JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

Lal et al. 2013 : 2 (6) 2392-2397

GENETIC VARIABILITY ASSESSMENT OF *FUSARIUM* WILT PATHOGEN RACES AFFECTING CHICKPEA USING MOLECULAR MARKERS

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ARTICLE INFO

Received 20. 10. 2012
Revised 4. 4. 2013
Accepted 8. 4. 2013
Published 1. 6. 2013

Regular article

ABSTRACT

Genetic diversity in Chickpea wilt pathogen has been characterized using 14 isolates of Fusarium oxysporum f. sp. ciceri (foc) collected from major pulse growing regions of India. Out of 247 bands produced by 24 Random Amplified Polymorphic DNA (RAPD) primers in Foc isolates, 210 (85%) were polymorphic. A maximum of 14 amplicons were generated by primer OPF 05 whereas minimum 7 amplicons were generated by primer K7. A total of 24 alleles were produced by twelve simple sequence repeat (SSR) primers with an average of two alleles per marker in foc isolates. The maximum number of 4 alleles was obtained with primer SSR 12. SSR amplicon size ranged from 100 to 400 bp. The Unweighted Pair Group Method with Arithmetic Mean (UPGMA) cluster analysis based on RAPD and SSR profiles grouped the fourteen foc isolates into four major clusters. The universal Inter Transcribed Spacer (ITS) primer pair amplified 630 bp bands in all fourteen foc isolates while significant length polymorphism was obtained only when analysed by restriction digestion with EcoRI and MspI enzymes. The cluster analysis of ITS-RFLP grouped all 14 Foc isolates into three major clusters. The cluster analysis using RAPD, SSR and ITS-RFLP markers show the grouping of Fusarium isolates strictly according to their cultural characteristics and degree of pathogenicity and not the geographical origin. This information will be helpful for pathologists and plant breeders to design effective resistance breeding programs in chickpea taking into account the diversity in wilt pathogen.

Keywords: Chickpea, *Fusarium*, genetic diversity, ITS, polymorphism**,** RAPD**,** rDNA, SSR

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is among the world's most important pulse crops which suffer heavy yield losses up to 90% due to vascular wilt caused by *Fusarium*. The disease is prevalent in almost all pulses growing areas of the world, including the Indian subcontinent, Iran, Peru, Syria, Ethiopia, Mexico, Spain, Tunisia, Turkey, and the United States (**Nene *et al.*, 1989**). Although much progress has been made in developing chickpea lines with resistance to biotic constraints and tolerance to abiotic stresses, yield loss in this crop is very high due to the high incidence of diseases and insect-pests.

Fusarium wilt of chickpea is caused by *F. oxysporum* f. sp. *ciceri*. Pathogenic isolates of *F. oxysporum* often display a high degree of host specificity and may be subdivided into *formae speciales* based on the plant species affected and into races based on the host cultivars attacked (**Armstrong and Armstrong, 1968; Alves-Santos *et al.*, 2002**). Eight races of the pathogen have been reported, of which six (1A, 2, 3, 4, 5, and 6) cause wilting syndrome and are economically more important when compared to races 0 and 1B/C that cause yellowing syndrome (**Haware and Nene, 1982**). The wilt pathogen is both soil and seed borne and difficult to eradicate as fungal chlamydospores survive in soil upto six years even in the absence of host plant (**Haware *et al.*, 1996**). It's near ubiquity in soil worldwide and its ecological activities indicate a much more diverse role in nature. Biochemistry and physiology of the *Fusarium*-plant interaction have been characterized extensively (**Heitefuss and Williams, 1976**), but definitive enquiry into identification of individual molecules essential for *Fusarium* pathogenesis to plants did not begin until molecular genetics technology became available for filamentous fungi (**Bennett and Lasure, 1991; Fincham, 1989; Timberlake and Marshall, 1989**). To develop effective strategy for management of wilt diseases, understanding of the molecular basis of pathogenesis and resistance mechanism is essentially required.

For integrated management of wilt, identification of isolates/races and developing strategy to incorporate resistance is very important. Infallible identification of races is critical to any resistance-breeding program as exact picture about the existence of number of physiological races in *Fusarium oxysporum* f. sp. *ciceri* is still not clear. Identification of any isolate/race by molecular tools like DNA fingerprinting is considered to be the most reliable method. Furthermore identification and classification of the race specific donors will help in pyramiding of resistance genes for developing varieties resistant against multiple races of pathogen.

