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Potential of microsatellites to distinguish four races of *Fusarium oxysporum* f. sp. *ciceri* prevalent in India

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Abstract *Fusarium oxysporum* f. sp. *ciceri*, the causal agent of chickpea wilt, is an important fungal pathogen in India. Thirteen oligonucleotide probes complementary to microsatellite loci, in combination with 11 restriction enzymes, were used to assess the potential of such markers to study genetic variability in four Indian races of the pathogen. Hybridisation patterns, which were dependent upon both the restriction enzyme and oligonucleotide probe used, revealed the presence of different repeat motifs in the *F. oxysporum* f. sp. *ciceri* genome. Among the restriction enzymes used, hexa-cutting enzymes were more informative than tetra- and penta-cutting enzymes, whereas tetranucleotide and trinucleotide repeats yielded better hybridisation patterns than dinucleotide repeats. Dependent upon the levels of polymorphism detected, we have identified $(AGT)_5$, $(ATC)_5$ and $(GATA)_4$ as the best fingerprinting probes for the *F. oxysporum* f. sp. *ciceri* races. The distribution of microsatellite repeats in the genome revealed races 1 and 4 to be closely related at a similarity index value of 76.6%, as compared to race 2 at a similarity value of 67.3%; race 3 was very distinct at a similarity value of 26.7%. Our study demonstrates the potential of oligonucleotide probes for fingerprinting and studying variability in the *F. oxysporum* f. sp. *ciceri* races and represents a step towards the identification of potential race diagnostic markers.

Keywords *Fusarium oxysporum* · DNA fingerprinting · chickpea

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Introduction

Chickpea (*Cicer arietinum* L.) has been described as the world's third most important pulse crop, with India accounting for 75% of the world production (FAO 1993; Singh 1997). It is a major source of food protein for the largely vegetarian Indian population (Singh and Saxena 1992). However, fungal diseases and insects, in addition to abiotic stresses, are major impediments to chickpea production. Among the fungal diseases, chickpea wilt, caused by *Fusarium oxysporum* Schl. emend. Snyd. and Hans. f. sp. *ciceri* (Padwick), is widespread in several chickpea growing regions of the world, especially the Indian subcontinent, Ethiopia, Mexico, Spain and Tunisia (Nene et al. 1989). The pathogen is both soil- and seed-borne and difficult to eradicate as fungal chlamydospores survive in soil up to 6 years even in the absence of the host plant (Haware et al. 1996). The most effective and economical method for controlling chickpea wilt is the use of resistant cultivars, the effectiveness of which is threatened by appearance of pathogenic races able to overcome resistance genes (Kaiser et al.1994). It is desirable to determine the genetic variability existing within the pathogen population in order to breed for chickpea cultivars with durable resistance, as potentially resistant cultivars should have resistance to known variants of the pathogen.

F. oxysporum isolates of a given forma speciales are morphologically indistinguishable. Therefore, subspecific subdivision is usually based on physiological race reactions to a set of differential cultivars (Snyder and Hansen 1940) or on vegetative compatibility groups (VCGs) as determined by heterokaryon formation between anastomosing, nitrate non-utilising (nit) mutants (Puhalla 1985). Seven races of the pathogen have been reported world-wide on the basis of the differential disease reactions of ten chickpea lines to isolates of the pathogen (Haware et al.1990). *F. oxysporum* f. sp. *ciceri* races 1 to 4 have been reported to be present in India (Haware and Nene 1982), races 0 and 5 in Spain (Jiménez-Díaz et al. 1989) and race 6 in California, USA (Phillips 1988). In

India, the races are geographically distinct. Race 1 is widespread in central and peninsular India and race 2 in northern India. Both of these races appear to be more virulent than the others. Races 3 and 4 are location-specific and are prevalent in the Punjab and Haryana states of India (Haware et al. 1992).

The classical method of race identification (Haware and Nene 1982) requires at least 40 days for complete analysis, is labour-intensive and is sensitive to variations in the environment. With the advent of molecular marker technology, it is possible to overcome these limitations and provide additional information for fungal characterisation (Ouellett and Seifert 1993). Moreover, molecular markers can be useful, if correlated to race, in identifying isolates, monitoring disease demographics or investigating evolutionary relationships, especially if the relationship is maintained over time and with increased sample size (Elias et al. 1993). Earlier studies on serological, electrophoretic and biochemical variability have revealed that the four physiologic races of *F. oxysporum* f. sp. *ciceri* have a close antigenic relationship, common isozyme patterns for catalases, esterases, and peroxidases and also similar proteins; hence, these may not be very useful for characterising individual races (Desai et al. 1992a,b). Nogales Moncada et al. (1993) have reported that all the races of *F. oxysporum* f. sp. *ciceri* are represented by a single vegetative compatibility group.

The utility of DNA markers in detecting polymorphism is well established in several animal, plant and fungal systems (Weising et al. 1991). DNA fingerprinting involves the use of minisatellites and microsatellites, which are hypervariable and dispersed in the form of long arrays of short tandem repeat units throughout the genome (Jeffreys et al. 1985; Tautz and Renz 1984). Since 1988 (Epplen 1988), synthetic oligonucleotides complementary to simple repetitive DNA sequence motifs have been extensively used for multilocus fingerprinting in several systems (Weising et al. 1995). DNA fingerprinting has facilitated the studies of population structure, epidemiology and systematics for fungi pathogenic to plants and animals (Rosewich and Mc Donald 1994). In addition to restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) markers (Hamer et al. 1989; Kistler et al. 1991; Levy et al. 1991; Milgroom et al. 1992), which have been successfully used to estimate the genetic diversity in fungal pathogens, microsatellites have found application in variability analyses in fungi (DeScenzo and Harrington 1994) such as *Ascochyta rabei* (Geistlinger et al. 1997a,b), *Sclerospora graminicola* (Sastry et al. 1995) and *Letosphaeria maculans* (Meyer et al.1992). DNA marker-based studies in *F. oxysporum* f. sp. *ciceri* races have mainly focussed on the use of RAPD-PCR (polymerase chain reaction) and mitochondrial DNA-restriction fragment length polymorphism approaches (Kelly et al.1994; Pérez-Artés et al. 1995). However, there are no reports of any analysis of *F. oxysporum* f. sp. *ciceri* races using microsatellite markers.

In the study reported here we have used simple sequence repeats (di-, tri-, and tetranucleotide repeats), which hybridise to microsatellite loci in the genome, with the objectives to distinguish between the four *F. oxysporum* f. sp. *ciceri* races predominant in India and to assess their potential for DNA fingerprinting. To the best of our knowledge, this is the first report of the use of microsatellite markers for DNA fingerprinting of *F. oxysporum* f. sp. *ciceri* races.

Materials and methods

Sampling of isolates

Single conidial isolates of the four Indian races of *Fusarium oxysporum f.* sp. *ciceri* (races 1, 2, 3, and 4) were used in the present work. The fungi were isolated from roots of chickpea plants showing typical wilt symptoms, and cultures were obtained by isolating single germinating conidia on water-agar medium and subculturing on potato dextrose agar medium. These isolates, collected from various wilt hotspots in India, were further characterised by conventional methods in the Legumes Pathology Laboratory at ICRISAT (International Crops Research Institute for the Semi-Arid Tropics, Patancheru, India) from where they were obtained.

