Characterization of *Fusarium oxysporum* f. sp. *phaseoli* by Pathogenic Races, VCGs, RFLPs, and RAPD

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ABSTRACT

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Isolates of *Fusarium oxysporum* were obtained from the vascular tissue of *Phaseolus* spp. and characterized by pathogenicity, vegetative compatibility, restriction fragment length polymorphisms (RFLPs), and random amplified polymorphic DNA (RAPD) analyses. Eleven isolates were *F. oxysporum* f. sp. *phaseoli* and comprised five pathogenic races. Two races, nine vegetative compatibility groups (VCGs), and three heterokaryon self-incompatible isolates are new reports for this forma specialis. Pathogenic and nonpathogenic isolates were not found in the same VCGs and, in some cases, there was a correspondence of VCG to race and place of origin. RFLPs with five different restriction enzymes and RAPD with four different primers indicated genetic diversity in *F. oxysporum* f. sp. *phaseoli*. The banding patterns showed a correspondence to the VCGs, but not to the pathogenic races. RFLPs and RAPD markers could be used to distinguish isolates that were nonpathogenic, self-incompatible, and from obscure origins. The use of all four methods combined was effective in characterizing and providing insight into the complex relationship of *F. oxysporum* f. sp. *phaseoli*.

Fusarium oxysporum Schlecht. emend. Snyd. and Hans. f. sp. *phaseoli* Kendr. and Snyd. causes "bean yellows" or wilt disease in *Phaseolus vulgaris* L. *F. oxysporum* f. sp. *phaseoli* was first described by Harter in 1929 in California (3) and has since been reported in other bean-growing regions of the world including Brazil (45,46), Colombia, Peru (39), other regions of the United States (4), and Costa Rica (3). Particularly in South America, vascular wilt of bean is a disease of economic significance. In Europe, this disease has been described in the Netherlands (45) and was first reported in Italy in 1983 (2). A similar wilt disease also has been described in Britain on *P. coccineus* (3).

F. oxysporum isolates of a given forma specialis are not distinguishable morphologically, and subspecific subdivision is usually based upon pathogenic or physiological race reactions on a set of differential cultivars (49) or by the determination of vegetative compatibility groups (VCGs) obtained from heterokaryon formation between anastomosing nitrate nonutilizing (*nit*) mutants (43). The disadvantages of these techniques are that they are time consuming, labor intensive, and subject to varying environmental or culture growth conditions during the experiments. In theory, molecular biology techniques such as restriction fragment length polymorphisms (RFLPs) and random amplified polymorphic DNA (RAPD) (51) overcome all these limitations and provide additional information for fungal characterization (12,38). Moreover, these characters could be useful, if correlated to race or VCG, in identifying isolates, monitoring disease demographics, or investigating evolutionary relationships, especially if the relationship is maintained over time and with increased sample size (14,17,18,31). These molecular methods have been used successfully to characterize other formae speciales of *F. oxysporum* (24,35) and to determine a genetic relationship to pathogenicity (5,14,33,36).

Ribeiro and Hagedorn (45,46) identified diverse pathogenic races of *F. oxysporum* f. sp. *phaseoli*: a race from Brazil and a race consisting of two different isolates, one originating from the United States (ATCC 18131) and the other from the Netherlands. Other studies reported a race from Colombia (39) and a race from Italy that exhibited similar race reactions to isolate ATCC 18131 when tested together (3). Since these experiments were not uniform, it was difficult to discern the inter-relationship among these isolates and races. Research on other *F. oxysporum* f. sp. *phaseoli* characteristics, besides physiological race, has not yet been performed and is generally lacking.

The objectives of this study were to characterize isolates of *F. oxysporum* obtained from bean affected by vascular wilt using physiological races, VCGs, RFLPs, and RAPD analysis to determine i) if these techniques could characterize the isolates; ii) if the genetic diversity could be detected, and distinguish the isolates within *F. oxysporum* f. sp. *phaseoli*; and iii) if the genetic and pathogenic characteristics are correlated.

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MATERIAL AND METHODS

Fungal isolates. Isolates of *F. oxysporum* f. sp. *phaseoli* were obtained from previous studies (3,39,46) and requested from fungal collections around the world. Out of the 32 isolates received, some were misidentified as *F. oxysporum* and others were duplicates. Consequently, 20 representative isolates were selected for detailed examination (Table 1). Each isolate was identified with a *F. oxysporum* f. sp. *phaseoli* (Fop) code (that is referred to hereafter) and subcultured from a single microconidium. Conidia stocks were stored in sterile, 20% glycerol or on silica gel at –65°C and on potato-dextrose agar (PDA) (Difco Laboratories, Detroit) slants under mineral oil and deposited in the fungal collection at the Institute of Plant Pathology at the University of Naples, Portici, Italy.

Pathogenicity tests and race determination. PDA plates were inoculated with a single spore inoculum for each *F. oxysporum* f. sp. *phaseoli* isolate, and cultures were incubated at 24°C in the dark for 10 days. Plates were flooded with sterile, distilled water, conidia were scraped from the colonies, and a suspension of 10^6 conidia per milliliter was obtained. Isolates were screened for pathogenicity on eight *Phaseolus* cultivars from a differential set used at Centro Internacional de Agricultura Tropical (CIAT): A211, BAT 477, Calima, ICA 032, IPA 1, Mortino, RIZ 30, and TIB 3042 (39); plus cultivars HF 465-63-1 and Pinto were initially screened with the fungi. Isolates that did not produce disease symptoms on these cultivars were further screened on an additional set of 17 *Phaseolus* cultivars. Seeds were germinated in vermiculite, and 5- to 7-day-old seedlings were inoculated by a root dip procedure modified from Pastor-Corrales and Abawi (39). Twelve plants of each cultivar were inoculated with each isolate, as well as with a control of distilled water. Seedlings were transplanted in sterile soil and maintained in the greenhouse at diurnal temperatures of 18/26°C.

