Molecular Plant Pathology

Differentiation of *Fusarium oxysporum* f. sp. *vasinfectum* Races on Cotton by Random Amplified Polymorphic DNA (RAPD) Analysis

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We thank the following for kindly providing strains of *Fusarium oxysporum* f. sp. *vasinfectum*: J. C. Follin (CIRAD-CA, Montpellier, France); G. H. Daï (Hebei Academy Agriculture and Forest Science, Beijing, China); A. Varma (Indian Agricultural Research Institute, New Delhi, India); M. A. Rutherford (International Mycological Institute, Kew, England); L. G. Portenko (Academy of Science, Dushanbe, Tadjikistan); J. Devay (University of California, Davis); G. Ibrahim (Agricultural Research Corporation, Wad Medani, Sudan); and J. Katan (Hebrew University, Jerusalem, Israel). We thank H. C. Kistler (University of Florida, Gainesville) and M. Nicole (ORSTOM, Montpellier, France) for previous review of this manuscript.

Accepted for publication 16 February 1994.

ABSTRACT

Assigbetse, K. B., Fernandez, D., Dubois, M. P., and Geiger, J.-P. 1994. Differentiation of *Fusarium oxysporum* f. sp. *vasinfectum* races on cotton by random amplified polymorphic DNA (RAPD) analysis. Phytopathology 84:622-626.

We used pathogenicity and random amplified polymorphic DNA (RAPD) markers to assess genetic diversity among 46 isolates of *Fusarium oxysporum* f. sp. *vasinfectum* of worldwide origin. Based on pathogenicity tests on five differential cotton cultivars and species, isolates were differentiated into three races (A, 3, and 4), restricted to defined geographic areas. The amount of genetic variation was evaluated by polymerase chain reaction amplification with a set of 11 random 10-mer primers. All amplifications revealed scorable polymorphisms among the isolates, and a total of 83 band positions was scored (1/0) for the 11 primers tested. Genetic distances between each of the isolates were calculated, and cluster analysis was used to generate a dendrogram showing relationships between them. Isolates clustered into three groups corresponding to their pathological reactions. We suggest that RAPD markers can be a quick and reliable alternative for differentiating isolates of *F. o. vasinfectum* into their respective pathogenicity group.

Additional keywords: Fusarium wilt of cotton, DNA markers.

Wilt of cotton (*Gossypium* spp.) is a vascular disease caused by the soilborne pathogen *Fusarium oxysporum* Schlechtend.:Fr. f. sp. *vasinfectum* (Atk.) W.C. Snyder & H.N. Hans. The disease is widespread and causes substantial crop losses in most of the major cotton-producing areas of the world.

Currently, six distinct races, restricted to defined geographic areas, have been described for this wilt pathogen. Races 1 and 2 were described in the United States and Tanzania, race 3 in Egypt, Sudan, and Israel, race 4 in India, race 5 in Sudan, and race 6 in Brazil and Paraguay (1–4,7,17,18). *F. o. vasinfectum* has a wide host range, encompassing plants in the Leguminosae, Malvaceae, and Solanaceae. So far, races 1, 2, and 6 have been distinguished only by their pathogenicity on alfalfa (*Medicago sativa*) and tobacco (*Nicotiana tabacum*) (2,3).

Determination of both host specificity and genetic diversity in *F. o. vasinfectum* populations are of great importance in plant breeding for resistance. Assessment of genetic diversity in *F. o. vasinfectum* is needed to determine whether races constitute genetically distinct groups and to obtain molecular markers for differentiating them.

The modified polymerase chain reaction (PCR) with single primers of arbitrary nucleotide sequence and requiring no prior sequence information have proved useful in detecting intraspecific polymorphisms among organisms (27,28). This amplification technique (arbitrarily primed PCR or random amplified polymorphic DNA [RAPD]) can generate specific DNA fragments useful for genome mapping, identification of isolates, and applications in molecular ecology (14). For plant pathogenic fungi, RAPD analysis can provide markers to differentiate races of *F. solani* f. sp. *cucurbitae* (6), *F. o. pisi* (11), *Gremmeniella abietina* (16), aggressive and nonaggressive isolates of *Phoma lingam* (12,24,29), and isolates with different geographic origins of *Colletotrichum graminicola* (13). Recently, Haemmerli et al (15) characterized isolates of *Discula umbrinella* from different hosts and using RAPD analysis detected multiple infections in the same leaf.

