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Identification of pathogenic races 0, 1B/C, 5, and 6 of *Fusarium oxysporum* f. sp. *ciceris* with random amplified polymorphic DNA (RAPD)

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Abstract

Ninety-nine isolates of *Fusarium oxysporum* f. sp. *ciceris* (Foc), representative of the two pathotypes (yellowing and wilt) and the eight races described (races 0, 1A, 1B/C, 2, 3, 4, 5, and 6), were used in this study. Sixty isolates were analyzed by the RAPD technique using DNA bulks for each race and 40 primers. Bands presumably specific for a DNA bulk were identified and this specificity was confirmed by further RAPD analysis of individual isolates in each DNA bulk. Primers OPI-09, OPI-18, OPF-06, OPF-10, and OPF-12 generated RAPD marker bands for races 0, 1B/C, 2, 3, 4, 5, and 6. The reliability and utility of this procedure was validated in 'blind trials' using 39 new Foc isolates. Ten of the 39 isolates had already been typed to race by pathogenicity tests and 29 were typed both by pathogenicity and RAPD testing in this study. In these 'blind trials', we assigned the 39 new isolates to a race solely on the basis of their RAPD haplotype. Thus, we concluded that Foc races 0, 1B/C, 5, and 6 can be characterized by the RAPD markers. Cluster analysis of the RAPD data set resulted in three clusters of isolates within Foc. The yellowing isolates were grouped in two distinct clusters which correspond to races 0 and 1B/C. The wilt isolates constitute a third cluster that included races 1A, 2, 3, 4, 5, and 6. These results provide a means of studying the distribution of Foc races, to assist in the early detection of introduced race(s) and to facilitate the efficient deployment of available host resistance.

Introduction

Knowledge of the nature of variation and distribution of pathogenic races is required for the efficient disease management through the use of resistant cultivars. In some pathosystems, race identification by pathogenicity tests requires considerable effort and can be less informative than the use of neutral genetic markers. Neutral markers are especially useful to identify races for pathogens with asexual reproduction since no recombination occurs and the entire pathogen genome is effectively linked (Milgroom and Fry, 1997).

Fusarium wilt, caused by the asexual soilborne fungus *Fusarium oxysporum* Schelchtend.: Fr. f. sp. *ciceris* (Padwick) Matuo & K. Sato (Foc), is one of the most damaging chickpea diseases worldwide (Jalali and Chand, 1992). This pathogen can cause complete crop loss (Halila and Strange, 1996; Haware and Nene, 1980), with average annual yield losses of 10–15% (Jalali and Chand, 1992; Trapero-Casas and Jiménez-Díaz, 1985). The most practical and cost-efficient method for management of this disease is through the use of resistant cultivars (Jalali and Chand, 1992); however, pathogenic variability in Foc limits the

Table 1. Geographic and race information of isolates of *F. oxysporum* f. sp. *ciceris* (Foc), grouped by method of race classification, and of nonpathogenic isolates of *F. oxysporum* (Fo), used in this study

Isolate(s)[a]	Origin[b]	Race by	
		Pathogenicity test	RAPD analysis[c]
Group A			
Foc-7932, -82115, -8317, -8601, -8602, -8604, -8733, -8901, -8911 to -8914, -90111, -9198, -91100, -91114	Spain	0	NT
Foc-1987-B	United States	0	NT
Foc-USA 1633-2RIJ	United States	1A	NT
Foc-1992 R1N	India	1A	NT
Foc-8905, -8924	Spain	6	NT
Foc-1987 T	United States	6	NT
Group B			
Foc-7802, -7952, -7982, -8207, -82108, -82113, -8310, -8413, -8415, -8717, -9018 (JG62), -9018 (PV1), -91105, -91108	Spain	0	0
Foc-8250[d]	Spain	NP	NP
Foc-1987-W17, -USA 3-1 (JG62)	United States	1B/C	1B/C
Foc-7989	India	1A	*
Foc-9166, -9168	Morocco	1A	*
Foc-8605, -1992 R2N	India	2	2
Foc-8606, -1992 R3N	India	3	3
Foc-8607, -1992 R4N	India	4	4
Foc-8012, -8257, -8408, -8508, -9035	Spain	5	5
Foc-USA 1-1 (JG62), -1987-W6-1	United States	5	5
Foc-9164	Morocco	6	6
Foc-8272, -8720, -9023, -9093 (PV1)	Spain	6	6
Group C			
Foc-8503[e], -9032	Spain	0	0 (W)
Foc-9165	Morocco	1A	* (W)
Foc-9027 (PV1)	Spain	1A	* (W)
Foc-USA SD-8415	United States	1A	* (W)
Foc-9094 (JG62)	Morocco	5	5 (W)
Foc-USA 6-1 (JG62), -USA 14201	United States	5	5 (W)
Foc-9170	Morocco	6	6 (W)
Foc-TONINI	United States	6	6 (W)
Group D			
Foc-cc21C, -cc62R, -cc63K	Israel	0	0 (U)
Foc-L96-5 to -L96-7, -L96-9 to -L96-11	Lebanon	0	0 (U)
Foc-Sy96-14-1, -Sy96-18-3, -Sy96-19-1	Syria	0	0 (U)
Foc-9601, -9603 to -9606	Tunisia	0	0 (U)
Foc-T96-1-3	Turkey	0	0 (U)
Foc-cc22D	Israel	1B/C	1B/C (U)
Foc-Sy96-10-1	Syria	1B/C	1B/C (U)
Foc-9602	Tunisia	1B/C	1B/C (U)
Foc-T96-1-1, -T96-1-2, -T96-2-1, -T96-2-2	Turkey	1B/C	1B/C (U)
Foc-9620, -9622, -9628, -9632	Israel	6	6 (U)
Group E			
Fo-9009, -9081, -90105	Spain	NP	NP

[a]Isolates in groups A–D were characterized to race by: A, RAPD analysis using DNA bulks (see Material and methods: approach 1); B, RAPD analysis using DNA bulks and single isolate DNA (see Material and methods: approach 2); C, RAPD analysis in a 'blind trial'; D, pathogenicity tests and RAPD analysis in double 'blind trials'. Group E: nonpathogenic isolates of *F. oxysporum*.

