decayed and fungus-rotten) or other reasons that need to be investigated further.

In the present study, we have optimized reaction conditions through few modifications, including redesigning the primers (Agd3 and Agd4) used in the diagnostic PCR assay of Martinez-Torres *et al.*[2]. Primers specific to *An. culicifacies* were designed (AcAgd3 and AcAgd4) based on the mismatches observed in the Agd3 and Agd4 region of the para-type sodium channel gene sequence of *An. culicifacies* in comparison with that of *An. gambiae*. This PCR assay has potential in the detection of *kdr* resistance among *An. culicifacies* populations in India, but it needs to be further tested on a larger number of samples against each species of *Anopheles*. Since *kdr* mechanism of DDT resistance appears to be operating only among a part of the population, there is a need to understand other mechanisms conferring pyrethroid/DDT resistance among them and develop multiplex PCR assays.

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Pathogenic and molecular characterization of Indian isolates of *Fusarium oxysporum* f. sp. *ciceris* causing chickpea wilt

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Wilt (*Fusarium oxysporum* f. sp. *ciceris*) is considered as one of the major factors for low productivity of chickpea (*Cicer arietinum*). The present study was conducted to determine pathogenic and genetic variability of isolates of *F. oxysporum* f. sp. *ciceris* collected from different parts of India. Pathogenic virulence study of 25 isolates of the pathogen on international set of differential cultivars revealed the existence of three new races of the pathogen in India, besides characterization of new isolates in the known four races of the pathogen. Genetic variability within 24 isolates representing seven races of *F. oxysporum* f. sp. *ciceris* was assessed by RAPD, with a set of 40 ten-mer primers. UPGMA cluster analysis divided the isolates into seven distinct clusters at 0.55 genetic similarities. The most virulent isolate obtained from wilt sick field of IARI (MB-4C), New Delhi was distinct from others.

Keywords: Chickpea, Indian isolates, pathogens, wilt.

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CHICKPEA (*Cicer arietinum* L.) is one of the most important pulse crops cultivated in many countries of Asia and Africa. In addition to its importance as a food crop, it is valued for its beneficial effects in improving soil fertility and thus sustainability and profitability of production systems[1]. Fusarium wilt caused by *Fusarium oxysporum* f. sp. *ciceris* (Padwick) Matuo & K. Sato, is one of the major factors limiting chickpea production worldwide[2]. The disease is widespread in chickpea-growing areas of the world and is reported from at least 33 countries[3], causing 10–15% annual losses[4].

The use of resistant cultivars is one of the most practical and cost-effective strategies for managing *Fusarium* wilt, but deployment of resistant varieties has not been extensive because of undesirable agronomic characteristics[5]. Moreover, the high pathogenic variability in *F. oxysporum* f. sp. *ciceris* may limit the effectiveness of resistance[6,7]. Pathotypes have been differentiated into two groups based on the distinct yellowing or wilting syndromes[8].

Presently, 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[6,7]. Races 0 and 1B/C induce yellowing symptoms, whereas the remaining races induce wilting[9–11]. The eight races have distinct geographic distribution. Races 1–4 have been reported from India, whereas 0, 1B/C, 5 and 6 are found in the Mediterranean region and USA[10]. Identification of pathogenic races has been mostly by use of differential reaction to selected host genotypes. Several biotechnological tools like RAPD, RFLP and SSR have been increasingly used to study variability in pathogenic population of *F. oxysporum* f. sp. *ciceris*[1,10–12].

Race-specific RAPD markers were cloned and sequenced, and primers for specific PCR were developed that differentiated races 0, 1A, 5 and 6, occurring in the Mediterranean region[12]. Races causing yellowing symptoms are not prevalent in India. An attempt was made by Sivaramakrishnan *et al.*[1] to establish genetic variability in wilt-causing Indian races of *F. oxysporum* f. sp. *ciceris*[1]. They observed high level of DNA polymorphism and suggested the rapid evolution of new recombinants of the pathogen in chickpea-growing fields. Study about structure of pathogen population is pre-requisite for designing a cost effective management strategy for such a devastating disease. Since this disease is primarily managed by use of resistant cultivars, characterization and identification of pathogenic races of *F. oxysporum* f. sp. *ciceris* in a given area is important for disease resistance breeding and for the effective use of cultivars[10]. In all earlier studies, cultures of *F. oxysporum* f. sp. *ciceris* were either taken from culture collections where they were deposited in 1982 or isolated[6] from areas reported to have specific races of the pathogen in 1982. Attempts were not made earlier to test the reaction of the pathogen on standard differentials prior to molecular characterization; therefore, the present study was taken up with the aim to correlate cultural and pathogenic variability of isolates of pathogen collected from different chickpea-growing regions of India with molecular characterization.

