Characterization by RAPD-PCR of Races of *Fusarium oxysporum* f.sp. *ciceri* Infecting Chickpea

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Abstract

Isolates of *Fusarium oxysporum* f.sp. *ciceri* from chickpea (*Cicer arietnum* L.) can be characterized as races and wilt and yellowing pathotypes by pathogenicity tests on varieties of differentials chickpea. This study shows that, DNA bands generated by RAPD-PCR can be used to assign *Fusarium oxysporum* f.sp. *ciceri* isolates to pathotype and pathogenic race.

Keywords: *Cicer arietnum* L.; fusarium wilt; identification; RAPD-PCR analysis; genetic finger print

INTRODUCTION

Fusarium wilt caused by the soil-borne pathogen *Fusarium oxysporum* Schl. emnd. Snyd. Hans. f.sp. *ciceri* (Padwick) Snyd & Hans is a major limiting factor for chickpea production in most of chickpea-growing regions in the world (NENE & REDDY 1987). Natural population of *Fusarium oxysporum* f.sp. *ciceri* are diverse in terms of pathogenic variability, and can be characterized into pathotypes (yellowing and wilt) or races by means of biological typing (JIMENEZ-DIAZ *et al*. 1989).

Fusarium oxysporum has about 80 formae specials (pathotypes specific to species), and several are subdivided into races (specific to cultivar within a species). The identified specialized formae that cause wilt disease on chickpea is *F. oxysporum* f.sp. *ciceri* (BOOTH 1971).The traditional criteria used to differentiate these genera and their species are based on plant, host, symptoms, colony appearance, morphologic characterization of their conidia and telemorph (CORBIERE & BOUZNAD 1998).

The recent development of random amplified polymorphic DNA (RAPD) has allowed a rapid generation of reliable reproducible DNA fragment that showed of great use in identifying pathogenic variation in several fungal plant pathogens, including formae specials of *F. oxysporum* (JIMENEZ-GASCO *et al*. 1998).

The main objectives of this work was to determine if RAPDs could be of useful for the characterization of pathotypes and races of *F. oxysporum* f.sp. *ciceri* isolates infecting chickpea.

MATERIALS AND METHODS

Fungal strains. The *Fusarium* strains used in this study obtained from various sources of diseased chickpea plants from wide range of geographical origins and Governorate over all Egypt. All cultures were derived from single spore. Data are listed in Table 1.

Table 1. Geographical origin, and year of collection of *Fusarium oxysporum* isolates used in this study (Host of origin in all cases was chickpea. All isolates were isolated in 1996)

Isolates No.	Geographic origin	Isolates No.	Geographic origin
1	El-Sharkia	8	Kafer El-Shiekh
2	El-Sharkia	9	El-Sharkia
3	El-Sharkia	10	El-Sharkia
4	Kafer El-Shiekh	11	El-Sharkia
5	Kafer El-Shiekh	12	El-Sharkia
6	Nubaria	13	El-Sharkia
7	El-Behira		

Isolation and identification of the pathogen. Roots and basal stems of diseased chickpea plants showing typical symptoms of wilt disease washed carefully with running tap water. Plants in the diseased samples were cut into small pieces, surface sterilized by immersing in 2% sodium hypochlorite for 3 min, and washed twice in sterilized distilled water, then dried between two sterilized filter paper and plated on PDA medium, then incubated at 25°C for 7 days. Emerged fungi were isolated and purified on PDA plats, then identified according to (BOOTH 1971; ALEXOPOULOS & MIMS 1979; NELSON *et al*. 1983), then maintained on PDA slants and kept in refrigerator at 5°C for further studies.

Pathogen variability. Pathogen variability of *F. oxysporum* isolates was determined previously using the spore suspension technique (ABOU-ZEID *et al*. 1999).

Race identification. For race identification of chickpea wilt pathogen, 10 chickpea cultivars obtained from the Germplasm Unit of the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) were sown as the seed source (ABOU-ZEID *et al*. 1999) (as shown in Table 2).