[b]Isolates from Tunisia were provided by Dr. M.H. Halila, Institute Nacionale de la Recherche Agronomique, Ariana, Tunisia; isolates from Israel were a gift from Professor J. Katan, Department of Plant Pathology and Microbiology, The Hebrew University of Jerusalem, Israel; isolates from Lebanon, Syria, and Turkey were provided by Dr. C. Akem, ICARDA, Aleppo, Syria.

effectiveness of this strategy (Haware and Nene, 1982; Jiménez-Díaz et al., 1993; Trapero-Casas and Jiménez-Díaz, 1985). Therefore, the identification of pathogenic races of Foc is important for disease resistance breeding, and for the efficient use of available resistant cultivars. Eight Foc races (races 0, 1A, 1B/C, 2, 3, 4, 5, and 6) have been characterized by pathogenicity tests on a set of 10 differential cultivars (Haware and Nene, 1982; Jiménez-Díaz et al., 1993). Two pathotypes, causing yellowing and wilting, have been differentiated (Trapero-Casas and Jiménez-Díaz, 1985). Races 1A, 2, 3, 4, 5, and 6 induce the wilt syndrome (severe chlorosis and flaccidity combined with vascular discoloration followed by plant death within 20 days of inoculation). Races 0 and 1B/C induce the yellowing syndrome (progressive foliar chlorosis with vascular discoloration followed by plant death within 40 days of inoculation) (Jiménez-Díaz et al., 1993; Trapero-Casas and Jiménez-Díaz, 1985). Races 1A, 2, 3, and 4 were first identified in India (Haware and Nene, 1982). To our knowledge, 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 (Halila and Strange, 1996; Jiménez-Díaz et al., 1993; Jiménez-Díaz, unpublished).

Pathogenicity tests are cumbersome, time-consuming (50–60 days), require extensive facilities, and are influenced by variability inherent in the experimental system. Furthermore, pathogenicity data alone provide no information about genetic diversity within, or relatedness among, races of the pathogen. Knowledge of genetic diversity is needed for resistance deployment to be effective and to identify shifts in race or population structure that might occur (McDonald, 1997). Consequently, improved methods are needed for the rapid and informative characterization of Foc races.

Random amplified polymorphic DNA (RAPD) analysis (Williams et al., 1990) has been applied widely in the detection and genetic characterization of phytopathogenic fungi (Brown, 1998; Miller, 1996), including race differentiation in several *formae speciales* of *F. oxysporum*, i.e., f. sp. *cubense* (Bentley et al., 1994), *dianthi* (Manulis et al., 1994; Migheli et al., 1998), *pisi* (Grajal-Martín et al., 1993), and *vasinfectum* (Assigbetse et al., 1994). In previous studies, RAPD analysis was used with primers based on either known ribosomal DNA sequences or sequencing primers to characterize and differentiate the yellowing and wilt pathotypes in Foc, without achieving satisfactory identification of pathogenic races (Kelly et al., 1994). However, those results in that study proved useful for *in-planta* and in-soil detection of the wilt-inducing pathotype (García-Pedrajas et al., 1999; Kelly et al., 1998). The objectives of the present study were: (1) to test whether different pathogenic races of Foc could be differentiated by RAPD analysis and (2) to analyze the genetic relationship and variability between and within representative isolates of these races.

Materials and methods

Fungal isolates

Ninety-nine Foc isolates representative of the eight described races and from a wide geographic range of the pathogen, as well as three nonpathogenic *F. oxysporum* isolates obtained from roots of healthy chickpeas, were used (Table 1). All Foc isolates except for 29 from Israel, Lebanon, Syria, Tunisia, and Turkey (Group D in Table 1) had been previously characterized to race (Jiménez-Díaz et al., 1993; Kelly et al., 1994). All 99 fungal isolates were obtained from the culture collection of Departamento de Protección de Cultivos, Instituto de Agricultura Sostenible, CSIC, Córdoba, Spain. Isolates Foc-9601 to -9606 originated from Tunisia and were provided by Dr. M.H. Halila (Institute Nationale de la Recherche Agronomique, Ariana, Tunisia). Isolates Foc-9620 to -9632, -cc21C, -cc22D, -cc62R, and -cc63K originated from Israel and were a gift from Professor J. Katan (Department of Plant Pathology and Microbiology, The Hebrew University of Jerusalem, Israel). Isolates labelled L, Sy, and T, originated from Lebanon, Syria, and Turkey, respectively, and were provided by Dr. C. Akem (ICARDA, Aleppo, Syria). Isolates were stored as monoconidial cultures in sterile soil in test tubes at 4 °C in the dark. Active cultures of isolates were obtained by

[c] NT = not tested; NP = nonpathogenic; U, W = race identity of isolates was unknown (U) or known prior to RAPD analysis but information was withheld (W) to completion of analysis. An asterisk indicates that no RAPD markers were found for race 1A.
[d] Isolate originally classified as race 0 (Cabrera de la Colina, 1986) but latter confirmed as nonpathogenic *F. oxysporum* (Kelly et al., 1994).
[e] Isolate originally classified as race 5 (Cabrera de la Colina, 1986) but latter confirmed as race 0 by pathogenicity tests in the present study.

subculturing stored cultures on potato-dextrose agar at 25 °C and a 12-h photoperiod of fluorescent and near-UV light at 36 μE m^{-2} s^{-1}. For DNA extraction, 1 ml of conidial suspension (5×10^6 conidia/ml) was transferred to flasks containing 100 ml of potato-dextrose broth. Cultures were incubated on an orbital shaker at 125 rpm, 25 °C, and 12 h light for 4 days. Mycelia were harvested by filtration through sterile cloth, washed with sterile water, lyophilized and stored at −20 °C.

