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Full Length Research Paper

Genetic diversity in Indian isolates of *Fusarium oxysporum* f. sp. *ciceris*, chickpea wilt pathogen

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Forty-eight isolates of FOC collected from different chickpea growing regions in India were evaluated for genetic variations using amplified fragment length polymorphism (AFLP). Out of 48 isolates, 41 were found pathogenic and seven non-pathogenic. Pathogenic isolates differ in their virulence however; there was no apparent correlation between geographical origin and virulence of the isolates. The genetic variation was evaluated by the AFLP analysis. A total 339 fragments were scored following selective amplification with five E*co*R1 and M*se*1 primer combinations E-TC/M-CAT, E-TC/M-CAC, E-AC/M-CAG, E-TA/MCAG, E-TA/M-CAG, out of which 331 fragments were polymorphic. UPGMA cluster analysis and principle coordinate analysis distinctly classified 48 isolates into two major groups; pathogenic and non-pathogenic. The pathogenic isolates could be further clustered into six major groups at 0.77 genetic similarities. Region specific grouping was observed with in few isolates. The results of the present study provide evidence of the high discriminatory power of AFLP analysis, suggesting the applicability of this method to the molecular characterization of *Fusarium oxysporum* f.sp. *ciceri*s.

Key words: AFLP, *Cicer arietinum*, fusarium wilt, variability.

INTRODUCTION

Fusarium wilt of chickpea caused by *Fusarium oxysporum* f.sp. *ciceris* (Padwick) Matuo and Sauto (FOC) is a major constraint to chickpea production world-wide. The disease is widespread in the chickpea growing areas of the world and is reported from at least 33 countries (Nene et al., 1996), causing 10 - 90% annual losses (Singh and Dahiya, 1973; Jalali and Chand, 1992). It is more important between the latitudes 30˚N and 30˚S where the chickpea-growing season is dry and warm.

The use of resistant cultivars is one of the most practical and cost-efficient strategies for managing this disease. However, the efficiency of resistant cultivars in disease management is limited by high pathogenic variability in FOC. FOC have extreme genotypic and phenotypic variability and can adapt to a wide range of environmental conditions. Eight races (race 0, 1A, 1B/C, 2, 3, 4, 5 and 6) of the pathogen have been identified based on a set of differential chickpea cultivars (Haware and Nene, 1982; Jimnez Diaz et al., 1993). All the races have distinct geographical locations; races 1 - 4 have been reported from India; whereas 0, 1B/C, 5 and 6 are found in the Mediterranean region and USA (Trapero-Casas and Jimnez-Diaz, 1985; Jimnez Gasco et al., 2001; Phillips, 1988). Frequent reoccurrence of virulent forms of FOC have affected chickpea production and exhausted valuable genetic resources available with them. Therefore, accurate and rapid identification of pathogen is necessary for appropriate management of this disease. Identification of pathogenic races has been mostly done by use of differential reaction to selected host genotypes. This method of race identification is expensive, time consuming and takes at least 40 days to complete (Bhatti and Kraft, 1992; Kelly, 1994). Therefore, it would be desirable to have a more rapid, cheaper and less labor-intensive method of distinguishing pathotypes.