Three to five weeks after inoculation, plants were evaluated for external symptoms according to the CIAT disease severity rating scale (39), ranging from 1 (no symptoms) to 9 (severe wilting or stunting and vascular discoloration). Stem slices were obtained from plants with no apparent symptoms, or slight vascular discoloration, from heights of 0.5, 5, and 10 cm above soil level. Samples were surface-sterilized, plated on acidified PDA plates, and then incubated for 14 days to detect the pathogen in the symptomless plants. The experiment was repeated three times over a 3-year period.

Classification of the races was determined by the disease reactions of the 10 differential host cultivars of *P. vulgaris* to the *F. oxysporum* f. sp. *phaseoli* isolates. Isolates with similar disease reactions were grouped together to comprise a race (Table 2). The two races of *F. oxysporum* f. sp. *phaseoli* initially described by Ribeiro and Hagedorn (45) were used as a reference point for differentiating and naming the subsequent races (Table 1).

VCGs. *nit* mutants were generated as described by Puhalla (43) and Correll et al. (10) on PDA plus chlorate (PDC) or minimal media (MM) plus chlorate (MMC) and characterized as *nit*1, *nit*3, or NitM as determined by growth on basal media supplemented with ammonium, nitrate, nitrite, or hypoxanthine.

One *nit* mutant of each type was randomly selected for each isolate, and pairings were conducted between *nit*1 and NitM mutants, since these produced the most rapid and obvious heterokaryon formations (10). Complementation tests were performed in 60-mm petri plates containing MM between *nit*1 × NitM mutants and reciprocal pairings for each *F. oxysporum* f. sp. *phaseoli* isolate to itself, as well as in all possible pairwise combinations with the other isolates. Plates were incubated at 20°C and then scored after 10 and 28 days. Isolates were considered to be vegetatively compatible if the complementation of the *nit* mutants resulted in the formation of a prototrophic heterokaryon with dense aerial growth at the zone of the anastomosing mycelia. If mutants from the same isolate had not complemented, they were considered as self-incompatible and not placed in a VCG. However, if isolates were self-compatible, but had not complemented with any other isolates, they were considered as a single member of a VCG. Each pairing was performed in duplicate and repeated twice at different times.

TABLE 1. *Fusarium oxysporum* isolates used (listed in order of race) noting code, isolate annotation, source, and geographical origin; pathogenic races and vegetative compatibility groups as determined in this study

Code[a]	Isolate annotation	Source[b]	Geographical origin	Race[c]	VCG[d]
Fop 1	ATCC 18131/FOP-SC	ATCC	United States (South Carolina)	1	0161
Fop 11	FC 1789	NA	Italy (Portici)	1	0165
Fop 2	ATCC 42145/2107-A	ATCC	Brazil	2	0162
Fop 3	Fo 5 BRA	MPC	Brazil	2	0162
Fop 6	Fo 28 COL	MPC	Colombia	3	0163
Fop 7	Fo 10 COL	MPC	Colombia	3	0164
Fop 8	Fo 45 COL	MPC	Colombia	3	0164
Fop 28	FOP-CL25	HFS	Colombia	3	0164
Fop 30	FOP-CO/ATCC 90245	HFS	United States (Colorado)	4	016–
Fop 31	No. 9	EK	Greece (Kavala)	5	0165
Fop 32	No. 10	EK	Greece (Kastoria)	5	0165
Fo 4	CCM F419	CCM	ex-Czechoslovakia	np	216–
Fo 9	IFO 9970	IFO	Japan	np	216–
Fo 10	IPV BO	UB	Italy (Bologna)	np	2161
Fo 13	F 4	DP	Poland (Lublin)	np	2162
Fo 15	F 10	DP	Poland (Lublin)	np	2162
Fo 16	F 13	DP	Poland (Lublin)	np	2163
Fo 19	F 18	DP	Poland (Lublin)	np	2163
Fo 25	F 50	DP	Poland (Lublin)	np	2163
Fo 17	F 14	DP	Poland (Lublin)	np	2164

[a] Identification code of isolate in Portici collection. Fop = *F. oxysporum* f. sp. *phaseoli*; Fo = *F. oxysporum*, as determined by pathogenicity tests in this study.

[b] Contributing sources of isolates: American Type Culture Collection (ATCC); M. Pastor-Corrales (MPC); H. F. Schwartz (HFS); Czechoslovakian Collection of Microorganisms (CCM); E. Kalonira (EK) and T. C. Tjamos; University of Bologna (UB); University of Naples, Portici (NA); Institute for Fermentation, Osaka (IFO); and D. Pieta (DP).

[c] Pathogenic races as determined by pathogenicity tests in this study (Table 2). np = nonpathogenic.

[d] Vegetative compatibility groups (VCGs) as determined in this study and numbered according to Puhalla (43), in which 016# = VCG code for *F. oxysporum* f. sp. *phaseoli* (Fop); and 216# = an arbitrary VCG code for non-Fop isolates of this study (numbers >2,000 are for nonpathogenic isolates). 016– and 216– are artificial groups containing vegetatively self-incompatible isolates that did not form heterokaryons upon pairing with themselves or with any of the other isolates.

DNA extraction, restriction digests, and Southern blotting. A 50-µl glycerol-spore suspension (10^6 spore per milliliter) was used to inoculate 150 ml of potato-dextrose broth in 250-ml flasks and grown for 1 week at 25°C with agitation at 100 rpm. Mycelia were collected onto Miracloth (Calbiochem-Behring, La Jolla, CA) by vacuum filtration, washed with sterile distilled water, immersed in liquid nitrogen, lyophilized, and stored at –20°C until needed. DNA was isolated from a 500-mg sample of powdered material using a modified method of Raeder and Broda (44).