Pathogenicity tests

For 'blind trials' in this study, the 29 uncharacterized isolates (Group D, in Table 1) were characterized by pathogenicity tests on the differential cultivars P 2245, JG 62, BG 212, C 104, JG 74, CPS 1, WR 315, Annigeri, Chafa, K850 3/27, 12071/10054, and ICCV 4 (Haware and Nene, 1982; Jiménez-Díaz et al., 1993).

Inoculum was increased in a cornmeal-sand mixture (CMS) (Trapero-Casas and Jiménez-Díaz, 1985) at 25 °C and a 12-h photoperiod of fluorescent and near-UV light at 36 μE m^{-2} s^{-1} for 2 weeks. The infested CMS was mixed thoroughly with autoclaved soil (clay loam/sand/peat, 1 : 1 : 1, v/v/v) at a rate of 1 : 12 (w/w). Seeds were surface-disinfested, germinated, and sown into 15-cm diameter clay pots (four plants per pot) filled with the infested soil mixture. Control plants were grown in a mixture of noninfested CMS and autoclaved soil. Plants were grown in a growth chamber adjusted to 24 ± 1 °C, 60–90% relative humidity, and a 14-h photoperiod of fluorescent light at 360 μE m^{-2} s^{-1}, and fertilized weekly with 100 ml of a nutrient solution (Hoagland and Arnon, 1950). Plants were observed daily for symptom development. Disease reactions were assessed by symptom severity on a 0–4 scale according to percentage of foliage affected (0 = 0%, 1 = 1–33%, 2 = 34–66%, 3 = 67–100%, 4 = dead plant) at 7-day intervals for 2 months. Scores <1 were considered as resistant and scores >3 were considered as susceptible reactions. Intermediate scores were considered as moderately susceptible reactions. Upon termination of the experiments, *Fusarium* strains were isolated from stem segments of symptomless plants by placing tissue on V8 juice-oxgall-PCNB agar *Fusarium*-selective medium (Bouhot and Rouxel, 1971). Two replicated experiments were conducted following a randomized complete block design with three replicated pots for each isolate–cultivar combination.

DNA extraction and RAPD analysis

Genomic DNA of each fungal isolate was extracted from 50 mg of lyophilized ground mycelium (Raeder and Broda, 1985). Aliquots of samples were analyzed on 0.7% agarose gels in Tris–acetate EDTA buffer (40 mM Tris–acetate and 1 mM EDTA, pH 8.0) to estimate the concentration and quality of the DNA. Samples were diluted with sterile water to a final concentration of 50–100 ng/μl for PCR reactions.

RAPD reactions (Williams et al., 1990) were carried out with 40, 10-mer oligonucleotide primers corresponding to the OPF and OPI primers sets (Operon Technology, Alameda, CA). Each reaction mixture (25 μl) consisted of 0.5 μM of primer, 200 μl M of each dNTP, 2.5 μl of 10× reaction buffer (50 μM KC1, 10 mM Tris–HC1, pH 9.0 [25 °C], 1% v/v triton X-100), 2 U of *Taq* DNA Polymerase (Promega, Madison, WI), 1.5 mM $MgC1_2$ and 50–100 ng of fungal DNA. Amplifications were performed in different thermocyclers: Perkin-Elmer 2400, Perkin-Elmer 9600 (Perkin-Elmer, Norwalk, CT) and PTC 100 (MJ Research Inc., Watertown, MA), programmed for 4 min of denaturation at 94 °C, followed by 30 cycles of 1 min of annealing at 37 °C, 3 min of extension at 72 °C and denaturation for 1 min at 94 °C. The final cycle consisted of 1 min of annealing followed by 6 min at 72 °C to produce fully double-stranded DNA fragments. Temperature between annealing and extension increased at 0.6 °C/s. Amplification products were separated by electrophoresis on 1.5% agarose gels at 1.5 V cm^{-1}, stained with ethidium bromide and visualized under UV light. The 0.1-kb DNA ladder XIV size marker was used for electrophoresis (Boehinger-Manheim, Barcelona, Spain). All reactions were repeated at least twice and always included negative controls (no template DNA).

RAPD analyses were of two approaches (1 and 2) in a sequence, to facilitate selection of appropriate primers that would produce informative amplifications. Approach 1 included analysis of DNA bulks of each race. To do this, equal amounts of DNA from every isolate comprising a given Foc race were combined. A total of 60 Foc isolates in eight bulked samples were examined. These consisted of 32 isolates of race 0, five of race 1A, two of each of races 1B/C, 2, 3, and 4, seven of race 5 and eight of race 6 (Table 1). Each DNA bulk was amplified using the 40 Operon primers. Subsequently, any primer that generated DNA bands polymorphic for a race was used to analyze the DNA

from arbitrarily selected individual components of each DNA bulk, to confirm that the putative RAPD markers were present in all of the isolates of one race. A total of 38 Foc isolates were used in approach 2 (Table 1).