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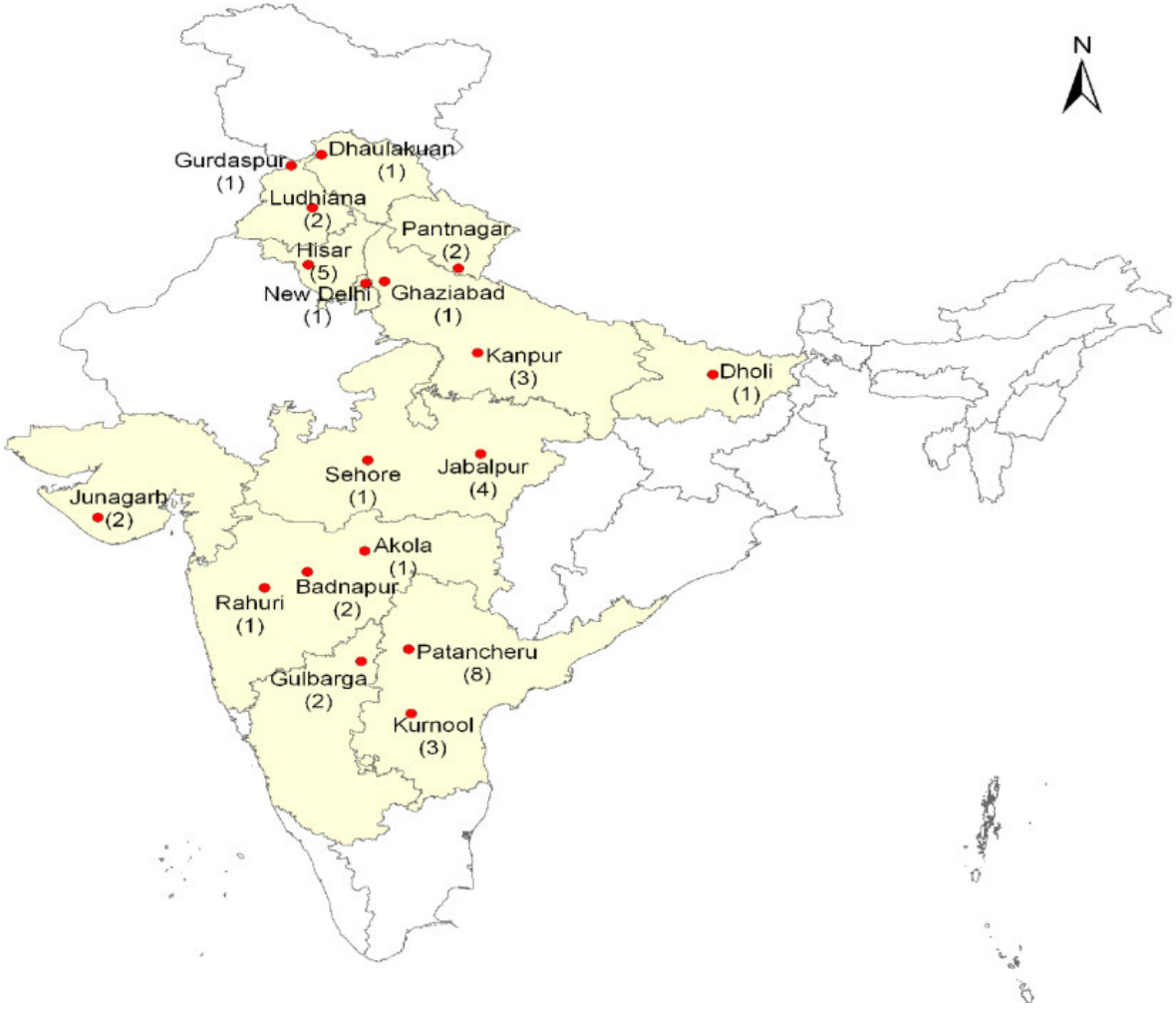

Figure 1. *F. oxysporum* f.sp. *ciceris* isolates collected from diverse agro-ecological regions in India. (Numbers in parentheses indicates the total isolates collected from particular location).

In the recent years, several types of molecular marker systems such as RAPD (random amplified polymorphic DNA), RFLP (restriction fragment length polymorphism), SSR (simple sequence repeat) and amplified fragment length polymorphism (AFLP) have been increasingly used to study the variability in pathogenic populations of FOC (Jimnez Gasco et al., 2001; Jimnez Gasco and Jimnez-Diaz, 2003; Kelley et al., 1994). Among different marker systems, AFLP technique is generic and provided genome wide DNA profiling (Vos et al., 1995). The utility, repeatability, and efficiency of the AFLP technique have made the marker system more suitable for broader applications in the analysis of *Fusarium* populations (Abd-Elsalam et al., 2002; Kelley et al., 1994).

The present study therefore was undertaken to assess the extent of genetic diversity in *F. oxysporum* f.sp. *ciceris* isolates collected from the diverse agro-climatic chickpea growing regions of India using AFLP markers.

MATERIALS AND METHODS

Fungal isolates

A total forty-eight isolates of *F. oxysporum* f. sp. *ciceris* (FOC) were obtained from wilt-infected chickpea plants from different agro-climatic regions of India during 1995 - 2006 (Figure 1). Isolations were made from the wilted plants on potato dextrose agar (PDA) medium. The fungus was identified according to the identification keys of *F. oxysporum*. All the isolates were single spored and was stored in tubes containing PDA at 4°C. The details of geographical origin of the isolates are presented in Table 1.