Genomic DNA was digested with *Bgl*II, *Hae*III (Promega, Madison, WI), *Taq*I, *Msp*I, or *Hin*dIII (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) according to manufacturer's recommendations; electrophoresed (48); photographed; and transferred by vacuum-blotter (Bio-Rad Laboratories, Richmond, CA) onto nylon membrane (Nytran; Schleicher and Schuell, Dassel, Germany) for each of the restriction digests. Each experiment was repeated three or more times using DNA from different isolations.

RFLP probe and hybridization. A 1.5-kb nonradioactive probe (digoxigenin-11-labeled dUTP; Boehringer Mannheim Biochemicals, Indianapolis, IN) was obtained by amplifying genomic DNA from Fop 6 with a primer designed for a pyruvate dehydrogenase gene of *Trichoderma harzianum* (ED2: 5′-TGTCCATCTGGATGTTTCCC-3′; M. Lorito, S. L. Woo, and F. Scala, *unpublished data*). Other hybridizations were performed with a digoxigenin-labeled, 1.3-kb fragment of the D4 probe described by Manicom et al. (33,34) that contained a random fragment from *F. oxysporum* f. sp. *dianthi*. Nonradioactive hybridizations and detection were conducted on the membranes from each of the restriction digests, according to the instructions for the DIG DNA Labeling and Detection Kit (Boehringer Mannheim Biochemicals).

RAPD analysis. Initially, genomic DNA from two pathogenic isolates (Fop 3 and Fop 8) and one nonpathogenic isolate (Fo 17) were selected to screen 45 primers obtained from various sources to determine if banding patterns produced by the arbitrary amplification could differentiate between the isolates. Primers were not useful when there were no amplification products, too many products, or monomorphic products. The following four primers were selected on the basis of reproducible results obtained for use throughout the study: ED3 = 5′-ATGGCCACTTCTTGG-3′; ED13 = 5′-ATGGCCACCTCGTGG-3′; ED24 = 5′-ATGGCAACTTCGTGG-3′; and ED15 = 5′-ATGGCAACCTCGTGG-3′. Additional preliminary testing was conducted to determine the optimal concentrations and manufacturer sources of the reaction components. Only the most intense bands were considered for the analysis.

RAPD reactions were performed in a total volume of 25 µl containing a 1× polymerase chain reaction (PCR) reaction mixture (Promega); 2.5 mM $MgCl_2$; 50 µM of each dNTP (Promega); 0.3 µM of primer; 50 ng of genomic DNA; and 0.5 units of *Taq* polymerase (Stratagene Inc., La Jolla, CA). A negative control using water instead of the target DNA was included to test for contamination. Amplification conditions of the thermocycler (Perkin-Elmer Cetus, Foster City, CA) were one cycle for 3 min at 94°C and 1 min at 55°C; followed by 34 cycles of 1 min at 94°C, 1 min at 55°C, and 3 min at 72°C. The final cycle was 1 min at 94°C, 1 min at 55°C, and 7 min at 72°C. Samples were electrophoresed in a 2% agarose gel (1% agarose + 1% Wide Range/Standard 3:1; Sigma Chemical Co., St. Louis). All experiments were repeated at least three times, by different researchers.

Statistical analysis. Computer images of the gels from both the RFLPs and RAPD analysis were scored for each *F. oxysporum* f. sp. *phaseoli* isolate using the program NCSA GelReader (37), and the major bands were considered for the statistical analysis. A square binary matrix, based on the presence or absence of bands (1/0), of the data was subjected to cluster analysis by agglomerative classification using program NCLAS in the multivariate statistical software package SYN-TAX IV (41). An unweighted paired group method with arithmetic (UPGMA) averages based on simple matching similarity coefficients was used to generate a dendrogram.

RESULTS

Five pathogenic races were found among 11 *F. oxysporum* f. sp. *phaseoli* isolates, as differentiated by 10 *P. vulgaris* cultivars (Tables 1 and 2). Nine isolates were nonpathogenic and will, hereafter, be referred to as *F. oxysporum* (Fo). Control plants did not develop symptoms, and the fungus was not found vascularly. Three races corresponded to geographic origins: race 2 isolates were from Brazil (Fop 2 and Fop 3); race 3 isolates were from Colombia (Fop 6, Fop 7, Fop 8, and Fop 28); and race 5 isolates were from Greece (Fop 31 and Fop 32). The race 4 isolate was from Colorado (Fop 30), and the race 1 isolates were from South Carolina (Fop 1) and Italy (Fop 11). The nonpathogenic *F. oxysporum* were largely comprised of isolates from Poland.

Similar reactions to the pathogens were observed between cultivars A211 and RIZ 30; BAT 477 and Pinto; and among Calima, ICA 032, and Mortino (Table 2). Only cultivars susceptible to race 3 exhibited a dense growth of spores on the plant stem (10 to 15 cm above the stem base) 1 month after inoculation (data not shown).

Mutants resistant to chlorate were obtained at a mean frequency of 0 to 2.1 sectors per colony on PDC and 0 to 1.33 sectors per colony on MMC (depending upon the isolate considered; data not shown). Among the chlorate-resistant sectors, 22 to 47% (in all cases, the frequencies depended upon the isolate considered) were

TABLE 2. Five races of *Fusarium oxysporum* f. sp. *phaseoli* as determined by the differential response of 10 *Phaseolus vulgaris* cultivars

Cultivars	Races of *F. oxysporum* f. sp. *phaseoli*[a]				
	Race 1	Race 2	Race 3[b]	Race 4	Race 5
A211/RIZ 30	S	S	R	S	S
BAT 477/Pinto	S	R	R	S	S
Calima/ICA 032/Mortino	R	R	S	S	R
IPA 1	R	S	R	S	S
TIB 3042	R	R	S	S	S
HF 465-63-1	R	R	R	R	R

[a] S = susceptible, R = resistant.