Data analysis

Seven primers, four from set OPF and three from set OPI produced consistent, informative polymorphisms in the RAPD analyses. RAPDs from individual Foc isolates in the DNA bulk for a race and selected isolates of the 'blind trials' were used to study the relationship among isolates and between races. A binary matrix of combined data from seven primers for the 57 Foc isolates and four *F. oxysporum* isolates was prepared by scoring bands for presence or absence. DNA bands of the same mobility (molecular weight) were assumed to be identical. The NTSYSpc2.0 (Rohlf, 1988) software package was used to cluster the isolates by an unweighted paired group method with arithmetic averages (UPGMA), based on Jaccard's similarity coefficient (Sneath and Sokal, 1973). This coefficient disregards negative matches between pairs of isolates and provides a more accurate picture of relatedness.

RAPD data were also used for analysis of molecular variance (AMOVA) (Excoffier et al., 1992) to identify and analyze genetic variation among isolates. This procedure converts a phenotypic distance matrix into an equivalent analysis of variance, and allows estimation of variance components for RAPD haplotypes by partitioning the variance among and within samples. The level of significance for AMOVA values was computed by nonparametric permutational procedures.

Results

RAPD analysis using DNA bulk from isolates of a race

DNA bulks proved useful for dealing with the large number of isolate–primer combinations in our study. No band was amplified in any of the control reactions. For most primers, RAPD profiles were reproducible across experiments using both DNA extracted from same lyophilized mycelia and DNA from mycelia of newly grown cultures of the Foc isolates. Also, these RAPD profiles were reproduced when amplifications were carried out by different researchers in the same laboratory and in a different laboratory, i.e., Microbial Physiology Group, King's College, University of London. Of the 40 primers used on bulk DNAs, 12 (OPF-05, -06, -08, -10, -12, -16, -18, -19; and OPI-01, -09, -18, -19) produced 31 distinct polymorphic DNA bands. Putative RAPD markers were identified for all of the Foc races in the study except race 1A. For race 1A, RAPD analysis of DNA bulk using primer OPI-01 produced bands of 1.2 and 2.7 kb that were also amplified in DNA bulk of race 6 isolates.

RAPD analysis using DNA from individual isolates of race

Additional RAPD analyses were carried out using the 12 primers and template DNA from individual isolates that made up the DNA bulk of a race. Seven of the 12 primers amplified a total of 17 DNA bands producing the identical RAPD profile for all isolates of every race (Figure 1). This confirmed the specificity of the DNA bands as markers for Foc races 0, 1B/C, 2, 3, 4, 5, and 6 (Table 2). An exception was found with isolate Foc-8250, a representative of race 0. RAPD profiles from Foc-8250 were completely different from profiles obtained from other 31 race 0 isolates regardless of primers or DNA (bulk, single isolate) used (Figure 1, lane 28). This discrepancy was addressed by means of an additional pathogenicity test which indicated that Foc-8250 was not pathogenic to chickpea (Kelly et al., 1994).

Reliability of race characterization by RAPD analysis

The RAPD methodology for race identification was validated in a 'blind test' by using 10 new Foc isolates arbitrarily chosen from our culture collection. These isolates had already been typed to race by pathogenicity tests (labelled 'W' in Table 1). Three nonpathogenic isolates of *F. oxysporum* were also examined. Isolates were characterized to race by RAPD analysis, and then compared to the previous race characterization. RAPD analysis correctly identified the race to which the Foc isolates belonged. The following RAPD bands were diagnostic for Foc races: race 0, 0.9 kb (OPI-09); race 1B/C, 0.53 kb (OPI-09), 0.53 kb (OPI-18), 1.9 kb (OPF-06), 0.51 kb and 1.1 kb (OPF-10); race 2, 0.9 kb (OPF-12); race 3, 2.0 kb (OPF-06); race 4, 0.95 kb (OPF-10); race 5, 0.9 kb (OPF-10); and race