DNA isolation

The fungal cultures were maintained on Potato Dextrose Agar (PDA slants) at 5°C and multiplied on MYG liquid medium (0.3% malt extract, 0.5% yeast extract, 1% glucose) at 28°C, 160 rpm for 48 h. The mycelial mass was harvested by vacuum filtration on sterile Whatmann No.1. filter discs and washed thoroughly in two to three volumes of sterile water. Either fresh or frozen tissue was used for DNA extraction using a modified CTAB DNA extraction protocol. The fungal mycelium, ground to a fine powder using a mortar-pestle, was suspended in a buffer containing 25 m*M* TRIS-HCl pH 8, 10 m*M* EDTA, and 1% BME. EDTA and SDS were added at final concentrations of 60 m*M* and 1%, respectively, and the suspension was incubated at 57°C for 10 min with intermediate mixing. The slurry was reincubated at 57°C for 10 min with NaCl and CTAB (final concentrations of 1.4 *M* and 2%, respectively), followed by two rounds of chloroform:isoamylalcohol (24:1) extractions. The aqueous supernatant was aspirated and precipitated with 0.6 vol. isopropanol. The pellet obtained after centrifugation was dissolved in high salt TE buffer (10 m*M* TRIS-HCl, pH 8.0, 1 m*M* EDTA, 1 *M* NaCl) and reprecipitated with 2.5 vol. absolute EtOH. The pellet was washed with 70% EtOH, dried under vacuum, dissolved in TE buffer (10 mM TRIS-HCl, 1 m*M* EDTA, pH 8.0) and incubated with RNase A at a concentration of 100µg/ml at 37°C for 1.0 h. The concentration of the DNA was estimated by visual comparison with known concentrations of standard bacteriophage lambda DNA (Bangalore Genei, India) by agarose gel electrophoresis.

Microsatellite hybridisation

Fungal DNA (10µg) was digested to completion with several hexa-cutting restriction enzymes, tetra-cutting restriction enzymes and a penta-cutting restriction enzyme according to the manufacturers' instructions (Promega, USA, Bangalore Genei, India). DNA fragments thus obtained were separated by electrophoresis on 0.8% or 1.2% agarose gels run in 1 × TAE buffer (0.04 *M* TRIS acetate; 0.001 *M* EDTA, pH 8.0) depending on the restriction enzyme used. DNA was transferred from the agarose gels to Hybond N membrane (Amersham, UK) using a vacuum blotting apparatus (LKB) as described by Sambrook et al. (1989). In a few cases, gels were dried *in vacuo* and the dried gels were used for in-gel

hybridisations, as described by Ali et al. (1986). Dinucleotides $(AT)_{10}$, $(CT)_{10}$ and $(TG)_{10}$, trinucleotides $(ACA)_5$, $(ACC)_5$, $(ACG)_5$, $(ACT)_5$, $(AGC)_5$,$(AGG)_5$, $(AGT)_5$ and $(ATC)_5$ and tetranucleotides $(GACA)_4$ and $(GATA)_4$, synthesised on a gene assembler plus (Pharmacia), desalted on NAP-5 column and purified on 20% denaturing polyacrylamide gels, were used for the hybridisation studies. Oligonucleotide probes were end-labelled as described by Sambrook et al. (1989) using γ-[^{32}P]-ATP. The DNA blots were used for prehybridisation and hybridisation according to the manufacturers' instructions, while dry gels were used for hybridisation as described by Ali et al. (1986). Hybridisation was carried out overnight in a hybridisation oven at (Tm–5)°C (Miyada and Wallace 1987) by adding a labelled probe (10^7–10^8 cpm/μg DNA) directly to the prehybridisation solution. The blots/dry gels after hybridisation were washed at various stringent conditions, exposed to X-ray films in the presence of intensifying screens and incubated at –70°C. After adequate exposure, autoradiograms were developed.

Analysis of molecular data

Band positions on the autoradiograms were visually determined, and pair-wise comparisons of degree of band sharing were made. Fingerprint patterns were transformed into a binary matrix (1 for presence and 0 for absence of a band at a particular position). Similarity index values were calculated by Nei's method (Nei and Li 1979), as S.I.= 2Nab /Na+Nb, where Na= total number of bands present in lane a, Nb = total number of bands present in lane b, and Nab= number of bands common to lanes a and b. Computer software TAXAN, version 2.0 (D. Swartz, University of Maryland, USA, 1980) was used to calculate genetic distance values from simple matching of bands, and a phenogram was constructed by the unweighted pair group method using arithmetic averages (UPGMA) (Sneath and Sokal 1973). To determine robustness of the dendrogram, we bootstrapped the data with 1000 replications using the computer programme WINBOOT (IRRI, Manila, Philippines). The probability of an identical match by chance (for each enzyme-probe combination) by which the two genotypes would show an identical band pattern was calculated as $(X_D)^n$, where X_D represents the average similarity index value for all pair-wise comparisons and n represents the number of total bands shared per probe (Wetton et al. 1987). $(X_D)^n$ values were then compared to categorise the oligonucleotide probes used.

Results

General features of restriction endonuclease digestion of genomic DNA of *F. oxysporum* f. sp. *ciceri* races and hybridisation with oligonucleotide probes

Eleven restriction enzymes, seven hexa-cutting (*Bam*HI, *Dra*I, *Eco*RI, *Eco*RV, *Hind*III, *Pst*I and *Pvu*II), one penta-cutting (*Hinf*I) and three tetra-cutting enzymes (*Alu*I, *Hae*III, and *Taq*I), were selected out of several restriction enzymes used in the initial trials, as they revealed good DNA digestion for all four races. Genomic DNA digests in the 4- to 23-kb size range were obtained with hexa-cutting enzymes, whereas tetra- and penta-cutting restriction enzymes yielded a smear in the 0.4- to 6.0-kb size range. Genomic DNA digests with *Eco*RV, *Hind*III and *Pvu*II depicted bands superimposed on smears in agarose gels, indicating the presence of repetitive DNA. A digestion smear below 2.0 kb with *Alu*I reflected the abundance of *Alu*I restriction sites in the *F. oxysporum* f. sp. *ciceri* genome.

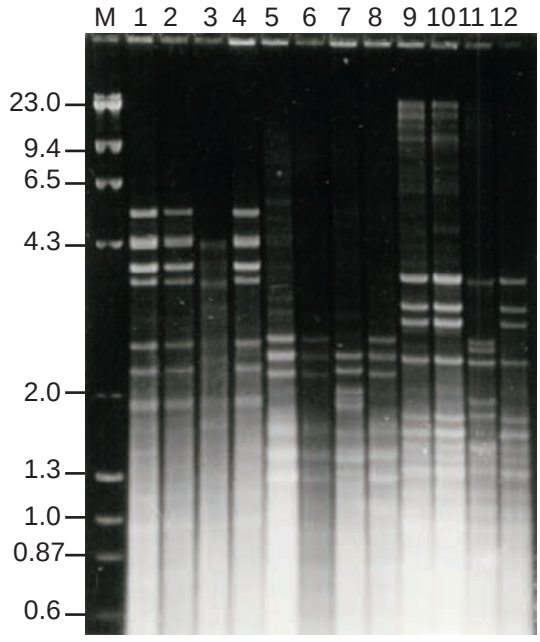

Fig. 1 Agarose gel electrophoresis of genomic DNA digests of *Fusarium oxysporum* f. sp. *ciceri* races 1, 2, 3, and 4 with restriction enzymes *Hae*III, *Hinf*I, and *Taq*I. *Lanes 1–4* Pattern for *Hae*III with races 1, 2, 3 and 4, respectively, *lanes 5–8* and *9–12* patterns for *Hinf*I and *Taq*I, respectively, for races 1, 2, 3 and 4 in the same order, *Lane M* λ/*Hind*III molecular-weight marker (in kilobases)

