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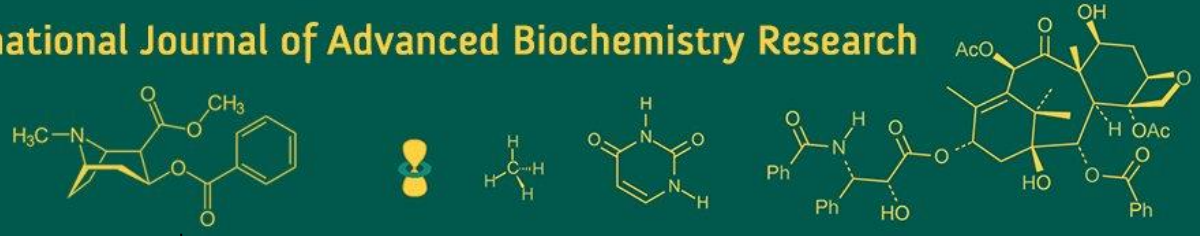

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Race identification and differentiation of *Fusarium oxysporum* f. sp. *ciceri* causing chickpea wilt

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Abstract
Fusarium oxysporum f. sp. *Ciceri* (FOC) caused vascular wilt is one of the main causes of reduced productivity in chickpeas. Identification and differentiation of races is crucial for integrated management of wilt. Differential screening of chickpea using several genotypes of the crop were the primitive method of race identification and this is very time consuming and laborious method. Molecular identification and characterization of four different races of *Fusarium oxysporum* f. sp. *ciceri* was conducted using different marker techniques, like ITS (Inter transcribed spacers), SCAR (Sequence characterized amplified region) and FDP (Fibrinogen degradation products) primers. Among 11 FDP primers and three SCAR primers used in the experiment most of them were used to differentiate races of pathogen more effectively. FDP 25 and FDP 3 differentiated FOC1, whereas FDP 11, FDP 12, FDP 14, FocR0-M15 and FocR6-O2 was able to differentiate FOC2 from other races. FOC3 is distinguished by FDP 22 and FDP 3 differentiate FOC 4 with a band size of 400 bp. These primer combinations can be used to distinguish between the many races of *F. oxysporum f. sp. ciceri*.

Keywords: Chickpea, *Fusarium oxysporum* f. sp. *ciceri*, ITS, FDP, SCAR

Introduction
Chickpea (*Cicer arietinum* L.) is a self-pollinating, true diploid (2n=2x=16) cool season leguminous crop with a genome size of approximately 740 Mbp (Arumuganathan and Earle, 1991) [1] and is one of the oldest legume crops which is widely consumed across the world. It ranks second after common bean among the food grain legumes worldwide. It is grown in semi-arid parts of around fifty countries in temperate and subtropical regions of the world (Varshney *et al*. 2014) [23]. The crop may have been originally cultivated in Turkey 7500 years ago, and it most likely originated in southern Turkey and nearby Syria (Singh and Ocampo, 1997) [25].

Chickpeas are farmed over 99 lakh hectares of land in India. The nation's highest productivity level in 2021-2022 was 1086 kg/ha, which led to a harvest of 107 lakh tonnes, a record-breaking amount. Madhya Pradesh contributed significantly, placing top in both categories with 28% of the nation's total chickpea growing area and 34% of its total production. This is followed by Maharashtra (20% and 18%), Rajasthan (19% and 18%) and Karnataka (10% and 6%). Approximately 97% of the country's chickpea crop is produced by ten states, including Madhya Pradesh, Maharashtra, Rajasthan, Karnataka, Uttar Pradesh, Andhra Pradesh, Gujarat, Chhattisgarh, and Jharkhand (GOI, 2022).