[b] Cultivars susceptible to race 3 also had fungal sporulation on their stems 1 month after inoculation.

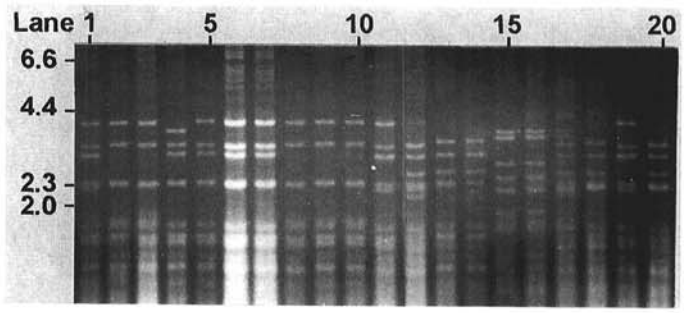

Fig. 1. Restriction fragment length polymorphism (RFLP) patterns of genomic DNA digested with *Taq*I from 11 *Fusarium oxysporum* f. sp. *phaseoli* (Fop) and nine nonpathogenic *F. oxysporum* (Fo) isolates in a 1% agarose gel stained with ethidium bromide. Numbers on the left indicate fragment sizes in kilobase pairs. Isolates are the following: lane 1, Fop 1 (race 1, VCG 0161); lane 2, Fop 2 (race 2, VCG 0162); lane 3, Fop 3 (race 2, VCG 0162); lane 4, Fop 6 (race 3, VCG 0163); lane 5, Fop 7 (race 3, VCG 0164); lane 6, Fop 8 (race 3, VCG 0164); lane 7, Fop 28 (race 3, VCG 0164); lane 8, Fop 11 (race 1, VCG 0165); lane 9, Fop 31 (race 5, VCG 0165); lane 10, Fop 32 (race 5, VCG 0165); lane 11, Fop 30 (race 4, VCG 016–); lane 12, Fo 10 (VCG 2161); lane 13, Fo 13 (VCG 2162); lane 14, Fo 15 (VCG 2162); lane 15, Fo 16 (VCG 2163); lane 16, Fo 19 (VCG 2163); lane 17, Fo 25 (VCG 2163); lane 18, Fo 17 (VCG 2164); lane 19, Fo 4 (VCG 216–); and lane 20, Fo 9 (VCG 216–).

chlorate resistant but utilized nitrate (CRUN) and had wild-type growth on MM (27). The relative frequencies of the *nit* mutants recovered from PDC were 60 to 86% for *nit*1, 4 to 23% for *nit*3, and 0 to 15% for NitM mutants. From MMC, there were 58 to 62% for *nit*1, 5 to 25% for *nit*3, and 0 to 25% for NitM mutants.

Nine VCGs were found among 17 isolates, and three isolates were heterokaryon self-incompatible (Table 1). Heterokaryon formation was never observed between pathogenic and nonpathogenic isolates. Four isolates (Fop 1, Fop 6, Fo 10, and Fo 17) were self-compatible, but did not form heterokaryons upon anastomosis with any other isolates and constituted single isolate VCGs. Among the pathogenic isolates, there was a weak relationship of VCG to race and geographic origin: VCG 0162 contained both race 2 isolates from Brazil; VCG 0164 contained three of the four race 3 isolates from Colombia; VCG 0165 contained the race 1 isolate from Italy plus the two race 5 isolates from Greece. Two races contained more than one VCG: race 1 contained VCG 0161 and VCG 0165; and race 3 contained VCG 0163 and VCG 0164. Only VCG 0165 contained isolates belonging to more than one race (race 1 and 5). Among the nonpathogenic isolates from Poland, VCG 2162 contained Fo 13 and Fo 15, whereas VCG 2163 contained isolates Fo 16, Fo 19, and Fo 25.

RFLPs were noted in digests of genomic DNA from all 20 isolates (Fig. 1), and RFLP groups were identified for each of the five enzymes based on the similarity coefficients of the isolates (Table 3). These digests yielded 18 to 32 intense bands per enzyme that were reproducible and considered for characterization. The banding patterns that were clearly visible in ethidium bromide (EtBr)-stained gels after restriction digest with *Hae*III, *Msp*I, or *Taq*I (Fig. 1 and data not shown) corresponded to bands produced from hybridizations with the probes. However, in digests with *Bgl*II and *Hin*dIII, RFLPs could not be determined from the EtBr-stained gels and hybridizations with specific probes were required (data not shown). The ED2-derived probe hybridized with digested DNA from both the pathogenic and nonpathogenic isolates and produced 5 to 15 bands per isolate. The D4 probe produced fewer polymorphic bands, but produced high background on the membranes.

In general, the RFLP patterns corresponded to the VCGs and were not similar between the pathogenic and nonpathogenic isolates (Table 3). Among the pathogenic isolates, those from Brazil, Fop 2 and Fop 3 (both race 2, VCG 0162), had similar banding patterns upon digestion with four of the five restriction enzymes. The Colombian isolates, Fop 7 and Fop 8 (both race 3, VCG 0164), had similar RFLPs for all enzymes, whereas Fop 28 (race 3, VCG 0164) was similar to these two isolates upon digestion with three of the five enzymes. However, Fop 6 (race 3, VCG 0163) was similar for only one digest to the other Colombian isolates. The two Greek isolates, Fop 31 and Fop 32 (both race 5, VCG 0165), had similar RFLPs with three restriction digests and were similar to Fop 11 (race 1, VCG 0165) for two enzymes. The two American isolates, Fop 1 (race 1, VCG 1) and Fop 30 (race 4, VCG 016–) had similar RFLPs for three enzymes. Fo 4 was the only nonpathogenic isolate that showed similar banding patterns to pathogenic isolates Fop 1 and Fop 30.

