African Crop Science Journal, Vol. 21, No. 3, pp. 201 - 211
ISSN 1021-9730/2013 $4.00

GENETIC DIVERSITY OF *Fusarium* WILT RACES OF PIGEONPEA IN MAJOR REGIONS OF INDIA

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(Received 2 November, 2012; accepted 12 June, 2013)

ABSTRACT

Fusarium wilt is a serious fungal disease in pigeonpea (*Cajanus cajan*) which causes severe yield loses (up to 90%). Genetic diversity in pigeonpea wilt pathogen [*Fusarium udum* (*fud*)] was characterised using 14 isolates collected from major pulse growing regions of India. Twenty four RAPD primers generated a total of 226 bands (ranging 0.3 to 3.0 kb) in *F. udum* with an average of 9.4 bands per primer and a total of 27 alleles were produced by twelve SSR primers with an average of 2.25 alleles per marker. All isolates amplified a single band ranging from 100 to 450 bp. The universal ITS primer pair amplified 650 bp bands in all fourteen *fud* isolates, while significant length polymorphism was obtained only when analysed by restriction digestion with *Eco*RI and *Hind* III enzymes. Cluster analysis of ITS-RFLP grouped all 14 *Fud* isolates into three major clusters. Cluster analysis using various markers showed 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 programmes in pigeonpea taking into account the diversity in wilt pathogen.

Key Words: *Cajanus cajan, Fusarium*, RAPD, SSR

RÉSUMÉ

Le *fusarium* est une sérieuse maladies responsible de la phanaison du pois cajan (*Cajanus cajan*) induisant d'importantes pertes de rendements (jusqu'à 90%). La diversité génétique dans le pathogène de phanaison du pois cajan [*Fusarium udum* (*fud*)] était caractérisée utilisant 14 isolats collectés dans d'importantes régions indiennes productrices de la culture. Vingt quatre primers RAPD générés d'un total de 226 bandes (variant de 0.3à 3.0 kb) dans *F. udum* avec une moyenne de 9.4 bandes par primer et un total de 27 allèles étaient produits par douze primers SSR avec une moyenne de 2.25 allèles par marqueurs. Tous les isolats ont amplifié une bande unique variant de 100 à 450 bp. Le primer pair universel IT'S a amplifié 650 bp bandes dans tous les 14 *fud* isolats, pendant qu'un polymorphisme significatif de taille était seulement obtenu lorsqu'analysé par restriction-digestion avec enzymes *Eco*RI et *Hind* III. L'analyse par groupement de ITS-RFLP a groupé tous les 14 *Fud* isolats en trois groupes majeurs. L'utilisation des marqueurs a permis de montrer strictement le groupement d'isolats de fusarium suivant leurs caractéristiques culturales et degree de pathogénicité et non pas leur origine gvographique. Cette information pourra être aider les pathologists et améliorateurs des cultures à concevoir des programmes d'amélioration effective de résistance dans le pois cajan tenant en compte la diversité dans le pathogène de la phanaison.

Mots Clés: *Cajanus cajan, Fusarium*, RAPD, SSR

INTRODUCTION

Pigeonpea (*Cajanus cajan*) is among the world's most important pulse crops which suffer heavy yield losses up to 90% due to vascular wilt caused by *Fusarium udum*. 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 pigeonpea lines with resistance to biotic constraints and tolerance to abiotic stresses, yield loss in these crops is very high due to the high incidence of diseases and insect-pests (Nene and Sheila, 1990).

Fusarium wilt of pigeonpea is caused by *F. udum,* which is both soil and seed borne and difficult to eradicate as fungal chlamydospores survive in soil up to six years even in the absence of host plant. Biochemistry and physiology of the *Fusarium*-plant interaction have been characterised extensively, but definitive enquiry into identification of individual molecules essential for *Fusarium* pathogenesis to plants did not begin until molecular genetic technology became available for filamentous fungi (Timberlake and Marshall, 1989; Bennett and Lasure, 1991). To develop effective strategies for management of wilt diseases, understanding of the molecular basis of pathogenesis and resistance mechanism is essential.