Both biotic and abiotic stress have a negative impact on chickpea yield. The most notable diseases that cause significant economic loss are fusarium wilt, ascochyta blight, and botrytis grey mould. While ascochyta blight, fusarium wilt, BGM, stem rot, stunt, and root rot are more frequently found in Mediterranean nations, wilt and blight of chickpea are most destructive in temperate and tropical climates (Gurjar *et al*. 2011) [11]. *F. oxysporum* isolates mostly exhibit host specificity and they can be categorized into formae specialis depending on the host attacked (Alves-Santos *et al*. 2002) [2]. *Fusarium oxysporum* f. sp. *ciceri*, a deuteromycetes fungal pathogen, is the causal agent of chickpea wilt, which is the major limitation to chickpea production worldwide. The disease is prevalent across 32 nations on 6 continents. Chickpea wilt in India was originally observed by Butler in 1918, but Padwick accurately identified its aetiology in 1940. Eradication of Fusarium is challenging because the resting spores chlamydospores can survive in the soil for upto six years (Haware *et al*. 1996) [13].

Wilt disease is brought on by the pathogen entering the roots and causing root rots in the vascular system. The infection typically only damages living root tissues, kills the plant, and then spreads on the dead tissue. The collars may shrink unevenly and visible when the plants have been uprooted. When the roots are parted vertically, internal discoloration that extends to the stem as a result of infection of the xylem tissues of the root and stem may be noticed. The roots and pith do not appear to have rot from the outside. Transverse sections of the affected roots examined under a microscope reveal the fungus's hyphae and spores in the xylem, supporting the diagnosis of vascular wilt (Nene *et al*. 1978) [24]. It is difficult to distinguish the races of *F. oxysporum* f. sp. *ciceri* morphologically. The conventional approach to race identification comprises exposing several cultivars of chickpea to a certain race of *F. oxysporum* f. sp. *ciceri* and evaluating its pathogenicity. This procedure requires at least 40 days to analyse, and reactions may be impacted by a variety of environmental conditions, such as humidity and temperature (Haware and Nene, 1982) [12].

The most efficient and environmentally friendly way to treat the disease may instead be to utilise wilt resistant chickpea cultivars (Jalali and Chand, 1992) [15]. According to Haware and Nene (1982) [12], the considerable pathogenic heterogeneity in the *F. oxysporum* f. sp. *ciceri* may restrict the efficacy of resistance. Additionally, the poor agronomic traits of resistant cultivars have impeded their development (Honnareddy and Dubey, 2006) [14]. Developing an effective plan for treating wilt disease requires an understanding of the molecular underpinnings of pathogenesis and resistance mechanisms. For effective integrated management of wilt, isolates and races must be identified, and a plan must be created that takes resistance into account. It is yet unknown how many physiological races there are in *F. oxysporum* f. sp. *ciceri*, therefore any resistance-breeding strategy must be able to distinguish between them accurately.

The most accurate approach for identifying an isolate or race is through molecular techniques like DNA fingerprinting. It will help in the pyramiding of resistance genes for the formation of strains resistant to different races of disease if the race-specific donors are identified and categorised (Datta and Lal, 2013) [6]. Due to the growing significance of the chickpea Fusarium wilt disease and the use of DNA markers for pathogen identification, the current study aims to distinguish between the four *F. oxysporum* f. sp. *ciceri* races, which are found in India.

Materials and Methods

Purification and identification

The identification of isolated races of *F. oxysporum* f. sp. *ciceri* was done on basis of morphological characters as well as on molecular basis with internal transcribed spacer (ITS). Single-spore cultures of four races (FOC1, FOC2, FOC3 and FOC4) of *F. oxysporum* f. sp. *ciceri* representing four states (Maharastra, Uttar Pradesh, Haryana and Madhya Pradesh) in different pulse growing agroecological zones of India were selected for the present study. These races maintained in Division of Plant Pathology, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola, Maharashtra, India were taken for the present study. The cultures purified with single spore were preserved at 4^0C on potato dextrose agar slants. Through microscopic and visual inspection, the pure cultures were initially recognised based on morphological and cultural traits. Colony features of each races and its sporulation traits were typically used to observe cultural characteristics. Detailed morphological examination of fungal isolates was done under compound microscope and their identification was confirmed by comparing with relevant literature (Booth, 1971 and Booth, 1977) [4, 5].

Optimization of virulence analysis of *F. oxysporum* f. sp. *ciceri* races

Water culture inoculation and soil inoculation procedures were employed to standardise the virulence analysis, using single spore cultures of FOC1, FOC2, FOC3, and FOC4 on the wilt susceptible chickpea genotype JG-62. The experiment was conducted in a greenhouse with access to day light and an automatic cooling system was used to maintain the ambient temperature at 28 ± 2^0C during the experiment.