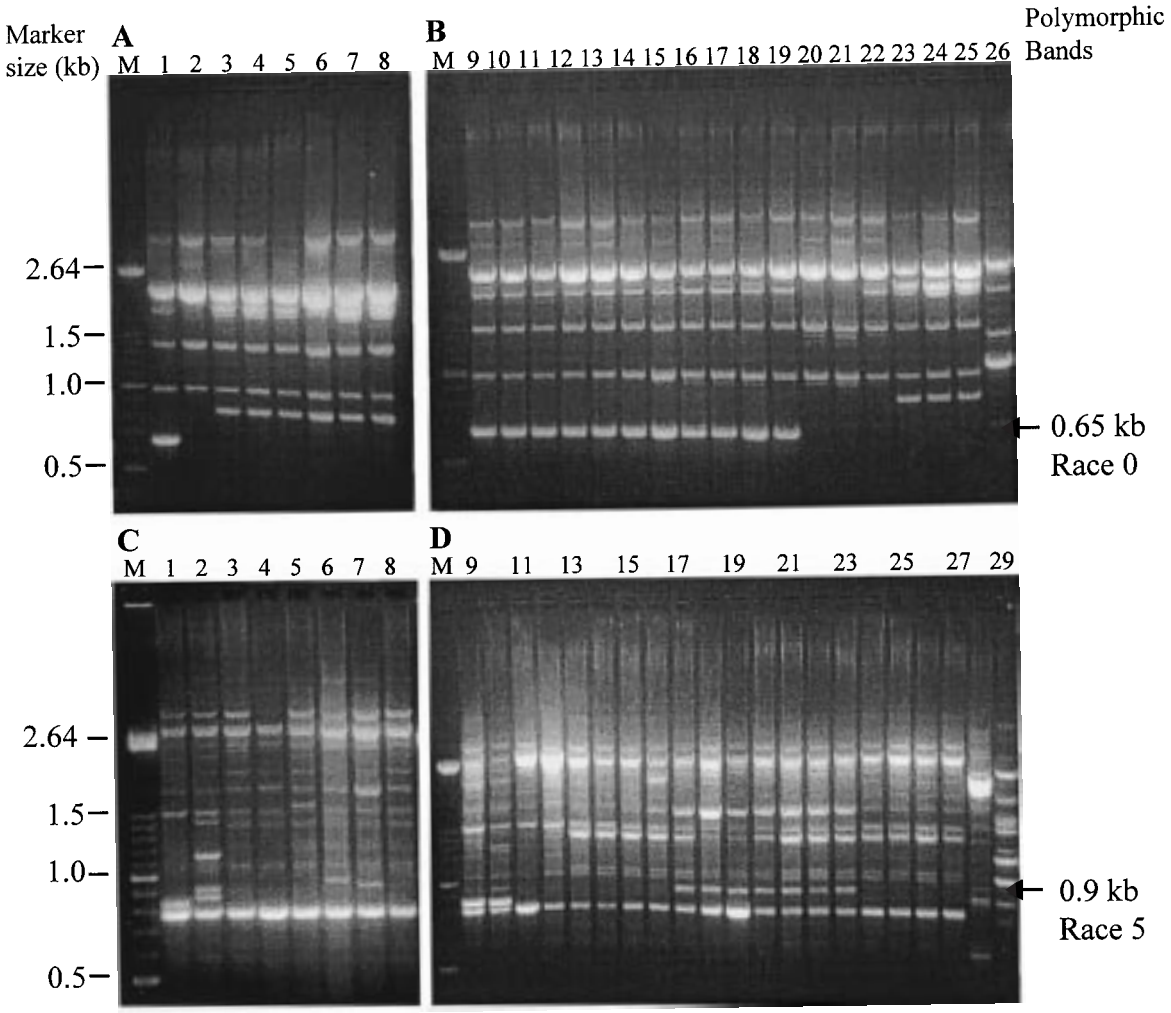

Figure 1. RAPDs generated by primer OPF-16 (A, B) and OPF-10 (C, D) using a DNA bulk of *F. oxysporum* f. sp. *ciceris* isolates of a race (A, C) and DNA from individual isolates representative of the race (B, D). DNA products were separated on a 1.5% agarose gel and stained with ethidium bromide. Numbers on the left side are the sizes of the 0.1-kb DNA ladder XIV (Boehringer-Mannheim) (lane M). Numbers on the right side are the sizes of the marker bands that identify races 0 (A and B) and 5 (C and D). A and C: lanes 1–8, DNA bulks for each of *F. oxysporum* f. sp. *ciceris* races 0, 1B/C, 1A, 2, 3, 4, 5, and 6. B: lanes 9–19, race 0 isolates Foc- 7802, -9018 (JG62), -82108, -8717, -7952, -8207, -9032, -9601, -91118, -9604, -82118; lanes 20–22, race 1B/C isolates Foc-USA 3–1 (JG62), -1987 W-17, -9602; lanes 23–26, race 1A isolate Foc-7989, race 5 isolate Foc-8012, race 6 isolate Foc-9093 (PV1), and nonpathogenic *F. oxysporum* isolate Fo-9009, respectively. D: lanes 9–12, race 0 isolates Foc-7802, -91108, -9018 (JG62), -9601; lanes 13–16, race 1A isolates Foc-9027 (PV1), -9165, -9166, -7989; lanes 17–23, race 5 isolates Foc-8012, -8508, -9035, -USA 1–1 (JG62), -USA 14201, -9094 (JG629), -1987-W6–1; lanes 24–27, race 6 isolates Foc-TONINI, -9023, -9164, -9093 (PV1); and lanes 28–29, nonpathogenic *F. oxysporum* isolates Fo-8250 and -9009, respectively.

6, 1.3 kb (OPI-09). Isolate Foc-8503 had been identified as race 5, but RAPD analysis resulted in a RAPD profile characteristic of race 0. When the pathogenicity tests were repeated, isolate Foc-8503 caused the typical yellowing syndrome (data not shown) and was thus reassigned to race 0. The three nonpathogenic *F. oxysporum* isolates had some similarities to Foc race 0 isolates. RAPD bands of 0.39 kb (primer OPF-12) and 0.65 kb (primer OPF-16), which identified race 0 in previous analyses, also were amplified with these primers in RAPD analysis using DNA from some nonpathogenic *F. oxysporum* isolates.

Table 2. Code and sequence of the seven primers which were selected for their ability to produce informative DNA polymorphisms for the identification of races of *F. oxysporum* f. sp. *ciceris* in random amplified polymorphic DNA (RAPD) analysis[a]

Code	Sequence (5′–3′)	Polymorphic amplified DNA band (kb)	Race
OPI-01	ACCTGGACAC	1.0	1A
		1.2	0, 1A, 1B/C, 6
		2.7	1A, 6
OPI-09	TGGAGAGCAG	0.53	1B/C
		0.9	0
		1.3	6
OPI-18	TGCCCAGCCT	0.53	1B/C
		1.0	2, 3, 4
OPF-06	GGGAATTCGG	1.9	1B/C
		2.0	3
OPF-10	GGAAGCTTGG	0.51	1B/C
		0.95	4
		0.9	5
		1.1	1B/C
OPF-12	ACGGTACCAG	0.39[b]	0
		0.9	2
OPF-16	GGAGTACTGG	0.65[b]	0

[a]RAPD analyses were performed with DNA from each individual isolate in DNA bulk of a race.
[b]Also amplified in RAPD analysis using DNA from some nonpathogenic isolates *F. oxysporum*.