Identification of species of the genus *Fusarium* is complicated on several aspects. Morphological characteristics such as shape and size of the macroconidia, presence or absence of microconidia and chlamydospores, and colony morphology are often insufficient to allow identification at the species level. In addition, these observations need some practices and are difficult for the non-specialist (Windels, 1991; Bluhm *et al.*, 2002). Although pathogenicity phenotypes can be useful for assessing genetic variation in fungal pathogens; pathogenicity markers are often limited in number and subject to host selection (Leung *et al.*, 1993). 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 programme as exact picture about the existence of number of physiological races in *Fusarium udum* 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* population (Kiprop *et al.*, 2002; Sivaramakrishnan *et al*., 2002). DNA fingerprinting has been successfully used for *Fusarium* in characterisation 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 important especially to pyramid several resistance genes in an elite genotype.

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) characterised 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 IGS primers and results indicated 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 *F. oxysporum* f. sp. *ciceri* has been reported using 40 RAPD primers (Jimenez-Gasco *et al.*, 2004).

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 *F. oxysporum ciceri* causing varied levels of wilting with differential host cultivars (Barve *et al.*, 2001). Bogale *et al.* (2005) showed that polymorphism revealed with 8 SSR markers was sufficient for study of genetic diversity in *F. oxysporum* complex.

The cluster of ribosomal DNA consists of tandem repeat of three coding (18S, 5.8S and 28S), and two noncoding 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). Inter Transcribed Spacer 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) showed 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 and sometimes RAPD patterns could reflect the variability of *formae speciales* (Clark *et al.*, 1998).

The aim of this study was to determine the genetic diversity of an isolate collection of *F. udum* recovered from diseased plants in wilt affected fields of pigeonpea from seven major pulse growing states of India using PCR based molecular markers and estimate the genetic relatedness.

MATERIALS AND METHODS

***Fusarium* isolates.** The *Fusarium udum* (*Fud*) isolates collected from wilt affected fields of pigeonpea 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 this 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

TABLE 1. RAPD primers used in the fingerprinting of *F. udum* 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'

TABLE 2. SSR primers used in the fingerprinting of *F. udum* isolates

Primer	Sequence 5'→3'	Tm (°C)	References
SSR 1	F: TGCTGTGTATGGATGGATGGR: CATGGTCGATAGCT	57	Bogale *et al.,* 2005
SSR 2	F: ACTTGGAGAAATGGGCTTCR: GGATGGAGTTTAATAAATCTGG	54	Bogale *et al.,* 2005
SSR 3	F: TGGCTGGGATACTGTGTAATTGR: TTAGCTTCAGCCCTTTGG	51	Bogale *et al.,* 2005
SSR 4	F: TATCGAGTCCGGCTTCCAGAACR: TTGCAATTACCTCCGATCCAC	48	Bogale *et al.,* 2005
SSR 5	F: GTGGACGAACACCTGCATCR: AGATCCTCCACCTCCACCTC	68	Bogale *et al.,* 2005
SSR 6	F: GGAGGATGAGCTCGATGAAGR: CTAAGCCTGCTACACCCTCG	68	Bogale *et al.,* 2005
SSR 7	F: CGTCTCTGAACCACCTTCATCR: TTCCTCCGTCCATCCTGAC	57	Bogale *et al.,* 2005
SSR 8	F: ACTGATTCACCGATCCTTGGR: GCTGGCCTGACTTGTTATTCG	57	Bogale *et al.,* 2005
SSR 9	F: GGTAGGAAATGACGAAGCTGACR: TGAGCACTCTAGCACTCCAAAC	57	Bogale *et al.,* 2005
SSR 10	F: CGAGCTAATGGTGGCAGGATR: AACAACAAAACGGCTCATCG	50	Giraud *et al.*, 2002
SSR 11	F: TATTTCGTGCAAGGACTTGGR: CTTGGTCCCTGGATATGGA	51	Giraud *et al.*, 2002
SSR 12	F: AAGCGCCAACAGAGATGACGAR: GACTGCCGAAACACCGAAA	55	Giraud *et al.*, 2002

TABLE 3. Repeat motifs, number of alleles and allele size from the SSR markers used in the fingerprinting of *F. udum* isolates

Marker name	Repeat motifs	Tm	Allele size (bp)	Number 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

ribosomal gene, was amplified using primers ITS1 (5'TCCGTAGGTGAACCTGCGG3') and ITS4 (5'TCCTCCGCTTTATTGATATG3').

