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MOLECULAR CHARACTERIZATION OF *FUSARIUM OXYSPORUM* F. SP. *CICERI* USING SCAR MARKERS

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Type Race-1
Type Race-2
Type Race-3
Type Race-4

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ABSTRACT
A study was development of putative SCAR markers from monomorphic bands of RAPD and ISSR primers. The races selected for studies were Type Race-1 (Hyderabad and Akola), Type Race-2, (Kanpur), Type Race-3, (Gurdaspur), Type Race-4 (New Delhi and Jabalpur). Primers used for developing SCAR marker for *Fusarium oxysporum* f.sp. *ciceri* were ISSR $(GA)_8$YT ,$(ATG)_6$,$(GA)_8$T) and RAPD OPA13 OPA18 and OPB14. These primers showed the monomorphic bands, these can be used for development of the putative of SCAR (Sequence Characterized Amplified Regions) markers for identification of *Fusarium oxysporum* f.sp.*ciceri*. The amplicon size 600 bp$(ATG)_6$,700 bp $(GA)_8$YT and 650 bp of ISSR and 750 bp(OPA18),1.4 kb (OPB14),1500 and 800 bp of OPA 13 were eluted, isolated, confirmed and sequenced. The primers were designed with the sequenced information of these two fragments using primer.3.software and putative SCAR markers were developed. These set of markers were validated against the isolates of the pathogen collected from different locations of India representing various races of the pathogen. Races 1and 4 showed close virulence patterns on chickpea differentials.

INTRODUCTION

Chickpea (Cicer arietinum L.) is one of the most important pulse crops cultivated in tropical and temperate regions. Low yield of chickpea is attributed to its susceptibility to several fungal, bacterial, and viral diseases. Among chickpea diseases, Fusarium wilt caused by *Fusarium oxysporum* f.sp. *ciceri* considered to be one of the most limiting factors of chickpea production (Haware and Nene, 1982). The fungus is both seed and soil-borne. The fungus colonizing xylem vessels could survive on crop residues (root and stem portions) buried in the soil for atleast 72 months (Haware *et al*., 1990). Biotechnology Centre, Post Graduate Institute, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola. *Fusarium oxysporum* f.sp. *ciceri* is a highly variable pathogen. Eight races of this pathogen have been reported, of which six (1A, 2, 3, 4, 5 and 6) cause wilting symptoms, whereas the races 0 and 1B/C cause yellowing syndrome (Gowda *et al.,* 2009). These races have distinct geographic distribution. Races 1-4 have been reported only from India whereas 0, 1B/C, 5, and 6 are found only in the Mediterranean region and USA (Kalaria *et al.,* 2004 andJimenez-Gasco *et al*., 2001).

The disease is wide spread in chickpea growing areas of the world and causes variable losses depending upon stages of crop infected. Early wilting causes 77-94 % losses, while late wilting causes 24-65 % loss (Haware *et al*., 1980).

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). Races 2, 3, and 4 have not yet been reported in other countries. Races 0, 1A, 1B/C, 5, and 6 have been found in California (USA) and Spain; races 0 and 1B/C in Syria, Tunisia, and Turkey; races 0, 1A, and 6 in Israel; races 1A and 6 in Morocco; and race 0 in Lebanon (Jimenez-Diaz *et al*., 1993).

The present study was aimed to understand the molecular differences in Indian races of *Fusarium oxysporum* f.sp. *ciceri* using SSR markers, which would help to identify new race and developing region specific resistant varieties and proper management of Fusarium wilt in chickpea.

MATERIALS AND METHODS

The present investigation entitled "Molecular characterization races of *Fusarium oxysporum* f.sp. *ciceri* causing chickpea wilt through SSR markers" was carried out in the laboratory of Department of Plant Pathology and Biotechnology Centre, Post Graduate Institute, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola in 2012-2013. Development of SCAR (Sequence Characterized Amplified Regions) markers for

identification of *Fusariumoxysporum*f.sp.*ciceri* and identify new race and developing region specific resistant varieties and proper management of Fusarium wilt in chickpea.

Isolates

The isolates of races of *Fusarium oxysporum* f.sp. ciceri viz. Race- 1 (Hyderabad and Akola), Race- 2 (Kanpur), Race- 3 (Gurdaspur), Race- 4 (New Delhi and Jabalpur) was obtained from ITCC (Indian Type Culture Collection), Division of Mycology and Plant Pathology, IARI, New Delhi; ICRISAT, Hyderabad and Department of Plant Pathology, Dr. PDKV, Akola (M.S.). The *Fusarium oxysporum* f.sp. *ciceri* specific Sequence Characterized Amplified Region (SCAR) primer pair Foc0-12 f-GGCGTTTCGCAGCCTTACAATGAAG and Foc0-12 r-GACTCCTTTTTCCCGAGGTAGGTCAGAT were used for the confirmation of the isolates (Jimenez Gasco *et al.,* 2003). 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 of Jimenez Gasco and Jimenez- Diaz (2003).

DNA Extraction

Genomic DNA of the fungal isolates was purified from mycelium as described by CTAB method of DNA extraction by Murray and Thompson (1980). Ground mycelium was suspended in an extraction buffer (100 mM Tris- HCl [pH 8.0], 1.4 M NaCl, 20 mM EDTA, 2% CTAB).Mercapthoethanol was added to the above content. The mixture was homogenized with equilibrated chloroform/isoamyl alcohol (24:1), and centrifuged for 15 min at 8000 rpm (4ºC). Then nucleic acid was precipitated by adding cold Isopropanol and centrifuged at 8000 rpm for 15 min and pellet was resuspended in TE buffer and stored at -20ºC. The solution was treated with RNAse enzyme (10.51μl for 200μL) at 37ºC for 1hr. DNA samples were analyzed on 1.2% agarose gels in 1x TBE buffer to estimate the concentration and quality of the extracted DNA. For PCR reactions, samples were diluted to a final concentration of 25 to 50 ng/ìl in sterile water.