However, genomic DNA digests of the four *F. oxysporum* f. sp. *ciceri* races revealed discrete bands in the size ranges of 1.8–6.0 kb for *Hae*III, 1.3–2.8 kb for *Hinf*I, and 2.5–4.2 kb for *Taq*I (Fig. 1). In general, the patterns obtained for race 3 were always different from those for the other three races, as exemplified in Fig. 1. For assessing the potential of oligonucleotides as fingerprinting probes, we used 60 enzyme-probe combinations for hybridisation, including three dinucleotide, eight trinucleotide and two tetranucleotide repeats as probes. However, the similarity matrix and dendrogram were constructed using data obtained from 21 such combinations, that exhibited distinct and scorable hybridisation bands. Intense signals were obtained with almost all of the oligonucleotide probes used, indicating the abundance of these repeat motifs in the *F. oxysporum* f. sp. *ciceri* genome. It was interesting to note that some di- and trinucleotide repeats revealed bands superimposed on a smear, whereas other dinucleotide repeats and most of the tri- and tetranucleotide repeats exhibited discrete bands.

Hybridisation patterns with specific enzyme-probe combinations

Hybridisation of genomic DNA digested with *Bam*HI, *Eco*RV, *Hind*III, *Pst*I and *Pvu*II with different oligonucleotide probes yielded bands ranging from 0.7 kb to 21.0 kb, whereas lower molecular-weight fragments,

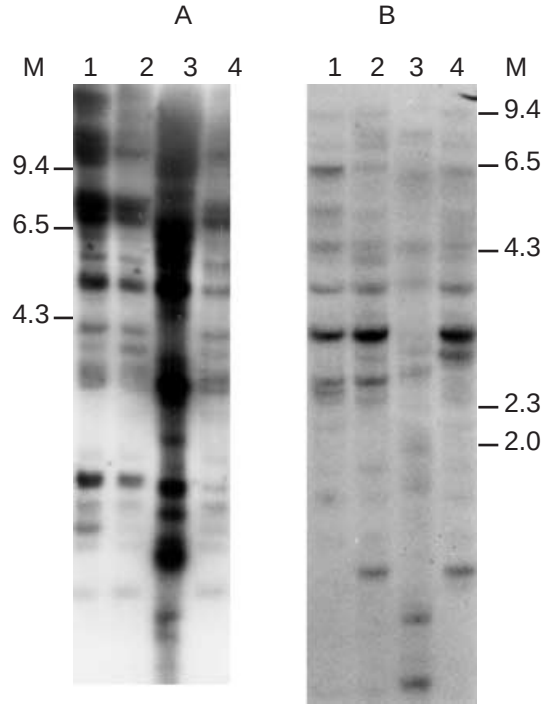

Fig. 2A, B Hybridisation profiles of genomic DNA of *Fusarium oxysporum* f. sp. *ciceri*. *Lanes 1–4* Races 1, 2, 3, and 4 digested with restriction enzyme *Bam*HI and probed with $(TG)_{10}$ (**A**) and digested with *Eco*RV and probed with $(AGT)_5$ (**B**). *Lane M* λ/*Hind*III molecular-weight marker (in kilobases)

ranging from 0.6–2.0 kb were seen with tetra- and penta-cutting restriction enzymes. However, in case of *Hinf*I/$(GATA)_4$, hybridisation bands ranging from 0.6 kb to 5.0 kb were obtained. Figure 2A depicts the hybridisation of $(TG)_{10}$ with *Bam*HI-digested *F. oxysporum* f. sp. *ciceri* genomic DNA, revealing discrete bands (1.7–21.0 kb) superimposed on a smear, the latter persisting even after stringent washes. A higher number of scorable bands can be observed for races 1, 2 and 4 (Fig. 2A, lanes 1, 2 and 4), than for race 3 (Fig. 2A, lane 3). The banding pattern is almost monomorphic for races 1, 2, and 4, whereas bands in race 3 are highly polymorphic. Figure 2B is a representative hybridisation profile of a trinucleotide repeat $(AGT)_5$ with *Eco*RV digests of genomic DNA of races 1 to 4 (lanes 1–4). Prominent hybridisation bands were detected in the 0.7- to 9.4-kb size range for the *Eco*RV digests. The polymorphic bands obtained in *Fusarium oxysporum* f. sp. *ciceri* races indicate the informativeness of such combinations for their fingerprinting. Figure 3A and B represents the hybridisation patterns of *Bam*HI-digested genomic DNAs of four races with $(GATA)_4$ and $(ATC)_5$, respectively, where the same dry gel was used in both cases. Intense signals obtained for race 3/$(ATC)_5$ (Fig. 3B, lane 3) as compared to race 3/$(GATA)_4$ (Fig. 3A, lane 3) reflect the higher frequency of $(ATC)_n$ repeats as compared to $(GATA)_n$ repeats in its genome.

Table 1 summarises the data on number of bands obtained upon hybridisation with various enzyme-probe combinations in the four *F. oxysporum* f. sp. *ciceri* races. In most cases, fewer bands were obtained in race 3 than for the other three races, with only two exceptions, *Bam*HI/$(ATC)_5$ and *Bam*HI/$(AGC)_5$ (Table 1).

Table 1 Number of bands obtained in the four *Fusarium oxysporum* f. sp. *ciceri* races upon hybridisation with various enzyme-probe combinations

Oligo-nucleotide probe	Restriction enzyme	Number of bands obtained per race			
		*Foc*1	*Foc*2	*Foc*3	*Foc*4
$(CT)_{10}$	*Bam*HI	7	7	4	7
	*Eco*RV	11	11	8	13
	*Hind*III	5	8	6	6
$(TG)_{10}$	*Bam*HI	20	18	10	19
	*Hae*III	12	11	11	12
$(ACC)_5$	*Eco*RV	8	8	8	9
$(ATC)_5$	*Eco*RV	7	7	5	7
	*Bam*HI	18	18	19	17
	*Dra*I	10	10	4	10
$(AGC)_5$	*Eco*RV	13	12	9	12
	*Hind*III	14	13	13	14
	*Bam*HI	9	10	12	9
	*Pvu*II	8	10	6	8
$(AGT)_5$	*Hind*III	11	11	4	11
	*Pst*I	12	13	6	13
	*Hae*III	6	6	2	9
	*Dra*I	7	7	6	8
	*Eco*RV	17	18	13	19
	*Pvu*II	18	15	11	17
$(GATA)_4$	*Hinf*I	8	8	5	8
	*Bam*HI	21	20	9	16

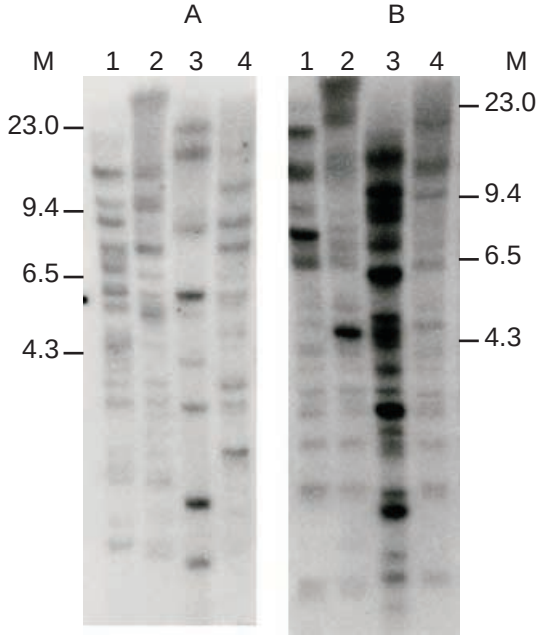

Fig. 3A, B Hybridisation profiles of genomic DNA of *Fusarium oxysporum* f. sp. *ciceri*. *Lanes 1–4* Races 1, 2, 3, and 4 digested with restriction enzyme *Bam*HI and probed with $(GATA)_4$ (**A**) and digested with *Bam*HI and probed with $(ATC)_5$ (**B**). *Lane M* λ/*Hind*III molecular-weight marker (in kilobases)