Collection and maintenance of fungal isolates. The pathogens were isolated from fourth-node stem sections taken from wilted pigeonpea plants collected from different agro-climatic regions of India according to the procedure described by Tullu *et al.* (1998). The re-isolated pathogens were colonised on Whatman no. 1 filter paper, dried in a transfer hood, and aseptically cut into small pieces (3-5 mm). The colonised 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 characterised at the laboratories of Department of Life Sciences, and Department of Biochemistry, I.B.S.B.T, C.S.J.M. University, Kanpur in India. Single spore culture of fungus was obtained by serial dilution method.

Isolation and purification of genomic DNA of isolates. A single spore culture of each isolates was grown on PDA medium at 28 °C and stored at 4 °C until use. Mycelia for genomic DNA

extraction were grown in 250 ml of PDB at 28 °C for 5 days. After vacuum filtration, the mycelia were lyophilised 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^{-1}) 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 (28 °C). 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 ml^{-1} to be used as template.

DNA fingerprinting. 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'TCCTCCGCTTTATTGATATG3'). 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, and 72 °C for 1 min. The amplification was completed with a final extension at 72°C for 5 min.

Electrophoresis and visualisation of amplified bands were 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^{-1}) and photographed under UV light. Unambiguous polymorphic DNA band were scored as "1" for presence and "0" for absence. UPGMA dendrogram was generated using software programme NTSYS-PC (Version 2.02), based on Jaccards similarity coefficient (Rohlf, 1998).

RESULTS

RAPD amplification. Twenty four RAPD primers used for diversity analysis of 14 *F. udum* isolates generated a total of 226 bands with an average of 9.4 bands per primer. The RAPD amplicon size ranged from 0.3 kb to 3.0 kb. RAPD profile obtained with primer OPA 11 is shown in Figure 1a.

A dendrogram based on UPGMA analysis depicted the grouping of isolates into three major clusters namely cluster I, II and III (Fig. 2a). The major cluster I further divided into two sub-clusters IA and IB. Sub-cluster IA comprised of two isolates *Fud* 1 and *Fud* 4 from Uttar Pradesh which share common characteristic of medium