Wilted chickpea plants were collected from different agroclimatic regions of India (Table 1) and associated fungus was isolated on potato dextrose agar (PDA) (potato 200 g, dextrose 20 g, agar 20 g and water 1 l) medium. Single spore culture of fungus was obtained by serial dilution method. Isolated fungus was identified as *F. oxysporum* f. sp. *ciceris* and its pathogenicity tested[13] on chickpea cultivar, JG 62. The original cultures of known races of the pathogen, deposited in Indian Type Culture Collection (ITCC), Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi (Table 1) were also included in the study. Further studies were performed with 25 isolates of *F. oxysporum* f. sp. *ciceris*. Cultures on PDA slants were stored at 4°C for use.

Virulence analysis of 25 isolates of *F. oxysporum* f. sp. *ciceris* was carried out on a set of ten international differential cultivars, viz. C104, JG 74, CPS-1, BG 212, WR 315, Annigeri, Chafa, L 550, K 850 and JG 62 in the greenhouse[6]. Ten seeds of each cultivar were sown in 15 cm diameter surface-sterilized plastic pots (0.1% mercuric chloride) filled with 2 kg sterilized soil (three subsequent sterilizations at 1.1 kg/cm^2 for 1 h for 3 days), inoculated with the 14-day-old culture of the pathogen multiplied on sand maize meal water medium (90 g sand, 10 g maize meal

Table 1. Isolates of *F. oxysporum* f. sp. *ciceris* collected from different agroclimatic regions of India

Isolate number	Place of collection
F_1	Dharwad, Karnataka
F_2	Hisar, Haryana
F_3	Ganganagar, Rajasthan
F_4	Kanpur, Uttar Pradesh
F_5	Junagarh, Gujarat
F_6	Anand, Gujarat
F_7	Anand, Gujarat
F_8	Anand, Gujarat
F_9	Badnapur, Maharashtra
F_{10}	Badnapur, Maharashtra
F_{11}	MB-15, IARI, New Delhi
F_{12}	Ranchi, Jharkhand
F_{13}	Dholi, Bihar
F_{14}	Bangalore, Karnataka
F_{15}	Ludhiana, Punjab
F_{16}	Ranchi, Jharkhand
F_{17}	Jaipur, Rajasthan
F_{18}	Jaipur, Rajasthan
F_{19}	Jabalpur, Madhya Pradesh
F_{20}	Ranchi, Jharkhand
F_{21}	Udaipur, Rajasthan
F_{22}	New Delhi
F_{23}	Kanpur, Uttar Pradesh
F_{24}	Gurdaspur, Punjab
F_{25}	MB4C, IARI, New Delhi

F_{21} to F_{24} were obtained from Indian Type Culture Collection, Indian Agricultural Research Institute, New Delhi, and others from wilted chickpea plants.

and 20 ml distilled, sterilized water) @ 50 g kg^{-1} soil, seven days before sowing[13]. The experiment was conducted in three replications. Uninoculated pots served as control. The incidence of wilt was recorded at 15 days interval up to crop maturity. Reactions were graded as resistant (0–20% wilt), moderately susceptible (>20 to 50% wilt) and susceptible (>50% wilt)[6].

Pure cultures of 24 isolates of *F. oxysporum* f. sp. *ciceris* were grown on potato dextrose broth for 10 days at 25 ± 1°C. Mycelial mats were harvested by filtration through Whatman no. 1 filter paper and washed with sterile water. The harvested mycelia were either used immediately for DNA extraction or stored at – 80°C until use. DNA was extracted by the cetyldimethylethyl ammonium bromide (CTAB) method[14].

The RAPD–PCR reaction mixture (25 µl) consisted of 30 ng template DNA, 0.16 µl (3 units) Taq polymerase (Bangalore Genei, India), 5 mM of $MgCl_2$, 100 mM of each dNTP (Bangalore Genei, India) and 4 mM of primer (OPB and OPN, Operon Technologies, Inc, USA) in 1X reaction buffer (Bangalore Genei). Amplification was performed in a thermal cycler (Bio-Rad Gene Cycler™) at 94°C for 2 min for initial denaturation followed by 40 cycles at 92°C for 1 min denaturation, annealing at 37°C for 1 min and extension at 72°C for 2 min with an elongation of 72°C for 5 min. Amplification products were resolved by electrophoresis on agarose horizontal gel (1.2%) in 1X TAE buffer stained with ethidium bromide and photographed under UV light. A 1 kb (Bangalore Genei, India) ladder was used as marker. Forty different primer OPB and OPN series (Operon Technologies, Inc, USA) were tested. Primers that gave reproducible and scorable amplifications were used in the analysis. All experiments were repeated twice.