The potential of this technique for identifying DNA markers related to the intraspecific diversification of the pathogens has led us to investigate the genetic diversity within *F. o. vasinfectum*. The aim of this study was to examine the relationships between pathogenicity and these anonymous genetic markers within a collection of 46 isolates from Africa, America, and Asia.

MATERIALS AND METHODS

Hosts. Cotton plants of the cultivars Isa 205 and Acala S.J. (*G. hirsutum* L.), Ashmouni 106 and Sakel (*G. barbadense* L.), and CG17 (*G. arboreum* L.) were used as host ranges for *F. o. vasinfectum* race determination (1–3,17). Seeds of cotton cultivars were obtained from the CIRAD-CA (Montpellier, France).

Fungal cultures. Forty-six isolates of *F. o. vasinfectum* were collected from different cotton-growing regions throughout the world. The geographic origin and host species are presented in Table 1. One strain sent to us (strain 122 originating from Tanzania) was not identified as *F. oxysporum* after microscopic examination. We decided to include strain 122 in our analyses as an outgroup species. All cultures were single-spored and maintained on potato-dextrose agar (PDA) slants. Inoculum was prepared from 5-day-old cultures on PDA.

Inoculation of plants and scoring of the symptoms. Fifteen-day-old plants were uprooted and inoculated by dipping the roots for 5 min in a conidial suspension of *F. o. vasinfectum* (10^6 conidia ml^{-1}) (5). For each isolate, 10 plants were inoculated. Control plants were treated with sterile water. Plants were maintained for 3 wk in a greenhouse (25 C night, 30 C day, 80% relative humidity, and 12-h photoperiod). Wilted plants were scored, and severity of wilt symptoms was assessed on the leaves by a wilt index (WI) (9). Reisolation of the fungus from the hypocotyl

of inoculated plants was conducted. Data from pathogenicity tests were statistically treated by analysis of variance. Pathogenicity tests were conducted twice for all the isolates.

Genomic DNA extraction. Isolates were grown in 200 ml of GYP medium (glucose 2%, yeast extract 0.5%, and peptone 0.5%) (10) for 5 days at 25 C. The mycelium was harvested by filtration and freeze-dried for 48 h. Total DNA extraction was performed by miniprep procedure (22), and the DNA was dissolved in TE buffer (10 mM Tris HCl, pH 7.5; 0.1 mM EDTA) to a final concentration of 5 ng μl^{-1}.

RAPD primers. The primers used are listed in Table 2 and were obtained from kit F, Operon Technologies (Alameda, CA).

Amplification conditions. Amplification reactions were performed in a total volume of 25 μl, containing 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 50 μM each of dATP, dCTP, dGTP, and dTTP, 15 pmol of primer, 25 ng of genomic DNA, and 1 U of *Taq* polymerase (Promega, Charbonnières, France). Negative controls, in which DNA template solution was replaced by water, were performed in all experiments to test for contamination. The amplification was performed with a DNA thermal cycler (PHC-3, Techne, Cambridge, England) programmed as follows: one cycle for 5 min at 95 C (before the addition of the *Taq* polymerase), followed by 45 cycles of 1 min at 94 C, 1 min at 34 C, and 2 min at 72 C. A cycle with 15 min at 72 C was conducted after the 45 cycles. Twenty microliters of the amplification products was separated by electrophoresis on 1.4% agarose gels stained with ethidium bromide and photographed under UV lights.