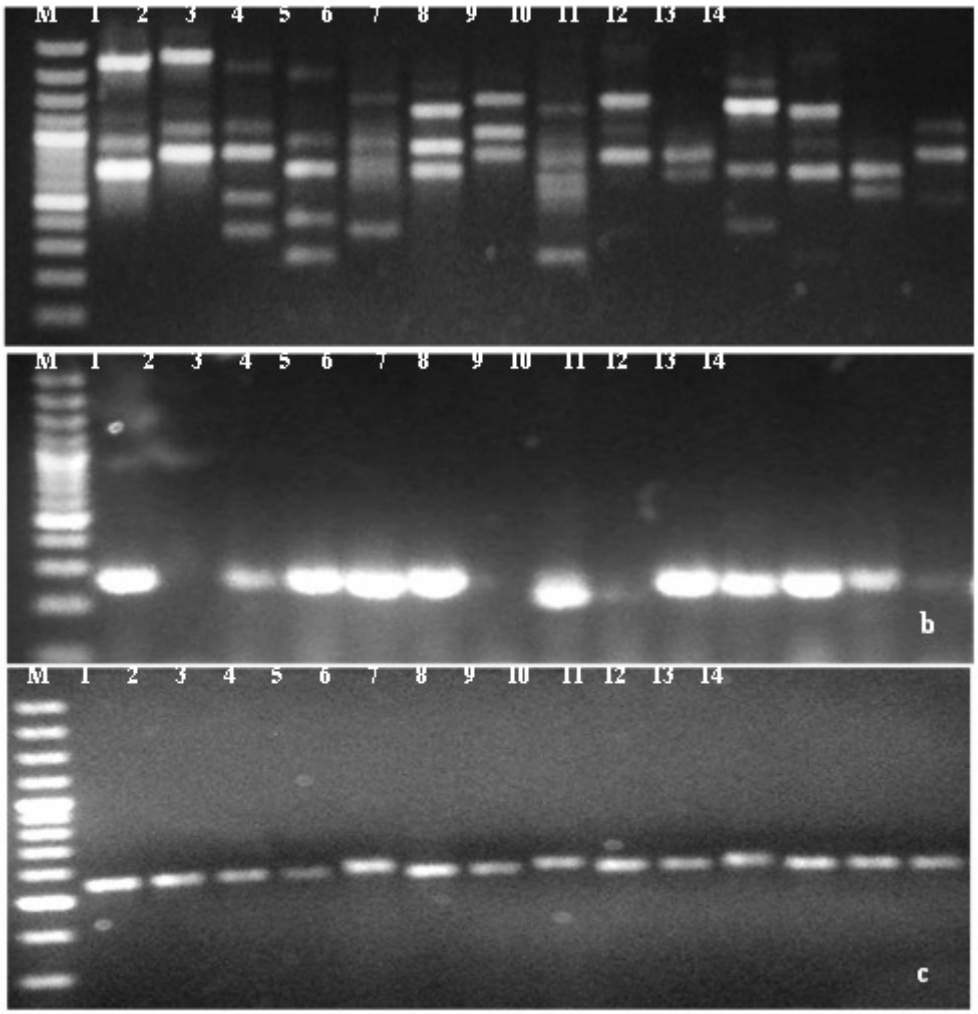

Figure 1. Amplification profile of *Fusarium udum* isolates obtained with different molecular markers. (a) Amplification profile of *Fusarium udum* isolates obtained with RAPD marker OPA11. (b) SSR profile of *Fusarium udum* isolates obtained with SSR 1 (c) Amplification profile of ITS region with ITS-1and ITS-4 primers pairs. Lanes M: 100 bp DNA ladder, 1: Fud 1, 2: Fud 4, 3: Fud 6, 4: Fud 7, 5: Fud9, 6: Fud11, 7: Fud 14, 8: Fud, 17, 9: Fud 18, 10: Fud 19, 11: Fud 20, 12: Fud 22, 13: Fud 23, 14: Fud27

mycelium growth rate. Both shared 60% genetic similarity value and bootstrap replication obtained between the two was 45.1. Cluster IB was further sub-divided into two clusters, IB1 and IB2. Sub-cluster IB1 comprised of two isolates *Fud* 6 and *Fud* 14 which shared 64% of genetic similarity and 40.7 bootstrap values. The maximum similarity of 69% was found between *Fud* 7 (Bihar) and *Fud* 20 (MP) with 66.3 bootstrap replication value. Isolate *Fud* 9 showed distinctness from all other isolates of cluster I and fell separately. The average bootstrap replication value among *Fud* 7, *Fud* 20, *Fud* 18 and *Fud* 9 was 94.0. Cluster II comprised of four isolates *viz. Fud* 22, *Fud* 23 and *Fud* 27 and *Fud* 17. *Fud* 22 (Madhya Pradesh) and *Fud* 23 (Tamilnadu) were in same cluster with second highest similarity (68%) and highest bootstrap replication 99.8.

Isolate *Fud* 27 (Tamilnadu) clustered separately and showed 57.3% average similarity and 94.0 bootstrap value with *Fud* 22 and *Fud* 23. *Fud* 17 and *Fud* 11 were most diverse from other 12 isolates and did not fall in any particular distinct cluster.