Table 2 Potential of various oligonucleotide probes used for fingerprinting *Fusarium oxysporum* f. sp. *ciceri* races

Probe	GC content (%)	Enzyme	Average similarity index (X_D)	Average no. of bands per lane (*n*)	Probability of identical match by chance [$(X_D)^n$]
$(CT)_{10}$	50	*Bam*HI	0.4878	6.25	1.12×10^{-2} [a]
		*Eco*RV	0.4866	10.75	4.33×10^{-4} [b]
		*Hind*III	0.4898	6.25	1.15×10^{-2} [a]
$(TG)_{10}$	50	*Bam*HI	0.4893	16.75	6.32×10^{-6} [c]
		*Hae*III	0.5396	11.50	8.29×10^{-4} [b]
$(ACC)_5$	66	*Eco*RV	0.6073	8.25	1.63×10^{-2} [a]
$(AGC)_5$	66	*Eco*RV	0.5000	11.50	3.45×10^{-4} [b]
		*Hind*III	0.4708	13.50	3.83×10^{-5} [c]
		*Bam*HI	0.5080	10.00	1.14×10^{-3} [b]
		*Pvu*II	0.4583	8.00	1.94×10^{-3} [b]
$(AGT)_5$	33.3	*Hind*III	0.4683	9.25	8.96×10^{-4} [b]
		*Pst*I	0.4566	11.00	1.79×10^{-4} [b]
		*Hae*III	0.4488	5.75	9.98×10^{-3} [b]
		*Dra*I	0.4795	7.00	5.82×10^{-3} [b]
		*Eco*RV	0.4950	16.75	7.66×10^{-6} [c]
		*Pvu*II	0.4585	15.25	6.84×10^{-6} [c]
$(ATC)_5$	33	*Eco*RV	0.4705	6.50	7.44×10^{-3} [b]
		*Bam*HI	0.5166	18.00	6.86×10^{-6} [c]
		*Dra*I	0.4666	8.50	1.53×10^{-3} [b]
$(GATA)_4$	25	*Hinf*I	0.4825	7.25	5.07×10^{-3} [b]
		*Bam*HI	0.4796	16.50	5.42×10^{-6} [c]

[a] Least informative enzyme probe combination, $(X_D)^n$ value above 10^{-3}

[b] Less informative enzyme probe combination, $(X_D)^n$ value between 10^{-5} and10^{-3}

[c] Informative enzyme probe combination, $(X_D)^n$ value below 10^{-5}

Fingerprinting with microsatellites identifies the presence of unique bands in the *F. oxysporum* f. sp. *ciceri* races

The 'Probability of identical match by chance' value, $(X_D)^n$, is an indicator of the degree of informativeness of an enzyme-probe combination for fingerprinting the races. In general, the lower the value, the less is the chance that any two races exhibit identical banding patterns with that combination and, hence, the greater the potential of that combination to identify each race discretely. Twenty-one combinations, which yielded clear and scorable banding patterns, were used for calculating the $(X_D)^n$ values. Based on these values, detailed in Table 2, enzyme-probe combinations for fingerprinting of *F. oxysporum* f. sp. *ciceri* races were arbitrarily categorised as (1) informative [$(X_D)^n$ value below 10^{-5})], (2) less informative [$(X_D)^n$ values between 10^{-3} and 10^{-5}] and (3) least informative [$(X_D)^n$ values above 10^{-3}]. Oligonucleotides $(TG)_{10}$, $(AGT)_5$, $(ATC)_5$ and $(GATA)_4$ revealed low $(X_D)^n$ values with many (see table) of the enzymes used in the present study and hence qualify as the best probes for fingerprinting of *F. oxysporum* f. sp. *ciceri* races (Table 2). $(AGC)_5$ revealed intermediate fingerprinting potential, while $(CT)_{10}$ and $(ACC)_5$ were uninformative as fingerprinting probes. Although the $(X_D)^n$ values were low for the *Bam*HI/$(TG)_{10}$ and *Hae*III/$(TG)_{10}$ combinations (6.32×10^{-6} and 8.3×10^{-4}, respectively), $(TG)_{10}$ is not a good fingerprinting probe for the *F. oxysporum* f. sp. *ciceri* races due to the grossly monomorphic banding pattern obtained for races 1, 2 and 4 (Fig. 2A). However, the low $(X_D)^n$ values obtained for the *Bam*HI/$(TG)_{10}$ and *Hae*III/$(TG)_{10}$ combinations could be due to the unique banding patterns of race 3 as compared to the other races (Fig. 2A).

Hybridisation profiles were further analysed for the presence of bands unique to *F. oxysporum* f. sp. *ciceri* races, as a step towards the identification of race-diagnostic markers. Preliminary studies revealed the presence of strong race 2 specific bands with $(ACG)_5$ and $(ATC)_5$. The molecular weights of these bands calculated using the SEQUAID II(T_M) version 3.5 programme (Kansas State University, USA, 1989) were 10.3, 2.5 and 12 kb when $(ACG)_5$ was hybridised to *Bam*HI, *Eco*RV and *Hind*III DNA digests, respectively, and 4.6, 6.3, and 7.1 kb, when $(ATC)_5$ was hybridised to *Bam*HI, *Eco*RV and *Hind*III digests in the same order. The band specific to race 1 with *Bam*HI/$(ATC)_5$ was approximately 7.2 kb, while the bands unique to race 3 were approximately 5.9, 3.4 and 2.5 kb, respectively (Fig. 3B). Similarly, a few unique bands observed with $(AGT)_5$/hexa-cutting restriction enzyme combinations could be potential candidates for furthering research towards the development of race-diagnostic markers for *F. oxysporum* f. sp. *ciceri*. However, in our analysis, no bands specific to race 4 were identified.