To further validate the RAPD methodology for race identification of Foc, we also conducted a double 'blind' study of the 29 nontyped isolates from Israel, Lebanon, Syria, Tunisia and Turkey (Table 1). Each isolate was typed by RAPD analysis using primers OPI-01, OPI-09, OPI-18, OPF-06, OPF-10, OPF-12, and OPF-16, as well as by pathogenicity tests on differential cultivars. Results from both assays indicated a 100% match in the race characterization of each isolate. All 21 Foc isolates from Lebanon, Syria, Tunisia, and Turkey were of the yellowing pathotype, of which 15 isolates were race 0 and six were race 1B/C (Table 1). Similarly, of eight Foc isolates from Israel, four isolates were of the yellowing pathotype (three race 0 and one race 1B/C) and four were of the wilt pathotype and were characterized as race 6 (Table 1).

Analysis of RAPD data

RAPD analysis resulted in 160 bands. UPGMA analysis of the RAPD data separated the Foc isolates into three main clusters (I, II, III), each of which shared about 72% similarity and correlated well with the yellowing- or wilt-inducing nature of isolates (Figure 2). The nonpathogenic *F. oxysporum* isolates did not group closely with the Foc isolates. All isolates of race 0, yellowing-inducing Foc isolates, constituted one cluster (I) showing approximately 82% average similarity. Race 1B/C isolates, which also induce yellowing symptoms, were grouped together in another small cluster (II). Yellowing-inducing isolates, including race 0 and race 1B/C isolates, shared about 73% average similarity. All wilt-inducing Foc isolates, including isolates of races 1A, 2, 3, 4, 5, and 6, formed a third cluster (III) sharing approximately 82% average similarity. These wilt-inducing isolates were distributed between several subclusters with similarity ranging from 80% to 100%. Isolates of races 2, 3, and 4 from India joined into separate single linkages separated from races 1A, 5 and 6, within the wilt-inducing isolates grouping (Figure 2). There was no association between clustering in the RAPD dendrogram and geographic origin of isolates, except for Foc isolates from India.