Among the nonpathogenic isolates, the Polish isolates Fo 13 and Fo 15 (both VCG 2163) had similar RFLP patterns following digestion with all five enzymes. Other isolates from Poland, Fo 16 and Fo 19 (both VCG 2163), had similar RFLPs for three enzymes. Fo 25, also in VCG 2163, had no identities with Fo 16 and Fo 19. However, in analytical electrophoresis runs, it was noted that undigested nucleic acid preparations from Fo 25 occasionally exhibited seven sharp bands (0.85 to 2.3 kb) below the band of genomic DNA (data not shown). Further examinations of these bands revealed that they were double-stranded RNA (52).

TABLE 3. Restriction fragment length polymorphism (RFLP) patterns of five restriction enzymes and random amplified polymorphic DNA (RAPD) patterns of four primers from 11 *Fusarium oxysporum* f. sp. *phaseoli* and nine *F. oxysporum* isolates with corresponding vegetative compatibility groups (VCGs) and pathogenic races, listed in order of VCG

			RFLPs[a]					RAPD analysis[b]			
Code[c]	VCG[d]	Race[e]	*Bgl*II	*Hae*III	*Hin*dIII	*Msp*I	*Taq*I	ED3	ED13	ED24	ED15
Fop 1	0161	1	I	I a	I	I a	I a	I a	I a	I a	I
Fop 2	0162	2	II a	II	II	II	II a	II a	II a	II a	II
Fop 3	0162	2	II a	II	III	II	II a	II a	II a	II a	III
Fop 6	0163	3	I	I	IV a	III	III	I a	III	I a	IV
Fop 7	0164	3	III	III a	IV	II b	I b	III a	IV a	III	IV a
Fop 8	0164	3	III	III a	IV a	II b	I b	III a	IV a	IV	IV a
Fop 28	0164	3	IV	III	V a	II b	I b	III a	IV a	V	IV
Fop 11	0165	1	V	IV	III	IV	IV a	IV a	V a	VI	V
Fop 31	0165	5	VI	V a	VI	II b	IV a	IV a	V a	VII a	V
Fop 32	0165	5	V	V a	VII	II b	IV a	V	V a	VII a	V
Fop 30	016–	4	VII	I a	V a	I a	I a	I a	VI	VIII	I a
Fo 10	2161	np	VIII	VI	V	V	V	VI	VII	IX a	VI
Fo 13	2162	np	IX a	VII a	VIII a	VI	VI a	VII	V a	X	VII
Fo 15	2162	np	IX a	VII a	VIII a	VI	VI	VIII	I a	XI	VIII
Fo 16	2163	np	X	VIII	IX a	VII	VII a	IX	VIII	IX a	IX a
Fo 19	2163	np	X	IX	IX a	VIII	VII a	X	IX a	XII a	IX a
Fo 25	2163	np	XI	X	X	IX	VIII	XI	IX a	XII a	X
Fo 17	2164	np	XII	XI	XI	IX	VI a	XII	X	XII a	XI
Fo 4	216–	np	I	I a	IV	I a	I a	I a	I a	XIII	I a
Fo 9	216–	np	XIII	XII	I	X	VI	XIII	XI	XII a	XII

[a] RFLPs observed among the 20 *Fusarium* isolates upon digestion with each restriction enzyme. Isolates with the same number for a given restriction digest have RFLP patterns with similarity coefficients ≥0.70; and those with the same numbers and letters have banding patterns with similarity coefficients >0.85.

[b] RAPD analysis of 20 *Fusarium* isolates upon polymerase chain reaction amplification with each primer. Isolates with the same number for a given primer amplification have RAPD patterns with similarity coefficients ≥0.70; and those with the same numbers and letters have banding patterns with similarity coefficients >0.85. Primer sequences: ED3 = 5′-ATGGCCACTTCTTGG-3′; ED13 = 5′-ATGGCCACCTCGTGG-3′; ED24 = 5′-ATGGCAACTTCGTGG-3′; and ED15 = 5′-ATGGCAACCTCGTGG-3′.

[c] Identification code of isolate. Fop = *F. oxysporum* f. sp. *phaseoli*; Fo = *F. oxysporum*, as determined by pathogenicity tests in this study.

[d] Vegetative compatibility groups (VCGs) as determined in this study and numbered according to Puhalla (43), in which 016# = VCG code for *F. oxysporum* f. sp. *phaseoli* (Fop) and 216# = an arbitrary VCG code for non-Fop isolates. 016– and 216– are artificial groups containing vegetatively self-incompatible isolates that did not form heterokaryons upon pairing with any of the other isolates.

[e] Pathogenic races as determined by pathogenicity tests in this study (Table 2). np = nonpathogenic.

Our RAPD analysis produced distinct banding patterns that were not commonly shared between the pathogenic and nonpathogenic isolates and corresponded well to the VCGs among the pathogenic isolates (Figs. 2 and 3). RAPD patterns differentiated the 20 isolates into groupings similar to those of the RFLPs (Table 3). Amplification with the four random primers produced bands ranging from 0.37 to 2.8 kb in size. Primer ED3 generated 14 polymorphic bands out of a total of 15 informative bands; primer ED13 and primer ED24 generated 11 polymorphic bands from a total of 12 informative bands; and primer ED15 generated 20 polymorphic bands from 22 informative bands. Similar banding patterns were observed among pathogenic isolates of the same VCG for three of the four primers (Table 3): Fop 2 and Fop 3 (VCG 0162); Fop 7, Fop 8, and Fop 28 (VCG 0164); and Fop 11 and Fop 31, plus Fop 31 and Fop 32 (VCG 0165). In addition, the RAPD pattern of Fop 1 (VCG 0161) was similar to that of pathogenic isolate Fop 30 (VCG 016–), as well as that of nonpathogenic isolate Fo 4 (VCG 216–).

