	Race 2	Sehore, MP
I-4	Race 3	Dongargaon, Narsingpur, MP
I-20	Race 4	Indore, MP
I-13	Race 4	Jabalpur, MP
I-80	Race 5	Dharampur, CG
I-1	New Variants	Kawardha, CG

amplified markers range from 150-500bp. Maximum number of bands, i.e. 4 were scored by primer 10 while minimum number of bands i.e. 1 were scored by Primer 9. Out of 24 bands amplified by SSR markers, 18 (75%) were found to be polymorphic and remaining 6 were monomorphic. Average

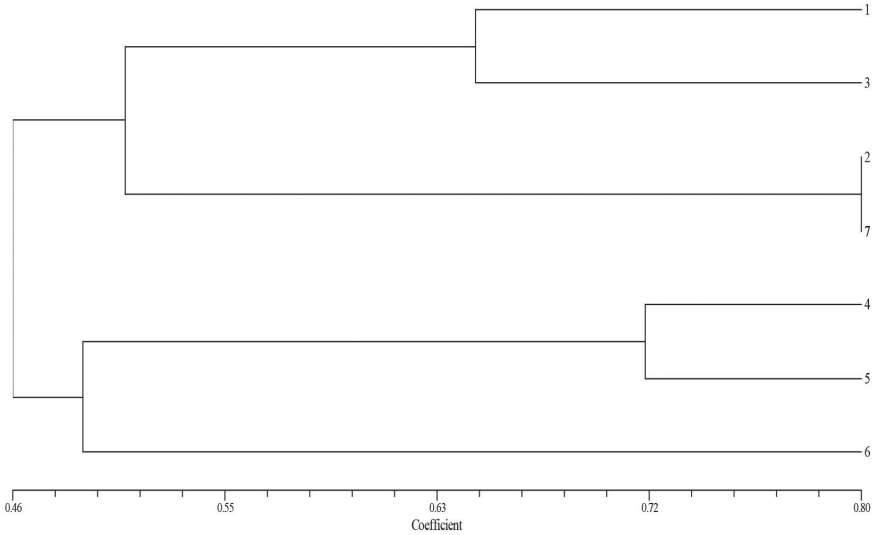

Fig. 1. Dendrogram on the basis of SSR similarity matrix data by unweighted pair group with average (UPGMA) cluster analysis.

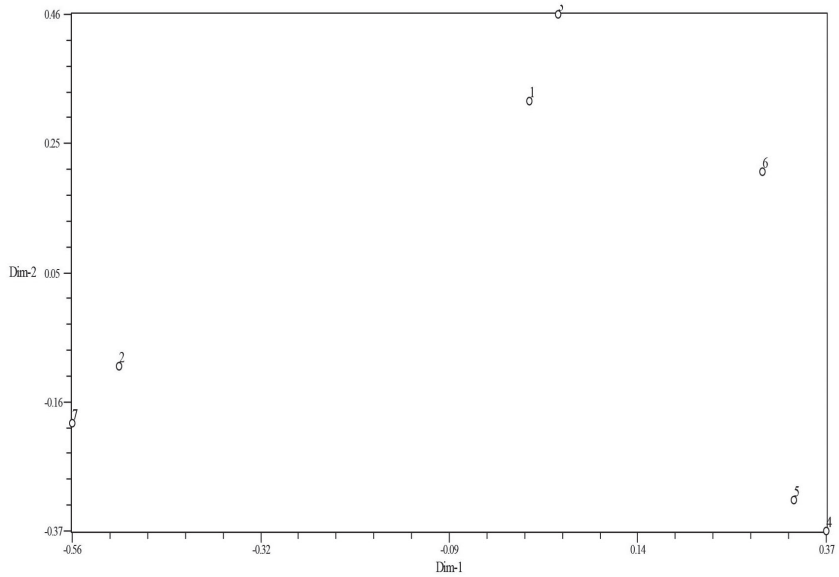

Fig. 2. Two dimensional plot of seven isolates of *F. oxysporum* PCA (principal component analysis) based on SSR UPGMA

numbers of bands per primer were 3 while average numbers of polymorphic bands per primer were 2.25. Out of 8 SSR primers used in this study, primers 1 were amplified monomorphic banding pattern while, primers 7 were amplified polymorphic banding pattern.

During the present study 5 (20.8%) specific bands were amplified by 4 primers. Among these primers, primer 9 and primer 10 amplified one specific band in isolates I-80 and I-4 respectively. Primer 9 amplified a specific allele of about 250bp in isolate I-80, primer 10 amplified specific alleles in isolate I-4 of about 170bp, primer 1 amplified a specific marker in isolates I-19 and I-1 of about 270bp, Most of the markers amplified specific alleles of different molecular sizes in isolates I-28 and race I-80. These markers can be used in race discrimination.

Table 2. SSR primers used in the fingerprinting of *Fusarium* isolates.