A comparison at DNA sequences level provides accurate classification of fungal species to elucidate the evolutionary and ecological relationships among diverse species. In recent years, numerous DNA based methods have been increasingly used to study variability in pathogenic *Fusarium* populations (**Sivaramakrishnan *et al.*, 2002**). DNA fingerprinting has been successfully used for *Fusarium* in characterization of individual isolates and grouping them into standard racial classes. For breeding of resistant crop varieties, knowledge about the pathogen races in that particular crop area is very important especially to pyramid several resistance genes in an elite genotype. So far, eight races of the pathogen (race 0, 1A, 1B/C, 2, 3, 4, 5 and 6) have been identified by reaction on a set of differential chickpea cultivars (**Jimenez-Diaz *et al.*, 1989**). Races 1 to 4 have been reported from India, whereas 0, 1B/C, 5 and 6 are found in the Mediterranean region and USA. Random Amplified Polymorphic DNA (RAPD) offers several advantages and reduces the time needed for race identification in disease plants, provides genetic information on isolates, allowing for their fingerprinting. **Gherbawy *et al.*, 2002** used RAPD technique for identifying *Fusarium subglutinans*, *F. proliferatum* and *F. verticillioides* strains isolated from maize in Austria. **Pasquali *et al.*, 2003** characterized isolates of *Fusarium oxysporum* pathogenic on *Argyranthemum frutescens* L. using RAPD technique. Genetic similarity between isolates of *F. oxysporum* f. sp. *ciceri* was studied using 40 RAPD and 2 Intergenic spacer region (IGS) primers and results indicate that there was little genetic variability among the isolates collected from the different locations in India (**Singh *et al.*, 2006**). **Cramer *et al.*, 2003** reported specific RAPD banding which distinguish among races *F. oxysporum* f. sp. *phaseoli* and *F. oxysporum* f. sp. *betae*. Identification of pathogenic races 0, 1B/C, 5 and 6 of *Fusarium oxysporum* f. sp. *ciceri* has been reported using 40 RAPD primers (**Jimenez-Gasco *et al.*, 2004**). Similarly, simple sequence repeats (SSRs) provide a powerful tool for taxonomic and population genetic studies (**Britz *et al.*, 2002**). Simple sequence repeats have been used as genetic markers in numerous DNA-fingerprinting and PCR fingerprinting experiments for strain typing of a variety of filamentous fungi and yeasts without prior knowledge of their abundance and distribution in the investigated fungal genomes (**Meyer *et al.*, 2003**). SSR markers distinguished the four races of *Fusarium oxysporum ciceri* causing varied levels of wilting with differential host cultivars (**Barve *et***

al., 2001). **Bogale *et al.*, 2005** have shown that the polymorphism revealed with 8 SSR markers was sufficient for study of genetic diversity in *Fusarium oxysporum* complex.

The cluster of ribosomal DNA consists of tandem repeat of three coding (18S, 5.8S and the 28S), and two noncoding Inter Transcribed Spacer (ITS) and Intergenic spacer (IGS). Designing primers from the rDNA region has far superior reliability compared to the use of random non-defined probes or primers. These markers occur in multiple copies with up to 200 copies per haploid genome arranged in tandem repeats and are most effective in detecting polymorphism (**Yao *et al.*, 1992**). ITS regions have been used successfully to generate specific primers capable of differentiating closely related fungal species (**Bryan *et al.*, 1996**). Taxon-selective ITS amplification has already been used for detection of the fungal pathogens such as *Fusarium* and *Verticillium* spp. (**Nazar *et al.*, 1991**). **O'Donnell, 1992** found a surprising level of divergence for ITS sequences within the species of *F. sambucinum*. **Chakrabarti *et al.*, 2000** have shown that digestion of amplified IGS region with EcoR1 produced similar bands for both race1 and race 4 of *F. oxysporum* f. sp. *ciceri* but individual and distinctive banding patterns were observed for race 2 and race 3. Genetic diversity studies could reveal the adaptive potential of pathogenic populations (**Huertas-González *et al.*, 1999**) and sometimes RAPD patterns could reflect the variability of *formae speciales* (**Clark *et al.*, 1998**). The aim of this study was to analyze the diversity of an isolate collection of *F. oxysporum* recovered from diseased plants. The diversity of isolates was assessed by genetic analysis using RAPD, SSR markers and the restriction fragment lemgth polymorphism (RFLP) analysis of Inter transcribed sequence (ITS) of the nuclear ribosomal DNA.