The correspondence of the RAPD patterns to the VCG groupings was not as obvious among the nonpathogenic isolates as it was with the RFLPs (Table 3). Isolates of the same VCG, Fo 13 and Fo 15 (VCG 2162) were never similar; Fo 16 and Fo 19 (VCG 2163) were similar only with primer ED15; and Fo 19 and Fo 25 (VCG 2163) were similar with two primers. Amplification with primer ED2 frequently produced similar banding patterns among isolates of different VCGs, whereas primer ED13 frequently produced similar banding patterns between pathogenic and nonpathogenic isolates.

In the RAPD analysis, it was possible to note bands that were generally associated more with the pathogenic isolates than with the nonpathogenic isolates (Fig. 2). The following bands were distinct in the pathogenic isolates, but faint or absent in the nonpathogenic isolates: a pair of 0.55- and 0.48-kb bands together for primer ED3 (Figs. 2 and 3); a 1.0-kb band for ED13; a 1.1-kb band for ED24; and an intense pair of 1.1- and 1.5-kb bands together for ED15.

Separate cluster analyses were executed using combinations of a single mtDNA plus a single nuDNA digest from the RFLPs, with all four primers from the RAPD analysis. The dendrograms generally produced groupings of the isolates by VCGs and separated the pathogenic and nonpathogenic isolates (Fig. 4 and data not shown). A representative dendrogram using *Hin*dIII, *Msp*I, and the four primers (Fig. 4) produced isolate groups with high similarity coefficients (sc) that corresponded to the VCGs: Fop 7, Fop 8 (sc = 0.94), plus Fop 28 (sc = 0.85) of VCG 0164; Fo 16 and Fo 19 (sc = 0.88) of VCG 2163; Fop 31 and Fop 32 (sc =

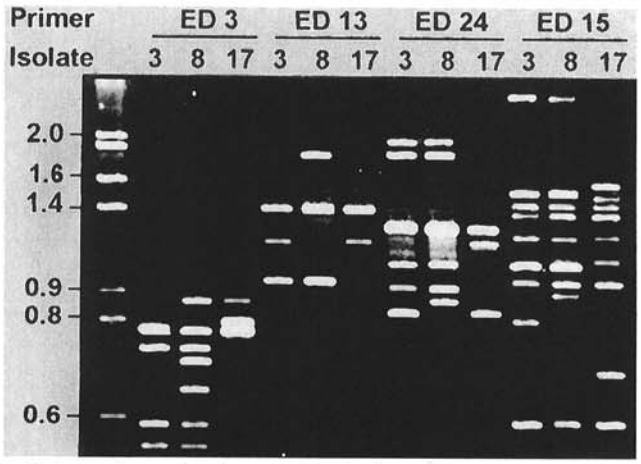

Fig. 2. Comparison of random amplified polymorphic DNA patterns for two *Fusarium oxysporum* f. sp. *phaseoli* (Fop) isolates, Fop 3 (race 2, VCG 0162) and Fop 8 (race 3, VCG 0164), and one nonpathogenic *F. oxysporum* (Fo) isolate, Fo 17 (VCG 2164), amplified with the primers used in this study: ED3 = 5′-ATGGCCACTTCTTGG-3′; ED13 = 5′-ATGGCCACCTCGTGG-3′; ED24 = 5′-ATGGCAACTTCGTGG-3′; and ED15 = 5′-ATGGCAACCTCGTGG-3′. DNA electrophoresis was conducted in a 2% agarose gel that was stained with ethidium bromide. Lane 1 is a molecular marker of λ-DNA digested with *Eco*RI and *Hin*dIII; fragment sizes in kilobase pairs are indicated on the left.

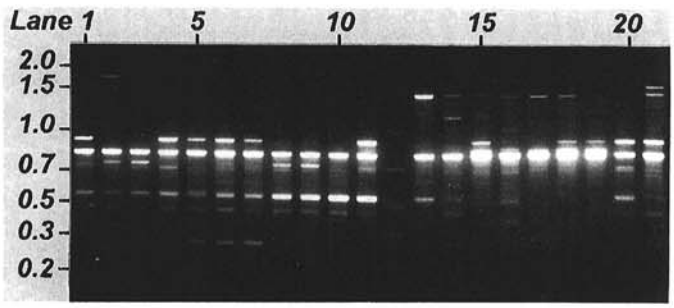

Fig. 3. Random amplified polymorphic DNA patterns of genomic DNA amplified with primer ED3 (5′-ATGGCCACTTCTTGG-3′) from 11 *Fusarium oxysporum* f. sp. *phaseoli* (Fop) and nine nonpathogenic *F. oxysporum* (Fo) isolates in a 2% agarose gel stained with ethidium bromide. Numbers on the left indicate fragment sizes in kilobase pairs. Lane 12 is a molecular marker of 50- to 2,000-bp λ-DNA ladder (Bio-Rad Laboratories). Isolates are the following: lane 1, Fop 1 (race 1, VCG 0161); lane 2, Fop 2 (race 2, VCG 0162); lane 3, Fop 3 (race 2, VCG 0162); lane 4, Fop 6 (race 3, VCG 0163); lane 5, Fop 7 (race 3, VCG 0164); lane 6, Fop 8 (race 3, VCG 0164); lane 7, Fop 28 (race 3, VCG 0164); lane 8, Fop 11 (race 1, VCG 0165); lane 9, Fop 31 (race 5, VCG 0165); lane 10, Fop 32 (race 5, VCG 0165); lane 11, Fop 30 (race 4, VCG 016–); lane 13, Fo 10 (VCG 2161); lane 14, Fo 13 (VCG 2162); lane 15, Fo 15 (VCG 2162); lane 16, Fo 16 (VCG 2163); lane 17, Fo 19 (VCG 2163); lane 18, Fo 25 (VCG 2163); lane 19, Fo 17 (VCG 2164); lane 20, Fo 4 (VCG 216–); and lane 21, Fo 9 (VCG 216–).