Genomic DNA extraction

Genomic DNA was extracted from four selected races of *F. oxysporum* f. sp. *ciceri* by CTAB (cetyltrimethyl ammonium bromide; 5M NaCl; 0.5M EDTA, 1M TrisHCl, pH 8.0; pH 8.0 and 2% CTAB) method (Murray and Thompson, 1980) with some modifications. The liquid broth culture of each race was grown in a conical flask. A 7 mm piece of culture disc, cut out from the fringe of a seven day old culture of each race cultured in a Petri plate, was added to 100 ml of potato dextrose broth. For seven days, the inoculated broth was incubated at 28±2 °C. The mycelial mat was dried at room temperature after being filtered through Whatman no. 1 filter paper. The dried mycelium quickly frozen in liquid nitrogen (-196 °C) and crushed into powder with the help of sterilized mortar and pestle. Immediately after adding pre-warmed (65 °C) extraction buffer, the powder was homogenised and put into 2 ml eppendorf tubes. The tubes were gently mixed every 15 minutes while being incubated in a hot water bath for one hour (65 °C). After taking the tubes out of the hot water bath, gently combine them before centrifuging them at room temperature for 15 minutes at 12000 rpm.

Transfer upper aqueous layer into eppendorf tubes and equal volume of chloroform: isoamyl alcohol (24:1) was added. The tubes were mixed gently for few minutes and centrifuged at 12000 rpm for 15 minutes. A fresh tube was used to transfer the aqueous phase. Equal volume of ice chilled isopropanol was added to each tube and allowed to precipitate overnight at 4 °C. Centrifuge the tubes at 10,000 rpm for 8 minutes the following day. At this point, a pellet will be seen, and the supernatant will be decanted. The pellet was twice centrifuged at 10,000 rpm for 8 minutes with ethanol (70%) before being appropriately dissolved in TE (Tris-EDTA) buffer (20-50 µl depending on the size of the pellet obtained).

Unwanted RNA was removed from the DNA by treating it with RNase at 37 °C for 1 hour. A 1:1 phenol: Chloroform mixture was added, and the mixture was centrifuged at 12,000 rpm. Equal volume of chloroform was added after the upper aqueous layer was moved to another tube. By adding 100% ethanol, the aqueous layer was separated and DNA was precipitated. Following two ethanol (70%) washes, the pellet was resuspended in TE buffer. The finished DNA is kept at -20^0C for long-term storage. DNA purity and concentration of DNA in sample was estimated by spectrophotometer at 260 nm as well as on 0.8% agarose gel electrophoresis.

Confirmation of *F. oxysporum* f. sp. *ciceri* by ITS

PCR amplification of Internal transcribed spacer regions using universal ITS oligonucleotides (ITS 1-5'

TCCGTAGGTGAACCTGCGG 3' and ITS 4 5' TCCTCCGCTTATTG ATATGC 3') were carried out for the confirmation of isolated pathogen as *F. oxysporum* f. sp. *ciceri* on molecular level (White *et al.* 1990) [31]. All PCR reactions were carried out in a 0.2 ml PCR tubes with a total volume of 12.5 µl for ITS primers in thermal cycler. Each amplification reaction contained 2.0 µl of template DNA, 1.0 µl each of forward and reverse primer, 0.3 µl of dNTPs, 0.3 µl Taq DNA polymerase (5 U/µl), 2.5 µl 10X PCR buffer with $MgCl_2$ and 5.4 µl ddH_2O. PCR cycles were performed with initial denaturation of 94^0C for 5 min, followed by 30 cycles of 94 °C for 1 min denaturation, 55^0C for 1 min annealing and 72^0C for 30 s extension. Final extension was carried out at 72 °C for 10 min. The amplified PCR products were separated on 2% agarose gel run in 1X TBE (Tris Borate EDTA) buffer and viewed in UV transilluminator after staining with ethidium bromide (EtBr).