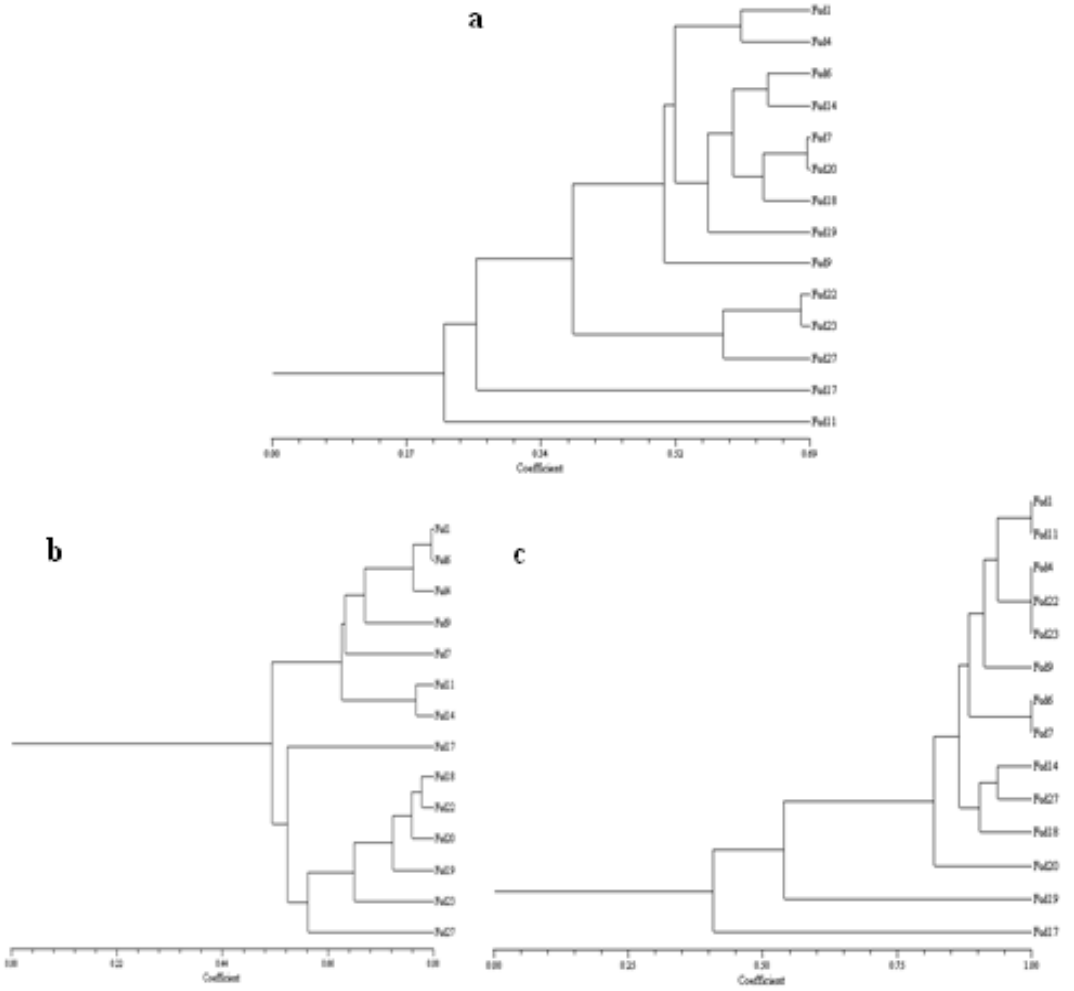

Figure 2. UPGMA cluster analysis showing relationship between *F. udum* isolates using different molecular markers RAPD (a), SSR (b) and ITS-RFLP (c).

SSR amplification. Twelve SSR primers were used for amplification of loci of 14 isolates of *Fusarium udum*. A total of 27 alleles were produced by SSR primers with an average of 2.25 alleles per marker. All isolates amplified a single band ranging from 100 to 450 bp. Maximum number of five alleles were amplified by primer SSR 9. SSR amplification pattern of SSR 1 (Fig. 1b). The dendrogram based on Jaccards similarity coefficient depicted two major clusters I and II (Fig. 2b). In cluster I, the maximum similarity (88.0%) was found between *Fud* 1 (Uttar Pradesh) and *Fud* 6 (Bihar) with the bootstrap replication of 65.0 followed by *Fud* 4 (Uttar Pradesh) and *Fud* 6 (Bihar). Isolate *Fud* 11 (Andhra Pradesh) and *Fud* 14 (Karnataka) grouped close together and shared second highest similarity value (84.0%) due to their high pathogenesis. Cluster II comprised of 7 isolates *Fud* 17, *Fud* 18, *Fud* 19, *Fud* 20, *Fud* 22, *Fud* 23 and *Fud* 27. Isolates *Fud* 18 (Maharashtra) and *Fud* 22 (Madhya Pradesh) clustered together and showed 85% similarity value with 38.8 bootstrap replication due to their high pathogenicity. Isolate *Fud* 17 fell distinctly from all other isolates with bootstrap value of 48.9. Isolates *Fud* 23 and *Fud* 27 were depicted in cluster II with less similarity value from rest of all other isolates of group.