Genetic relationship among the four *Fusarium oxysporum* f. sp. *ciceri* races

Virulence reactions of individual *Fusarium oxysporum* f. sp. *ciceri* races are known to differ with different chickpea hosts. A knowledge of virulence-related differences in pathogenic races at the molecular level is important for breeding wilt-resistant chickpea varieties. Examination of genetic relatedness in the four races is a step towards this objective. Microsatellite markers, being multilocus probes, facilitate such studies as compared to hybridisation-based single locus markers like RFLPs. A

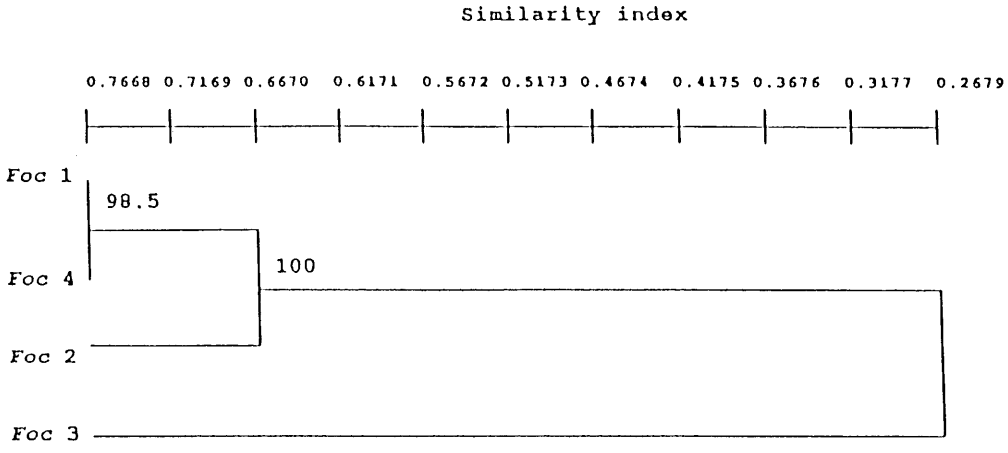

Fig. 4 Phenogram depicting the relationship between *Fusarium oxysporum* f. sp. *ciceri* races 1, 2, 3 and 4 (*Foc*1, 2, 3 and 4) based on microsatellite fingerprinting analysis using the UPGMA algorithm as described in the text. Bootstrap values calculated using the WINBOOT programme are shown at the nodes

Table 3 Enzyme-probe combinations showing relatedness in the standard races of *Fusarium oxysporum* f. sp. *ciceri* (*Foc*)[a] as % similarity values (based on band sharing)

*Foc*1 and 4	*Foc*1 and 2	*Foc*2 and 4	Others (*Foc*1, 2 & 4)
*Hinf*I/$(GATA)_4$ 78.9	*Hind*III/$(AGT)_5$ 72.0	*Pst*I/$(AGT)_5$ 70.4	*Eco*RV/$(CT)_{10}$ 76.0
*Eco*RV/$(ATC)_5$ 76.4	*Eco*RV/$(ACC)_5$ 100[b]	*Eco*RV/$(AGC)_5$ 100[c]	
*Hind*III/$(AGC)_5$ 76.4	*Hae*III/$(AGT)_5$ 69.0	*Dra*I/$(ATC)_5$ 70.0	
*Bam*HI/$(GATA)_4$ 63.4	*Pvu*II/$(AGT)_5$ 80.5	*Bam*HI/$(CT)_{10}$ 85.7	
*Bam*HI/$(AGC)_5$ 90.4		*Eco*RV/$(AGT)_5$ 85.2	
*Bam*HI/$(ATC)_5$ 87.5		*Bam*HI/$(TG)_{10}$ 96.8	
*Dra*I/$(AGT)_5$ 81.2		*Hae*III/$(TG)_{10}$ 95.2	
*Pvu*II/$(AGC)_5$ 62.0			
*Hind*III/$(CT)_{10}$ 93.0			

[a] *Foc*3 has not been considered here as it always shows a different pattern from the other races
[b] For *Eco*RV/$(ACC)_5$, races 1 and 2 are identical and related to race 4 at 92.8%
[c] For *Eco*RV/$(AGC)_5$, races 2 and 4 are identical and related to race 1 at 95%

more complete picture of relatedness of the four races can be obtained by compiling data from individual enzyme-probe combinations to create a superimposed dendrogram. Therefore, similarity index values from the hybridisation data of the 21 enzyme-probe combinations were averaged, and a dendrogram was constructed (Fig. 4). Bootstrapping of this dendrogram using the WINBOOT software indicated very high (95–100%) bootstrap values, thereby confirming the robustness of the dendrogram. Analysis of the dendrogram revealed that *F. oxysporum* f. sp. *ciceri* races 1 and 4 are closely related (similarity index value of 76.6%) – this group is then related to race 2 (similarity index value of 67.3%) – whereas race 3 is distinct from them (similarity index value of 26.7%). These results corroborate well with cultural characteristics and the symptoms caused by race 3 on chickpea lines, which are different from those of the other three races (Haware, unpublished)

Organisation of microsatellite repeats in the four races

Analysis of the distribution of individual microsatellite repeats in *F. oxysporum* f. sp. *ciceri* races can be instrumental in identifying potential race-specific markers. The hybridisation data and dendrograms obtained for individual enzyme-probe combinations were, therefore, analysed and the combinations classified based on the closest pair of races denoted by that combination (Table 3). The comparison was made only among races 1, 2 and 4 because race 3 always showed a completely distinct pattern with almost each enzyme-probe combination studied. Races 1 and 4 were the closest, with more combinations, however, either 1 oligonucleotide probe in combination with different restriction enzymes or 1 restriction enzyme in combination with different probes revealed various levels of similarities in *F. oxysporum* f. sp. *ciceri* races 1, 2 and 4 (Table 3). This could be due to different loci in the *F. oxysporum* f. sp. *ciceri* genome getting highlighted in each enzyme-probe hybridisation profile. $(AGT)_5$ exhibited different similarity values among races 1, 2 and 4 with different restriction enzymes, indicating the varied organisation of $(AGT)_n$ repeats in the *F. oxysporum f. sp. ciceri* genome, thus making it a candidate probe to identify each race individually (Table 3). However, the *Eco*RV/$(ACC)_5$ combination indicated that races 1 and 2 are identical and related to race 4 at a high similarity value of 92.8% (Table 3). Similarly, the *Eco*RV/$(AGC)_5$ combination revealed that races 2 and 4 are identical and related to race 1 at a 95% similarity val-

ue (Table 3), indicating that the distribution of $(ACC)_5$ and $(AGC)_5$ repeats with the *Eco*RV sites flanking them may be highly conserved in the three *F. oxysporum* f. sp. *ciceri* races.

Discussion

F. oxysporum f. sp. *ciceri*, one of the most important fungal pathogens of chickpea, damages annually between 10% and 100% of the plants sown, resulting in reduced crop production. Haware and Nene (1982) classified the isolates of *F. oxysporum* f. sp. *ciceri* into races based on their virulence reactions to a set of chickpea differential cultivars. The prevalence of different races all over the world makes it essential to identify region-specific races in order to devise strategies for conferring resistance against them in the respective areas. Previous attempts to study variability in different races, based on biochemical and serological parameters (Desai et al. 1992a,b) as well as determination of vegetative compatibility groups, (Nogales-Moncada et al. 1993) revealed a lack of correlation between physiological races and the variations observed.

Various hybridisation-based approaches, including RFLP with endogenous single-locus probes (Manicom et al. 1990; McDonald and Martinez 1991), RFLP with endogenous multilocus probes (Hamer et al. 1989; Kistler et al. 1991), RFLP with minisatellite probes (Meyer et al. 1991), RFLP with multilocus oligonucleotide probes (Kaemmer et al. 1992; Meyer et al. 1991; Morjane et al. 1994; Weising et al. 1991), PCR-based approaches including RAPD (Fischer et al. 1995; Jungehulsing and Tudzynski 1997), DNA amplification fingerprinting (Kaemmer et al. 1992), microsatellite primed PCR (Geistlinger et al. 1997a; Hantula et al. 1996) and amplified fragment length polymorphisms (AFLPs) (Julian et al. 1999; Van der Lee et al. 1997) have been used for the characterisation of DNA polymorphism in fungal genomes.