MATERIAL AND METHODS

Fusarium isolates

The *Fusarium oxysporum* f. sp. *ciceri* (*Foc*) isolates collected from wilt affected fields of chickpea from seven major pulse growing states of India were used as experimental material in the present study (Table 1). The isolates were selected based on the variability in their cultural, morphological and pathogenic characters. For facilitating easy identification of the isolates, the original isolate numbers have been used throughout the text.

Molecular markers

A total of 40 RAPD primers and 12 SSR markers were used in the present study. The RAPD primers from Operon series and SSR primers based on reported primer sequences were got synthesized from Operon Technologies, USA. Tables 2 and 3 show the Tm, source and sequence details of RAPD and SSR primers. The nuclear rDNA ITS region, including ITS2 and the 5.8S ribosomal gene, was amplified using primers ITS1 (5'TCCGTAGGTGAACCTGCGG3') and ITS4 (5'TCCTCCGCTTTATTGATATG3').

Table 1 *Fusarium oxysporum* f. sp. *ciceri* isolates used in the present study.

Isolates	Place of collection	Characteristics	Pathogenicity
Foc 17	Jhansi (Uttar Pradesh)	Long size/medium growth	Moderate
Foc 42	Mau (Uttar Pradesh)	Small size/slow growth	Moderate
Foc 92	Kanpur (Uttar Pradesh)	Medium size/medium growth	High
Foc 66	Lam Guntur (Andhra Pradesh)	Medium size/medium growth	High
Foc 65	Anantpur (Andhra Pradesh)	Medium size/medium growth	High
Foc 80	Bangalore (Karnataka)	Small size/slow growth	High
Foc 73	Gulberga (Karnataka)	Large size/slow growth	High
Foc 6	Narsinghpur (Madhya Pradesh)	Large size/fast growth	Moderate
Foc 56	Raisen (Madhya Pradesh)	Medium size/fast growth	High
Foc 91	Bharuch (Gujarat)	Medium size/slow growth	High
Foc 60	Porbandar (Gujarat)	Large size/fast growth	High
Foc 58	Bilaspur (Chhattisgarh)	Large size/fast growth	High
Foc 79	Raipur (Chhattisgarh)	Medium size/fast growth	High
Foc 88	Amravati (Maharastra)	Medium size/ medium growth	Moderate

Table 2 RAPD primers used in the fingerprinting of *Fusarium oxysporum* f. sp. *ciceri* isolates.

Primer	Sequence 5'→3'	Primer	Sequence 5'→3'
K 1	5' TGCGTGCTTG 3'	P 19	5' GCGGCATTGT 3'
K 2	5'ACTTCGCCAC 3'	P21	5' CCAGACAAGC 3'
K 3	5' GGCTCATGTG 3'	OPD 11	5'AGCGCCATTG 3'
K 4	5' CAAACGTGGG 3'	OPD 13	5'GGGGTGACGA 3'
K 5	5'CGAGGTCGACG3'	OPD 16	5' AGGGCGTAAG 3'
K 6	5'CACCGCCCCAA 3'	OPA 3	5' AGTCAGCCAC 3'
K 7	5'GTCCTCAGTCCC 3'	OPA 4	5' AATCGGGCTG 3'
P1	5' CGTTGGATGC 3'	OPA 7	5' GAAACGGGTG 3'
P2	5' TACGGCTGGC 3'	OPA 11	5' CAATCGCCGT 3'
P3	5' GCGGCATTGT 3'	OPA 12	5' TCGGCGATAG 3'
P 8	5' CAGGCCCTTC 3'	OPF 01	5'ACGGATCCTG3'
P 17	5' TACGGCTGGC 3'	OPF 05	5'CCGAATTCCC 3'

Collection and maintenance of the fungal isolates

The pathogens were isolated from fourth-node stem sections taken from wilted chickpea plants collected from different agro-climatic regions of India according to the procedure described by **Tullu *et al.*, 1998**. The re-isolated pathogens were colonized on filter paper, dried in the transfer hood, and aseptically cut into small pieces. The colonized filter paper pieces were placed in potato-dextrose broth and incubated to produce liquid cultures of the pathogen. The liquid cultures were filtered through cheese cloth to remove mycelia. The spore suspension was pelleted by centrifugation. After discarding the supernatant, the conidia were washed with sterile water to adjust the spore suspension to 1 x 10^6 spores ml^{-1} with a haemocytometer. These isolates were further characterized at the laboratories of Department of Life Sciences, and Department of Biochemistry, I.B.S.B.T, C.S.J.M. University, Kanpur. Single spore culture of fungus was obtained by serial dilution method.

