J. Mycopathol. Res, 50(1) : 43-47 (2012)

Molecular marker based RAPD analysis of *Fusarium oxysporum* f.sp. *pisi* causing wilt of pea

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Received : 22. 02 2011 Accepted : 21. 10. 2011 Published : 30.04.2012

Six isolates of *Fusarium oxysporum* f. sp. *pisi* were isolated from rhizosphere soil of pea from different locations of Uttar Pradesh. Morphological variability with respect to colony characters, radial growth of mycelium and size of conidia existed among the isolates. The variation with respect to pigmentation in medium was also observed which varied from normal white to violet brown in colour. The micro and macro conidia produced by different isolate of *F. oxysporum* f. sp. *pisi* also showed variation in their size and shape. The genetic variation was evaluated by polymerase chain reaction (PCR) amplification with a set of 20 RAPD primers and the results revealed that 90% of the amplified fragments in each case were polymorphic. Genetic similarity between each of the isolates was calculated and results indicated that there was more genetic variability among the isolates collected from the different locations.

Key words: *Fusarium oxysporum* f. sp. *pisi*, *Fusarium* wilt of pea, RAPD

INTRODUCTION

Fusarium wilt of pea caused by *Fusarium oxysporum* f. sp. *pisi* is one of the most destructive disease of pea, distributed all over the country. The pathogen is soil borne in nature. The different species of *Fusarium* have been isolated from different location of the country and variability among the isolates is existed in nature. The pathogen can easily be identified in culture media based on morphological characters. Currently, 10 races of wilt fungus have been reported and designated as races 2 to race 11. The race 2 is predominates in Illinois (Anonymous, 1988).

Molecular biology has brought many powerful new tools to give the taxonomic position of fungi including the potential for rapid identification, methods for rapid determination of virulence or toxicity of strains, and the means to elucidate the relationships among fungal species. Molecular methods have also been used to distinguish between closely related species with few morphological differences and to distinguish strains (or even specific isolates) within a species. Genetic characterization of *F. oxysporum* f. sp *pisi* isolates causing wilt disease to the pea plants is important for development of efficient management practices through use of resistant cultivars. Therefore, to find out the genetic diversity at molecular level and its further application on the respect of plant disease management, the present study as Molecular marker based RAPD analysis of *Fusarium oxysporum* f.sp. *pisi* causing wilt of pea has been undertaken.

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MATERIALS AND METHODS

Isolation of *Fusarium oxysporum* f.sp. *pisi*. wilt affected pea (*Pisum sativum*) plants were collected from different places of Uttar Pradesh and associated pathogen was isolated on Potato Dextrose Agar (potato 200 g. dextrose 20 g. agar 20 g. and water 1000 ml) medium. The pathogen was isolated by serial dilution method as described by Lilly and Barnett (1951). F1 to F6. Isolated fungus was identified as *Fusarium oxysporum* f. sp. *pisi* and its pathogenicity was tested on pea cultivar. The isolated pathogens were designated as F1 (Allahabad), F2 (Fatehpur), F3 (Varanasi), F4 (Kanpur), F5 (Basti) and F6 (Jaunpur).

Morphological variation

Morphological characters like shape, size of micro-

conidia, macroconidia and chlamydospores were examined with temporary slides prepared in lactophenol and cotton blue under calibrated compound microspore to determine the variation in morphology of the isolates. The characters such as radial growth, pigmentation and sporulation of all isolates were also observed on PDA medium in Petri dishes having equal quantity of sterilized medium in three replications for each isolate. The observation on radial growth was recorded 6 days after incubation at 25 ± 1°C and sporulation was counted following the serial dilution method (Lilly, and Barnett, 1951).

Molecular variability of Fusarium oxysporum f.sp pisi based on RAPD analysis

Production of mycelial mat

Different isolates of *F.oxysporum* were grown in 1000 ml conical flask containing 400 ml of PDB (Potato Dextrose Broth) medium separately. Two agar plugs from actively growing colony of *F.oxysporum* were transferred to each flask aseptically in a laminar flow. The flasks were incubated at 25 ± 1°C for 21 days. The mycelial mat was collected by passing the fluid through 3 layers cheese cloth.