Pathogenicity test

The pathogenicity of all the 48 isolates of FOC was tested in the greenhouse following root dip inoculation technique (Pande et al., 2007). This is a modification of method given by Nene et al. (1981). The pathogenicity was tested on the chickpea cultivar JG 62, highly susceptible to fusarium wilt. Seeds of JG 62 were surface sterilized

Table1. Isolates of *Fusarium oxysporum f.sp. ciceris,* their place of collection and pathogenicity.

S. No	Isolate name	Location	State	Year of collection	Incubation period (days)	Disease development (days)
Pathogenic isolates						
1	Foc 1	ICRISAT, Patancheru	Andhra Pradesh	1995	10	11
2	Foc 2	ICRISAT, Patancheru	Andhra Pradesh	1995	11.5	16.5
3	Foc 3	ICRISAT, Patancheru	Andhra Pradesh	1995	9	14.5
4	Foc 4	ICRISAT, Patancheru	Andhra Pradesh	2000	14.5	17
5	Foc 5	ICRISAT, Patancheru	Andhra Pradesh	2001	10	13
6	Foc 6	ICRISAT, Patancheru	Andhra Pradesh	2004	10	15
7	Foc 7	Hisar	Haryana	2002	8	14
8	Foc 8	Hisar	Haryana	2003	10	15
9	Foc 9	Hisar	Haryana	2004	10	14
10	Foc 10	Hisar	Haryana	2004	10	14
11	Foc 11	Dholi	Bihar	2005	11	15
12	Foc 12	Dhaulakuan	Himachal Pradesh	2005	12	14
13	Foc 13	Gulberga	Karnataka	2001	17	37
14	Foc 14	Junagarh	Gujarat	2005	10	14
15	Foc 15	Junagarh	Gujarat	2005	11	20
16	Foc 16	Sehore	Madhya Pradesh	2005	10	14
17	Foc 17	Badnapur	Maharashtra	2005	13	15
18	Foc 18	Badnapur	Maharashtra	2005	10	15
19	Foc 20	Rahuri	Maharashtra	2004	14	21
20	Foc 21	New Delhi	Delhi	2005	14	21
21	Foc 22	Ludhiana	Punjab	2002	9	14
22	Foc 23	Ludhiana	Punjab	2005	12	15
23	Foc 25	Kanpur	Uttar Pradesh	2003	13	17
24	Foc 26	Kanpur	Uttar Pradesh	2004	13	22
25	Foc 28	Pantnagar	Uttaranchal	2004	11.5	15
26	Foc 29	Pantnagar	Uttaranchal	2004	14	24
27	Foc 30	Ghaziabad	Uttar Pradesh	2005	11	14
28	Foc 31	Kurnool	Andhra Pradesh	2006	13	16
29	Foc 32	Kurnool	Andhra Pradesh	2006	12	29.5
30	Foc 33	Akola	Maharashtra	2006	11	14
31	Foc 34	Jabalpur,	Madhya Pradesh	2006	11	14
32	Foc 35	Jabalpur	Madhya Pradesh	2006	12	14
33	Foc 36	Jabalpur	Madhya Pradesh	2006	10	13

Table 1. Contd.

34	Foc 37	Jabalpur	Madhya Pradesh	2006	11	13
35	Foc 38	ICRISAT, Patancheru	Andhra Pradesh	2005	12	16.5
36	Foc 39	Dharwad	Karnataka	2007	17	27
37	Foc 40	Race 1, Hyderabad	Andhra Pradesh	1980	13	34
38	Foc 41	Race 2, Kanpur	Uttar Pradesh	1980	16.5	18.5
39	Foc 42	Race 3, Gurdaspur	Punjab	1980	12	37
40	Foc 43	Race 4, Hisar	Haryana	1980	20	18.5
41	Foc 44	Kurnool	Andhra Pradesh	2006	12	15.0
Non-pathogenic isolates						
42	NP FOC 1	ICRISAT	Andhra Pradesh	1995	-	-
43	NP FOC 2	Warangal	Andhra Pradesh	2005	-	-
44	NP FOC 3	Jabalpur	Madhya Pradesh	2003	-	-
45	NP FOC 4	Hisar	Haryana	2004	-	-
46	NP FOC 5	Dharwad	Karnataka	2006	-	-
47	NP FOC 6	Barielly	Uttar Pradesh	2006	-	-
48	NP FOC 7	Kanpur	Uttar Pradesh	2006	-	-
CD (0.05%)					**1. 6**	**2.1**