RAPD assays. Amplification reactions were conducted with each primer on the DNA of the 46 *F. o. vasinfectum* isolates and of the *Fusarium* sp., strain 122. All amplification reactions were conducted at least twice, in two separate experiments, for

TABLE 1. Code and geographic origin of the 46 *Fusarium oxysporum* f. sp. *vasinfectum* isolates and one isolate of a *Fusarium* sp., pathogenicity tests, race classification, and random amplified polymorphic DNA (RAPD) group determined in this study

		Pathogenicity reactions[x]						
		G. hirsutum		*G. barbadense*		*G. arboreum*		
Isolates	Origin	Isa 205	Acala S.J.	Ashmouni 106	Sakel	CG17	Race[y]	RAPD group
ATCC16421	USA	+	+	+	+	−	A (R1)	I
ATCC16611	USA	+	+	+	+	−	A (R2)	I
3F90	USA	+	+	+	+	−	A	I
Pu	Peru	+	+	+	+	−	A	I
9	Tanzania	+	+	+	+	−	A	I
11	Tanzania	+	+	+	+	−	A	I
13	Tanzania	+	+	+	+	−	A	I
15	Tanzania	+	+	+	+	−	A	I
34	Tanzania	+	+	+	+	−	A	I
189	Tanzania	+	+	+	+	−	A	I
J218	Ivory Coast	+	+	+	+	−	A	I
Fm8	Ivory Coast	+	+	+	+	−	A	I
Ci	Ivory Coast	+	+	+	+	−	A	I
Ci5	Ivory Coast	+	+	+	+	−	A	I
Cip	Ivory Coast	+	+	+	+	−	A	I
Cian	Ivory Coast	+	+	+	+	−	A	I
Cysa	Ivory Coast	+	+	+	+	−	A	I
Okra	Ivory Coast	+	+	+	+	−	A	I
Apl	Benin	+	+	+	+	−	A	I
Bn	Benin	+	+	+	+	−	A	I
ATCC36198	Brazil	+	+	+	+	−	A (R6)	I
Arg	Argentina	+	+	+	+	−	A	I
Pa	Paraguay	+	+	+	+	−	A	I
48	Zimbabwe	+	+	+	+	−	A	I
Bir	Unknown	+	+	+	+	−	A	I
ATCC16612	Egypt	−	−	−	+	+	3	II
S1	Sudan	−	−	−	+	+	3	II
S2	Sudan	−	−	−	+	+	3	II
S3	Sudan	−	−	−	+	+	3	II
S4	Sudan	−	−	−	+	+	3	II
S5	Sudan	−	−	−	+	+	3	II
S6	Sudan	−	−	−	+	+	3	II
S7	Sudan	−	−	−	+	+	3	II
S8	Sudan	−	−	−	+	+	3	II
I348	Israel	−	−	−	+	+	3	II
Mh3	Israel	−	−	−	+	+	3	II
Fil69	Israel	−	−	−	+	+	3	II
ATCC16613	India	−	−	−	−	+	4	III
40	Uzbekistan	−	−	−	−	+	4	III
CH1	China	−	−	−	−	+	4	III
CH3	China	−	−	−	−	+	4	III
CH4	China	−	−	−	−	+	4	III
CH5	China	−	−	−	−	+	4	III
CH6	China	−	−	−	−	+	4	III
CH7	China	−	−	−	−	+	4	III
CH8	China	−	−	−	−	+	4	III
Fusarium sp. 122	Tanzania	−	−	−	−	−	NP	OG[z]

[x] − = negative symptom (wilt index = 0); + = positive symptom (wilt index = 100). Reactions on *Gossypium* spp. cultivars.
[y] Race A refers to former races 1, 2, or 6 (3) (R1, R2, or R6, respectively). NP = nonpathogen.
[z] OG = outgroup.

each isolate. For the five ATCC isolates (American Type Culture Collection; representative of the races [3]), RAPD assays were replicated several times by three of the four authors.