Extraction of genomic DNA

The fungal cell wall was disrupted by grounded with pestle and mortar in liquid nitrogen. The powdered mycelium was then transferred to an extraction buffer that contained detergent cetyl-trimethyl ammonium bromide (CTAB) and 2 ß-mercaptroethanol, EDTA and polyvinyl pyrolidone (PVP) and incubated for 1 hr at 60 °C followed by centrifugation at 12000 rpm for 15 minutes. The supernatant was then extracted with equal volume of phenol : chloroform : isoamyl alcohol (25 : 24 : 1) and centrifuged at 12000 rpm for 10 minutes ,the aqueous phase was then transferred in a fresh tube and the DNA was precipitated with chilled (100%) ethanol. DNA was pelleted by centrifuging at 12000 rpm for 15 minutes and washed in 70% ethanol by centrifugation. The pellets were air dried and suspended in TE buffer (pH 8.0).

Qualitative and quantitative estimation of DNA

The qualitative and quantitative estimation of DNA was done by extraction of total genomic DNA from different isolates of *F.oxysporum* as per the above procedure followed by RNAse treatment . Genomic DNA was re-suspended in 100 µl. 1X TE buffer and incubated at 37 °C for 30 min with RNAse. After incubation, the sample was reextracted with PCI (phenol : chloroform : isoamylalcohol 25:24:1) solution and RNA free DNA was precipitated with chilled ethanol as described earlier. Quantification of DNA was done with 0.8% agarose gel electrophoresis. Working concentration of DNA was adjusted to 20 ng/µl and stored at 4°C. The DNA from all the isolates produced clear sharp bands, indicating good quality of DNA.

RAPD analysis

The procedure described by Williams *et al.* (1990) with minor modification was used for carrying out PCR reaction to produce RAPD profiles. Amplification of DNA fragments was carried out by the PCR using 10-mer arbitrary primers. The reaction mixture consisted of 200 µm of dNTP mix (permentas) 15 p mol of primer (Operon), 5 U/µl of Taq polymerase (fermentas) and 25 mM $MgCl_2$. DNA amplifications were performed in thermocycler with one cycle of initial denaturation at 94°C for 15 min, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 35 °C for 2 min, extension at 72 °C for 1 min and final extension at 72 °C for 10 min.

Amplified products together with marker (lambda DNA/ Eco PI + Hind III double digest; Bangalore, Genei) were resolved by 1.5% agarose gel electrophoresis (60 Vcm-1). Gels were photographed by Gel documentation system (Uvitec).

Data analysis

Comparison of each profile for each primer was done on the basis of the presence or absence (1/0) of amplified bands. Bands of the same length were scored as identical. Analysis was based on the simple matching index which measures the proportion of common data (either 0 or 1) between the isolates.

RESULTS AND DISCUSSION

Morphological variability

The results presented in the Table 1 showed that

Table 1. The isolate of *Fusarium oxysporum f.* sp. *pisi* collected from different places in Uttar Pradesh.

Isolate No.	Place of collection
F_1	Sainy, Allahabad.
F_2	Khaga, Fatehpur.
F_3	IVRI, Varanasi.
F_4	Kalyanpur, Kanpur.
F_5	Gaur, Basti.
F_6	Dobhi, Jaunpur

revealed high level of morphological and cultural diversity among the isolates of *Fusarium oxysporum* f. sp. *pisi*. The data presented in the Table 3 revealed that the micro and macro conidia produced by different isolates of *Fusarium* also showed variation in their size and shape. The highest length and width of macro conidia was produced by the isolate F_6 which is 36.0 x 4.1 µm. The minimum length and width of macro conidia was found in F_5 and from the table it was also cleared that septation on macro conidia also showed variation. The highest number of septa with 1-3 was found in the isolate F_3 and F_6. The rest of the isolates showed at par in respect of septa formation. The morphological and pathological variability were also re-

Table 2 : Colony characters, radial growth, diameter of chlamydospores and sporulation of different isolates of *F. oxysporum* f. sp. *pisi*

Isolate	Colony characters	Radial growth (mm)	Diameter chlamydospores (µm)	Sporulation conidia / ml (x 10^7)
F_1	Mycelium aerial, violet	50.00	5.5–70.0	2.3
F_2	Mycelium aerial,violet	80.00	5.5–7.5	1.3
F_3	Mycelium aerial, white	45.20	7.5–9.0	1.8
F_4	Mycelium aerial, dirty white	71.00	6.0–7.5	1.5
F_5	Mycelium aerial, pinkish	53.10	6.0–7.5	0.8
F_6	Mycelium aerial, brown	84.50	6.0–75.0	1.5

the isolate of *F. oxysporum* f. sp. *pisi* showed variability with respect to colony characters, radial growth of mycelium and size of conidia. The variation with respect to pigmentation in medium was also observed which varied from normal white to violet brown in colour. The pigmentation of various colours like violet, white, pinkish and brown were prominent in isolates F_1 (Allahabad), F_2 (Fatepur), F_3 (Varanasi), F_4 (Kanpur), F_5 (Basti), and F_6 (Jaunpur) respectively. It was also found that the intensity of colour varied with age of the fungi and temperature in growth chamber.