Specific PCR reactions

The PCR reaction mixture (12.5μL) contain PCR reaction buffer 10X without 1.25μL, 25mM $Mgcl_2$,1.25 μL 10 mM each DNTPs 0.75 μl , 5 unit μl^{-1} Taq DNA Polymerase 0.5 μl, 10 μM Primer 2.0 μl ,10ng/ μl DNA 2.0 μl .. Cycling profiles consisted of an initial step of 5 min at 94°C, 40 cycles of 1 min at 94°C, 32°C 1 min of annealing temperature, and 30 s at 72°C, followed by a final step of 4 min at 72°C. Annealing temperatures were 32°C for the *F. oxysporum* f. sp. *ciceris*-specific RAPD primer OPA13,OPA18,OPB14 and (48-68)°C for specific ISSR primer $(GA)_8T$,$(GA)_8YT$,$(ATG)_6$ and visualized as described above for the RAPD and ISSR reactions.

Electrophoresis of RAPD and ISSR Marker

The ISSR and analysis was carried out in horizontal gel electrophoresis. The PCR products were separated electrophoretically in 1.2% agarose gel using 1x TBE buffer. The gel was stained with ethidium bromide (Kumari *et al.,* 2014 and Sambrook *et al.,* 1989).

The cleaned and dried electrosphoresis assembly was used for ISSR and separately. The gel tray was wiped and cleaned with methanol. The agarose gel solution was prepared by mixing agarose in 1x TBE Buffer. This mixture was heated in microwave oven for 3 min. The ethidium bromide was added to the gel solution as staining agent. The gel solution was then poured in the gel-casting tray and combs were placed in the gel and allowed to set. The 1x TBE buffer was used as the tank buffer. After the casting of the gel the combs were removed and the gel was placed in electrophoresis assembly with 1x TBE buffer. The gel was pre-run for 15 min. The care was taken while handling the gel as the ethidium bromide added is highly mutagenic. After the pre-run, the ISSR-PCR and product was mixed with 5 μL 6x dye and the mixture was loaded in the wells, along with the 1 Kb DNA ladder in the first well. The gel was run for 2hrs. at 70v. After the run, the gel was removed carefully from the unit and observed under Gel Doc instrument.

Excising and dissolving the gel

The Excising and dissolving gel was done as per the described below by the quixgen gel extraction kit. Methods was done by using Equilibrated water bath or heat block to 50ºC. Excised