Similar studies on other plant pathogenic fungi have focussed on the use of molecular approaches to characterise genetic diversity within and between different isolates, as exemplified in the cases of the rice pathogen *Magnaporthe grisea* (Hamer et al. 1989; Levy et al. 1991), the barley pathogen *Erysiphe graminis* (Brown et al. 1990), the wheat pathogen *Septoria tritica* (McDonald and Martinez 1991) and the chickpea pathogen *Ascochyta rabei* (Kaemmer et al. 1992; Morjane et al. 1994; Weising et al. 1991). Molecular approaches have also been employed for variability analysis of *Fusarium oxysporum* f. sp. *ciceri*. However, the mitochondrial DNA-RFLP approach has not been able to distinguish variations related to the races (Pérez-Artés et al. 1995). Although the use of RAPD markers (Kelly et al. 1994) can distinguish wilt-inducing isolates of *F. oxysporum* f. sp. *ciceri* from the non-wilt-inducing isolates (causing yellowing symptoms), it has not been possible to further dissect this cluster of wilt-inducing isolates. Such attempts have emphasised the need for additional molecular approaches to further decipher variations in the wilt-inducing *F. oxysporum* f. sp. *ciceri* isolates. The use of microsatellite markers in our analysis enabled us to distinguish the four races of *F. oxysporum* f. sp. *ciceri* causing varied levels of wilting with differential host (chickpea) cultivars. Not only race 3, which is distinct from the other races, but also races 1, 2 and 4 can be differentiated based on simple-sequence repeat (SSR) hybridisations carried out in our analysis. Although the potential of this marker system to reveal variations among the four races of *F. oxysporum* f sp. *ciceri* has been demonstrated, investigations on additional isolates from each race should further support our analysis. DNA fingerprinting using microsatellite markers has been carried out in several plant pathogens, including the downy mildew pathogen *Sclerospora graminicola* (Sastry et al. 1995), the chickpea blight pathogen *Ascochyta rabei* (Kaemmer et al. 1992; Weising et al. 1991) and *Letosphaeria maculans* (Meyer et al. 1992). Although the human minisatellite PV47 could distinguish a particular pathotype in *Sclerospora graminicola*, fingerprints generated using the oligonucleotide $(GATA)_4$ were the most effective in distinguishing the six isolates examined by Sastry et al. (1995), and these were stable even after ten asexual generations of the pathogen. Also, repetitive DNA probes from a genomic library were unable to detect polymorphism in the downy mildew pathogen unlike in *Magnaportha grisea*, in which they are the main source of polymorphism (Sastry et al. 1995). In the chickpea blight pathogen *Ascochyta rabiei*, Weising et al. (1991) reported the detection of variation among isolates using DNA fingerprinting with oligonucleotides $(GATA)_4$, $(GTG)_5$, $(CA)_8$ and $(TCC)_5$. Geistlinger et al. (1997a,b) used oligonucleotides to study the variability between *A. rabiei* isolates from different geographical regions and mating types to find suitable mating partners for the production of a mapping population. They further studied the Mendelian inheritance of microsatellite markers in progeny of a sexual cross between two mating types (Geistlinger et al. 1997b). In filamentous fungi such as *Penicillium*, *Aspergillus*, and *Trichoderma*, oligonucleotide probes such as $(GATA)_4$ along with the M13 minisatellite probe have revealed informative DNA fingerprint patterns (Meyer et al. 1991). The present study has deployed the use of reliable, multilocus, hypervariable oligonucleotide probes complementary to microsatellite loci to distinguish pathogenic races in *F. oxysporum* f. sp. *ciceri*.

Informativeness of the enzymes and probes used

Based on the analysis of data from the 21 enzyme-probe combinations used in our study, we deduced that the fingerprint profiles were dependent on both the restriction enzymes, and the microsatellite repeats used for hybridisation and that any one of these two components alone was not that informative. The enzyme-probe combinations, based on their potential to reveal polymorphism among the four *F. oxysporum* f. sp. *ciceri* races, could be

categorised as informative, less informative and least informative combinations. Our analysis revealed that oligonucleotide probes with a low GC content were more informative than those with a higher GC content, since the most informative probes, namely $(AGT)_5$, $(ATC)_5$ and $(GATA)_4$, had a GC content below 40% (Table 2). Our results are in agreement with those of Geistlinger et al. (1997b) who reported that oligonucleotides with a higher AT content reveal more polymorphic banding patterns than oligonucleotides with a lower AT content. In our studies, more distinct, polymorphic banding patterns were obtained with most of the hexa-cutting enzymes as compared to tetra- and penta-cutting enzymes. In contrast, *Ascochyta* fingerprinting patterns obtained with tetra-cutting restriction enzymes were more informative and easier to screen than those generated by the hexa-cutting enzyme *Eco*RI (Geistlinger et al. 1997b).

Another interesting observation was that genomic DNA digestion of the four races with *Hae*III, *Hinf*I and *Taq*I produced discrete bands in the molecular-weight ranges 2.2–6.0 kb, 2.2–2.8 kb, and 2.5–4.2 kb, respectively (Fig. 1). However, $(TG)_{10}$, $(AGT)_5$ and $(GATA)_4$ did not hybridise to any of the above mentioned bands, as they produced signals that were smaller than 2.0 kb. This indicated that the discrete bands produced by *Hae*III, *Hinf*I, and *Taq*I digestion of *F. oxysporum* f. sp. *ciceri* genomic DNA did not contain microsatellites $(TG)_{10}$, $(AGT)_5$ and $(GATA)_4$. However, they could represent other microsatellites not included in our studies or repetitive DNA, such as satellite DNA, ribosomal DNA repeats, mitochondrial DNA (mtDNA) repeat sequences, etc. This can only be confirmed by using these bands individually as probes.

Relationships among the races at the molecular level

One of the important applications of DNA fingerprinting is to differentiate pathogenic strains from each other and from the non-pathogenic strains. In the cases of fungi with a defined race concept, DNA markers can be used to analyse genetic relationships among the races (Hong et al. 1996; Woo et al. 1996). Our data revealed that *F. oxysporum* f. sp. *ciceri* races 1 and 4 were genetically more similar to each other than to race 2, while race 3 was distinct. Similar results based on a 6.3-kb rDNA probe have been presented by Mukherjee et al. (1997). This observation of similarity between races 1 and 4 is significant because of their geographic distribution and varied virulence characteristics. Race 1 is widespread throughout the country, while race 4 is prevalent in localised regions of the Punjab State of India. Hence, the genomic regions in the two races that we detected probably do not include virulence loci.

Another interesting feature evident from our study is the distinctness of race 3 as revealed by its unique banding patterns and relatively fewer hybridisation signals as compared to the other races (Table 1). This implied a relatively simpler distribution of microsatellite repeats in the race 3 genome. In a similar study on *Fusarium oxysporum* f. sp. *pisi* using RAPD markers, Grajal-Martin et al. (1993) reported race 2 to be very different from isolates of races 1, 5 and 6.

Is pathogenicity related to variation in the mtDNA sequence?