Differentiation of races of *F. oxysporum* f. sp. *ciceri*

Fibrinogen degradation product (FDP) and sequence characterized amplified region (SCAR) primers were used for the differentiation of four different races of *F. oxysporum* f. sp. *ciceri*. The amplification reaction was carried out with total volume of 20 µl reaction mixture for each sample. The mixture contained reagents including template DNA 1 µl, Primer (Forward+Reverse) 2 µl, dNTP 0.4 µl, Taq Polymerase 0.2 µl, 10X PCR Buffer with $MgCl_2$ 3.5 µl and 12.9 µl ddH_2O. PCR was run using the program: Initial denaturation at 94 °C for 5 min, 28 cycles of denaturation at 94 °C for 2 min, annealing at 52-55 °C (depending on the primer as given in Table 1) for 1 min and elongation at 72^0C for1 min. Followed by final extension of 72 °C for 10 min and holding temperature at 4 °C. The amplified PCR products were separated on 2% agarose gel run in 1X TBE buffer and viewed in UV transilluminator after staining with ethidium bromide (EtBr).

Table 1: List of primers used for racial differentiation of *F. oxysporum* f. sp. *Cicero*

	Primer	Nucleotide sequence (5' to 3')	Annealing temperature (T_m) °C
1	ITS	1F: TCCGTAGGTGAACCTGCGG	55
		4R: TCCTCCGCTTATTG ATATGC	55
2	FDP-2	F: CTTGGTGTTGGGATCTGTGTGCAA	57
		R: ACAAATTACAACTCGGGCCCGAGA	57
3	FDP-3	F: CAGCAGTGAGGAATATTGGTCAATG	57
		R: GCGGATCATCGAATTAAATAACAT	56
4	FDP-4	F: ATGGGTAAGGAAGACAAGAC	51
		R: GGAAGTACCAGTGATCATGTT	52
5	FDP-5	F: GAGACAAGCATATGACTACTG	50
		R: AATACAAGCACGCCGACAC	51
6	FDP-9	F: CCAGACTTCCACTGCGTGTC	56
		R: CACGCCAGGACTGCCTCGT	58
7	FDP-11	F: GGAGAGCAGGACAGCAAAGACTA	57
		R: GGAGAGCAGCTACCCTAGATACACC	61
8	FDP-12	F: GAGAGCAGGGTCAGCGTAGATAG	59
		R: GCAGCAGAAGAGGAAGAAAATGTA	54
9	FDP-14	F: GAGCAGTCAATGGCAATGG	51
		R: AGAGCAGGGTCAGCGTAGATA	54
10	FDP-22	F: ACACTGTTTGGGACCGAATCA	52
		R: ATAGAAGAGCCCATCCGATAA	50
11	FDP-25	F: ATGGGTAAGGAAGACAAGAC	50
		R: GGAGGTACCAGTCATCATGTT	51
12	FDP-29	F: ATGGGTAAGGAGGACAAGAC	52
		R: GGAAGTACCAGTGATCATGTT	51
13	FocR0-M15	F: GGAGAGCAGGACAGCAAAGACTA	57
		R: GAGAGCAGCTACCCTAGATACACC	59
14	FocR6-O2	F: GAGCAGTCAATGGCAATGG	51
		R: AGAGCAGGGTCAGCGTAGATA	54
15	FocR1B/C-N5	F: GAGAGCAGGGTCAGCGTAGATAG	59
		R: GCAGCAGAAGAGGAAGAAAATGTA	54

Results and discussion

Identification of *F. oxysporum* f. sp. *ciceri* isolates

On the basis of morphological characteristics described by Booth (1971 and 1977) [4, 5], the pure culture produced from single spore cultures were recognised as *F. oxysporum* f. sp. *ciceri.* These isolates were identified based on microscopic examinations because they possessed septate and hyaline mycelium. The microconidia that observed were non septate, oval to cylindrical in shape, and were between 3.5-9.0 X 2.1-5.0 micrometres. Macroconidia had 3 to 5 septa and measured 11.5-20.4 X 2-5.5 micrometres. They were foot-shaped at one end, pointed at the other, and had thin walls. These spore characteristics improved the capacity to distinguish between races. Old cultures produced terminal or intercalary chlamydospores with smooth to rough walls. These races shared morphological traits with *F. oxysporum* f. sp. *ciceri*, which had previously been characterised by a number of researchers (Dubey *et al.* 2010 and Om Gupta *et al.* 1986) [7, 26].