Isolation and purification of genomic DNA of *Fusarium* isolates

Single spore culture of each isolates was grown on Potato Dextrose Agar (PDA) medium at 28°C and stored at 4°C until used. Mycelia for genomic DNA extraction were grown in 250 ml of PDB at 28°C for 5 days. After vacuum filtration, the mycelia were lyophilized and stored at -20°C. Genomic DNA for PCR was extracted using a modified method of **Kim *et al.*, 1992**. Prepared mycelium (approximately 0.5 g) was suspended in extraction buffer (4 M NaCl, 1 M Tris-HCl (pH 8.0), 0.5 M EDTA, 2% PVP) and extracted with phenol: chloroform: isoamyl alcohol (25:24:1) and chloroform/ isoamyl alcohol [24:1]. DNA was then precipitated by adding 2.5 volumes of absolute ethanol and pelleted by centrifugation for 10 min at 10,000 rpm (Sorvall SS 34 rotor). The pellet was washed with 70 % ethanol, air dried and resuspended in 1 mM TE buffer (10 mM Tris-HCl, 1 mM EDTA (pH 8.0)). DNA concentration and purity were measured using a spectrophotometer (BioRad SmartSpec plus) at 260 nm and 280 nm. RNA was degraded with the treatment of RNase A (50 µg/ml) for 30 min at 37°C. Proteins were removed by phenol-chloroform extraction. Equal volume of phenol: chloroform: iso-amyl alcohol (25:24:1) was added, and mixed and the tubes were spun at 10,000 rpm for 5 minutes at room temperature. Aqueous phase extracted twice with chloroform: iso-amyl alcohol (24:1) was collected after centrifugation. DNA was precipitated by adding two volumes of chilled absolute alcohol, pelleted and dissolved in $T_{10}E_1$ buffer. Genomic DNA samples were purified and quantified to 25 ng /µl to be used as template.

Table 3 SSR primers used in the fingerprinting of *Fusarium oxysporum* f. sp. *ciceri* isolates.

Primer	Sequence 5'→3'	Tm (°C)	References
SSR 1	**F:** TGCTGTGTATGGATGGATGG **R:** CATGGTCGATAGCT	57	Bogale *et al.,* 2005
SSR 2	**F:** ACTTGGAGAAATGGGCTTC **R:** GGATGGAGTTTAATAAATCTGG	54	Bogale *et al.,* 2005
SSR 3	**F:** TGGCTGGGATACTGTGTAATTG **R:** TTAGCTTCAGCCCTTTGG	51	Bogale *et al.,* 2005
SSR 4	**F:** TATCGAGTCCGGCTTCCAGAAC **R:** TTGCAATTACCTCCGATCCAC	48	Bogale *et al.,* 2005
SSR 5	**F:** GTGGACGAACACCTGCATC **R:** AGATCCTCCACCTCCACCTC	68	Bogale *et al.,* 2005
SSR 6	**F:** GGAGGATGAGCTCGATGAAG **R:** CTAAGCCTGCTACACCCTCG	68	Bogale *et al.,* 2005
SSR 7	**F:** CGTCTCTGAACCACCTTCATC **R:** TTCCTCCGTCCATCCTGAC	57	Bogale *et al.,* 2005
SSR 8	**F:** ACTGATTCACCGATCCTTGG **R:** GCTGGCCTGACTTGTTATTCG	57	Bogale *et al.,* 2005
SSR 9	**F:** GGTAGGAAATGACGAAGCTGAC **R:** TGAGCACTCTAGCACTCCAAAC	57	Bogale *et al.,* 2005
SSR 10	**F:** CGAGCTAATGGTGGCAGGAT **R:** AACAACAAAACGGCTCATCG	50	Giraud *et al.,* 2002
SSR 11	**F:** TATTTCGTGCAAGGACTTGG **R:** CTTGGTCCCTGGATATGGA	51	Giraud *et al.,* 2002
SSR 12	**F:** AAGCGCCAACAGAGATGACGA **R:** GACTGCCGAAACACCGAAA	55	Giraud *et al.,* 2002

DNA fingerprinting using RAPD and SSR markers

Polymerase chain reaction was performed in a 25 µl volume containing, *Taq* polymerase assay buffer (10 mM Tris-HCl pH 8.0, 2.5 mM $MgCl_2$), 0.2 mM of each dNTP, 0.6 units of *Taq* polymerase (Bangalore Genei, Bengaluru, India), 20 pmoles of primer and 25 ng of DNA. The PCR regime comprised of an initial denaturation at 94°C for 3 mins followed by 40 cycles of denaturation at 94°C for 1 min, annealing of primers at suitable temperature for 1 min, extension at 72°C for 2 mins. Final extension was given at 72°C for 7 mins and reaction was held at 4°C. Amplification was performed using Biometra Thermal Cycler gradient (USA). Amplified products were resolved on 1.5 and 2% agarose gel at 45 V using 1X TBE buffer. PCR amplification with each primer was repeated twice before scoring for presence or absence of bands.