Several hybridisation-based markers have been used in plant pathogenic fungi to link aggressiveness or pathogenicity with DNA polymorphism (Meyer et al. 1992; Weising et al. 1991). Pérez-Artés et al. (1995) demonstrated the absence of mtDNA-RFLPs in seven races of *F. oxysporum* f. sp. *ciceri* reported worldwide on the basis of the digestion of 40.5-kb mtDNA fragment with eight restriction enzymes. The identical restriction pattern for the seven races indicated extensive conservation in the gene composition of the mtDNA of the races, suggesting that it might not be responsible for pathogenic diversity observed (Pérez-Artés et al. 1995). In contrast to this, mitochondrial RFLPs were reported in *F. oxysporum* f. sp. *niveum* (Kim et al. 1991, 1992) and *F. oxysporum* f. sp. *melonis* (Jacobson and Gordon 1990), though these were not correlated with pathological race or geographic region of origin. Reports on genomic DNA digestions of *Fusarium* and other genera with *Hae*III suggest that small DNA fragments resulting in a smear were derived from nuclear DNA, while the remaining bands most exclusively represented mtDNA fragments (Bridge et al. 1993). Bridge et al. (1993) correlated such mtDNA polymorphism obtained upon *Hae*III digestion with vegetative compatibility groups. The molecular size range of the bands obtained upon *Hae*III digestions of *F. oxysporum* f. sp. *ciceri* genomic DNA in our studies was similar to that obtained by Bridge et al. (1993). It is possible that these fragments correspond to mtDNA. If these bands are indeed mtDNA fragments, then the variation in banding pattern observed for race 3 (Fig. 1) may have some correlation with pathogenic diversity for this race. Although this needs to be analysed further using both mtDNA-specific, as well as virulence-related probes, such variations upon total DNA restriction with *Hae*III have been correlated to a differentiation of two races, namely races 1 and 4 of *F. oxysporum* f. sp. *cubense*, by Thomas et al. (1994).

In summary, the informativeness of the fingerprints obtained for *F. oxysporum* f. sp. *ciceri* was dependent on the enzyme used for DNA digestion as well as the oligonucleotide probe used for hybridisation. The enzyme-probe combinations revealed various levels of polymorphism, and based on these they were classified into three categories. Based on the fingerprinting data, *F. oxysporum* f. sp. *ciceri* race 3 was very different from the other three races. Races 1 and 4, though closely related, could be distinguished from each other using the microsatellites $(AGT)_5$, $(ATC)_5$ and $(GATA)_4$. Thus, our analysis could distinguish each of the four Indian races of *F. oxysporum* f. sp. *ciceri* and represents an important step to-

wards developing race-diagnostic markers for *Fusarium oxysporum* f. sp. *ciceri*.

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References

Ali S, Müller CR, Epplen JT (1986) DNA fingerprinting by oligonucleotide probes specific for simple repeats. Hum Genet 74:239–243

Bridge PD, Ismail MA, Rutherford MA (1993) An assessment of aesculin hydrolysis, vegetative compatibility and DNA polymorphism as criteria for characterising pathogenic races within *Fusarium oxysporum* f. sp. *vasinfectum*. Plant Pathol 42:264–269

Brown JKM, O'dell M, Simpson CG, Wolfe MS (1990) The use of DNA polymorphisms to test hypotheses about a population of *Erysiphe graminis* f. sp. *hordei*. Plant Pathol 39:391–401

Desai S, Nene YL, Jambunathan R, Ramachandra Reddy AG (1992a) Races of *Fusarium oxysporum* causing wilt in chickpea: biochemical variability. Indian Phytopathol 45:62–65

Desai S, Nene YL, Ramachandra Reddy AG (1992b) Races of *Fusarium oxysporum* causing wilt in chickpea – serological and electrophoretic variability. Indian Phytopathol 45:421–425

DeScenzo RA, Harrington TC (1994) Use of $(CAT)_5$ as a DNA fingerprinting probe for fungi. Phytopathology 84:534–540

Elias KS, Zamir D, Lichtman-Pleban T, Katan T (1993) Population structure of *Fusarium oxysporum* f. sp. *lycopersici*: restriction fragment length polymorphisms provide genetic evidence that vegetative compatibility group is an indicator of evolutionary origin. Mol Plant-Microbe Interaction 6:565–572

Epplen JT (1988) On simple repeated GATA/GACA sequences in animal genomes: a critical reappraisal. J Hered 79:409–417

FAO (1993) FAO yearbook production 1992, vol. 46. FAO, Rome, Italy, pp 105–115

Fischer C, Porta-Puglia A, Barz W (1995) RAPD analysis of pathogenic variability in *Ascochyta rabiei*. J Phytopathol 143: 601–607

Geistlinger J, Maqbool S, Kaiser WJ, Kahl G (1997a) Detection of microsatellite fingerprint markers and their Mendelian inheritance in *Ascochyta rabei*. Mycol Res 101:1113–1121

Geistlinger J, Weising K, Kaiser WJ, Kahl G (1997b) Allelic variation at a hypervariable compound microsatellite locus in the ascomycete *Ascochyta rabiei*. Mol Gen Genet 256:298–305

Grajal-Martin MJ, Simon CJ, Muehlbauer FJ (1993) Use of random amplified polymorphic DNA to characterise race 2 of *Fusarium oxysporum* f. sp. *pisi*. Phytopathology 83:612–614

Hamer JE, Farrall L, Orbach MJ, Valent B, Chumley FG (1989) Host species-specific conservation of a family of repeated DNA sequences in the genome of a fungal plant pathogen. Proc Natl Acad Sci USA 86:9981–9985

Hantula J, Dusabenyagasani M, Hamelin RC (1996) Random amplified microsatellites (RAMS) a novel method for characterizing genetic variation within fungi. Eur J For Pathol 26: 159–166

Haware MP, Nene YL (1982) Races of *Fusarium oxysporum* f. sp. *ciceri*. Plant Dis 66:809–810

Haware MP, Nene YL, Natarajan M (1996) Survival of *Fusarium oxysporum* f. sp. *ciceri* in soil in the absence of chickpea. Phytopathologie Mediterranea 35:9–12

Haware MP, Jiménez Díaz RM, Amin KS, Phillips JC, Halila H (1990) Integrated management of wilt and root rots of chickpea. In: Chickpea in the nineties: Proc 2nd Int Workshop Chickpea Improve. ICRISAT Center, Patancheru, India ICRISAT Publication, pp 129–133

Haware MP, Nene YL, Pundir RPS, Narayana Rao J (1992) Screening of world chickpea germplasm for resistance to Fusarium wilt. Field Crops Res 30:147–154

Hong SM, Kang KY, Kim NS, Kang SW, Kim HK (1996) Random amplified polymorphic DNA and restriction fragment length polymorphism analysis to differentiate races of the rice blast fungus, *Pyricularia oryza*, in Korea. Mol Cells 6:346–351

Jacobson DJ, Gordon TR (1990) Variability of mitochondrial DNA as an indicator of relationships between populations of *Fusarium oxysporum* f. sp. *melonis*. Mycol Res 94:734–744

Jeffreys AJ, Wilson V, Thein SL (1985) Hypervariable ''minisatellite'' regions in the human DNA. Nature 314:67–73

Jiménez-Díaz RM, Trapero-Casas A, Cabrera de la Colina J (1989) Races of *Fusarium oxysporum* f. sp. *ciceri* infecting chickpeas in Southern Spain. In: Tjamos EC, Beckman CH (eds) Vascular wilt diseases of plants. NATO ASI series H, vol. 28, pp 515–520

Julian MC, Acero J, Salazar O, Keijer J, Rubio V (1999) Mating type-correlated molecular markers and demonstration of heterokaryosis in the phytopathogenic fungus *Thanatephorus cucumeris* (*Rhizoctonia solani*) AG 1-IC by AFLP DNA fingerprint analysis. J Biotechnol 67:49–56

Jungehülsing U, Tudzynski P (1997) Analysis of genetic diversity in *Claviceps purpurea* by RAPD markers. Mycol Res 101:1–6