On the other hand, maximum radial growth was found in the isolate F_6 which is 84.5 mm against 45.20 mm in case of F_3. The F_1, F_2, F_4, and F_5 were showing 50.00 mm, 80.00 mm, 71.8 mm and 53.10 mm radial growth, respectively. Similarly highest diameter of chlamydospores ranging between 7.5-9.0 µm was found in the isolate F_3 and least in the isolate F_2 (5.5 -7.5 µm). The maximum sporulation of conidia was found in F_1 isolate which is 2.3 X 10^7 conidia/ml followed by F_3 (1.8 X 10^7) F_6 (1.5 X 10^7) and F_2 (1.3 X 10^7) isolates. The present findings

Table 3. Size and septation in micro and macro conidia produced by isolates of *F. oxysporum* f. sp. *pisi*

Isolate	Microconidia		Macroconidia	
	L x W (average µm)	Septation	L x W (average µm)	Septation
F1	6.0x3.1	0	18X3.7	1-2
F2	5.0X2.5	0	4.0X3.6	1-2
F3	10.0X3.0	0	20.1X3.8	1-3
F4	6.0X3.0	0	19.2X5.0	1-2
F5	6.0X2.5	0	14.5X4.0	1-2
F6	12.0X2.0	0	36.0X4.1	1-3

ported in seven isolates of *Fusarium* sp. by El-Fadly, (2008). Honnareddy and Dubey (2007) also showed the variability in 25 isolates of *F. o.* f. sp. *ciceris* with respect to colony characters, radial growth and size of conidia.

Molecular variability

The result presented in the table showed that the number of reproducible band amplified were 112, out of which 109 were found to be polymorphic and the rest 3 is monomorphic hence, the percentage

Table 4. OPZ primers used for RAPD amplification and their corresponding PCR products of *Fusarium oxysporum* f.sp. *pisi*

Name of primer	Sequence of Primer	Amplified product	Total No. of bands	No. of Polymorphic bands	No. of Monomorphic bands
Z 1	5'TCGGATCCGT3'	YES	10	10	0
Z 2	CCTGAACGGA	YES	5	5	0
Z 3	GGCTGTGTGG	YES	2	2	0
Z 4	CCAGCCTCAG	NO	-	-	-
Z 5	TCCGCATACC	YES	4	3	1
Z 6	CCTTCGGAGG	YES	5	5	0
Z 7	CACGAGTCTC	YES	7	7	0
Z 8	TCGCTCGTAG	YES	9	9	0
Z 9	CCTTGACCCC	YES	2	2	0
Z 10	ACTCTGGGGA	YES	8	8	0
Z 11	TCCAGCGCGT	YES	6	6	0
Z 12	GATCCCGGTG	YES	6	6	0
Z 13	CCCGAAGCAA	YES	8	6	2
Z 14	CACGGCTTCC	YES	2	2	0
Z 15	TCCGCTAGTC	YES	3	3	0
Z 16	AGGCGAACCT	YES	8	8	0
Z 17	CACGCAGATG	YES	8	8	0
Z 18	CCGACGTTGA	YES	9	9	0
Z 19	ACACTCTCGG	YES	4	4	0
Z 20	CATCACCCCT	YES	6	6	0
TOTAL			112	109	3