DNA bands that could be scored unequivocally for presence or absence were included in the analysis. Binary matrices were analysed by NTSYS–PC (version 2.0; Exeter Biological Software, Setauket, NY). Jaccard's coefficients were clustered to generate a dendrogram using SHAN clustering program, selecting unweighted pair group method with arithmetic average (UPGMA) using the NTSYS program[15].

Twenty-one isolates of *F. oxysporum* f. sp. *ciceris* isolated from wilted chickpea plants collected from different parts of India and four isolates procured from ITCC, IARI, New Delhi, were purified through single-spore method and on the basis of morphological characters identified as *F. oxysporum* f. sp. *ciceris*[16]. All the isolates proved pathogenic on susceptible chickpea cultivar JG 64 under artificial inoculation. Characteristic wilt symptoms such as drooping of plants and yellowing of lower leaves were observed. Internal discoloration of root tissues was also conspicuous in wilted plants[17].

Pathogenic variability of 25 isolates of *F. oxysporum* f. sp. *ciceris* tested on ten differential cultivars revealed that the isolates were highly variable. Pathogenic variability of this pathogen has been well demonstrated since 1982, and the existence of four races in India has been reported[6]. In the present study, the isolates were grouped into seven categories based on disease reactions on the ten differential cultivars (Table 2). The reactions of isolates on differentials were different from earlier reports. Bangalore (F_{14}) and Dharwad (F_1) came under one group as race 1. These isolates were distinguished from the others using K 850 along with C 104, JG 74, CPS 1, BG 212 and WR 315 as resistant reaction. Earlier, the Hyderabad isolate was designated as race 1 by CPS 1 and BG 212 (resistant). Kanpur (F_4 and F_{23} original race 2), Ganganagar (F_3), Junagarh (F_5) and Udaipur (F_{21}) were grouped together and designated as race 2. These isolates were distinguished by WR 315 and JG 74 (resistant to pathogen), whereas Haware and Nene[6] recorded susceptible reaction with Kanpur isolates on JG 74. Isolates of Gurdaspur and Ludhiana were distinguished using cultivars C104 and JG 74 (resistant to pathogen) and called as race 3, which was similar in reaction to earlier report[6]. Isolates of Hisar (F_2), Delhi (F_{11}, F_{22}, F_{25}), Dholi (F_{13}), Jaipur (F_{17}, F_{18}) and Jabalpur (F_{19}) were distinguished by cultivars WR 315 and BG 212 (resistant to pathogen) and designated as race 4. Isolates of Hisar and Jabalpur were also designated earlier as race 4 using cultivar CPS-1, as moderately susceptible to the pathogen[6]. In the present study, CPS-1 also gave moderately susceptible reactions against these isolates, but similar reactions were also observed with other isolates. Therefore, this cultivar CPS-1 could not be considered as differential for this race. Three isolates collected from Anand (F_6, F_7, F_8) were distinguished by cultivar L 550 along with K 850, BG 212, JG 74 and C-104 and called as race 5. Two isolates from Badnapur (F_9 and F_{10}) were differentiated by Annigeri, which gave resistant reaction along with WR 315, CPS-1 and C-104 (resistant reaction to pathogen) and called as race 6. Three isolates from Ranchi (F_{12}, F_{16} and F_{20}) differentiated by Chafa along with WR 315, CPS-1 and C-104 (resistant to pathogen) were designated as race 7. Isolates collected from same place differed in their morphological characters, but were more or less similar in pathogenic virulence. Through the present findings, three new races of the pathogen in India have been established along with characterization and grouping of isolates from unreported places into known race groups. The isolate obtained from MB 4C plot, IARI, New Delhi was highly virulent with maximum wilt incidence in all the cultivars (13.3 to 100%). Haware and Nene[6] reported that the race prevalent in California was race 1, but later on it was reported[18] as race 6. Races 0 and 5 were reported from Spain[19]. Races 5 and 6 from southern Spain caused typical wilt symptoms similar to Indian isolates of *F. oxysporum* f. sp. *ciceris*[20]. Therefore, races 5–7 reported here may be similar to races 5 and 6 of southern Spain. It requires further confirmation along with original isolates. Races 0 and 1B/C induce yellowing symptoms (yellowing pathotype) and are found mainly in the Mediterranean re-

Table 2. Grouping of 25 isolates of *Fusarium oxysporum* f. sp. *ciceris* based on disease reactions on chickpea differentials.