DNA fingerprinting with ITS-RFLP markers

The nuclear rDNA ITS region, including ITS2 and the 5.8S ribosomal gene, was amplified using primers ITS1 (5'TCCGTAGGTGAACCTGCGG3') and ITS4 (5'TCCTCCGCTTATTGATATG3') (**White *et al.,* 1990**). The amplification was performed in 50 µl reaction volume with 0.1 mM of each dNTP and 0.5 µM of both forward and reverse primer. Biometra thermal cycler was programmed for initial denaturation at 94°C for 4 min, and 35 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, the amplification was completed with a final extension at 72°C for 5 min. Electrophoresis and visualization of amplified bands was done as described above. The restriction enzyme digestion analyses were performed using 15 µl of the amplified PCR product. The enzymes *Eco*RI and *Msp*I were used as per the manufacturers' specifications (New England Biolabs). The restriction fragments were size separated by electrophoresis on 2.5% agarose gel. The gels were stained with ethidium bromide (0.5 µg/ml) and photographed under UV light. Unambiguous polymorphic DNA band were scored as "1" for presence and "0" for absence. Unweighted Pair Group Method with Arithmetic average (UPGMA) dendrogram was generated using software programme NTSYS-PC (version 2.02), based on Jaccards similarity coefficient (**Rohlf, 1998**).

RESULTS AND DISCUSSION

RAPD amplification of *Fusarium oxysporum* f. sp. *ciceri* isolates

Out of 40 primers screened for amplification of DNA of *Fusarium* isolates, 16 resulted in either sub-optimal or non distinct amplification products. Therefore these were discarded and remaining 24 primers which generated reproducible RAPD patterns were used for subsequent analysis. A total of 247 bands were produced by 24 primers, out of which 85% (210) were polymorphic and 15% (37) were monomorphic. A maximum of fourteen amplicons were amplified by primer OPF05, whereas minimum seven amplicons were amplified by primer K7. RAPD profile obtained with primer P8 is shown in Figure 1a. The UPGMA cluster analysis based on RAPD profiles separated the fourteen isolates into four major clusters namely cluster I, II III and IV (Figure 2a). Isolates *Foc* 17, *Foc* 42 and *Foc* 92 grouped together in cluster I. Cluster II comprised of *Foc* 66, *Foc* 65, *Foc* 80 and *Foc* 73. *Foc* 6, *Foc* 91, *Foc* 79 and *Foc* 88 fell in cluster III while *Foc* 60, *Foc* 58 and *Foc* 56 grouped in cluster IV. The maximum genetic similarity of 65% was observed between *Foc* 79 (Chhattisgarh) and *Foc* 88 (Maharashtra) while a minimum of 13% similarity was found between *Foc* 17 and *Foc* 88. Isolate *Foc* 17 belonging to U.P. fell separate and totally demarcated from rest of the isolates.

Table 4 Repeat motifs, no. of alleles and allele size from the SSR markers used in the fingerprinting of *Fusarium oxysporum* f. sp. *ciceri* isolates.

Marker name	Repeat motifs	Tm(°C)	Allele size (bp)	No. of alleles
SSR 1	(GT)11(GA)6	57	225bp, 250, 300 bp	3
SSR 2	(TG)9	54	100 bp	1
SSR 3	(CA)9	51	100 bp	1
SSR 4	(AAC)6	48	200 bp	1
SSR 5	(GGC)7	68	150 bp, 400 bp	2
SSR 6	CTTGGAAGTGGTAGCGG)14	68	100 bp, 200 bp, 300 bp	3
SSR 7	(CCA)5	57	150 bp, 300 bp	2
SSR 8	(CA)21	57	250 bp	1
SSR 9	(CAACA)6	57	300 bp, 350 bp	3
SSR 10	$(AC)_{13}$	50	300 bp	1
SSR 11	$(AC)_{15}$	51	200 bp, 300 bp	2
SSR 12	$(AAG)_{28}$	55	100 bp, 200 bp, 300 bp, 400 bp,	4

SSR amplification of *Fusarium oxysporum* f. sp. *ciceri* isolates

Twelve SSR primers were used to generate amplification patterns in 14 *Foc* isolates. A total of 24 alleles were produced with an average of two alleles per marker. The maximum number of four alleles was obtained with primer SSR12 (Table 4). The SSR amplicon size ranged from 100-400 bp. SSR profile obtained with primer SSR1 is shown in Figure 1b. The cluster analysis grouped 14 *Foc* isolates into four major clusters demarcating cluster IV completely (Figure 2b).