Kaemmer D, Ramser J, Schön M, Weigand F, Saxena MC, Driesel AJ, Kahl G, Weising K (1992) DNA fingerprinting of fungal genomes: a case study with *Ascochyta rabiei*. Adv Mol Genet 5:225–270

Kaiser WJ, Alcalá-Jiménez AR, Hervás-Vargas A, Trapero-Casas JL, Jiménez-Díaz RM (1994) Screening of wild *Cicer* species for resistance to races 0 and 5 of *Fusarium oxysporum* f. sp. *ciceri*. Plant Dis 78:962–967

Kelly A, Alcalá-Jiménez AR, Bainbridge BW, Heale JB, Pérez-Artés E, Jiménez-Díaz RM (1994) Use of genetic fingerprinting and random amplified polymorphic DNA to characterise pathotypes of *Fusarium oxysporum* f. sp. *ciceris* infecting chickpea. Phytopathology 84:1293–1298

Kim DH, Martyn RD, Magill CW (1991) Mitochondrial DNA restriction fragment length polymorphisms in *Fusarium oxysporum* f. sp. *niveum*. Phytoparasitica 19:211–223

Kim DH, Martyn RD, Magill CW (1992) Restriction fragment length polymorphism groups and physical map of mitochondrial DNA from *Fusarium oxysporum* f. sp. *niveum*. Phytopathology 82:346–353

Kistler HC, Momol EA, Benny U (1991) Repetitive genomic sequences for determining relatedness among strains of *Fusarium oxysporum*. Phytopathology 81:331–336

Levy M, Romao J, Marchetti MA, Hamer JE (1991) DNA fingerprinting with a dispersed repeated sequence resolves pathotype diversity in the rice blast fungus. Plant Cell 3:95–102

Manicom BQ, Bar-Joseph M, Kotze JM, Becker MM (1990) A restriction fragment length polymorphism probe relating vegetative compatibility groups and pathogenicity in *Fusarium oxysporum* f. sp. *dianthi*. Phytopathology 80: 336–339

McDonald BA, Martinez JP (1991) DNA fingerprinting of the plant pathogenic fungus *Mycosphaerella graminicola* (anamorph *Septoria tritici*) Exp Mycol 15:146–158

Meyer W, Koch A, Niemann C, Beyermann B, Epplen JT, Börner T (1991) Differentiation of species and strains among filamentous fungi by DNA fingerprinting. Curr Genet 19:239–242

Meyer W, Lieckfeldt E, Wöstemeyer J, Börner T (1992) DNA fingerprinting for differentiating aggressivity groups of the rape seed pathogen *Letosphaeria maculans*. Mycol Res 96:651–657

Milgroom MG, Lipari SE, Powell WA (1992) DNA fingerprinting and analysis of population structure in the chestnut blight fungus *Cryphonectria parasitica*. Genetics 131:297–306

Miyada CG, Wallace RB (1987) Oligonucleotide hybridisation techniques. Methods in Enzymol 154:94–107

Morjane H, Geistlinger J, Harrabi M, Weising K, Kahl G (1994) Oligonucleotide fingerprinting detects genetic diversity among *Ascochyta rabei* isolates from a single chickpea field in Tunisia. Curr Genet 26:191–197

Mukherjee PK, Bhagwat A, Mitra J, Mitra R, Murthy NBK (1997) Restriction analysis of ribosomal DNA: a tool for differentiating between races of *Fusarium oxysporum* f. sp. *ciceris*. In: Indian Phytopathological Society – Golden Jubilee, Int Conf Integrated Plant Dis Manage Sustainable Agric. New Delhi, India, p 43 (abstr)

Nei M, Li WH, (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc Natl Acad Sci USA 76:5269–5273

Nene YL, Sheila VK, Sharma SB (1989) A world list of chickpea (*Cicer arietinum* L.) and pigeonpea (*Cajanus cajan* L.) Millsp. pathogens. ICRISAT legumes pathology progress report vol. 7, Patancheru, India

Nogales-Moncada AM, Pérez-Artés E, Jiménez-Díaz RM (1993) VCG diversity within *Fusarium oxysporum* f. sp. *ciceri*. In: 6th Int Conf Plant Pathol. International Plant Pathology Congress, Montreal, Canada, p 169 (abstr)

Ouellett T, Seifert KA (1993) Genetic characterisation of *Fusarium graminaem* strains using RAPD and PCR amplification. Phytopathology 83:1003–1007

Pérez-Artés E, Roncero MIG, Jiménez-Díaz RM (1995) Restriction fragment length polymorphism analysis of the mitochondrial DNA of *Fusarium oxysporum* f. sp. *ciceris*. J Phytopathol 43:105–109

Phillips JC (1988) A distinct race of chickpea wilt in California. Int Chickpea Newsl 18:19–21

Puhalla JE (1985) Classification of strains of *Fusarium oxysporum* on the basis of vegetative compatibility. Can J Bot 63:179–183

Rosewich UL, McDonald BA (1994) DNA Fingerprinting in Fungi Methods Mol Cell Biol 5:41–48

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, New York

Sastry JG, Ramakrishna W, Sivaramakrishnan S, Thakur RP, Gupta VS, Ranjekar PK (1995) DNA fingerprinting detects genetic variability in the pearl millet downy mildew pathogen *Sclerospora graminicola*. Theor Appl Genet 91:856–861

Singh KB (1997) Chickpea (*Cicer arietinum* L.) Field Crops Res 53:161–170

Singh KB, Saxena MC (1992) Disease resistance in chickpea. ICARDA, Aleppo, Syria

Sneath PHA, Sokal RR (1973) Numerical taxonomy. WH Freeman and Co, San Fransisco

Snyder WC, Hansen HN (1940) The species concept in *Fusarium*. Am J Bot 27:64–67

Tautz D, Renz M (1984) Simple sequences are ubiquitous repetitive components of eukaryotic genomes. Nucleic Acids Res 12: 4127–4138

Thomas V, Rutherford MA, Bridge PD (1994) Molecular differentiation of two races of *Fusarium oxysporum* special form *cubense*. Lett Appl Microbiol 18:193–196

Van der Lee T, De Witte I, Drenth A, Alfonso C, Govers F (1997) AFLP linkage map of the oomycete *Phytophthora infestans*. Fungal Genet Biol 21:278–291

Weising K, Kaemmer D, Epplen JT, Weigand F, Saxena M, Kahl G (1991) DNA fingerprinting of *Ascochyta rabei* with synthetic oligodeoxynucleotides. Curr Genet 19:483–489

Weising K, Ramser J, Kaemmer D, Kahl G, Epplen JT (1991) Oligonucleotide fingerprinting in plants and fungi. In: Burke T, Dolf G, Jeffreys AJ, Wolff R (eds) DNA fingerprinting: Approaches and Applications. Birkhâuser, Basel, pp 312–319

Weising K, Nybom H, Wolff K, Meyer W (1995) Genetic Variation at the DNA Level. In: Arbor A (ed) DNA fingerprinting in plants and fungi. CRC Press, Boca Raton, Fla., pp 13–15

Wetton JH, Carter RE, Parkin DT, Walters D (1987) Demographic study of a wild sparrow population by DNA fingerprinting. Nature 327:147–149

Woo SL, Zoina A, Del Sorbo G, Lorito M, Nanni B, Scala F, Noviello C (1996) Characterization of *Fusarium oxysporum* f. sp. *phaseoli* by pathogenic races, VCGs, RFLPs, and RAPD. Phytopathology 86:966–973