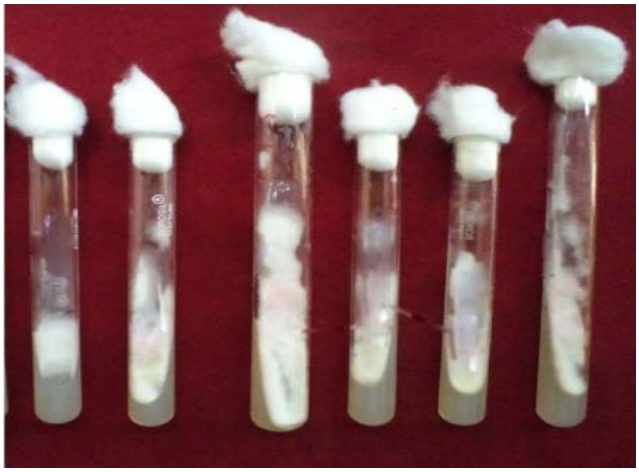


Figure 1A: Pure culture of isolates of *Fusarium oxysporum f. sp.* ciceri

Figure 1B: Cultures of *Fusarium oxysporum f. sp. ciceri* in patriplates

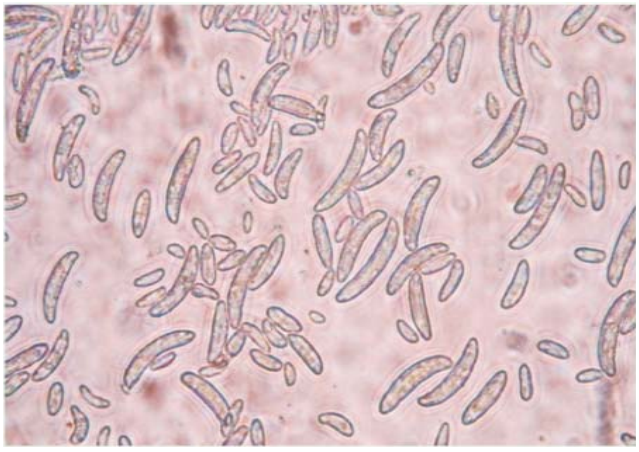


Figure 1C: Micro and macro conidia

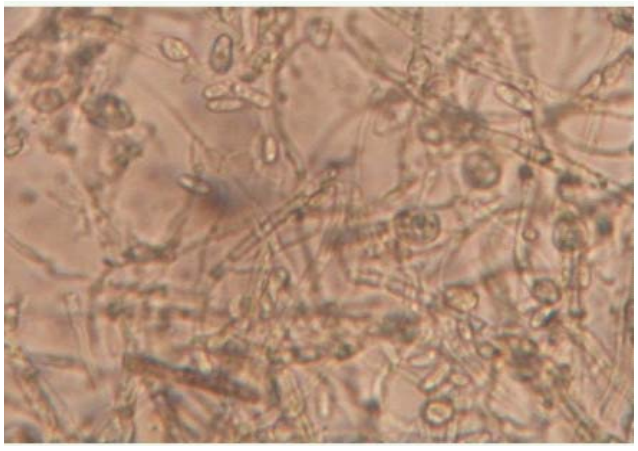
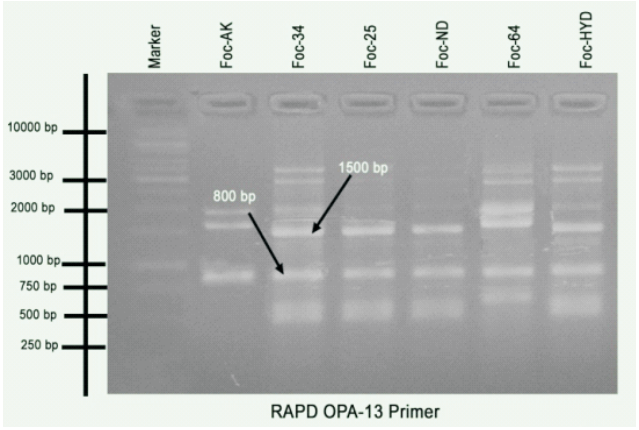


Figure 1D: Clamaidospores



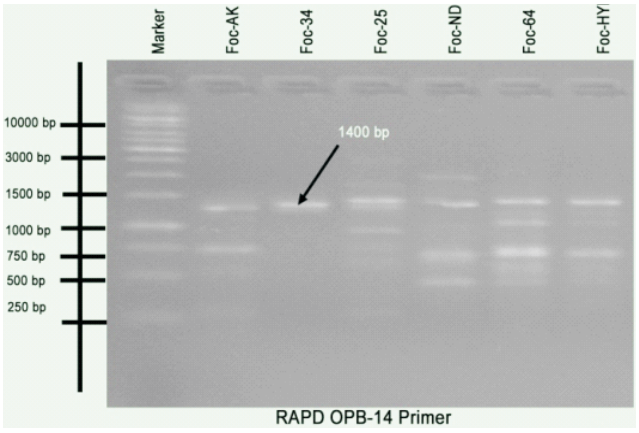
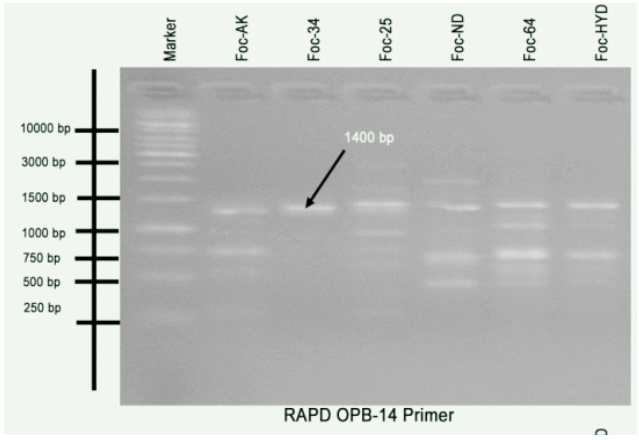


Figure 2: RAPD banding Pattern OPA-13, OPB-14 and OPA-18



a minimal area of gel containing the DNA fragment of interest. Weighed the gel slice containing the DNA fragment using a scale sensitive to 0.001 g.Added gel solubilizationBuffer(L3) to the excised gel in the tube size indicated in the following table:10.

Placed the tube with the gel slice and buffer L3 into a 50ºC water bath or heat block. Incubated the tube at 50ºC for 10 minutes. Incubated the tube every 3 minutes to mix and ensure gel dissolution.After the gel slice appeared dissolved,incubated the tube for an additional 5minutes.

Purified the DNA using a centrifuge or vaccum manifold.

Before starting of the experiment some procedure should be followed

Added ethanol to the wash buffer (w1) according to the level on the bottle.

Load: Pipet the dissolved gel piece onto a Quick Gel Extraction Column inside a Wash Tube.Used 1 colume per 400mg of agarose gel. Bind:Centrifuged the column at $>12000\times$g for 1 minute. Discard the flow-through and placed the column into the wash tube. Wash: Added 500μL Wash Buffer (W1) Containing ethanol to the column. Remove Buffer:Centrifuged the column at $>12000\times$ g for 1 minute. Discarded the flow-through and placed the column into the Wash Tube. Remove Ethanol: Centrifuged the column at maximum speed for 1-2 minutes. Discarded the flow-through. Elute:Placed the column into a Recovery Tube. Added 50 μL Elution Buffer (E5) to the centre of the column. Incubated the tube for 1 minute at room temperature. Collect the centrifuge tube at 12,000$\times$ g for 1 minute. Store: The elution tube contained the purified DNA. Stored the purified DNA at 4ºC for immediate use or at -20ºC for long–term storage.

Sequencing of RAPD and ISSR marker design of specific

SCAR primers

The six samples using primer OPA13, OPB14, OPA18, $(GA)_8YT$, $(GA)_8T$, $(ATG)_6$ eluted and purified from the gel and sent to the company SETLAB India in Pune- 411035 for sequencing and analyzing. A search for sequence similarities was performed with BLAST NCBI Primers were designed using the computer program Primer 3 software.