Cluster I comprised of *Foc* 17, *Foc* 42, *Foc* 92 and *Foc* 66. *Foc* 42 and *Foc* 92 clustered close to each other with similarity of 83% while *Foc* 66 shared 80% of genetic similarity with *Foc* 92. Cluster II consisted of *Foc* 65, *Foc* 80 and shared 82 % genetic similarity between them. *Foc* 73, *Foc* 6, *Foc* 56, *Foc* 91 fell in cluster III where *Foc* 6 and *Foc* 56 grouped closer with 84 % of similarity. Cluster IV contained closely grouping *Foc* 60, *Foc* 58 and *Foc* 79, *Foc* 88 with similarity values of 92 % and 88 %, respectively.

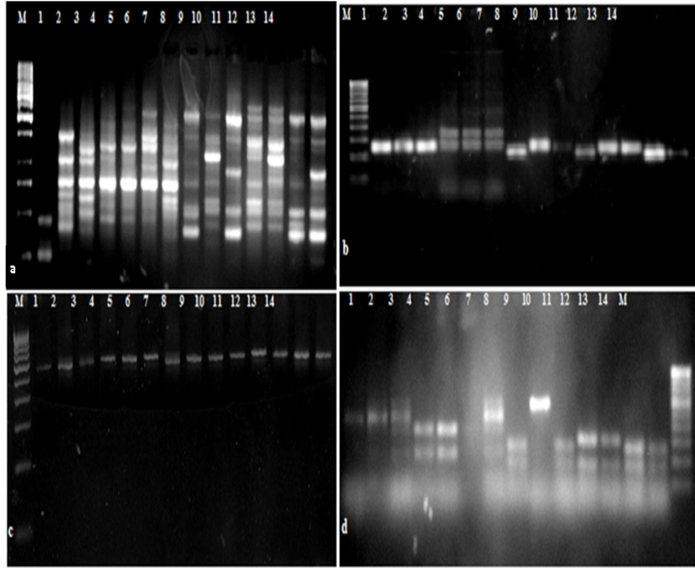
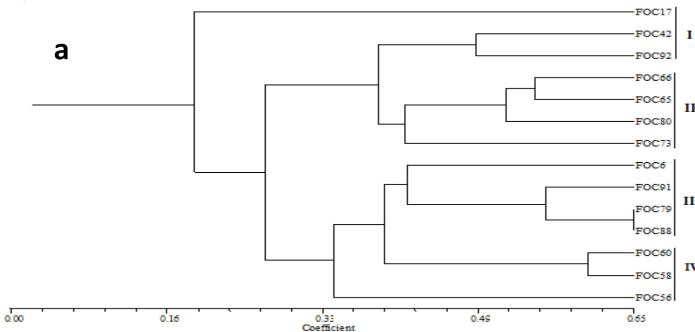


Figure 1 Amplification profile obtained with the different molecular markers in *Fusarium oxysporum* f. sp. *ciceri* isolates
a: RAPD profile obtained with the primer P-8
b: SSR profile obtained with SSR 1 primer pair
c: Amplification profile of ITS region with ITS-1and ITS-4 primers pairs
d: ITS-RFLP profile of rDNA with *Msp*I restriction endonuclease
Lanes M: 100 bp DNA ladder, 1: *Foc* 17, 2: *Foc* 42, 3: *Foc* 92, 4: *Foc* 66, 5: *Foc* 65, 6: *Foc* 80, 7: *Foc* 73, 8: *Foc* 6, 9: *Foc* 56,
10: *Foc* 91, 11: *Foc* 60, 12: *Foc* 58, 13: *Foc* 79, 14: Foc 88