Investigation of common bands. Sequence similarities representing amplified DNAs from different isolates were confirmed by Southern analysis of RAPD gels using PCR products as probes. DNA from individual RAPD bands was prepared as follows. A single RAPD band was excised from a 1% low-melting point agarose gel (Promega), purified with Prep-A-Gene kit (BioRad, Ivry-sur-Seine, France), and re-amplified with the appropriate primer under the same conditions. The re-amplified DNA was purified again in the same way before radiolabeling.

DNA from RAPD gels was transferred to nylon N+ membrane (Amersham, Les Ullis, France) by alkaline vacuum transfer (TE 80 TransVac, Hoefer Scientific Instruments, San Francisco).

RAPD-generated probes were labeled with ^{32}P-dCTP by random-priming (Megaprime kit, Amersham). Membrane-bound DNA fragments were hybridized to denatured probe at 65 C for 3 h in rapid-hybridization buffer (Amersham). Membranes were washed in 2× SSC (1× SSC is 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0), 0.1% sodium dodecyl sulfate (SDS) at room temperature for 20 min; 1× SSC, 0.1% SDS at 65 C twice for 15 min; and 0.1× SSC, 0.1% SDS at 65 C for 15 min. Filters were exposed to autoradiography film (β-max Hyperfilm, Amersham) at −80 C with intensifying screens (Amersham).

Cluster analysis. Comparison of each profile for each primer was done on the basis of the presence versus absence (1/0) of RAPD products of the same length. Bands of the same length were scored as identical. Analyses were based on the simple matching index (26), which measures the proportion of common discrete data (either 0 or 1) between the isolates. A dendrogram was derived from the distance matrix by the unweighted pair-group method algorithm (25) contained in the computer program package Phylip 3.4 (developed by J. Felsenstein, Department of Genetics, University of Washington, Seattle, in 1991).

RESULTS

Pathogenicity test. Three weeks after inoculation, all plants were either healthy (WI = 0) or wilted (WI = 100). Cultivars were designated as resistant or susceptible to a given isolate, respectively. Results of pathogenicity tests are given in Table 1. Control plants did not develop any symptoms (WI = 0). The 46 isolates collected from diverse geographic origins were classified into three groups on the basis of their virulence on the differential cultivars used.

A first group of 25 isolates was pathogenic to *G. hirsutum* and *G. barbadense* cultivars, but they were nonpathogenic to cultivar CG17 (*G. arboreum*). This group included isolates representative of races 1, 2, and 6. For convenience, we decided to assign isolates of this group to race A.

A second group of 12 isolates, including those from Sudan and Israel, as well as the isolate representative of race 3, was pathogenic only on CG17 and Sakel cultivars. These reactions corresponded to those described earlier for race 3 (3).

A third group of nine isolates, from China and Uzbekistan,

TABLE 2. Code and sequence of the 11 primers tested, with total number of amplified DNA fragments and number of polymorphic DNA fragments obtained with each primer in random amplified polymorphic DNA (RAPD) experiments

Code	Sequence 5′ to 3′	Amplified fragments	Polymorphic fragments
OPF-01	ACGGATCCTG	7	4
OPF-02	GAGGATCCCT	6	5
OPF-04	GGTGATCAGG	6	6
OPF-05	CCGAATTCCC	11	8
OPF-06	GGGAATTCGG	6	3
OPF-08	GGGATATCGG	3	2
OPF-10	GGAAGCTTGG	9	7
OPF-11	TTGGTACCCC	3	3
OPF-12	ACGGTACCAG	6	4
OPF-13	GGCTGCAGAA	5	4
OPF-14	TGCTGCAGGT	7	6