Data analysis

The gel images were captured and visualizaed in gel documentation system (Biorad).The data was scored as the presence (1) or absence (0) of individual band for each isolates in RAPD-PCR and ISSR-PCR analysis of races of *Fusarium oxysporum*.f.sp.*ciceri*.

The data was used to generate similarity coefficient using simple matching coefficient based on RAPD and ISSR bands scoring.

The sample for sequencing was sent to the company SETLAB INDIA in pune-411035.Sequence was obtained by analysis for the development of putative SCAR marker in BLAST and Tm calculator Primers selected for developing of SCAR marker are listed below.

RESULTS

List of the Primers selected for RAPD for developing SCAR marker

Three RAPD primers viz., OPA13, OPA18, OPB14 provided by Genaxy Scientific Pvt. Ltd. were used to evaluate the races of *Fusarium oxysporum* f. sp. *ciceri*. The PCR amplified product of each RAPD primer was resolved on 1.2% agarose gel and each ISSR primer was resolved on 1.8% agarose gel electrophoresis and the amplified product was compared with 1 Kb DNA ladder obtained from Fermentas.

The banding pattern observed in primer OPA-13 has presented in plate1. The primer amplified 2 amplicons among 5 races (6 isolates) of *Fusarium oxysporum* f.sp. *ciceri*.

The RAPD primer OPA- 13 showed the monomorphic banding pattern in all the races of *Fusarium oxysporum* f.sp. *ciceri* of the molecular weight1553 to1102 bp. The monomorphic band of 1500 and 800 bp was cut with the help of sharp spatula for elution of specific sequence from the band of interest.

The RAPD primer OPA- 18 showed the monomorphic banding pattern in all the races of *Fusarium oxysporum* f.sp. *ciceri* of the molecular weight 476 bp to1900 bp. The monomorphic band of 750 bp was cut with the help of sharp spatula for elution of specific sequence from the band of interest.

RAPD primer OPB-14 showed the monomorphic banding pattern in all the races of *Fusarium oxysporum* f.sp. *ciceri* of the molecular weight 232 bp to 2677 bp .The band showing monomorphic banding pattern with the expected size of 1.4 kb was cut with the help of sharp.

List of the primers selected of ISSR for developing SCAR marker

The ISSR primer GA_8T showed monomorphic banding pattern in all the races of *Fusarium oxysporum* f.sp. *ciceri*. The band showed monomorphic banding pattern with base pair 650 which was cut with the help of sharp spatula for elution of specific sequence from the band of interest.

The ISSR primer GA_8YT showed monomorphic banding pattern in all the races of *Fusarium oxysporum* f.sp. *ciceri*. The band showed monomorphic banding pattern with base pair 700 which was cut with the help of sharp spatula for elution of specific sequence from the band of interest.

The ISSR primer ATG_6 showed monomorphic banding pattern

Table 1: List of isolates of races of *Fusarium oxysporum* f. sp. ciceri

Sr.	RACES	LOCATION
1	Race- 1 (Foc-HYD)	Hyderabad
2	Race- 1 (Foc-AK)	Akola
3	Race- 2 (Foc-25)	Kanpur
4	Race- 3 (Foc-64)	Gurdaspur
5	Race- 4 (Foc-ND)	New Delhi
6	Race- 4 (Foc-34)	Jabalpur

Table 2: List and sequence of RAPD primers used in the study

Sr. No.	Primers	Sequence 5′ to 3′	Annealing temperature (ºC)
1.	OPA-13	CAGCACCCAC	29
2.	OPA-18	AGGTGACCGT	27
3.	OPB-14	TCCGCTCTGG	29

Table 3: List and sequence of ISSR primers used in the study

Sr. No.	Primers	Sequence 5′ to 3′	Annealing temperature (^{0}C)
1.	$(GA)_8T$	GAG AGA GAG AGA GAG AT	50
2.	$(GA)_8YT$	GAG AGA GAG AGA GAG AGC T	53
3.	$(ATG)_6$	ATG ATGATGATGATGATG	50

Table 4: Preparation of PCR master mix for RAPD

Sr. No.	Components	Concentration (stock)	Quantity (μl)
1	PCR Reaction Buffer	10X without $Mgcl_2$	1.25
2	$Mgcl_2$	25 mM	1.25
3	DNTPs	10 mM each	0.75
4	Taq DNA Polymerase	5 unit μl^{-1}	0.5
5	Primer	10 μM	2.0
6	DNA	10ng/ μl	2.0
7	Double distilled sterile water	-	4.75
		Total	12.5

Table 5: Programme for RAPD-PCR

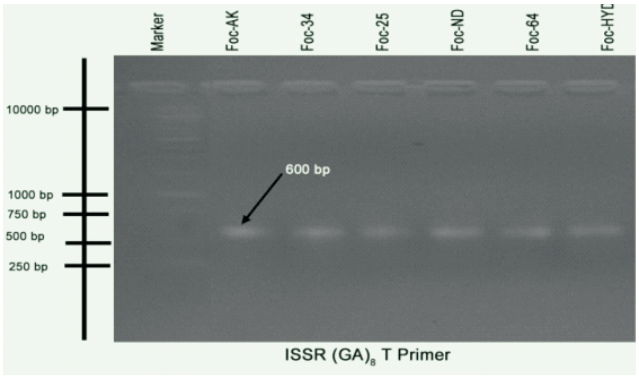
Step	Temp. °C	Duration	Cycles	Function
1	94	5 min	1	Initial denaturation
2	94	1 min	40 cycle	Denaturation
3	32	1 min		Annealing
4	72	2 min		Extention
5	75	5 min	1	Final elongation
6	4			Retention