DNA isolation. DNA was isolated from 50 mg tissue (*Fusarium oxysporum*) using Qiagen Kit for DNA extraction. The extracted DNA was dissolved in 100 μl of elution buffer and the concentration and purity of the obtained DNA was determined by using "Gene quantum" system-Pharmacia Biotech. The purity of the DNA for all samples were between 90–97%. The concentration was adjusted at 6 mg/μl for all samples using TE buffer pH 8.0.

RAPD technique. 30 mg/μl from the extracted DNA was used for amplification reaction the PCR. The PCR mixture contains tablet PCR beads (manufactured by Amessham Pharmacia Biotech.) that contain all of the necessary reagents. The primer and DNA extract are add to the tablet. The sequences of RAPD Analysis Primer 5 and 6 are 6-d (AACGCGCAAC)-3 and 6-d (CCCGTCAGCA)-3.

The total volume was completed to 25 μl using sterile distilled water. The amplification program was carried out as follows using PCR unit II biometra and consisted of the following:

a) Preheating at 95°C for 5 min
b) Three – step cycle repeated 44 times:
   - Denaturation at 95°C for 1 min
   - Annealing at 36°C for 1 min
   - Extension at 72°C for 2 min
c) Final extension at 72°C for 5 min
d) Hold at 4°C.

Then add 7 μl of 6X tracking buffer (manufactured by Qiagen Kit) to 25 μl of the amplification product.

Amplification product analysis. 1. The amplified DNA for all samples were electrophorated (15 μl) (using electrophoresis unit WIDE mini-sub-cell GT Bio-RAD on 1% agarose containing ethedium bromide at concentration 0.5 μg/ml).

2. The PCR products was separated and determined using polyacrylamide gel electrophoresis slabs (according to PIETER *et al*. 1995).

Using sub electrophoresis unit Hoefer SE600 series Pharmacia (8 μl) well form, each sample was loaded. The running was done at 2 melamper/sample constant current. The running took about 2 hours, the amplified

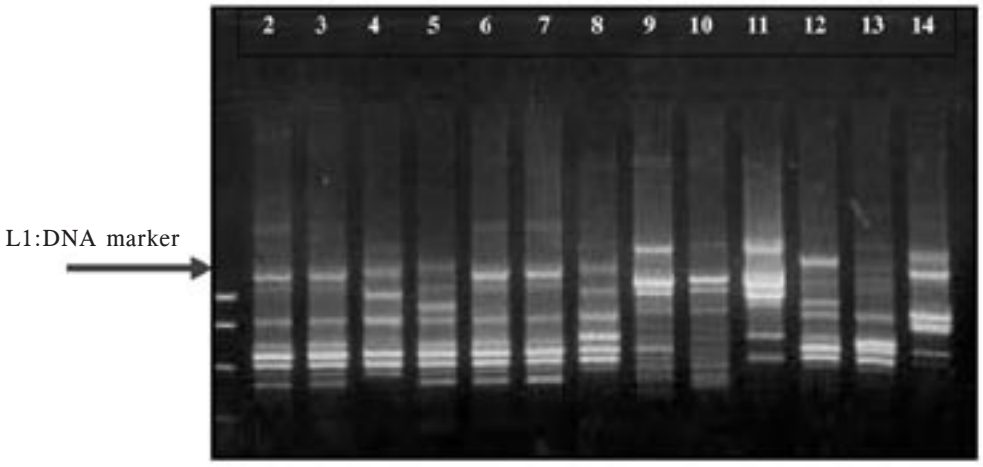

Figure 1. Electrophoresis analysis of RAPD for isolates of *Fusarium oxysporum* f.sp.*ciceri* using primer No. 6. L1: DNA marker, from L2–L14: *F. oxysporum* samples isolated from chickpea