Cultivar	Reactions to isolates from																									
	Dharwad		Bangalore		Ganganagar	Kanpur	Junagarh	Udaipur	Kanpur (ITCC)	Ludhiana	Gurdaspur (ITCC)	Hisar	Delhi (MB-15)	Dholi	Jaipur	Jaipur	Jabalpur	Delhi (ITCC)	Delhi (MB4C)	Anand	Anand	Anand	Badnapur	Badnapur	Ranchi	Ranchi	Ranchi
	F_1		F_{14}		F_3	F_4	F_5	F_{21}	F_{23}	F_{15}	F_{24}	F_2	F_{11}	F_{13}	F_{17}	F_{18}	F_{19}	F_{22}	F_{25}	F_6	F_7	F_8	F_9	F_{10}	F_{12}	F_{16}	F_{20}
	Race 1				Race 2				Race 3			Race 4								Race 5			Race 6		Race 7		
C-104	R		R		S	S	M	M	M	R	R	S	M	S	M	M	M	M	S	R	R	R	R	R	R	R	R
JG-74	R		R		R	R	R	R	R	R	R	S	M	S	M	M	S	M	S	R	R	R	S	S	M	M	M
CPS-1	R		R		S	S	M	S	M	S	S	S	M	M	M	M	M	M	S	S	M	M	R	R	R	R	R
BG-212	R		R		M	S	M	M	M	M	M	R	R	R	R	R	R	R	R	R	R	R	M	M	M	M	M
WR-315	R		R		R	R	R	R	R	M	M	R	R	R	R	R	R	R	R	M	M	M	R	R	R	R	R
Annigeri	S		S		S	S	S	S	S	S	S	S	S	M	M	M	S	S	S	M	M	M	R	R	S	M	M
Chafa	S		M		S	S	S	M	S	M	M	M	S	S	M	M	M	S	S	M	M	M	M	M	R	R	R
L-550	S		M		S	S	S	M	S	M	M	S	S	S	S	S	M	S	S	R	R	R	S	S	S	M	M
K-850	R		R		M	S	M	S	S	M	M	S	S	S	M	M	M	M	S	R	R	R	M	M	S	M	S
JG-62	S		S		S	S	S	S	M	S	S	S	S	S	S	S	S	M	S	S	S	M	S	S	S	S	M

R, Resistant (0–20% wilt); M, Moderately susceptible (>20–50% wilt); S, Susceptible (>50% wilt).

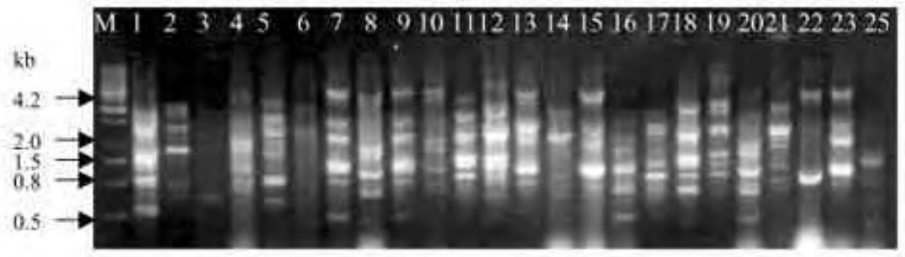

Figure 1. RAPD profiles of 24 isolates of *Fusarium oxysporum* f. sp. *ciceris* obtained with OPN1 primer. M, Marker weight marker (lambda DNA cut with *Eco*RI and *Hin*dIII) (Bangalore Genei, Bangalore, India). Lanes 1–25. Isolates of *F. oxysporum* f. sp. *ciceris*.

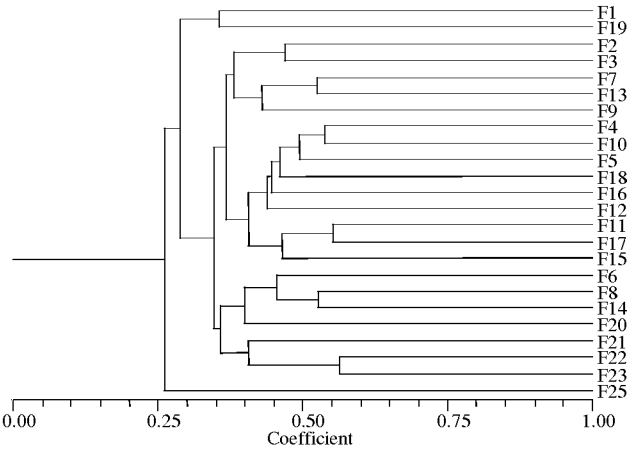

Figure 2. Dendrogram obtained from 24 isolates of *F. oxysporum* f. sp. *ciceris* with UPGMA based on Jaccard's coefficient. Branches are labelled by isolate number. The line below the dendrogram represents similarity index.

gion[5,10]. They are different from the new races reported in the present study.