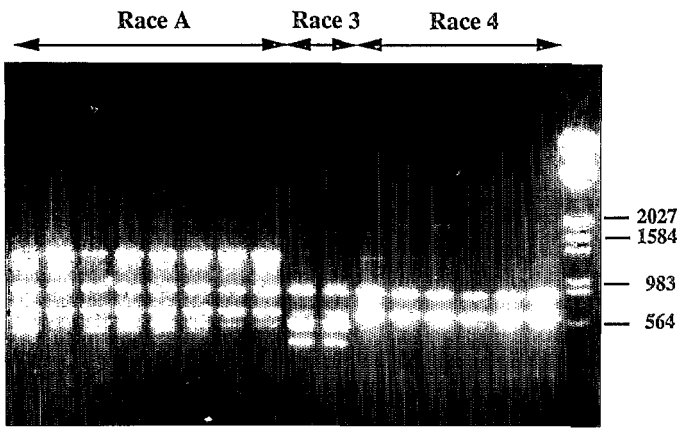

Fig. 1. Gel stained with ethidium bromide showing amplification products generated from the *Fusarium oxysporum* f. sp. *vasinfectum* isolates with primer OPF-06. Lanes, from left to right, show amplification products from isolates ATCC16421, ATCC16611, Ci, Pu, 11, 189, Bn, ATCC36198, ATCC16612, S1, ATCC16613, CH1, CH3, CH4, CH5, and 40. Last lane on right, a mixture of lambda DNA digested with *Eco*RI and *Hin*dIII used as molecular weight markers; the fragment size in base pairs is indicated on the right.

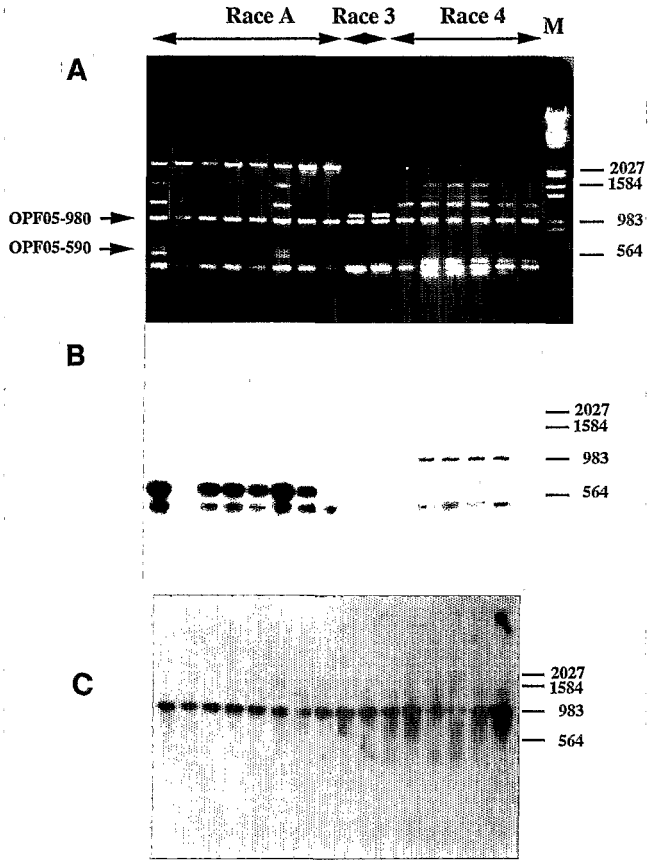

Fig. 2. Confirmation of common bands between *Fusarium oxysporum* f. sp. *vasinfectum* isolates with primer OPF-05 and random amplified polymorphic DNA bands OPF05-590 and OPF05-980. **A,** Gel stained with ethidium bromide. Lanes, from left to right, show amplification products from isolates ATCC16421, ATCC16611, Ci, Pu, 11, 189, Bn, ATCC36198, ATCC16612, S1, ATCC16613, CH1, CH3, CH4, CH5, and 40. Arrows indicate bands OPF05-1000, OPF05-980, and OPF05-590 excised from the gel for further amplification and radiolabeling for use as probes. **B and C,** Autoradiograph of Southern blot prepared from gel (**A**) probed with band OPF05-590 (**B**) and band OPF05-980 (**C**).

and one isolate representative of race 4, from India, was pathogenic only to CG17, corresponding to the pathological reactions of race 4 (3).

Strain 122 was nonpathogenic to all the cotton plants tested, demonstrating that it was not *F. o. vasinfectum*.