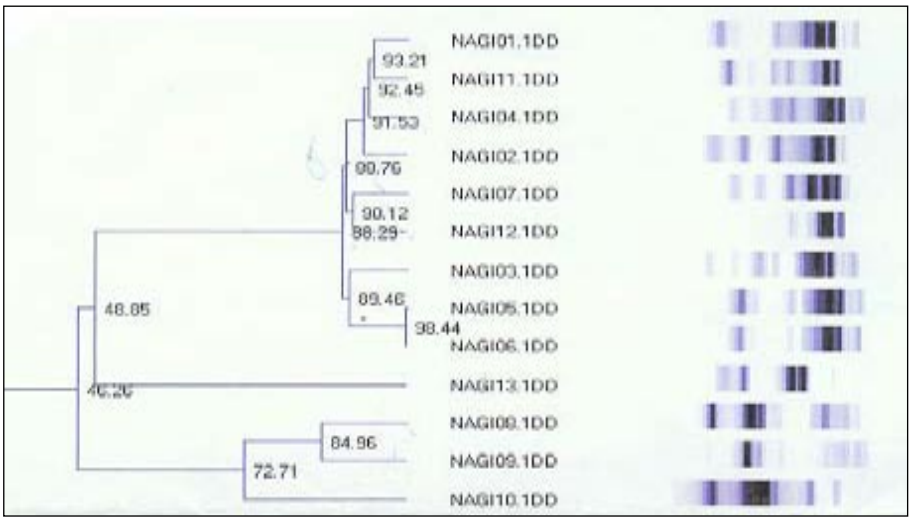

Figure 2. UPGMA cluster analysis of RAPD obtained from selected *Fusarium oxysporum* f.sp.*ciceri* using C 6 primer. Similarity values are indicated and final linkage for the subclusters are marked

pattern was developed using silver stain according to CARINS and MURRAY (1994).

Restriction fragment were compared in all pair wise combinations and the matrix was displayed as a dendogram using the unweighted pair-group method using arithmetic average (UPGMA).

Gel analysis. The polyacrylamide gel was scanned using gel documentation system (AAB advanced American Biotechnology 1166 E. Valencia Dr. Unit 6C, Fullerton CA 92631). The different molecular weights of bands were determined against PCR marker Promega G316A.

RESULTS

The clusters analysis of similarity coefficients based on RAPD profiles revealed discrete clusters of isolates possibly representing different lineages, among wilt inducing isolates of *Fusarium oxysporum* from chickpea. These corresponded to differences in pathogenicity characteristics and were distinguished from isolates representing formae specials of *F. oxysporum*.

DNA from 13 *F. oxysporum* f.sp. *ciceri* isolates were amplified using RAPD analysis primer 5 and

Figure 3. Electrophoresis analysis of RAPD for isolates of *Fusarium oxysporum* f.sp. *ciceri* using primer No. 5. L1: DNA marker, from L2–L14: *F. oxysporum* samples isolated from chickpea