Morphological variations among the races of *F. oxysporum* f. sp. *ciceri*

F. oxysporum f. sp. *ciceri* appearance ranged from thin, linear flat to thick, profuse, fluffy, aerial mycelium development with or without serrate edge. Thin flat linear white to dull white colour mycelium with moderate to fast growing was observed in FOC1. Thin, pink and moderate growing mycelium growth with slightly aerial appearance was recorded in FOC2. Thick yellow to orange mycelium with linear growth pattern was recorded in FOC3. Thick,

profuse, fluffy and aerial white colour mycelium with fast growth were observed in FOC4.

Pathogenicity and pathogenic variability

All four races showed pathogenic in nature in both water culture inoculation and soil inoculation procedures. The average withering expression was seen between 18 and 42 days after seeding. When *F. oxysporum* f. sp. *ciceri* was isolated from wilted plants at the end of the experiment, Koch's Postulates were confirmed. No correlation was found between virulence and geographical location. The way that each race expressed wilting differed from the others. Similar findings were made earlier by Giri (2002) [8] in isolates of *Fusarium udum* causing pigeonpea wilt, which demonstrated diversity in incubation periods and no correlation between virulence and symptom expression. In addition, Sharma *et al.* (2009) [29] found differences in the number of days needed for disease onset and disease incidence among isolates of *F. oxysporum* f. sp. *ciceri.*

Molecular confirmation of *F. oxysporum* f. sp. *ciceri*

The ITS-1 (forward) and ITS-4 (reverse) primer pair amplified a single DNA fragment of approximately 550 bp in all races of *F. oxysporum* f. sp. *ciceri* (figure 1). Similar findings were made by Gurjar *et al.* (2009) [10], with ITS primers to identify *F. oxysporum* f. sp. *ciceri* by amplification of its DNA to about 550 bp. Kaur *et al.* (2015) also employed ITS marker to identify isolates of *F. oxysporum* f. sp. *ciceri* by amplifying bands at 550 bp.

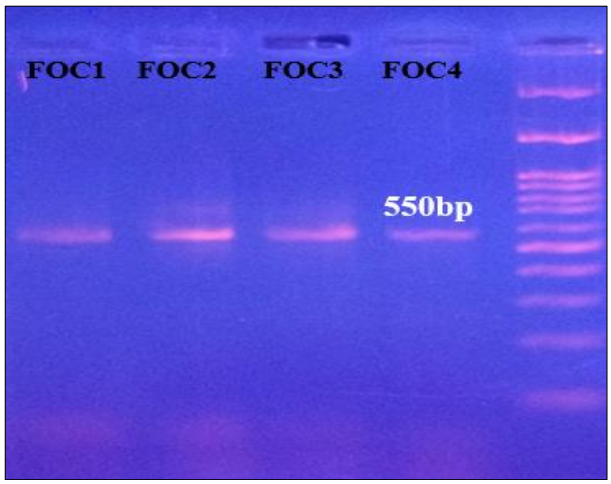

Fig 1: Identification of *F. oxysporum* f. sp. *ciceri* by using ITS primer

Race differentiation of *F. oxysporum* f. sp. *ciceri* by race specific primers

Pathogenic fungus used for current study have been differentiated according to race using several marker systems. A set of eleven FDP primers which targets the elongation factors and inter transcribed spacer regions and three locus-specific SCAR primers were used to differentiate the four races of *F. oxysporum* f. sp. *ciceri.* The main advantage of SCAR is that they are quick, easy to use and have high reproducibility. The bands produced by all the primers with four races of *F. oxysporum* f. sp. *ciceri* were mentioned in Table 2.