ITS-RFLP amplification. Universal primers ITS 1 and ITS 4 were used to amplify a region of the 5.8S rRNA gene. All the isolates amplified single amplicons of 650 bp (Fig. 1c). The amplified ITS was digested with restriction enzymes for extensive polymorphism in rRNA regions. The ITS regions were restricted with five different hexa cutter restriction enzymes (*Eco* RI, *Eco*RV, *Sma* I, *Pst* I and *Hind* III). Out of five restriction enzymes, two enzyme (*Eco* RI and *Hin*d III) restricted the ITS region. The two enzymes which had restriction sites in the ITS region revealed the extensive polymorphism in the isolates. The UPGMA cluster analysis based on ITS-RFLP profiles clustered 14 isolates into three major clusters (Fig. 2c). Eight isolates *Fud* 1, *Fud* 11, *Fud* 4, *Fud* 22, *Fud* 23, *Fud* 9, *Fud* 6 and *Fud* 7 grouped closely in cluster I while three isolates *Fud* 14, *Fud* 27 and *Fud* 18 fell in cluster II. *Fud* 14 and *Fud* 27 fell closer to each other with 67% of genetic similarity value. Three isolates *Fud* 20, *Fud* 19 and *Fud* 17 clustered separately from rest of the isolates in cluster III.

DISCUSSION

High level of genetic variability obtained in the study suggests that the isolates of *F. udum* are derived from genetically distinct clones. The exchange of contaminated seeds and cultures probably contributed to the existence of variable population of *F. udum* in wider geographical areas. Non-stability of most prominent genotypes of pigeonpea 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 this study, the genetic variability of 14 Indian isolates of *F. udum* was determined through RAPD, SSR and ITS-RFLP. It appears 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 (Jimenez-Gasco *et al*., 2004). *F. udum* isolates from the same site or diverse geographical origins have been shown to exhibit high variability in cultural characteristics (Reddy and Raju, 1993) and virulence or pathogenicity on pigeonpea genotypes (Reddy and Chaudhary, 1985; Gaur and Sharma, 1989). Two isolates *Fud* 1 and *Fud* 4 from Uttar Pradesh grouped together in same cluster and they shared common characteristic of medium mycelium growth rate. Studies of genetic diversity using isozyme markers revealed low variation in *F. udum* isolates (Shit and Sen Gupta, 1978).

There was no correlation between molecular diversity and geographical origin of the isolates. *F. udum* is a Deuteromycete and therefore natural populations of the pathogen may consist of clonal lineages produced by asexual reproduction. Using AFLP analysis, Gonzalez *et al.* (1998) classified *C. 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 (Gonzalez *et al.*, 1998). Koenig *et al.* (1997) identified 10 clonal lineages of *F. oxysporum* f. sp. *cubense* using RFLP analysis. 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.

Genome analysis of *F. udum* isolates by RAPD, SSR and ITS-RFLP in this study has provided evidence that this pathogenic fungus varies genetically. This variability should be taken into consideration in pigeonpea improvement programmes aimed at breeding for wilt disease resistance. 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 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 (Kiprop *et al.*, 2002; Sharma *et al.*, 2009).

CONCLUSION

Precise wilt resistance breeding programme in Pigeonpea requires authentic genetic identification of *Fusarium udum* isolates/races. 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. udum* was determined through PCR based markers (RAPD, SSR and ITS-RFLP). 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 characterisation 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.

ACKNOWLEDGEMENT

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 and pigeonpea genotypes. Thanks are due to Mr. Mayank Kaashyap and Mr. Prasoon P. Gupta for help in the conduct of experiments and statistical analysis.

REFERENCES

Barve, M.P., Haware, M.P., Sainani, M.N., Ranjekar, P.K. and Gupta, V.S. 2001. Potential of micro satellites to distinguish four races of *Fusarium oxysporum* f. sp. *ciceri* prevalent in India. *Theoretical and Applied Genetics* 102:138-147.