using 2% sodium hypochlorite for 2 min, rinsed in sterile water and germinated for eight days in plastic pots containing sterilized sand. The eight days old seedlings were carefully uprooted and the roots were washed under running water to remove excess sand. Root tips around 0.5 cm long were cut off to facilitate the entry of the pathogen into the roots. The roots of the seedlings were then dipped separately in the inoculum of each isolate (5×10^5 conidia ml^{-1}) for 1 - 2 min to enable conidia to adhere to the roots. Inoculated seedlings were transplanted in pre-irrigated sterile vertisol and sand (3:1) in pots and incubated at 25 ± 3ºC. Inoculated seedlings were observed regularly for incubation period and latent period. The experiment was repeated twice and Koch's postulates were proved.

Genomic DNA extraction

For DNA extraction, pure culture of all 48 isolates of FOC was grown on potato dextrose broth for four days at 25 ± 1°C. Mycelial mats were harvested by filtration through Mira cloth (Cal biochem, U. S. A.). The fungal mycelial mat was thoroughly and repeatedly washed with distilled water and dried using paper towels. Genomic DNA was isolated from each isolate by the cetyldimethylethyl ammonium bromide (CTAB) method (Murray and Thompson, 1980). Mycelial mat (2.0 g) was grinded in liquid nitrogen with the help of mortar and pestle. The powdered mass was mixed with 20 ml of extraction buffer (100 mM Tris-HCl, pH 8.0, 20 mM EDTA, 1.4 M NaCl and 2% CTAB). The contents were gently mixed by inversion and incubated at 65°C for 1 h in a water bath. The slurry was transferred to a 15 ml plastic tube and an equal volume of chloroform: isoamylalcohol (24:1) was added. The contents of the tube were mixed gently for 5 min and centrifuged for 5 min at 10,000 x g in a centrifuge. The aqueous phase was transferred to another tube and an equal volume of chloroform : isoamylalcohol (24:1) was added to the aqueous phase. The contents of the tube were mixed and centrifuged for 5 min as before. The aqueous phase was again transferred to another tube and the nucleic acid was precipitated by adding 0.6 volume of isopropanol. The solution was centrifuged for 10 min at 12,000 x g, and supernatant was decanted. The pellet was washed with 70% ethanol twice and suspended in $T_{50}E_{10}$ buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA). The DNA solution was treated with RNase (50 µg/ml, Sigma Co. MO, USA) at 37°C for 1 h. An equal volume of phenol: chloroform (1:1) was added to the solution after incubation, mixed well for 5 min and centrifuged at 12, 000 x g in a micro centrifuge (Eppendorf, USA). The aqueous phase was transferred to another tube and an equal volume of chloroform was added. The aqueous layer was separated and DNA was precipitated by adding 2.5 volume of abso-

lute ethanol. The DNA pellet was washed twice in 70% ethanol, vacuum-dried, and redissolved in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA).

Amplified fragment length polymorphism (AFLP)

AFLP was carried out using the commercial kit (Gibco BRL, USA) following the manufacturer's protocols with slight modifications as described below. Primary template DNA was prepared in a one-step restriction-ligation reaction. Fungal genomic DNA (400 ng) was digested with the two restriction endonucleases, *Eco*RI and *Mse*I at 37°C for 2 h and heated at 70°C for 15 min to inactivate the enzyme. The DNA fragments were ligated to E*co*RI and M*se*I adapters at 20°C for 2 h. After terminating the reaction, the ligation mixture was diluted 10 fold with TE buffer and the fragments were preamplified in a thermal cycler (MJ Research, USA) using a temperature cycle of 94°C for 30 s, 56°C for 60 s, and 72°C for 60 s in a total of 30 cycles.

Selective primers provided in the kit were used and the amplification was carried out according to the manufacturer's protocol. The three E*co*RI primer (E-TC, E- TA and E-AC) and five M*se*I (M-CAT, M-CAC, M-CAG, M-CTT and M-CTA) primers were used in five combinations (E-TC/M-CAT, E-TC/M-CAC, E-AC/M-CAG, E-TA/MCAG, E-TA/M-CAG) for amplification. Selective primers were provided by Life Technologies (USA).

The E*co*RI primer was labeled with [γ-^{32}P]-ATP (3000 Ci/mmol) and the DNA amplification was carried out as per the manufactures protocol. The PCR products in 5.0 µl sub-samples were separated by electrophoresis on 6% denaturing polyacrylamide DNA sequencing gel containing 7.5 M urea. Auto radiograms were obtained using Kodak X-Omat film.