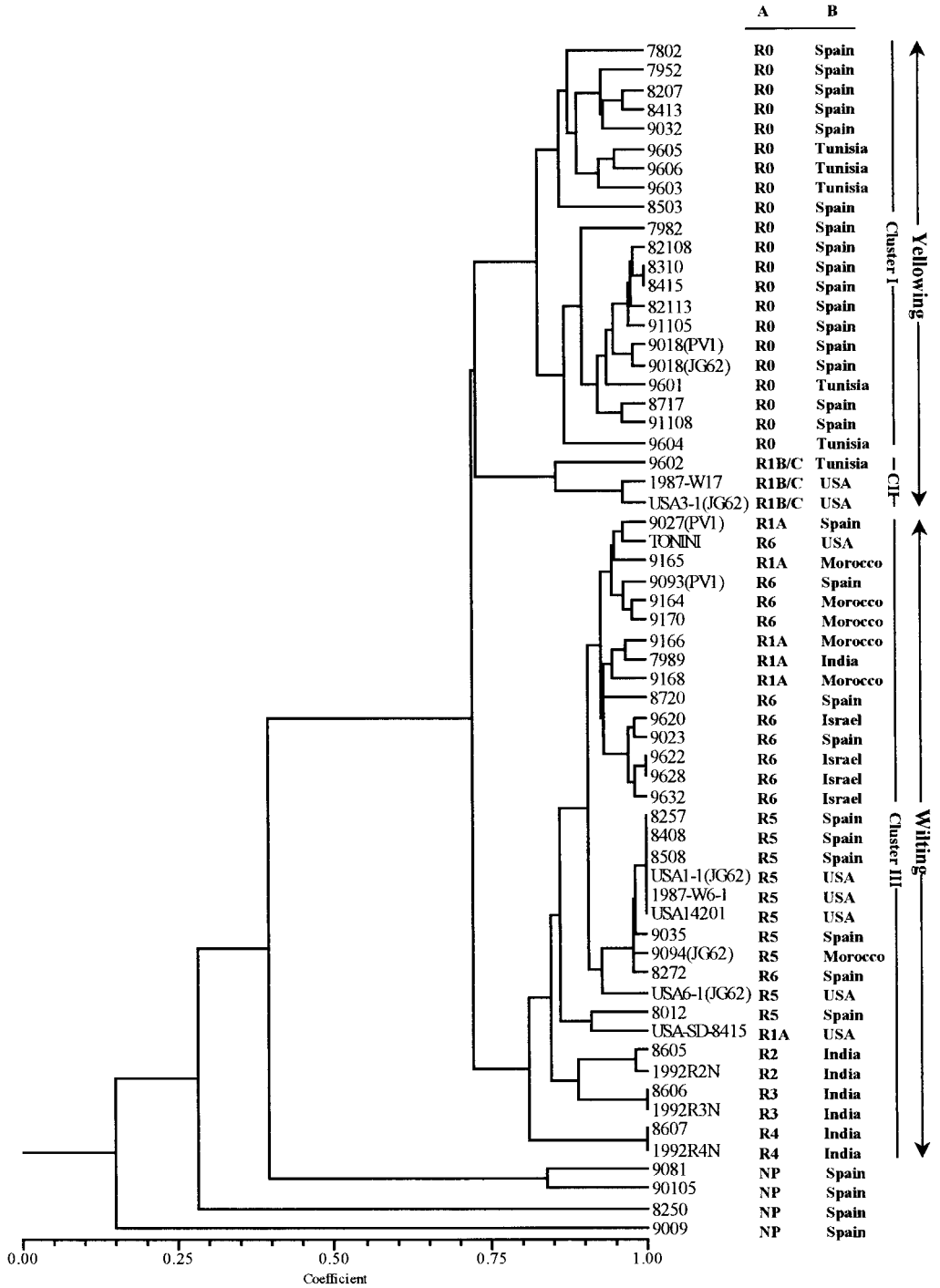

Figure 2. Dendrogram derived from RAPD analysis of 57 isolates *F. oxysporum* f. sp. *ciceris* and four isolates of nonpathogenic *F. oxysporum* using primers OPI-01, OPI-09, OPI-18, OPF-06, OPF-10, OPF-12, and OPF-16. The dendrogram was derived by UPGMA (unweighted paired group method with arithmetic averages). The bottom scale is the percentage of similarity by Jaccard's similarity coefficient. **A**, Race assignment of an isolate: R0 = race 0, R1A = race 1A, R1/B = race 1B/C, R2 = Race 2; R3 = race 3, R4 = race 4, R5 = race 5, R6 = race 6, NP = Nonpathogenic *F. oxysporum*. **B**, Geographic origin of isolates.

AMOVA analysis was used to examine genetic variation of Foc according to pathotype assignment (yellowing, wilt) and geographic origin of isolates (Table 3). AMOVA showed that differences between isolates of the two pathotypes were highly significant ($P < 0.001$). Within the yellowing pathotype genetic differences between races 0 and 1B/C were highly significant ($P < 0.001$) with some 54% of the total genetic variation attributable to race differences. AMOVA also indicated that there were no significant ($P = 0.1099$) differences between wilt-inducing isolates (races 1A, 5 and 6) from the Mediterranean region and the US. There were, however, highly significant ($P < 0.001$) differences between wilt-inducing isolates from India (races 1A, 2, 3, and 4) and those in the Mediterranean region and the US together (races 1A, 5 and 6). AMOVA indicated that about 36% of the total genetic variation was due to differences among wilt-inducing isolates (Table 3), and this proportion increased to 45% when race 1A from India was not included in the analysis (data not shown).

Discussion

The quick and proper identification of races in *F. oxysporum* f. sp. *ciceris* (Foc) is important for the development and efficient deployment of resistant chickpea cultivars. Our primary objective was to determine whether or not different Foc races could be distinguished by RAPD analysis. Results in the study support this hypothesis. Some of the RAPD primers amplified DNA fragments that were diagnostic for a race. The general applicability and reliability of our methods was confirmed by correctly identifying races of previously typed and untyped isolates in 'blind trials'. The low number of isolates representative of races 2, 3, and 4 that could be used in the study impose some limitations to the diagnostic reliability of markers for these races. Further work using a larger number of isolates will be needed to confirm the results for these races. Conversely, the higher number of isolates of races 0, 1B/C, 5, and 6 that were examined supports the utility of the identified RAPD markers for the characterization of those races.

In recent years, a number of studies failed to find a relationship between RAPD markers and pathogenic races in *formae speciales* of *F. oxysporum* (e.g., Woo et al., 1996). Other studies showed the utility of the RAPD technique for that purpose (e.g., Assigbetse et al., 1994; Grajal-Martín et al., 1993; Manulis et al., 1994; Migheli et al., 1998). Our findings indicate a clear relationship between the RAPD profiles and pathogenicity tests for Foc races 0, 1B/C, 5, and 6. A high genetic similarity between races 1A and 6 might explain both the lack of a diagnostic band for race 1A isolates, and the sharing of some RAPD markers between these two races. A similar case was reported by Woo et al. (1996) who found that some pathogenically diverse isolates of *F. oxysporum* f. sp. *phaseoli* had a similar banding pattern. Races 1A and 6 have similar virulence pattern on chickpea differentials (Halila and Strange, 1996; Jiménez-Díaz et al., 1993), and it is possible that misclassification of these isolates occurred during pathogenicity tests. It was assumed that an isolate's race had been correctly identified, and perhaps additional RAPD studies with other Foc race 1A isolates and additional primers are needed to resolve this issue. It is also possible that these two pathogenic races are not yet evolutionarily separated from each other to have developed diagnostic and separate pathogenicity traits.

The RAPD markers we identified have practical applications in the epidemiology and management of Fusarium wilt in chickpeas. This utility is indicated by the correct identification of two isolates (Foc-8250 and -8503) in the study, for which the RAPD patterns did not correspond to the expected identification. Isolate Foc-8250 was among the least virulent of all race 0 isolates tested for pathogenicity by Cabrera de la Colina (1986). Later, repeated pathogenicity tests under standardized conditions on susceptible chickpea lines revealed neither yellowing symptoms nor infection of the plant (Kelly et al., 1994). Therefore, both pathogenicity tests and the absence of RAPD markers for race 0 indicate that Foc-8250 is not pathogenic to chickpea. Similarly, isolate Foc-8503 was originally characterized as a race 5 isolate, but RAPD analysis and repeated pathogenicity tests have re-identified this isolate as race 0. An additional gain from our study is that races 0 and 1B/C, which induce the same yellowing symptom in pathogenicity tests, can be separated by RAPD markers. In previous studies, RAPD analyses using primers KS, P2 and P6 did not discriminate the two races but assigned them to the yellowing pathotype. The above are examples which highlight the constraints in pathogenicity assays that can be overcome by diagnostic RAPD markers.