Bennett, J.W. and Lasure, L.L. 1991. Growth media. pp. 441-458. In: *More gene manipulations in fungi*. Bennett, J.W. and Lasure, L.L. (Eds.) Academic Press, San Diego.

Bluhm, B.H., Flaherty, J.E., Cousin, M.A. and Woloshuk, C.P. 2002. Multiplex polymerase chain reaction assay for the differential detection of trichothecene- and fumonisin-producing species of *Fusarium* in cornmeal. *Journal of Food Protection* 65:1955-1961.

Bogale, M., Wingfield, B.D., Wingfield, M.J. and Steenkamp, E.J. 2005. Simple sequence repeat markers for species in the *Fusarium oxysporum* complex. *Molecular Ecology Notes* 5:622-624.

Bryan, G.T., Farrall, L., Orbach, M., Kang, S., Sweigard, J.A. and Valent, B. 1996. Functional analysis of the *Magnaporthe grisea* avirulence gene AVR2-YAMO. In: *Proceedings of the 8th International*

Congress on Molecular Plant-Microbe Interactions. Knoxville, USA.

Chakrabarti, A., Mukherjee, P.K., Sherkhane, P.D., Bhagwat, A.S. and Murthy, N.B.K. 2000. A simple and rapid molecular method for distinguishing between races of *Fusarium oxysporum* f. sp. *ciceri* from India. *Current Science* 80:571-575.

Clark, C.A., Hyun, J.W. and Hoy, M.W. 1998. Relationships among wilt inducing isolates of *Fusarium oxysporum* from sweet potato and tobacco. *Plant Diseases* 82:530-536.

Cramer, R.A., Byrne, P.F., Brick, M.A., Panella, L.,Wickliffe, E. and Schwartz, H.F. 2003. Characterization of *Fusarium oxysporum* isolates from common bean and sugar beet using pathogenicity assay and random-amplified polymorphic DNA markers. *Phytopathology* 151:352-560.

Gaur, V.K. and Sharma, L.C. 1989. Variability in single spore isolates of *Fusarium udum* Butler. *Mycopathology* 107:9-15.

Gherbawy, Y.A.M.H., Adler, A. and Prillinger, H. 2002. Genotypic identification of *Fusarium subglutinans*, *F. proliferatum* and *F. verticillioides* strains isolated from maize in Austria. *Mycobiology* 30:139-145.

Gonzalez, M., Rodriguez, M.E.Z., Jacabo, J.L., Hernandez, F., Acosta, J., Martinez, O. and Simpson, J. 1998. Characterization of Mexican isolates of *Colletotrichum lindemuthianum* by using differential cultivars and molecular markers. *Phytopathology* 88:292-299.

Jimenez-Gasco, M.M., Navas-Cortes, J.A. and Jimenez-Diaz, R.M. 2004. The *Fusarium oxysporum* f. sp. *ciceris*/*Cicer arietinum* pathosystem: A case study of the evolution of plant pathogenic fungi into races and pathotypes. *International Microbiology* 7: 95-104.

Kim, D.H., Martyn, R.D. and Magll, C.W. 1992. Restriction fragment length polymorphisms of mitochondrial DNAs from seven *Fusarium* species causing *Fusarium* head blight. *Japanese Journal of Genetics* 70:437-451.

Kiprop, E.K., Baudoin, J.P., Mwang'ombe, A.W., Kimani, P.M., Mergeai, G. and Maquet, A. 2002. Characterization of Kenyan Isolates of Fusarium udum from Pigeonpea [*Cajanus cajan* (L.) Millsp.] by Cultural Characteristics, Aggressiveness and AFLP Analysis. *Journal of Phytopathology* 150:517-525.

Koenig. R.L., Ploetz, R.C. and Kistler, H.C. 1997. *Fusarium oxysporum* f. sp. *cubense* consists of a small number of divergent and globally distributed clonal lineages. *Phytopathology* 87: 915-923.

Leung, H., Nelson, R.J. and Leach, J.E. 1993. Population structure of plant pathogenic fungi and bacteria. *Advances in Plant Pathology* 10:157-205.