Data analysis

For assessing the molecular genetic variation, AFLP profiles of 48 isolates were used to construct a binary matrix. Each AFLP fragment across all the AFLP primer combination was scored as present (1) or absent (0) across the isolates. Subsequently, 0 - 1 binary data was used to compute the similarity matrix using the Jaccard's similarity coefficient with the help of Numerical Taxonomy System Version 2.2 (NTSYSpc). The similarity matrix was used to construct the tree dendrogram by unweighted pair group method of arithmetic averages (UPGMA) using the SAHN (Sequential Agglomerative Hierarical Nested) cluster analysis module (Rohlf, 2000).

In order to assess the discriminatory feature of different primer combinations, resolving power (Rp) of the primers was also calculated using the following formula (Prevost and Wilkinson, 1999):

$$Rp = \sum I_b$$

where $I_b = 1 - (2 \times |0.5 - p)$, where p is the proportion of the 48 isolates containing the fragment. The I_b value was calculated for all bands that were scored in the study.

RESULTS AND DISCUSSION

Understanding of the pathogen's pathogenic and genetic diversity is an important prerequisite in developing and deploying varieties with durable resistance. Therefore, the present study was undertaken to assess the diversity in FOC isolates collected from different agro climatic regions of India.

Pathogenicity

Out of 48 isolates collected from different chickpea growing regions, 41 were found pathogenic. Characteristic wilt symptoms such as drooping of leaflets and yellowing of the leaves starting from apical part, progressing downward and final wilting of the whole plant were observed. Internal discoloration of the root vascular system was conspicuous in wilted plants. However, seven isolates were non-pathogenic and did not show any symptoms at all.

The disease development by pathogenic isolates on JG 62 revealed variability in virulence among isolates. Incubation period (IP) varied from 8 to 17 days and latent period (LP) from 11 to 37 days (Table 1). Majority of the isolates were highly virulent (60.67%) and caused wilting and death of the seedlings in 11 - 15 days. They include isolates from diverse regions; Andhra Pradesh, Bihar, Gujarat, Haryana, Himachal Pradesh, Punjab, Madhya Pradesh, Maharashtra, and Uttaranchal; 20% isolates showed seedling mortality in 16 - 20 days. The isolates in this group were from Andhra Pradesh, Gujarat, Uttar Pradesh and Hisar. Two isolates each from Karnataka (FOC 13 and 34), and Andhra Pradesh (FOC 32 and 40) and Foc 42 from Gurdaspur were less virulent and caused seedling death within 27 - 37 days. However, no apparent correlation between geographical origins and virulence of the isolates was found.

Pathogenic variability of FOC has been well documentted (Haware and Nene, 1982; Gupta et al., 1986; Honnareddy and Dubey, 2006; Rahman et al., 1998). Since majority of the isolates were highly virulent and distributed in most of the major chickpea growing regions, represents risk to the planting of local varieties because of their susceptible reaction. Different levels of virulence in the populations of *F. oxysporum* f.sp. *lentis* have also been reported by Belabid et al. (2004) and they also did not find any correlation between geographical origin and virulence of the isolates.

AFLP based diversity

A total 339 fragments were scored following selective amplification with five E*co*RI and M*se*I primer combinations; E-TC/M-CAT, E-TC/M-CAC, E-AC/M-CAG, E-TA/MCAG, E-TA/M-CAG, out of which 331 fragments were polymorphic (Table 2). A high level of polymorphism (95.23 - 98.61%) was observed among the 48 isolates (Table 2). This can be attributed to highly evolving nature of FOCs. Indeed, high levels of DNA polymorphisms were

Table 2. Summary of AFLP analysis of 48 isolates of *F. oxysporum* f.sp. *ciceris*

Primer combination	Total bands scored	Polymorphic bands	Polymorphism (%)	Resolving power (Rp)
E-TC / M-CAT	75	73	97.33	11.89
E-AC / M-CAG	74	72	97.29	6.90
E-TA / M-CTT	72	71	98.61	9.22
E-TA / M-CAG	63	60	95.23	8.05
E-TC / M-CAC	55	54	98.18	5.62
Average	67.8	66.2	97.3	8.3