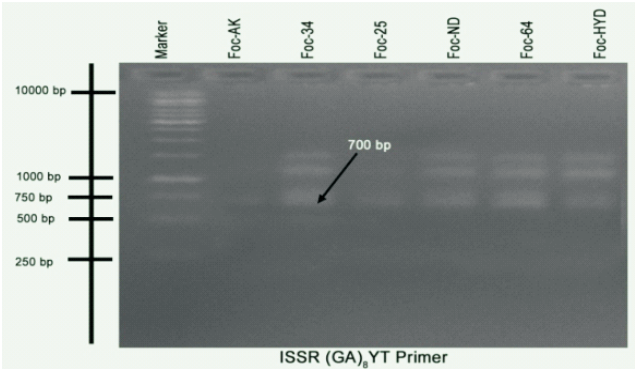
Table 6: Programme for ISSR-PCR Programme for ISSR-PCR

Step	Temp. °C	Duration	Cycles	Function
1	94	10 min	1	Initial denaturation
2	94	45 sec	40 cycles	Denaturation
3	48-68 (Depending on primers)	45 sec		Annealing
4	72	2 min		Extension
5	72	10 min	1	Final elongation
6	4			Retention

Table 7. Excising and dissolving the gel

Gel	Tube	Buffer L3 Volume
d″2% agarose	1.7- mL polypropylene	3:1(i.e.,1.2mL Buffer L3:400mg gel piece).
>2% agarose	5-mL polypropylene	6:1(i.e.,2.4mL Buffer L3:400mg gel piece).





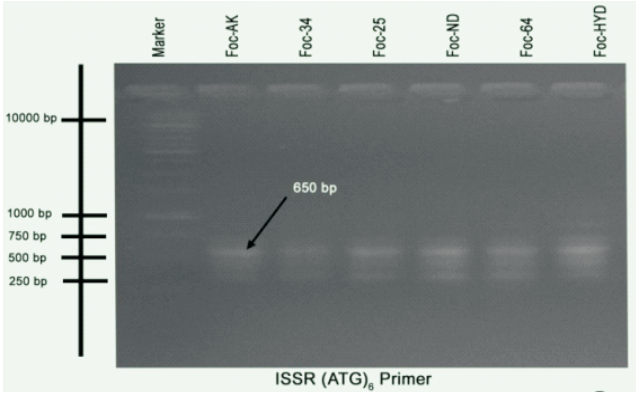


Figure 3: ISSR banding Pattern $(GA)_8$ YT, $(ATG)_6$ and $(GA)_8$ Primers

in all the races of *Fusarium oxysporum* f.sp. *ciceri*. The band showed monomorphic banding pattern with base pair 600 which was cut with the help of sharp spatula for elution of specific sequence

DNA amplification and sequence

Polymerase chain reaction (PCR) primers and amplification of these target base pair regions were purified using the quixagen gel extraction kits. All primers were synthesized by SETLAB INDIA Pune- 411035. Sequencing of PCR products was done by the faculty of SETLAB INDIA Pune- 411035

Genetic diversity of the isolates of *Fusarium oxysporum* f. sp. *ciceris* causing chickpea wilt for the developing of SCAR marker was studied by Durai(2012) for the isolates collected from 12 states representing different agro-ecological regions of India. It was determined through randomly amplified polymorphic DNA (RAPD) markers. Each group had the isolates from different states present in various agro-ecological regions of India. The RAPD primers, namely, OPA 7 and OPA 11 produced *Foc* specific fragment of H″1.3 kb and H″1.4 kb, respectively in all the isolates. These fragments were eluted, purified, cloned in pGEM-T Easy vector and sequenced. Primers were designed with sequence information of these two fragments using primer.3 software. Two sets of sequence characterized amplified region markers (SC-FOC 1 and SC-FOC 2) developed

Table 8: Results of Sequencing of RAPD Primer OPA 18(S1)

Description	Max score	Total score	Query cover	Evalue	Max indent	Accession
Fusariumoxysporum f.sp.*vasinfactum*25433 cont1.78,whole genome shotgun sequence	1005	1130	97%	0.0	93%	AGNC01000078.1
*Fusariumoxysporum*f.sp.*raphani* 544005 cont1.12, whole genome shotgun sequence	996	1035	97%	0.0	93%	AGNGO1000112.1
*Fusariumoxysporum*f.sp.*conglutinans* race 2 54008 cont1.180, whole genome shotgun sequence	996	996	97%	0.0	93%	AGNF01000180.1
Fusariumoxysporum f.sp.Fo5176 contig01776, whole genome shotgun sequence	996	996	97%	0.0	93%	AFQF01001689.1
*Fusariumoxysporum*f.sp.*Pisi* HDV247 cont1.79, whole genome shotgun sequence	993	1069	97%	0.0	93%	AGBI01000079.1
*Fusariumoxysporum*f.sp.*cubense* race 4 contig3433, whole genome shotgun sequence	991	991	97%	0.0	93%	AMGQ01003433.1
*Fusariumoxysporum*f.sp.*cubense* tropical race 4 54006 cont1.102, whole genome shotgun sequence	991	991	97%	0.0	93%	AGNDO1000102.1
*Fusariumoxysporum*f.sp.*melonis* 26406 cont1.124,whole genome shotgun sequence	985	1110	97%	0.0	93%	AGNE01000124.1
*Fusariumoxysporum*f.sp.*lycopersici* 4287 chromosome 2a cont2.541, whole genome shotgun sequence	985	985	97%	0.0	92%	AAXH01000541.1
*Fusariumoxysporum*f.sp.FOSC 3-a cont1.53, whole genome shotgun sequence	978	1055	97%	0.0	92%	AFML01000053.1
*Fusariumoxysporum*f.sp.*cubense* race 1 contig826, whole genome shotgun sequence	973	973	97%	0.0	92%	AMGP01000826.1
*Fusariumoxysporum*f.sp.*radicislycopersici* 26381 cont1.26, whole genome shotgun sequence	962	1085	97%	0.0	92%	AGNB01000026.1
*Fusariumoxysporum*f.sp.*lycopersici*MN25 cont1.10, whole genome shotgun sequence	957	1076	97%	0.0	92%	AGBH01000010.1
Fusariumoxysporum FO47 cont1.47, whole genome shotgun sequence	957	1113	97%	0.0	92%	AFMM01000047.1