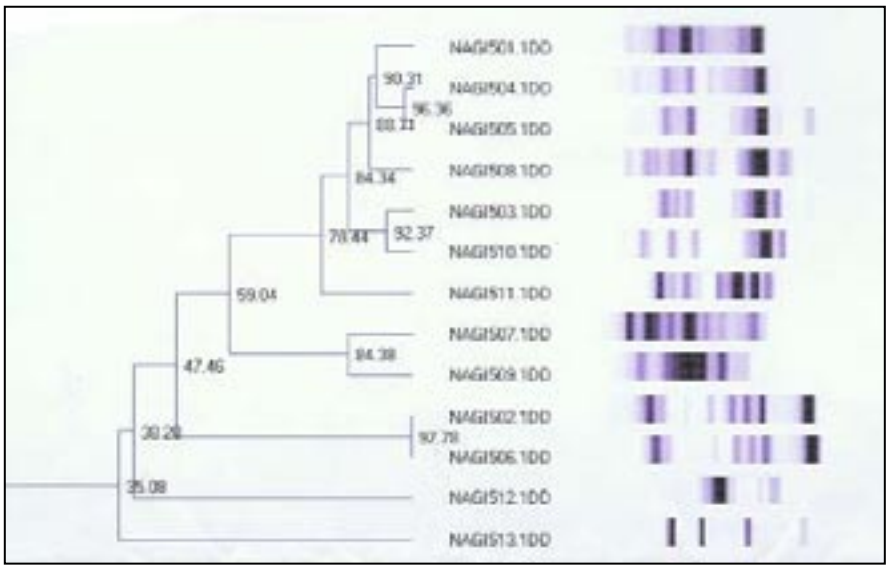

Figure 4. UPGMA cluster analysis of RAPD obtained from selected *Fusarium oxysporum* f.sp. *ciceri* using C 5 primer. Similarity values are indicated and final linkage for the subclusters are marked

primer 6, which identified that characterized *F. oxysporum* isolates as to pathotype. RAPD amplifications analysis observed that, from the 13 isolates of *F. oxysporum* f.sp. *ciceri* 9 isolates were identified as yellowing inducing pathotype according to their pathogenicity on chickpea plants, and 2 isolates identified as wilt – inducing pathotype, and these identification were confirmed in all cases by pathogenicity tests under controlled environmental conditions. Nine isolates were designated as race 0 and induce progressive foliar yellowing, isolate No. 10 was highly virulent and identified as a new race while isolate No. 11 distinguished as race 3.

In the case of primer No. 6, UPGMA analysis separated the isolates of chickpea into different distinct of clusters. All yellowing-inducing *F. oxysporum*

Table 2. Reaction of the 10 entries in the lentil nursery to *Fusarium oxysporum* f.sp. *ciceri*

No.	Nursery	Isolates No.										
		1	2	3	4	5	6	7	8	9	10	11
1	JG-62	R	R	R	R	R	R	R	R	R	S	S
2	C-104	R	R	R	R	R	R	R	R	R	S	R
3	GJ-74	M	M	M	M	S	M	S	S	M	S	R
4	CPS-1	R	R	R	R	R	R	R	R	R	S	N
5	BG-212	R	R	R	R	R	R	R	R	R	S	M
6	WR-315	R	R	R	R	R	R	R	R	R	S	S
7	Annigeri	R	R	R	R	R	R	R	R	R	S	M
8	Chafa	R	R	R	R	R	R	R	R	R	S	M
9	L-550	R	R	R	R	R	R	R	R	R	S	M
10	850-3/27	M	M	S	M	M	S	S	S	M	S	M

R – Resistant 0–20% wilted plants

M – Moderately susceptible 21–50% wilted plants

S – Susceptible > 50% wilted plants

f.sp. *ciceri* isolates were grouped together forming a separate cluster which included isolates No. 1, 4, 2, 7, 3, 5, and 6 and showing 88% similarity, and wilt-inducing isolates as isolate 10 (identified as new race) was grouped into subcluster showing 72% similarity with isolates 8 and 9 (Figures 1 and 2).

When using primer 5, it could divide the isolates into different clusters. However, isolate No. 13 could be divided as separate isolate, also No. 12 and isolate No. 11 the similarity between isolates could be due to the origin of the isolates (Figures 3 and 4).

DISCUSSION

The aim of this work was to determine whether races or pathotype could be characterized by RAPD analysis and to provide a methodology for the diagnosis of both the pathotype and race type. Results showed that amplified DNA bands can used reproducibly to assign a *Fusarium oxysporum* isolates to pathotype and pathogenic race and this agree with the result of their pathogenic variability (HAWARE & NENE 1982; CABRERA DE LA COLINA *et al*. 1985; HASSANIEN & ABOU-ZEID 1998), and this grouping correlated well with the degree of virulence of isolates of lentil race differentials (ABOU-ZEID *et al*. 1999).

Although the isolates produced the same reaction on chickpea cultivars as yellowing symptoms, they differ in the genome characterization as show in the cluster analysis using different type of primers. However, this molecular methods is very helpful to characterize isolates of *Fusarium oxysporum* species and may complement traditional characters for the better knowledge of the *Fusarium oxysporum* and be very useful to precise or re-define species of *F. oxysporum*.

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