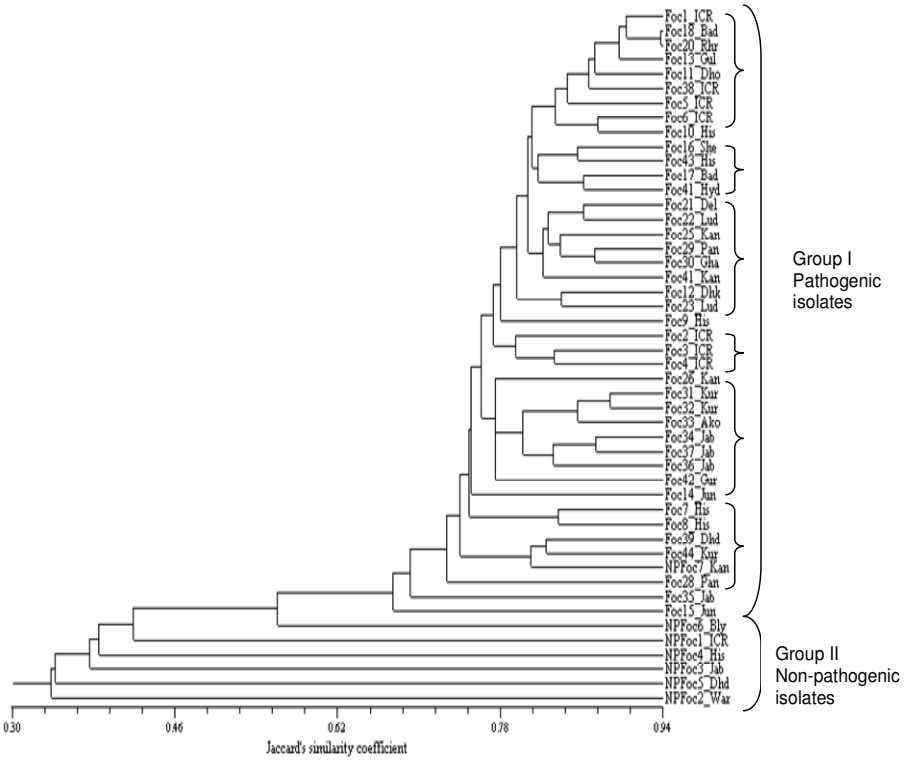

Figure 2. Genetic relationships among 48 isolates of *F. oxysporum* f.sp. *ciceris* based on AFLP analysis.

observed in FOC from different sources in several earlier reports (Barve et al., 2001; Honnareddy and Dubey, 2006; Sivaramakrishnan et al., 2002). Sivaramakrishnan et al. (2002) suggested the rapid evolution of new recombinants of the pathogen in chickpea growing fields.

In terms of investing the discriminatory feature of AFLP primer combinations, resolving power (Rp) was calculated for all the primer combinations. The Resolving Power (Rp) of the five-primer combination ranged from 5.62 (E-TC/M-CAC) to 11.89 (E-TC/M-CAT) with an average of 8.33 (Table 2). Primer combination E-TC/M-CAT had the high Rp value (11.89) and therefore it can be recommended as a useful primer combination for analyzing the FOC populations in future. Several studies have been conducted in the past on AFLP proofing of FOC populations, however it was difficult to compare the value of primer combinations from those studies. No report is available on the information of the Rp for AFLP primer pairs used for diversity assessment of FOC isolates. The Rp, however, seems to provide better estimate about the discriminatory power of the primer combination (Prevost and Wilkinson, 1999).

The UPGMA cluster analysis and principle coordinate analysis of the combined fingerprinting data classified the 48 isolates distinctly into two major groups; pathogenic and non-pathogenic (Figure 1 and 2). Pathogenic isolates