Table 9: Results Of RAPD Primer OPA-18(S2)

Description	Max score	Total score	Query cover	E	Max value	Accession indent
FusariumOxysporumf.sp.lycopersici 4287 chromosome 8 cont2.1000,whole genome shotgun sequence	874	874	90%	0	96%	AAXHO1001000.1
FusariumOxysporumf.sp. lycopersici MN25 cont1.145, whole genome shotgun sequence	854	854	89%	0	95%	AGBH01000
Fusariumoxysporum f. sp. melonis 26406 cont1.166, whole genome shotgun sequence	847	882	87%	0	96%	AGNE01000166.1
Fusariumoxysporum f. sp. radicis-lycopersici 26381 cont1.64, whole genome shotgun sequence	836	873	87%	0	96%	AGNB01000064.1
Fusariumoxysporum Fo47 cont1.53, whole genome shotgun sequence	823	860	85%	0	95%	AFMM01000053.1
Fusariumoxysporum f. sp. cubense race 1 contig591, whole genome shotgun sequence	778	778	86%	0	91%	AMGP01000591.1
Fusariumoxysporum f. sp. raphani 54005 cont1.380, whole genome shotgun sequence	778	778	85%	0	91%	AGNG01000380.1
Fusariumoxysporum f. sp. vasinfectum 25433 cont1.236, whole genome shotgun sequence	778	778	85%	0	91%	AGNC01000236.1
Fusariumoxysporum f. sp. pisi HDV247 cont1.144, whole genome shotgun sequence	778	778	85%	0	91%	AGBI01000144.1
Fusariumoxysporum f. sp. conglutinans race 2 54008 cont1.260, whole genome shotgun sequence	774	774	85%	0	91%	AGNF01000260.1
Fusariumoxysporum Fo5176 contig00934, whole genome shotgun sequence	774	774	85%	0	91%	AFQF01000897.1
Fusariumoxysporum f. sp. cubense tropical race 4 54006 cont1.167, whole genome shotgun sequence	756	756	85%	0	91%	AGND01000167.1
Fusariumoxysporum f. sp. cubense race 4 contig112, whole genome shotgun sequence	753	753	85%	0	90%	AMGQ01000112.1
Fusariumoxysporum FOSC 3-a cont1.82, whole genome shotgun sequence	742	742	85%	0	91%	AFML01000082.1

Table 10: Results Of Sequencing ISSR Primer $(GA)_8$ YT

Description	Max score	Total score	Query cover	E value	Max ident	Accession
Fusariumoxysporum f. sp. vasinfectum 25433 cont1.78, whole genome shotgun sequence	957	957	89%	0.0	93%	AGNC01000078.1
Fusariumoxysporum f. sp. raphani 54005 cont1.112, whole genome shotgun sequence	948	948	89%	0.0	93%	AGNG01000112.1
Fusariumoxysporum f. sp. conglutinans race 2 54008 cont1.180, whole genome shotgun sequence	948	948	89%	0.0	93%	AGNF01000180.1
Fusariumoxysporum f. sp. pisi HDV247 cont1.79, whole genome shotgun sequence	948	948	89%	0.0	93%	AGBI01000079.1
Fusariumoxysporum Fo5176 contig01776, whole genome shotgun sequence	948	948	89%	0.0	93%	AFQF01001689.1
Fusariumoxysporum f. sp. cubense race 4 contig3433, whole genome shotgun sequence	942	942	89%	0.0	93%	AMGQ01003433.1
Fusariumoxysporum f. sp. cubense tropical race 4 54006 cont1.102, whole genome shotgun sequence	942	942	89%	0.0	93%	AGND01000102.1
Fusariumoxysporum f. sp. melonis 26406 cont1.124, whole genome shotgun sequence	937	937	89%	0.0	92%	AGNE01000124.1
Fusariumoxysporum f. sp. lycopersici 4287 chromosome 2a cont2.541, whole genome shotgun sequence	937	937	89%	0.0	92%	AAXH01000541.1
Fusariumoxysporum FOSC 3-a cont1.53, whole genome shotgun sequence	930	930	89%	0.0	92%	AFML01000053.1
Fusariumoxysporum f. sp. cubense race 1 contig 826, whole genome shotgun sequence	924	924	89%	0.0	92%	AMGP01000826.1
Fusariumoxysporum f. sp. radicis-lycopersici 26381 cont1.26, whole genome shotgun sequence	913	913	89%	0.0	92%	AGNB01000026.1
Fusariumoxysporum f. sp. lycopersici MN25 cont1. 10, whole genome shotgun sequence	908	908	89%	0.0	92%	AGBH01000010.1
Fusariumoxysporum Fo47 cont1.47, whole genome shotgun sequence	908	908	89%	0.0	92%	AFMM01000047.
Fusariumoxysporum f. sp. vasinfectum 25433 cont1.78, whole genome shotgun sequence	957	957	89%	0.0	93%	AGNC01000078.1