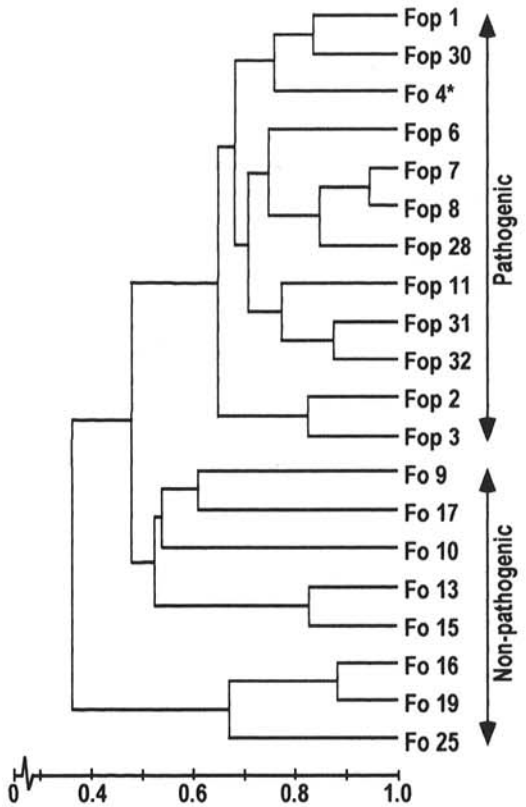

Fig. 4. Unweighted paired group method with arithmetic dendrogram showing relationships among the 11 *Fusarium oxysporum* f. sp. *phaseoli* (Fop) and nine nonpathogenic *F. oxysporum* (Fo) isolates derived from data of a *Hin*dIII and a *Msp*I digest plus the four random amplified polymorphic DNA (RAPD) primers. The scale on the bottom indicates the simple matching similarity coefficients obtained from the combined restriction fragment length polymorphism and RAPD data in the cluster analysis. Isolate Fo 4* is nonpathogenic. Isolates are coded as indicated in Table 1.

0.87), plus Fop 11 (sc = 0.77) of VCG 0165; Fop 2 and Fop 3 (sc = 0.83) of VCG 0162; and Fo 13 and Fo 15 (sc = 0.83) of VCG 2162. Although pathogenically diverse, Fop 1 and Fop 30 were similar (sc = 0.84) and grouped with Fo 4 (sc = 0.76). Fop 6 of VCG 0163 clustered at a greater distance to the other race 3 isolates (sc = 0.75). Fo 25 was not highly similar to the other members of VCG 2163 (sc = 0.67). However, isolates Fo 16, Fo 19, and Fo 25 formed a group that was clearly distinct from all other isolates.

DISCUSSION

The five physiological races of *F. oxysporum* f. sp. *phaseoli* comprised isolates originating from five different countries of three continents (Table 1). Although there was a correspondence between the race and the place of origin, it must be noted that the small number of isolates used in this study placed constraints on the ability to interpret the data. Inferences to the relationship among genetic similarities, pathotype specialization, and geographic location must be considered as hypothetical, and further investigations are required before conclusions may be drawn about this forma specialis.

Races 4 and 5 are new reports for *F. oxysporum* f. sp. *phaseoli*. Ribeiro and Hagedorn (46) found that *F. oxysporum* f. sp. *phaseoli* resistance in *Phaseolus* was race-specific and controlled by two single genes, one to a race from the United States (Fop 1, ATCC 18131) and the other to a race from Brazil (Fop 2, ATCC 42145/2107-A). Resistance was completely dominant with Fop 2 and incompletely dominant with Fop 1, with two alleles conferring resistance and susceptibility. More work is required with the cultivars and *F. oxysporum* f. sp. *phaseoli* races of the present study to determine if resistance to the new pathotypes is invoked by additional genes, loci, or conferred by alleles to the existing *F. oxysporum* f. sp. *phaseoli* loci. It is not surprising that more than one race may exist in the United States (race 1 and race 4), considering the large area and the occurrence of geographic barriers that would permit diverse evolution, as well as maintain the races as physically and genetically isolated. It is possible that the race 1 isolate from Italy (Fop 11) was introduced from the United States (or vice versa) as a contaminant of seed or plant material, thus explaining the distribution of this race across the Atlantic.

Ten *P. vulgaris* cultivars, all originating from South America, showed differential responses to the five *F. oxysporum* f. sp. *phaseoli* races. Among the 20 putative pathogenic isolates tested, nine were nonpathogenic on the cultivars. Three of these isolates were obtained from other *Phaseolus* species, Fo 9 from *P. angularis* and Fo 13 and Fo 15 from *P. coccineus*, and were possibly nonpathogenic to the *P. vulgaris* cultivars used (45). In the present work, Fop 1 and Fop 30 were ascribed to different pathogenic races, whereas in previous research using the same cultivars, it had been determined that Fop 30 and Fop 1 were similar in their pathogenic responses (47).

Seventeen isolates comprised nine VCGs, and three isolates were heterokaryon self-incompatible. It is conceivable that the genetic origins of isolates from the same VCG are similar, since alleles at each vegetative incompatibility loci need to be the same for heterokaryon complementation to occur (7,22,30,31,43). Unlike other studies in which a single VCG corresponded specifically to a forma specialis, suggesting genetic similarity, the occurrence of different VCGs in *F. oxysporum* f. sp. *phaseoli* indicates that there is more genetic variation within this forma specialis (26,43). In the present study, there were no common VCGs between pathogenic and nonpathogenic isolates, in agreement with the findings of Jacobson and Gordon for *F. oxysporum* f. sp. *melonis* (22). Furthermore, a direct one-to-one relationship between race and VCG was not observed in *F. oxysporum* f. sp. *phaseoli*, since the pathogenic isolates consisted of four races that contained one or two VCGs and one VCG (VCG 0165) that contained two races. In comparison, recent findings on *F. oxysporum* f. sp. *dianthi* showed that the races were always restricted to distinct VCGs, with the exception of one VCG that was comprised of two races (50).