High level of pathogenic variability in *F. oxysporum* f. sp. *ciceris* causing wilt of chickpea has also been reported by other workers in India[21–23]. Their observations supported the possibilities of existence of different races of the pathogen at the same place.

Genetic variation was detected among 24 isolates of *F. oxysporum* f. sp. *ciceris* using RAPD marker. Out of 40 primers from OPB and OPN series screened for amplification of DNA of 24 isolates of *F. oxysporum* f. sp. *ciceris*, 27 produced reproducible and scorable bands with high percentage of polymorphism. Number of bands obtained was specific to each primer and ranged from 2 to 10. Twenty-seven primers gave 135 bands, among which 122 bands were polymorphic. OPN series of primers were better than the OPB series for amplification of isolates of *F. oxysporum* f. sp. *ciceris*.

Out of 20 primers of the OPN series, 13 produced scorable and reproducible bands for all isolates. Amongst these, OPN1, OPN2, OPN3, OPN4, OPN5 and OPN9 gave more polymorphic bands (Figure 1). The size of amplification products in case of OPN series primers varied from 1 to 4 kb. Out of 20 primers of OPB screened, 14 gave scorable results among which, OPB 19 and OPB 15 gave better results compared to the other primers. The number of polymorphic bands obtained with the OPB series ranged from 2 to 7 and size of amplified products ranged from 1 to 3.5 kb.

The similarity matrix indicated that most of the isolates exhibited <50% similarity coefficient. Isolates F_7 (Anand) and F_{13} (Dholi), F_5 (Junagarh) and F_{14} (Bangalore), F_8 (Anand) and F_{14} (Bangalore), F_{17} (Jaipur) and F_{14} (Bangalore), F_{23} (Kanpur) and F_{22} (New Delhi) showed highest similarity coefficient from 50.6 to 56.3%. These similarity co-efficients were subjected to UPGMA. The dendrogram obtained after cluster analysis showed that at genetic similarity of 0.25, UPGMA analysis of RAPD banding patterns separated isolates into two distinct clusters. The first group included 23 isolates with genetic similarity of 0.15 to 0.56. The second group included only one isolate, i.e. F_{25} (MB4C, IARI, New Delhi), which is highly virulent causing 100% wilting to highly susceptible cultivar JG 62. At 0.55 genetic similarities, seven distinct groups were differentiated among 24 isolates of *F. oxysporum* f. sp. *ciceris* by RAPD markers (Figure 2). The RAPD analysis data suggested that the isolates of *F. oxysporum* f. sp. *ciceris* are derived from genetically distinct clones. Therefore, high level of genetic variability was observed. The exchange of contaminated seeds and cultures probably contributed to existence of variable population of *F. oxysporum* f. sp. *ciceris* 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.

The genetic variability of Indian isolates representing four races of *F. oxysporum* f. sp. *ciceris* was determined through RAPD and AFLP[1,24]. Races 1 and 2 were grouped into two separate clusters, whereas races 3 and 4 were grouped together into the third cluster. In RAPD, a fourth cluster was seen which did not match with any of the four races of pathogen. In the present study RAPD analysis also generated seven clusters at 0.55 genetic similarity, but they were not similar to the pathogenic virulence groups. Earlier, it was also reported that races 1 and 4 were closely related[24]. Therefore, it appeared that the RAPD-based

grouping was different from groups generated on the basis of virulence.

Seventy-two isolates of *F. oxysporum* f. sp. *ciceris* were grouped into two distinct clusters using RAPD–PCR, one for wilting and another yellowing syndrome causing isolates[11]. Specific primers were designed for detection of specific *F. oxysporum* f. sp. *ciceris* isolates. 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[10]. The present results are in agreement with this report. All the isolates except MB-4C, New Delhi (F_{25}) grouped in a single cluster at 0.35 genetic similarity. The SCAR primer for identification of race of the pathogen was developed, because RAPD analysis was not able to distinguish all the races separately[12]. Generalized race-specific patterns were not found through RAPD study[25].

The present study generated significant information in terms of pathogenic and genetic variability of *F. oxysporum* f. sp. *ciceris*, 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 RAPD markers are useful tools for analysing the structure of the pathogen population, but further studies are needed to make them complementary to each other. The international differentials which were developed during 1982 have to be standardized with new cultivars to get clear-cut differential reactions for changed population of *F. oxysporum* f. sp. *ciceris*.

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