ITS-RFLP amplification of *Fusarium oxysporum* f. sp. *ciceri* isolates

The universal ITS primer pair amplified 630 bp bands from all fourteen isolates (Figure 1c). Significant length polymorphism was not obtained unless analysed by digestion with restriction enzymes. Length polymorphism obtained after digestion of amplified ITS varied depending on the restriction enzyme and isolate used. Digestion of the amplified fragments by *EcoR*I and *Msp* I restriction enzymes revealed extensive polymorphism (Figure 1d). The two enzyme which had restriction sites in the ITS region revealed maximum polymorphism in three isolates. *Msp* I restriction enzymes could detect more variation in the restriction sites in all isolates. Isolates from U.P. (*Foc* 14, *Foc* 42, *Foc* 92) and two other isolates *Foc* 73 (Karnataka) and *Foc* 56 (M.P.) were not restricted by *Msp* I restriction enzymes. The cluster analysis of ITS-RFLP grouped all 14 *Foc* isolates into three major clusters (Figure 2c). Cluster I comprised of five isolates including three isolates from Uttar Pradesh (*Foc* 17, *Foc* 42 and *Foc* 92), one isolate from Karnataka (*Foc* 73) and one isolate from Madhya Pradesh (*Foc* 56). Cluster II was the largest cluster with seven *Foc* isolates. Within this cluster, two isolates from Andhra Pradesh, *Foc* 65 and *Foc* 66 shared close genetic similarities among themselves. Two isolates from Chhattisgarh (*Foc* 58 and *Foc* 79) also fell into cluster II. The other isolates within this cluster were *Foc* 60 (Gujarat), *Foc* 80 (Karnataka) and *Foc* 88 (Maharastra). The third cluster comprised of one isolate from Madhya Pradesh (*Foc* 6) and another isolate from Gujarat (*Foc* 91).



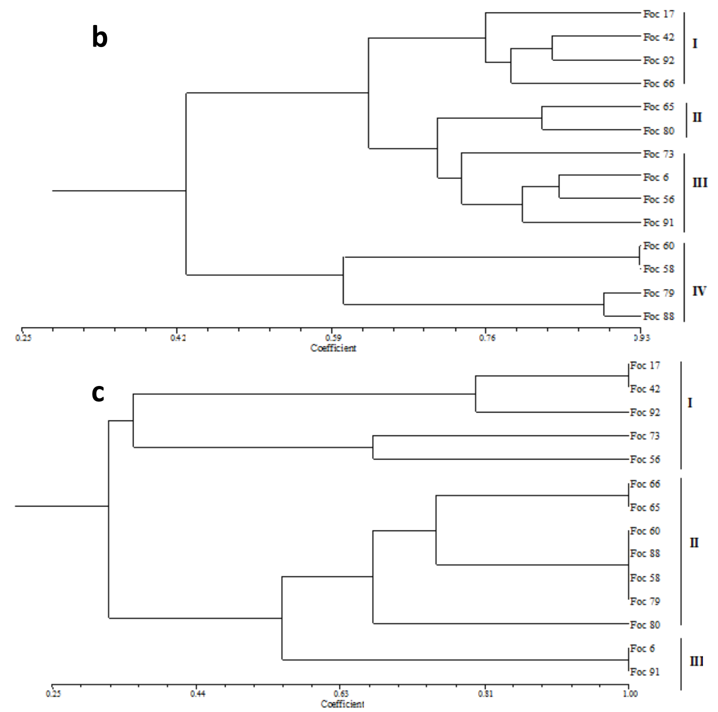


Figure 2 UPGMA cluster analysis showing relationship between *Fusarium oxysporum* f. sp. *ciceri* isolates using different molecular markers RAPD (a) ; SSR (b) and ITS-RFLP (c).

High level of genetic variability obtained in the study suggests that the isolates of *F. oxysporum* are derived from genetically distinct clones. The exchange of contaminated seeds and cultures probably contributed to existence of variable population of *F. oxysporum* f. sp. *ciceri* in wider geographical areas. Non-stability of most prominent genotypes of chickpea further supported the view that the pathotypes of this pathogen are not stable and parasexual recombination plays a major role in the evolution of races. In the present study, the genetic variability of 14 Indian isolates of *F. oxysporum* f. sp. *ciceri* was determined through RAPD, SSR and ITS-RFLP. It appeared that the fingerprinting-based grouping was different from groups generated on the basis of virulence or geographical origin. Variation in symptom types (pathotypes) and pathogenic races have been reported to correlate to different geographical regions and to polymorphisms in molecular markers.