Meyer, W., Castaneda, A., Jackson, S., Huynh, M. and Castaneda, E. 2003. The Ibero American cryptococcal study group. Molecular typing of Ibero American *Cryptococcus neoformans* isolates. *Emerging Infectious Diseases* 9:189-195.

Nazar, R.N., Hu, X., Schmidt, J., Culham, D. and Robb, J. 1991. Potential use of PCR-amplified ribosomal intergenic sequences in the detection and differentiation of *Verticillium* wilt pathogens. *Physiological and Molecular Plant Pathology* 39:1-11.

Nene, Y.L., Sheila, V.K. and Sharma, S.B. 1989. A world list of chickpea and pigeon pea Pathogens. ICRISAT Legume Pathol Prog Rep. Patancheru, India. p. 7.

Nene, Y.L. and Sheila, V.K. 1990. Pigeonpea: Geography and importance. pp. 1-10. In: *The Pigeonpea*. Nene, Y.L., Hall, S.D. and Sheila, V.K. (Eds.). CAB International, UK.

ÓDonnell, K. 1992. Ribosomal DNA internal transcribed spacers are highly divergent in the phytopathogenic ascomycete *Fusarium sambucinum* (*Gibberella pulicaris*). *Current Genetics* 22:213-220.

Pasquali, M., Acquadro, A., Balmas, V., Migheli, Q., Garibaldi, A. and Gullino, M.L. 2003. RAPD characterization of *Fusarium oxysporum* isolates pathogenic on *Argyranthemum frutescens* L. *Journal of Phytopathology* 151: 30-35.

Reddy, N.P.E. and Chaudhary, K.C.B. 1985. Variation in *Fusarium udum*. *Indian Phytopathology* 38:172-173.

Reddy, M.V. and Raju, T.N. 1993. Pathogenic variability in pigeonpea wilt pathogen *Fusarium udum*. pp. 32-34. In: *Plant Disease Problems in Central India*. Proceedings of Symposium, Central Zone, Indian

Phytopathology Society. Muralidharan, K. and Reddy, C.S. (Eds.). Directorate of Rice Research, Hyderabad, India.

Rohlf, F.J. 1998. NTSYS-PC: Numerical Taxonomy and Multivariate Analysis System. Version 2.01, Exeter Software: Setauket, New York.

Sharma, M., Varshney, R.K., Rao, J.N., Kannan, S., Hoisington, H. and Pande, S. 2009. Genetic diversity in Indian isolates of *Fusarium oxysporum* f. sp. *ciceris*, chickpea wilt pathogen. *African Journal of Biotechnology* 8:1016-1023.

Shit, S.K. and Sen Gupta, P.K. 1978. Possible existence of physiological races of *Fusarium oxysporum* f. sp. *udum*, the incitant of wilt of pigeonpea. *Indian Journal of Agricultural Sciences* 48:629-632.

Singh, B.P., Saikia, R., Yadav, M., Singh, R., Chauhan, V.S. and Arora, D.K. 2006. Molecular characterization of *Fusarium oxysporum* f. sp. *ciceri* causing wilt of chickpea. *African Journal of Biotechnology* 5:497-502.

Sivaramakrishnan, S., Kannan, S. and Singh, S.D. 2002. Detection of genetic variability in *Fusarium udum* using DNA markers. *Indian Phytopathology* 55:258-263.

Timberlake, W.E. and Marshall, M.A. 1989. Genetic engineering of filamentous fungi. *Science* 244: 1313-1317.

Tullu, A., Muehlbauer, F.J., Simon, C.J., Mayer, M.S., Kumar, J., Kaiser, W.J. and Kraft, J.M. 1998. Inheritance and linkage of a gene for resistance to race 4 of *Fusarium* wilt and RAPD markers in chickpea. *Euphytica* 102: 227-232.

Windels, C.E. 1991. Current status of *Fusarium* taxonomy. *Phytopathology* 81:1048-1051.

Yao, C., Frederiksen, R.A. and Magill, C.W. 1992. Length heterogeneity in ITS2 and the methylation status of CCGG and GCGC sites in the rRNA genes of the genus *Peronosclerospora*. *Current Genetics* 22: 415-420.