Table 2: Bands obtained in four races of *Fusarium oxysporum* f. sp. *ciceri* using ITS, FDP and SCAR

Primer	Expected band size	FOC1	FOC2	FOC3	FOC4
		(BP)			
ITS (Internal transcribed spacers)					
ITS 1 and 4	550	550	550	550	550
FDP (Fibrinogen degradation product)					
FDP-2	200	-	300	300	300
FDP-3	600	700	600	600	400
FDP-4	600	-	700	600	700
FDP-5	1500	-	2000	2000	2000
FDP-9	500	-	-	400	400
FDP-11	900	-	900	-	-
FDP-12	400	-	400	-	-
FDP-14	1000	-	1000	-	-
FDP-22	100	-	-	100	-
FDP-25	700	700	600	600	600
FDP-29	700	-	700	700	80
SCAR (Sequence characterized amplified region)					
FocR0-M15	900	-	900	-	-
FocR6-O2	900	-	800	-	-
FocR1B/C-N5	500	400	400	400	400

The ITS region of the ribosomal gene is targeted by FDP 2, which showed no bands in FOC1. Whereas in FOC2, FOC3 and FOC4 bands near to 200-300 bp are produced (figure 2a). The verticillium species-specific mitochondrial rRNA gene is the target of FDP 3 (Li *et al.* 1994) [20], in which polymorphic banding pattern around 700-500 bp was observed among all the races (figure 2b). FDP 4 targets the elongation factor 1 and polymorphic bands around 600-700 bp was observed in FOC2, FOC3 and FOC4. No bands are observed in FOC1 (figure 2c). Mbofung *et al.* (2007) [21] also reported same banding pattern in formae specialis of *F. oxysporum* using FDP 4. The band produced by FDP 5, which targets the intergenic spacer region produced bands ranged from 2000-2500 bp in FOC2, FOC3 and FOC4. FDP 9 produced band of 400 bp only in FOC 3 and 4 (figure 2d). FDP 11 and FDP 12 distinguishes FOC 2 with a specific band of 900 and 400 bp, respectively (figure 2e and 2f).

a) FDP 2 b) FDP 3 c) FDP 4

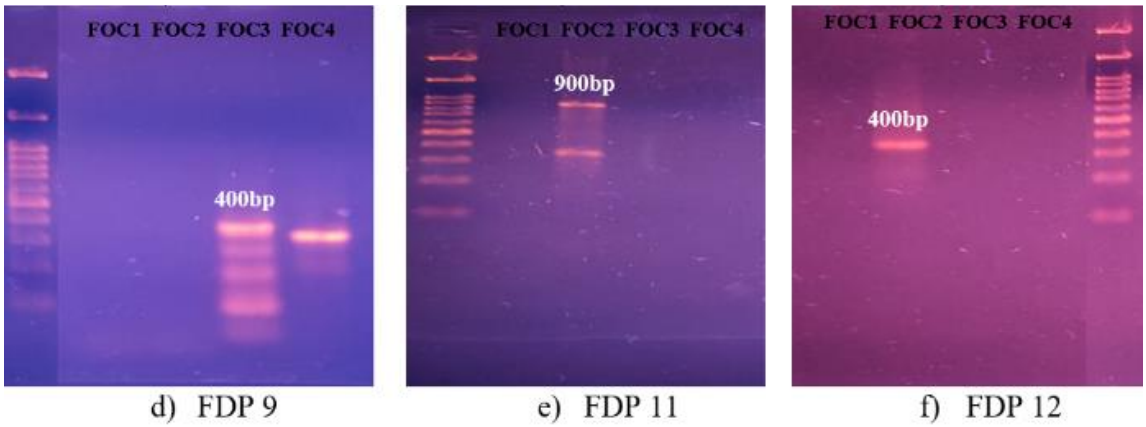

Fig 2: Molecular confirmation of races of *F. oxysporum* f. sp. *ciceri* by using FDP 2, 3, 4, 9, 11 and 12

FDP 14 also distinguish FOC 2 specifically by producing bands around 1000 bp (figure 3a). Ortiz *et al.* (2011) also reported 1000 bp band using FDP 14 for FOC 6. FDP 22 distinguishes FOC 3 with a specific band of around 100 bp (figure 3b). FDP 25 that targets the translation elongation factor region amplified monomorphic bands of 600-700bp in all the four races (figure 3c). FDP 29 targeting translational elongation factor (ef1/ef2) genes produce bands around 700-800 bp in FOC 2, 3 and 4, whereas no bands were observed in FOC1 (figure 3d). The translation elongation factor (EF-1a) has been the focus of Arif *et al.* (2012) [3] work to distinguish the *Fusarium solani* isolates. Sujata *et al.* (2021) also differentiated *F. oxysporum* f. sp. *ciceri* races using FDP 2, 3, 9, 14 and almost same banding pattern was observed.