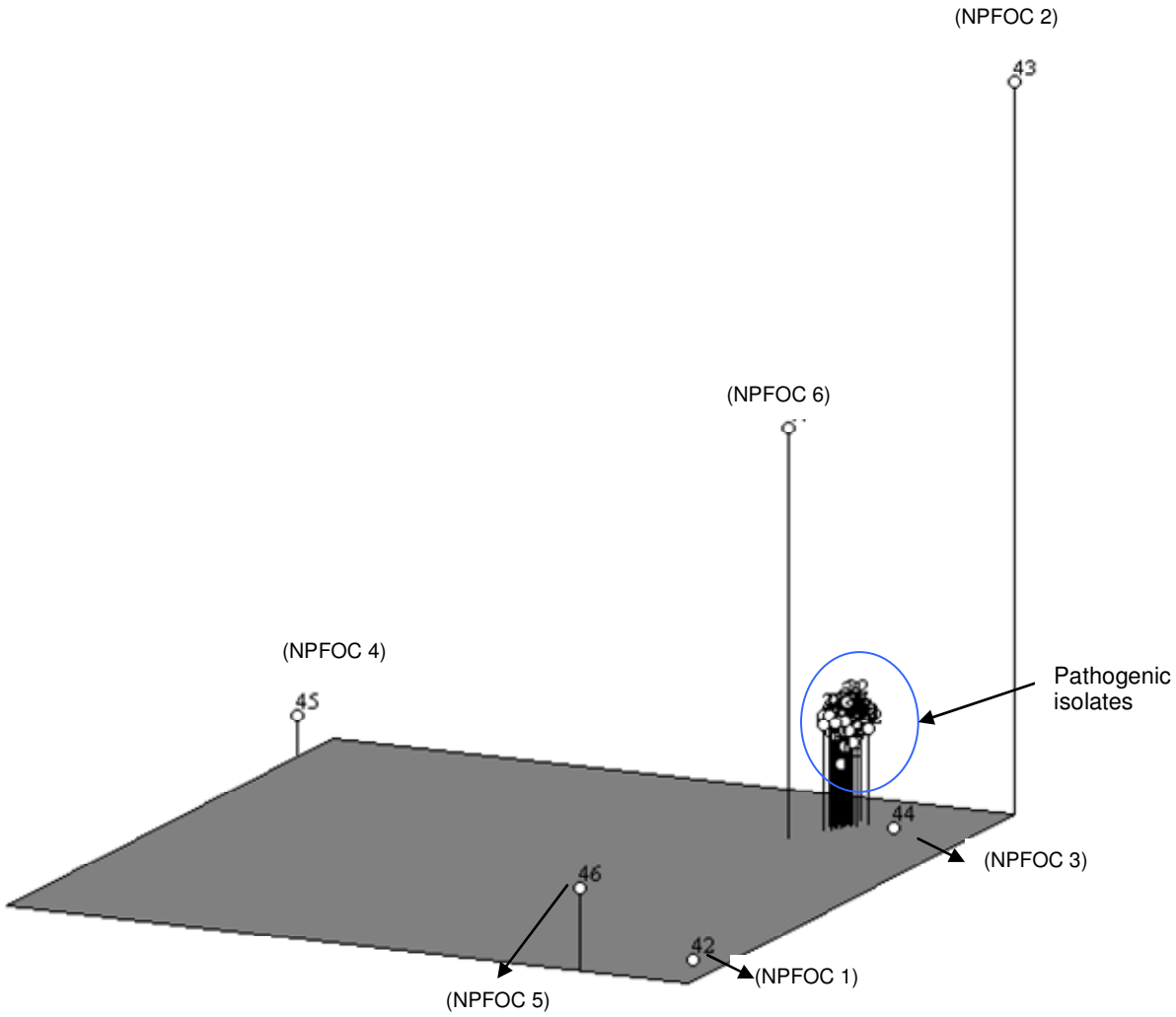

Figure 3. Principle coordinates analysis of 48 isolates of *F. oxysporum* f.sp. *ciceris*.

were clustered at one point and were clearly distinguishable from non-pathogenic isolates except non-pathogenic isolate from Kanpur (NPFOC 7). Jimnez-Gasco et al. (2004) also reported that DNA fingerprinting clearly delineated pathogenic and non-pathogenic isolates.

Genetic similarity coefficient among the 48 isolates varied from 0.33-0.93 (Figure 1). Furthermore, within pathogenic isolates, UPGMA cluster analysis grouped the isolates into six major groups at 0.77 genetic similarities. Region specific grouping was observed with in few isolates. For example group one includes nine isolates from southern part of India (Andhra Pradesh, Maharashtra, and Karnataka) except FOC 10 from Haryana. Group three includes isolates from northern part of India (New Delhi, Punjab, Uttar Pradesh, Uttaranchal, Himachal Pradesh, Haryana). Similar kind of region specific grouping was reported earlier also (Abdel-Satar et al., 2003; Belabid et al., 2004) isolates FOC 14 and 15 from Junagadh (Gujarat) and FOC 35 from Jabalpur (Madhya Pradesh) could not be grouped with other test isolates.

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

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