The present study generated significant information in terms of genetic variability of *F. oxysporum* f. sp. *ciceri*, which could be used further for development of area-specific resistant varieties of chickpea. The study also highlights the facts that both pathogenic virulence analysis and molecular markers are useful tools for analyzing the structure of the pathogen population, but further studies are needed to make them complementary to each other. Seventy-two isolates of *F. oxysporum* f. sp. *ciceri* were grouped into two distinct clusters using RAPD–PCR, one for wilting and another yellowing syndrome causing isolates (**Kelly *et al.*, 1994**). Ninety-nine isolates of *F. oxysporum* f. sp. *ciceris* were characterized by the RAPD marker and cluster analysis showed three groups of isolates. Races 0 and 1B/C grouped into two different clusters, whereas races 1–3, 5 and 6 were grouped into another cluster (**Jimenez-Gasco *et al.*, 2001**). One isolate from U.P. *Foc* 17 could not be grouped with other isolates and this result is in agreement with observations of **Honnareddy and Dubey, 2006**. The SCAR primer for identification of race of the pathogen was developed, because RAPD analysis was not able to distinguish all the races separately (**Jimenez-Gasco and Jimenez-Diaz, 2003**). Generalized race-specific patterns were not found through RAPD study (**Grajal-Martin *et al.*, 1993**).

Using AFLP analysis, **Gonzalez *et al.*, 1998** have classified *Colletotrichum lindemuthianum* isolates from Mexico into two major groups according to the type of common bean cultivar or system of cultivation from which they originated. The smaller subgroups generally associated with the geographical location from which they were obtained. **Koenig *et al.*, 1997** identified 10 clonal lineages of *F. oxysporum* f. sp. *cubense* using RFLP analysis and the two largest lineages had pantropical distribution, while the minor lineages were found only in limited geographical regions. AFLP based grouping of the isolates appeared independent of cultural and virulence traits. In order to determine the extent of genetic variation of this economically important fungus and relationships with cultural and pathogenic traits, more isolates from other countries and or geographical origins should be assayed using DNA based molecular techniques.
The traditional pathogenicity-based classification of isolates in *Fusarium* has several limitations. The use of vegetative compatibility tests, and various protein and DNA based techniques also has not solved the problem associated with

taxonomy of isolates of *Fusarium*. SSR markers are advantageous in that they are hypervariable, abundantly found in eukaryotic genomes, and co-dominant. These characters make SSRs very useful in taxonomic and population genetic studies. The high degree of polymorphism revealed using the SSR markers used in this study should be sufficient for studies focussed in understanding the genetic diversity amongst isolates of *Fusarium*. The SSR primers should be particularly useful because the fungus is one of the very common plant pathogens. Their application should also enhance understanding relatedness of *formae speciales* in the genus *Fusarium*.

The entire three marker techniques used in this study effectively separated the *Fusarium* isolates into distinct clades. The primers and restriction enzyme combinations used here resulted in extensive polymorphism due to lack and/or alteration in primer binding and restriction sites. None of the three techniques correlated geographical origin based grouping or based on pathogenicity. This suggests that phylogenetic groups do not necessarily correlate with pathogenic or geographic groups. Other studies on DNA fingerprinting of *Fusarium* wilt pathogens have also reported similar findings (**Bao *et al.*, 2002; Sharma *et al.*, 2009, Durai *et al.*, 2012**). This result is perhaps not surprising as *formae specials* are based on phenotypic characters which are influenced by a range of factors and linked to necessarily variations at DNA level.

CONCLUSION

For a precise wilt resistance breeding programme in Chickpea, genetic identification of *Fusarium* isolates/races is very important. The pathological and morphological data fail to provide exact picture about the existence of *Fusarium* races and need to be substantiated by molecular data to get clear picture about the genetic variability in isolates/races. Therefore, in the present study, the genetic variability of 14 Indian isolates of *F. oxysporum* f. sp. *ciceri* was determined through RAPD, SSR and ITS-RFLP marker techniques. All the three techniques effectively separated the isolates into distinct clades. None of the three techniques correlated with geographical origin based grouping or based on pathogenicity, suggesting that phylogenetic groups do not necessarily correlate with pathogenic or geographic groups. All the three PCR based markers prove well adapted for large scale characterization of genetic diversity of *Fusarium* natural populations and correlating inter- and intra-specific diversity with ecological traits such as antagonistic ability and compatibility with rhizosphere microflora. The study also highlights the fact that both pathogenic virulence analysis and molecular markers are useful tools for analysing the structure of the pathogen population but further studies are needed in *Fusarium* and other plant pathogenic fungal genera to make them complimentary to each other.

Acknowledgments: Authors are thankful to Dr. Vishwa Dhar, Network Leader, 'ICAR Wilt Network Project' and Dr. R.G. Chaudhary, Principal Scientist, Indian Institute of Pulses Research, Kanpur for sharing the *Fusarium* isolates and seeds of chickpea genotypes. Thanks are due to Mr. Mayank Kaashyap and Mr. Prasoon P. Gupta for help in the conduct of experiments and statistical analysis.

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