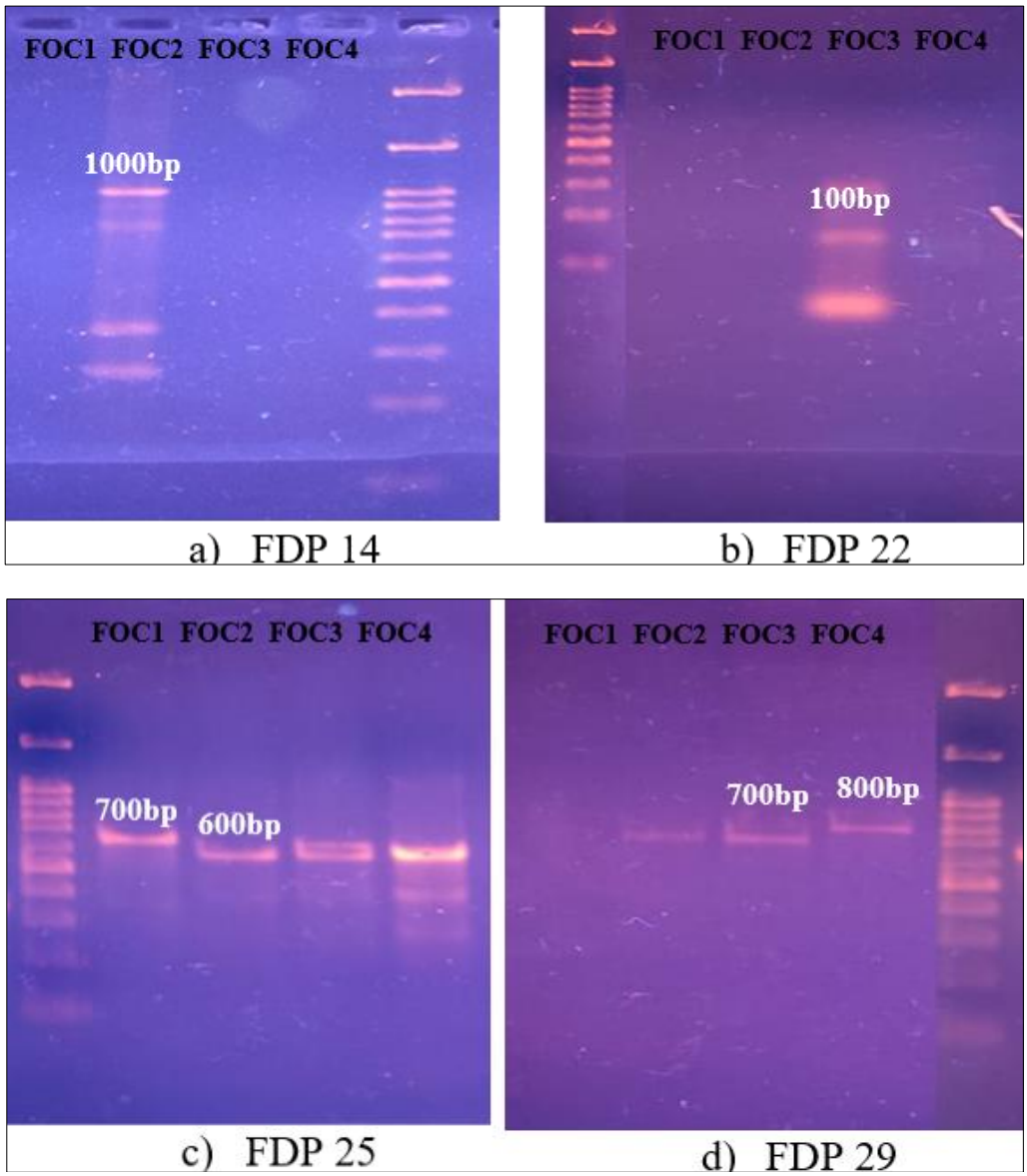

Fig 3: Molecular confirmation of races of *F. oxysporum* f. sp. *ciceri* by using FDP 14, 22, 25 and 29