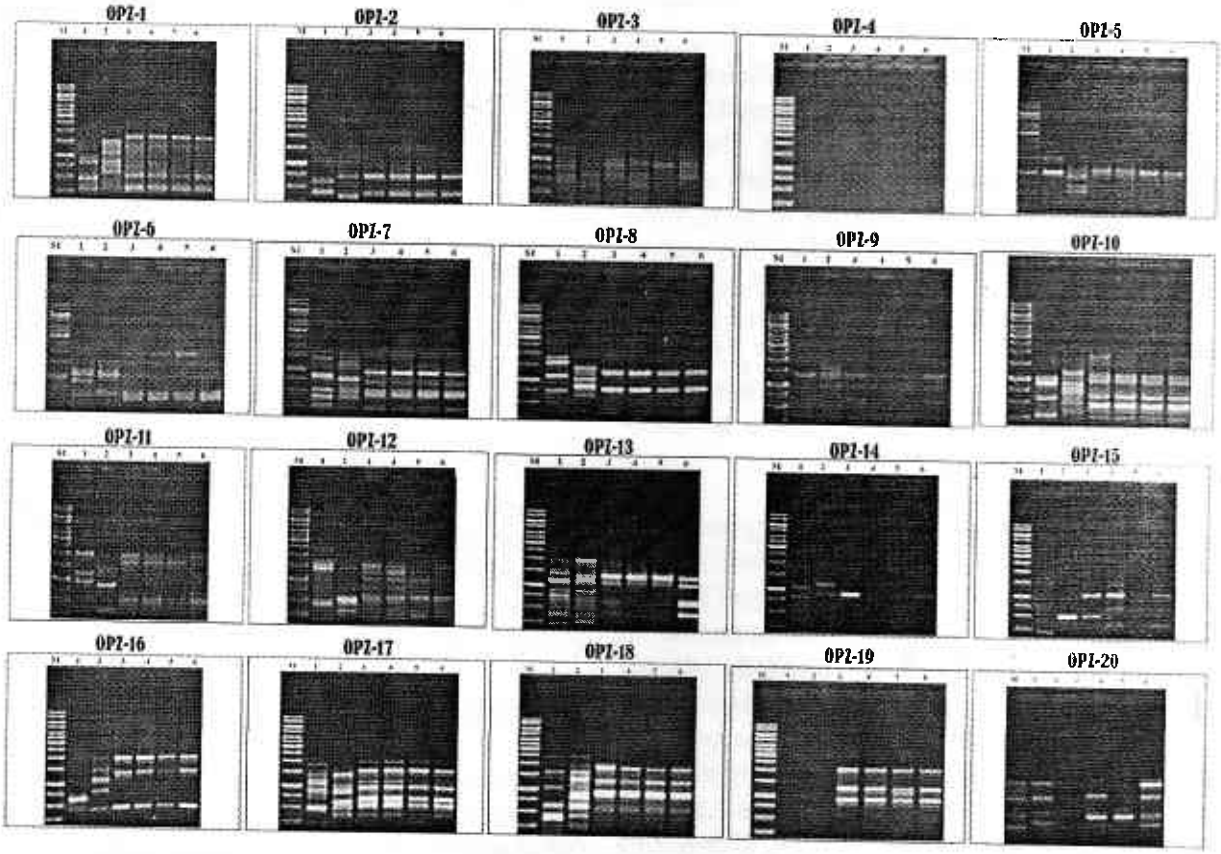

Fig. 1 : RAPD analysis of six isolates of *Fusarium oxysporum* f.sp *pisi* with 20 primers(OPZ-01-OPZ20).

of polymorphism is 97.32. The number of bands per primer ranged from maximum of 10 (given by OPZ-1) to minimum of 2 (given by OPZ-3,9&14) with an average of 6 bands per primer. Among OPZ primer in this study 8 RAPD primers (given by OPZ-1,7,8,10,13,16,17&18) produced average or above average amplified products (Table 2) Moreover, OPZ primers 5&13 showed unique monomorphic band and remaining primers showed polymorphic bands except OPZ primer 4 which did not give any amplification. From the table it was also cleared that the size of the amplified product varied from 200 bp or 0.2 kb to a maximum of 1480 bp or 1.48 kb. Pooja Sharma (2006) also found the genetics diversity in *Fusarium oxysporium* f. sp. *pisi* isolated from three agro-climatically distinct regions, sub-tropical, sub-humid and wet temperature of north western Himalayas based on cultural characteristics, native protein and esterase isozyme.

From the present finding it may be concluded that the high level of morphological, cultural and molecular variability existed among the isolates of *Fusarium oxysporum* f. sp. *pisi*. Based on the level of polymorphism detected by individual primers, informative primers had been identified. Those primers were identified where number of bands produced by those particular primers was to be more than average i.e. 6 and secondly they showed high polymorphism. Moreover, DNA polymorphism as revealed by different OPZ primers in case of *Fusarium oxysporum* had been depicted in figure.

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