Differentiation of isolates with RAPD markers. RAPD patterns were established for the 46 isolates of *F. o. vasinfectum* and *Fusarium* sp., strain 122, with the 11 primers listed in Table 2. These primers were chosen from the 20 tested because of the clear amplification pattern they produced (bright reproducible bands). Concentrations of DNA template, primer, and dXTP were determined in preliminary trials to get unambiguous amplification patterns. The profiles were reproducible from one experiment to another, with DNA newly extracted from the same culture and with DNA from newly cultivated mycelia.

Figure 1 shows amplification products generated with a primer (OPF-06). The size of amplified DNA fragments generated with the 11 primers ranged from 0.2 to 2.1 kb. All the primers revealed polymorphisms useful for classifying isolates. Amplification patterns for the *Fusarium* sp., strain 122, were very distinct from those of the *F. o. vasinfectum* isolates. Table 2 shows the total number of amplified fragments and the number of polymorphic fragments produced with each primer. By combining the results using 11 primers, 83 band positions were scored for presence versus absence (1/0) for all the isolates studied, and 46 were polymorphic. We verified that bands of the same length represented homologous sequences using amplification products from primer OPF-05, which displayed the most variability. We chose three of these RAPD fragments (two polymorphic and one common to all isolates) that were the common 980-bp band (OPF05-980), the 1,000-bp band (OPF05-1000), and the 590-bp band (OPF05-590). When hybridized with a blot of RAPD products, each probe hybridized to itself and to all bands of the same size that were amplified (Fig. 2). This experiment indicated that there was no co-migration of a nonhomologous fragment.

The combined data from all isolates were analyzed by a simple matching coefficient (25,26) to produce a dendrogram (Fig. 3). The *Fusarium* sp., strain 122, was included as an outgroup strain to create a rooted tree in cluster analysis. At a genetic distance of 0.2, three distinct groups were differentiated among the 46 *F. o. vasinfectum* isolates by RAPD markers. The first group, RAPD I, included 25 isolates with different origins (Africa and America) and exhibited slight differences in RAPD products. This group included all the isolates belonging to race A. A second group of 12 isolates, RAPD II, showed identical RAPD patterns whatever the primers used and included all isolates of race 3 from Sudan, Israel, and Egypt. The third group (RAPD III) displayed slight differences in the profiles and included the nine isolates of race 4 from China, Uzbekistan, and India.