Table 11: List of the putative SCAR markers

Sl. No	Putative primer	Nucleotide primer sequence	Tm	GC %	Nucleo-tide
1.	F OPA 18	GTGACCGTAATTATGGGCCTAACTTCCTCA	68.64	47	30
	R OPA-18	GTGACCGTTTCCCCTGCGTTATTTTATATC	67.94	43	30
2.	F $(GA)_8$YT	AGAGAGAGCTAAGCAGAGAT	48.65	45	20
	R $(GA)_8$YT	AGAGAGAGC TTCTCTCTCTC	47.80	50	20
3.	F $(GA)_8$YT	GAGAGAGAGAGCTAAGCAGAGAT	54.23	48	23
	R $(GA)_8$YT	GAGAGAGAGAGCTTCTCTCTCTC	53.71	52	23
4.	F $(GA)_8$YT	GAG AGA GAG AGA GAGAGCTAAGCAGAGAT	61.79	48	29
	R $(GA)_8$YT	GAG AGA GAG AGA GAGAGC TTCTCTCTCTC	61.63	52	29
5.	F $(GA)_8$YT	AGAGAGAGAGCTAAGCAGAGAT	52.26	45	22
	R $(GA)_8$YT	AGAGAGAGAGCTTCTCTCTCTC	51.63	50	22

F = Forward primer; R = Reverse primer

from the sequences of these fragments were found to be specific to *Foc* and produced an amplicon of 1.3 and 1.4 kb, respectively. These set of markers were validated against the isolates of the pathogen collected from different locations of India representing various races of the pathogen.

Sequencing Results

Sample was first sequenced and analysed from different isolates of each of race. Total 6 isolates consisting 4 races viz., Type Race -1(Hyderabad and Akola), Type Race -2 (Kanpur), Type Race -3 (Gurdaspur), Type Race -4 ($_{New}$ Delhi and Jabalpur) of *F. oxysporum* f. sp.*ciceri* were sequenced and analysed. Six primer were send for sequencing to SETLAB INDIA. Sequencing results were given below.

Similar study was reported by Najafiniya and Sharma (2011a) for the detection of *Fusarium oxysporum* f. sp. *cucumerinum* pathogenic groups for which specific PCR-based marker was developed. Specific random amplified polymorphic DNA (RAPD) markers which identified in four pathogenic groups I, II, III, and IV were cloned into PGem-Teasyvector. Cloned fragments were sequenced, and used for developing sequence characterized amplified regions (SCAR) primers for detection of pathogenic groups. *F. oxysporum* f. sp. *cucumerinum* isolates belonging to four pathogenic groups in India, cucumber nonpathogenic *F. oxysporum,F. oxysporum* f. sp. *moniliforme* and *melonis*, *Fusarium udum*, and isolate of *Alternaria* sp. were tested using developed specific primers. A single 1.320 kb, 770 bp, 1.119 kb, and 771 bp fragments were amplified from pathogenic groups I, II, III, and IV isolates, respectively. Results showed that the PCR based marker, used

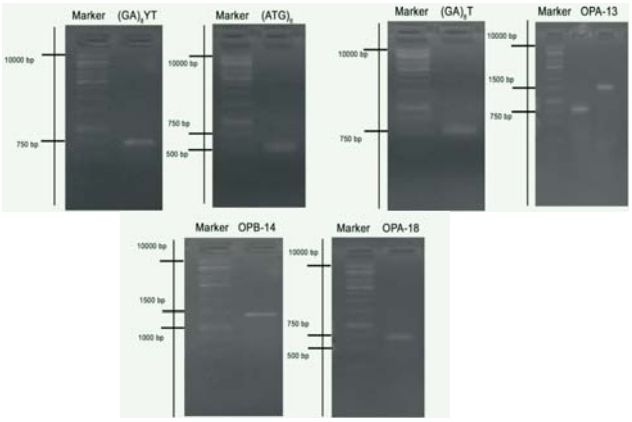


Figure 4: Confirmation of evaluated DNA of $GA)_8$ YT, $(ATG)_6$ and $(GA)_8$, OPA-13, OPA-14 and OPA- 18.

in this research work, could detect up to 1 ng of fungal genomic DNA ISSR primer $(GA)_8$YT and RAPD primer OPA 18 two showed the results of interest and evaluted by analyzing the data available at NCBI web site with BLAST as given below:

Similar study reported by Jiménez-Gasco and Jiménez-Díaz 2003) specific primers and polymerase chain reaction (PCR) assays that identify *Fusariums oxysporum* f. sp. *ciceris* and each of the *F. oxysporum* f. sp. *ciceris* pathogenic races 0, 1A, 5, and 6 were developed. *F.oxysporum* f. sp. *ciceris*- and race-specific random amplified polymorphic DNA (RAPD) markers were identified and sequence characterized amplified region (SCAR) primers for specific PCR were developed. *F. oxysporum* f. sp.*ciceris* isolates representing eight reported races from a wide geographic range, nonpathogenic *F. oxysporum* isolates, isolates of *F. oxysporum* f.sp. *lycopersici*, *melonis*, *niveum*,*phaseoli*, and *pisi*, and isolates of 47 different *Fusarium* spp. were tested using the SCAR markers developed. The specific primer pairs amplified a single 1,503-bp product from all *F.oxysporum* f. sp. *ciceris* isolates; and single 900- and 1,000-bp products were selectively amplified from race 0 and race 6 isolates, respectively. The specificity of these amplifications was confirmed by hybridization analysis of the PCR products. A joint use of race 0- and race 6-specific SCAR primers in a single-PCR reaction together with PCR assay using the race 6-specific primer pair correctly identified race 1A isolates for which no RAPD marker was found out previously. All the PCR assays described herein detected up to 0.1 ng of fungal genomic DNA.