Cluster analysis of the RAPD data showed a high genetic similarity among Foc isolates. All the

Table 3. Analysis of molecular variance, AMOVA, of random amplified polymorphic DNA data from 57 isolates of *F. oxysporum* f. sp. *ciceris* grouped according to pathotype (yellowing, wilt), pathogenic race (races 0, 1A, 1B/C, 2–6) and geographic origin

Source of variation[a]	Degree of freedom	Mean square deviation	Variance component[b]	*P* value[c]
Yellowing vs Wilt Pathotypes	1	2.043	0.0712 (52.2)	<0.001
Within	55	0.065	0.0653	
Race 0 vs Race 1B/C				
Among	1	0.459	0.0755 (54)	<0.001
Within	22	0.063	0.0632	
Wilting isolates from the Mediterr. and USA (Races 1A, 5, 6) vs wilting isolates from India (Races 1A, 2-4)				
Among	1	0.294	0.0226 (36)	<0.001
Within	33	0.041	0.0408	
Races 1A, 5, 6 (Mediterr.) vs Races 1A, 5, 6 (US)				
Among	1	0.069	0.0032	0.1099
Within	24	0.040	0.0397	

[a]Pathotypes are characterized according to symptoms induced in the host. The yellowing pathotype includes races 0 and 1B/C. The wilt pathotype includes races 1A, 2, 3, 4, 5 and 6. Geographic origin and race assignment of isolates are indicated in parentheses. Races 1A, 5 and 6 from the Mediterranean region are from Israel, Morocco and Spain. Races from the US are from California.
[b]Number in parentheses is percentage of the total.
[c]Probability computed by nonparametric procedures from 1,000 data permutations.

yellowing-inducing isolates were placed in two distinct clusters which correspond to race 0 and race 1B/C. Wilt-inducing isolates were placed in another large cluster grouping races 1A, 2, 3, 4, 5, and 6. This division of Foc isolates is consistent with that found by Kelly et al. (1994), who used only three RAPD primers and part of the same set of isolates in our study. Results from the cluster analysis also were consistent with those from AMOVA. Thus, we conclude that Foc is a highly race-structured, clonal pathogen group in which correlations between neutral genetic markers and pathogenicity can be used to infer conclusions about the genetic relationship between races.

Previous studies showed that all Foc races had the same mtDNA RFLP pattern (Pérez-Artés et al., 1995), belong to a single vegetative compatibility group (VCG) (Nogales-Moncada, 1997), and carry the same repetitive element (Kelly, 1996). Stable heterokaryons can be formed between a race 1B/C isolate from California and some nonpathogenic *F. oxysporum* isolates from chickpea roots (Nogales-Moncada, 1997). This suggests that asexual genetic exchange may occur between isolates of different races, and also between pathogenic and nonpathogenic isolates. Correll (1991) proposed several models to explain relationships between *formae speciales*, races, and VCGs in *F. oxysporum*, with the assumption that the primitive basis of the species was a parasitic, nonpathogenic, VCG-diverse population from which isolates could mutate to become virulent. Our results support this hypothesis and seem to fit into the model III pattern of race-VCG diversity, i.e., different races of a *forma specialis* originating from the same VCG. We found that race 0 isolates and some nonpathogenic *F. oxysporum* isolates share DNA bands amplified by several Operon primers, as did Pérez-Artés et al. (1996) using different nonpathogenic *F. oxysporum* isolates and primers KS, P2 and P6 (Kelly et al., 1994). Southern hybridization with a cloned yellowing-specific RAPD marker band as the probe demonstrated that some sequence similarity exists between DNA fragments of nonpathogenic *F. oxysporum* and yellowing-inducing Foc isolates (Pérez-Artés et al., 1996). That Foc isolates share a high genetic similarity within a VCG, but belong to different races, suggests that these isolates were clones or originated from a common parental origin, although they are geographically separated and pathogenically diverse at present (Leslie, 1993).

Race 0 has not been reported from the Indian subcontinent, but is common in the Mediterranean region (Halila and Strange, 1996; Jiménez-Díaz et al., 1993; Jiménez-Díaz, unpublished). Race 0 is not pathogenic to chickpea cv. JG 62, the universal suscept of wilting races, and is primarily pathogenic to 'kabuli' chickpeas (Jiménez-Díaz et al., 1993). 'Kabuli' chickpeas are grown extensively and traditionally in the Mediterranean region. In contrast, 'desi' chickpeas are grown

primarily in the Indian subcontinent and most 'desi' genotypes are resistant to race 0. Races 2, 3, and 4, reported only from India, are the most virulent races of the eight races (Halila and Strange, 1996; Haware and Nene, 1982; Jiménez-Díaz et al., 1993) and are genetically the most distant from other races (Figure 2, Table 3). This apparent correlation of fungal races together with host cultivars could be explained if the pathogen races are geographically isolated local adaptations to local germplasm.

In conclusion, the RAPD technique appears to be a powerful diagnostic tool for the identification of all the major Foc races present in the Mediterranean region. The molecular markers which were identified can be used to study the distribution of these Foc races, and to facilitate the efficient deployment of available host resistance. Also, these markers should assist in the early detection of introduced race(s), as well as of changes in the relative frequencies of different races that might occur in response to the use of resistant chickpea cultivars. Finally, the diagnostic DNA marker bands we identified can now be cloned and sequenced in order to develop race-specific probes and primers of use in dot-blot or specific PCR-based diagnostic tools.

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