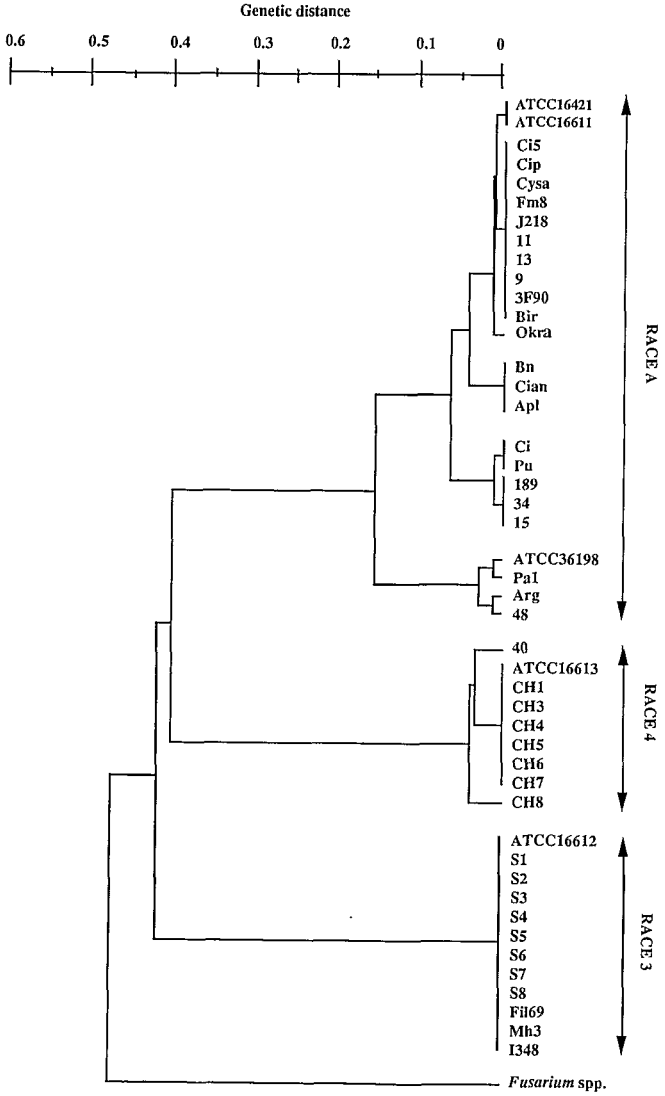

Fig. 3. Dendrogram showing relationships among the 46 *Fusarium oxysporum* f. sp. *vasinfectum* isolates and one isolate of *Fusarium* sp. Genetic distances were obtained by random amplified polymorphic DNA analysis with 11 primers.

DISCUSSION

We observed genetic diversity within a collection of 46 *F. o. vasinfectum* isolates of worldwide origin, based on pathogenicity and RAPD markers. Isolates were classified into three major pathogenicity groups on cotton, consistent with their geographic origin (1–3). Random amplified DNA patterns produced from genomic DNA reliably and unambiguously distinguished isolates of *F. o. vasinfectum* of each pathogenicity group.

In our experiment, we conducted a pathogenicity test on the same differential cotton species used previously (2,3). The five tested cultivars were inoculated with 41 isolates collected throughout the world and five isolates, deposited by Armstrong and Armstrong at ATCC (Rockville, MA), identified respectively as races 1, 2, 3, 4, and 6 (1–3) (no isolate representative of race 5 was available at ATCC). Three distinct virulence groups were recovered that corresponded to races 3, 4, and A, which grouped former races 1, 2, and 6 together. The definition of race A did not contradict Armstrong and Armstrong (2,3), who did not find differences among races 1, 2, and 6 on cotton. The geographic distribution of the races obtained confirmed and enlarged that previously described (1–3). Race A isolates originated from America and west African countries, whereas race 3 isolates were restricted to Israel, Egypt, and Sudan and race 4 isolates to Asian countries. However, none of the Sudan isolates we tested was race 5, despite a previous report (17).

The RAPD method revealed polymorphisms within isolates of *F. o. vasinfectum* and established DNA fingerprints useful for race characterization. We were able to differentiate the isolates into three main groups (RAPDs I, II, and III) directly related to both virulence and geographic origin (Table 1). The pathogenic specialization of *F. o. vasinfectum* on cotton, thus, is related to genetic diversity of isolates. The geographic isolation of each race in *F. o. vasinfectum* may have contributed to that genetic diversification revealed by RAPD analysis. Such correlation between race and genetic evolution is unusual within *F. oxysporum*. Most of the studies based on restriction fragment length polymorphism (RFLP) analyses of nuclear or mitochondrial DNA failed to characterize races (8,19–21,23), and RAPD analysis conducted on *F. o. pisi* isolates allowed differentiation of only one race (race 2) of four studied (11). In other phytopathogenic fungi, RAPD analyses have proved useful for detecting genomic polymorphisms directly related to host specialization (7,11,12, 16,24). The genetic basis of the polymorphisms generated by RAPD is not well-defined. Presence or absence of a specific band can arise from a point mutation as well as from a mutation event such as insertion or deletion of DNA sequences. Furthermore,

DNA fragments are amplified from unique and repetitive sequences (6,14,24) that are known to evolve at different rates in the genome.

Our results provide evidence that RAPD analysis can be used for differentiating and identifying *F. o. vasinfectum* isolates. Additionally, specific polymorphic RAPD fragments could be used to detect RFLPs and to generate race-specific probes (14). We suggest that RAPD markers may be used as a quick and reliable alternative for differentiating *F. o. vasinfectum* races on cotton.

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