Primers were designed with sequence information of these fragments using primer sequence and Tm calculater software. Two sets of sequence characterized amplified region markers (SC-FOC 1 and SC-FOC 2) developed from the sequences of these fragments were found to be specific to *Foc* and produced amplicons of base pair 750 and 700 of primer OPA18 and $(GA)_8$YT, respectively. These set of markers were validated against the isolates of the pathogen collected from different locations of India representing various races of the pathogen.

DISCUSSION

The chickpea wilt caused by *Fusarium oxysporum* f.sp. *ciceri*

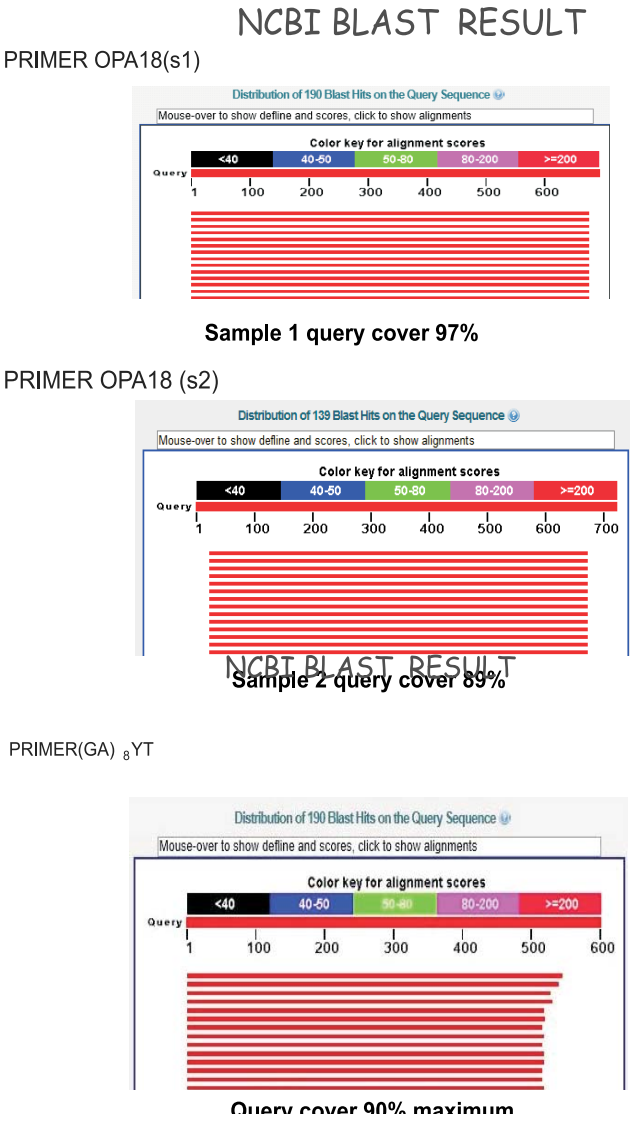


Figure 5: NCBI blast result of *Fusariums oxysporum* f. sp. *ciceris*

is one of the major constraint in chickpea production worldwide. The races of *Fusarium oxysporum* f.sp. *ciceri* were collected from Indian Type Culture collection (ITCC), New Delhi and International Crop Research Institute for Semi-Arid Tropics (ICRISAT), Hyderabad. Total 6 isolates consisting of 4 races *viz*., Type Race -1(Hyderabad and Akola), Type Race -2 (Kanpur), Type Race -3 (Gurdaspur) and Type Race -4 (New Delhi and Jabalpur) and one isolate from Akola were used.

Genetic characterization of *F. oxysporum* f. sp. ciceris races is important for the efficient management of Fusarium wilt through use of resistant cultivars in chickpea-growing areas. This study demonstrated that SCAR primers developed from RAPD markers (Gasco J *et al.*, 2001) can be used to unambiguously identify *F. oxysporum f. sp.* ciceris races 1, 2, 3, and 4, which are prevalent in the Indian condition (Mamta Sharma *et al.*, 2009). Also, these SCAR primers proved useful in discriminating *F. oxysporum f. sp.* ciceris from other diverse formae speciales of this species, other *Fusarium spp.*, and nonpathogenic *F.*

oxysporum. The SCAR primer pairs amplified a single diagnostic PCR product from all isolates of the target forma specialis and races irrespective of the geographic origin. An additional benefit from the present study concerns the molecular identification of race 2 of the pathogen, for which no RAPD marker was found previously (Gasco J *et al.,* 2001). This can be achieved through two independent PCR assays, one using the SCAR primers which amplified a single PCR product from races 1 and 4 and another assay using the race 3-specific primers. Races 1and 4 showed close virulence patterns on chickpea differentials (Halila, H. M et al., 1996 and Diaz J *et al.,* 1993) and high genetic similarities by RAPD analyses (Gasco J *et al.,* 2001). This could explain them occurrence of a common specific PCR marker.

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