Primer	Sequence 5′-3′	Melting temp ^{0}C
SSR1 F	TGCTGTGTATGGATGGATGG	54.7
SSR1 R	CATGGTCGATAGCTTGTCTCAG	55.1
SSR2 F	ACTTGGAGGAAATGGGCTTC	55.1
SSR2 R	GGATGGCGTTTAATAAATCTGG	51.9
SSR3 F	TGGCTGGGATACTGTGTAATTG	54.7
SSR3R	TTAGCTTCAGAGCCCTTTGG	54.8
SSR5F	GTGGACGAACACCTGCATC	56.3
SSR5R	AGATCCTCCACCTCCACCTC	58.0
SSR6F	GGAGGATGAGCTCGATGAAG	54.8
SSR6R	CTAAGCCTGCTACACCCTCG	57.3
SSR9F	GGTAGGAAATGACGAAGCTGAC	55.5
SSR9R	TGAGCACTCTAGCACTCCAAAC	56.9
SSR10F	GACAAGCAAGCGATAGGAAA	52.9
SSR10R	CTTGATAGCACGGACCGACG	57.9
SSR16F	AAGCGCCAACAGAGATGACGA	59.1
SSR16R	GACTGCCGAAACACCGAAA	55.8
SSR19F	ACGGTGAGAATGCGAGGTAG	56.7
SSR19R	AAACCACAGAAGCAGAGAGGAG	56.7
SSR20F	AGTATCCTCCACACCATGTTCC	56.4
SSR20R	TCCTCAACTGTCCTATCCAGGT	57.0
SSR22F	GTCCATCGAAAATCTCAAGACC	53.5
SSR22R	TGGATCACCTCTCCCATCTC	55.7
SSR25F	AAGATAGCATGTTCCACGGC	55.4
SSR25R	TGTTTCATCGTGTTTGTGTCTG	53.8

Cluster analysis of SSR markers

Based on electrophoretic banding pattern of SSR primers, pair wise genetic similarity among different races for genetic diversity was estimated and dendogram was generated using Unweighted Pair Group method with "UPGMA" sub programme of "NTSYS"-pc "(Fig. 1). Cluster analysis revealed that races of FO under study fell in to two groups, major group A and minor group B.

Major group A divided into two sub groups, first subgroup containing two isolates namely I-19 and I-4. Second sub group had also two isolates I-28 and I-1 respectively. Group B divided into two sub group, first sub group containing two isolates I-20 and I-13. Second sub group having only one isolate i.e. I-80. These diverged from other accessions were placed at end of the cluster.

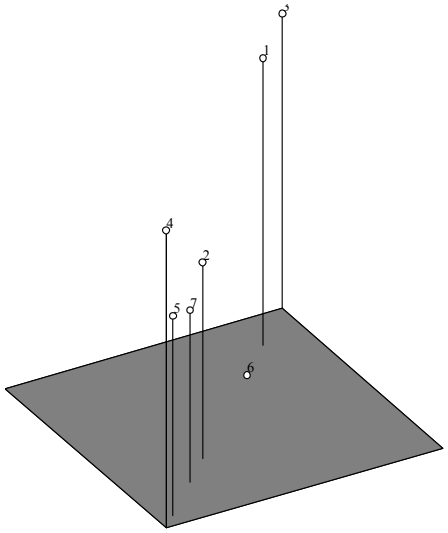

Fig. 3. Three dimensional plot of seven isolates*F. oxysporum* PCA (principal component analysis) based on SSR UPGMA

Table 3. Jaccard's similarity co-efficient among *F. oxysporum* races using SSR data

	1	2	3	4	5	6	7
1	1.000						
2	0.600	1.000					
3	0.647	0.500	1.000				
4	0.5	0.4370	0.500	1.000			
5	0.562	0.500	0.470	0.714	1.000		
6	0.473	0.411	0.473	0.421	0.562	1.000	
7	0.466	0.800	0.466	0.400	0.461	0.375	1.000

Table 4. Total number of alleles, monomorphic alleles, polymorphic alleles, expected heterozygosity and pic value among *F. oxysporum* using SSR markers

Sr	Primer	Total alleles	Monomorphic alleles	Polymorphic alleles	He	Pic
1	Primer 1	3	0	3	0.5714	0.5015
2	Primer2	3	1	2	0.5124	0.4442
3	Primer6	3	0	3	0.6328	0.5556
4	Primer9	1	1	0	0.0000	0.0000
5	Primer10	4	1	3	0.6272	0.5758
6	Primer 16	3	1	2	0.5833	0.5295
7	Primer 19	3	1	2	0.5124	0.4442
8	Primer 22	3	0	3	0.6328	0.5556

He= Expected Heterozygosity, Pic= polymorphic information content

Jaccard's similarity co-efficient

The similarity co-efficient of the seven isolates offocbased on SSR markers ranged from 0.7 to 0.3 among all the genotypes. Accessions 4 and 5 showed the highest similarity index (0.7), while the lowest (0.3) between 6 and 7 (Table 1).

Principle component analysis (PCA) and three dimensional scaling

Principle components analysis of seven isolates of foc races was carried out according to the similarity coefficient. Since molecular markers reveal natural site of variation at the DNA level, this implies that most of the variation measured in morphological and biochemical traits were due to the genetic effects. It has been also suggested that if two or three first component account for changes higher than 80%, the PCA will have high efficiency. In this analysis isolates I-19 and isolates I-4 were placed closely isolates I-28 and I-1 were placed together and also isolates I-13 and I-20 were placed closely and isolate I-80 was diverse from all other races.

Three dimensions scaling of seven isolates of focraces also showed similarity according to principle components analysis. In this accessions divided in to two groups, first group contain only three isolates namely I-9, I-4 and I-80 were divers from other accessions. Group B contained four isolates namely I-28, I-20, I-13 and I-1. Similarity according to principle components analysis race 5 and 4 were placed closely and race 6 was diverse from other races.

The importance of understanding genetic diversity in any fungal species collection is critical for their effective management. Although tremendous progress has been made in terms of availability of molecular markers to study the genetic diversity in Foc availability of sound, reliable and cost-effective marker platform is still lacking.

In conclusion, the present study generated significant information in terms of pathogenic and genetic variability of FOC. The study also highlights the fact that genetic analysis using SSR marker is useful tool and have high discriminatory power for studying the diversity in *F. oxysporum* f.sp. *ciceri*.

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