To determine *F. oxysporum* f. sp. *cucumerinum*, Lievens *et al.* (2007) [19] created unique SCAR primers. A set of SCAR primers used by Jimenez *et al.* (2003) [17] to identify the *F. oxysporum* f. sp. *ciceri* races was also used in the current study. SCAR primers FocR0-M15 and FocR6-O2 distinguishes FOC 2 with a specific band of 900 and 800 bp, respectively (Figure 4a and 4b). Amplified monomorphic bands around 400bp was obtained from SCAR primer FocR1B/C-N5 with all the four races (figure 4c). The outcomes compliments the result of Poornima *et al.* (2021), but were different from what Jimenez *et al.* (2003) [17] reported. Although the expected band length was the same, but the race in which the anticipated banding pattern observed was variable.

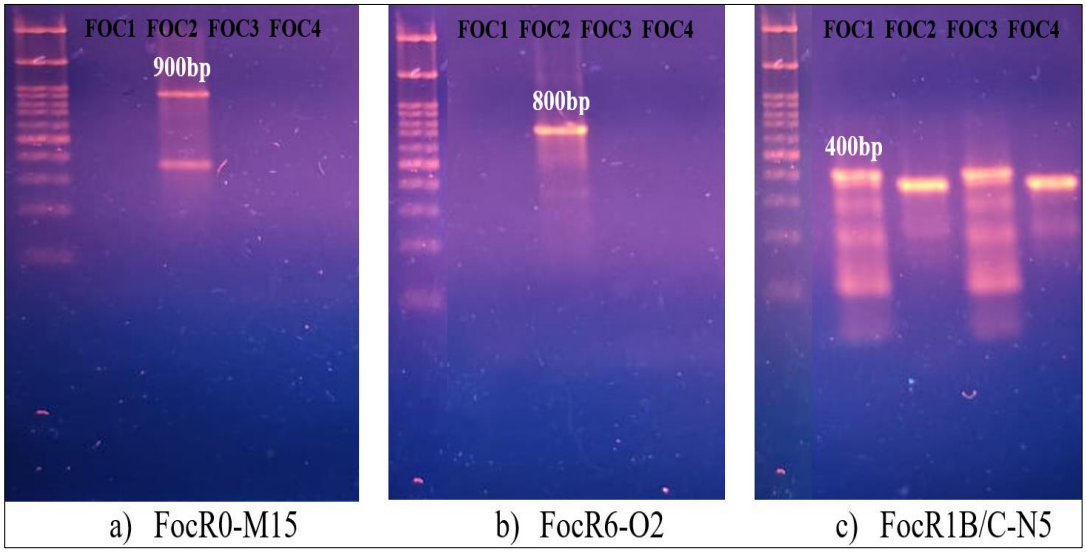

Fig 4: Molecular confirmation of races of *F. oxysporum* f. sp. *ciceri* by using SCAR primers

Conclusion

FOC 1 can be distinguished from other races with FDP 25 and FDP 3 with a band size of 700 bp for both primers. FDP 11, FDP 12, FDP 14, FocR0-M15 and FocR6-O2 which yields specific band of 900, 400, 1000, 900 and 800 bp, respectively can be able to differentiate FOC2 from other races. FOC 3 is distinguished by FDP 22 with a band size of 100 bp from all other races. FDP 3 differentiate FOC 4 with a band size of 400 bp. Poornima *et al.* (2021) also differentiated six races of FOC using FDP, SCAR and CAPS primer. Sujata *et al.* (2021) also differentiated FOC races using FDP 2, 3, 9, 14 and almost same banding pattern was observed.

The scientific community can utilise these primer combinations to distinguish between the many races of *F. oxysporum f. sp. ciceri*. Additionally, the use of these primers on various *F. oxysporum* f. sp. *ciceri* races gathered across India can provide insight into the diversification of various racial groups. The aforementioned initiatives enable high-throughput DNA fingerprinting of Foc races, which is particularly important for plant-pathogen interaction investigations and plant breeding initiatives that focus on chickpea genotypes resistance responses.

Declarations

Availability of data and materials: Not applicable

Competing interests: The authors declare that there is no competing interests

Acknowledgement: Not applicable

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Authors contribution: The authors contributed in interpretation of results and manuscript preparation

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