The genetic diversity among the 20 isolates was further supported by the variable banding patterns observed in the RFLP and RAPD analyses (Table 3). Pathogenic and nonpathogenic isolates rarely shared the same DNA patterns (Fo 4 was an exception). A similar relationship was noted among the pathogenic isolates for banding patterns obtained from both the RFLP and RAPD analyses. Moreover, the correspondence between RFLP and RAPD markers was not as apparent among the nonpathogenic isolates such as Fo 13 and Fo 15 (Table 3). Fo 16 and Fo 19 were similar, but differed from Fo 25 for RFLPs; however, Fo 19 and Fo 25 were similar, but differed from Fo 16 for the RAPD analysis. Possibly, there were problems in the interpretation of Fo 25 RFLPs, since dsRNA bands were not readily distinguishable from DNA bands; but with RAPD analysis this may not have been problematic, since the dsRNA did not amplify (data not shown). Other researchers have also reported that a relationship observable between the VCGs, or races, and the RFLPs was not always apparent in the RAPD analysis (28).

Where there was a good correspondence of the RFLP and RAPD banding patterns to the VCGs (Table 3 and Fig. 4), we concluded that these isolates were genetically similar. Several researchers have noted a strong correlation between the restriction patterns of mtDNA and VCGs in *F. oxysporum* f. sp. *melonis* and indicated that, to be vegetatively compatible, the isolates must have a high degree of genetic homology (16,20,21). Similar results have been reported in *F. oxysporum* f. sp. *gladioli*, *F. oxysporum* f. sp. *lycopersici*, and *F. oxysporum* f. sp. *dianthi* (14,33,36). As reported by Guthrie et al. (19), the use of combined data in the present study (RFLPs and RAPD analysis) was most useful in determining the relationship of the molecular markers to the VCGs. However, to avoid an oversampling bias of RFLPs at the same locus (8), only one restriction digest was selected for the mtDNA, and one restriction digest was selected for the nuDNA at the time of the cluster analysis.

Other studies on *F. oxysporum* from carnation and muskmelon observed that there were no similarities between pathogenic and nonpathogenic isolates when analyzed by RAPD markers or RFLPs (17,35), which was generally in accordance to our results. The exception was nonpathogenic isolate Fo 4, since it had both RFLPs and RAPD patterns similar to pathogenic isolates (Figs. 1, 3, and 4). This isolate was obtained from the Czechoslovakian Collection of Microorganisms as a *F. oxysporum* f. sp. *phaseoli* from an undesignated geographical origin, but it had always proven to be nonpathogenic (as well as self-incompatible) upon repeated testing throughout our study. It may be hypothesized that Fo 4 has common origins with Fop 1 and Fop 30, since the banding patterns were similar to both American isolates. Vegetative compatibility testing could not be performed among these isolates, since both Fo 4 and Fop 30 were heterokaryon self-incompatible. However, this and other studies have found that molecular characters such as RFLPs and RAPD markers are useful in characterizing nonpathogenic or self-incompatible isolates that could not be characterized by other methods or in determining relationships among isolates coming from unknown sources (15,21).

Our findings indicate that there was not a clear relationship between the results of the molecular analyses and the pathogenicity tests. This is consistent with the conclusions of others who have found that races are not monophyletic and that isolates of the same race are not necessarily closely related (6,21,40). Fop 30 often had banding patterns similar to Fop 1, indicating that these isolates may be genetically similar although pathogenically diverse. In a previous study, these isolates were pathogenically similar (47), thus suggesting that the change in pathogenicity may have occurred recently as a result of storage conditions on artifi-

cial substrate (42). In addition, Fop 11 did not have RFLPs or RAPD patterns similar to Fop 1, although they belong to the same race. Since these two isolates were not vegetatively compatible, they probably have diverse genetic origins and their pathogenic similarity may have developed independently (31). Correll (9) proposed several models to explain VCG diversity and the relationship to pathogenic races within *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. The relationship of Fop 1 and Fop 11 may fit with model II, which proposes that the isolates from a polyphyletic origin and diverse VCGs mutated to become virulent members of the same race. Although Aloj et al. (3) found that Fop 1 and Fop 11 were different races, our experiments indicated that these two isolates always belonged to the same race. In other studies, variable disease responses have been observed depending upon the growing conditions, age of the host plants, and method of inoculation (11,42). This variability highlights some of the major constraints in performing greenhouse assays—constraints that molecular techniques should overcome.

Model III of Correll (9) proposes that the VCG origin of different virulent races within a forma specialis may be monophyletic, as indicated by similar RFLP patterns observed among different races of *F. oxysporum* f. sp. *melonis* (22) and *F. oxysporum* f. sp. *lycopersici* (14,36). In the present study, Fop 11, Fop 31, and Fop 32 have the same VCG (0165) and similar DNA patterns, but belong to different races. Possibly, these isolates were clones or originated from a common parental origin, although they are geographically separated and pathogenically diverse at present (31). However, Italy (Fop 11) and Greece (Fop 31 and Fop 32) are geographically and economically close (i.e., movement of agricultural goods), and this may lower the isolation barrier, thus providing an opportunity for distribution of common genetic material (isolates of the same VCG) and subsequent evolution of different pathogenic races.

Initial results from this study suggest that the VCG-race relationship may be complex within *F. oxysporum* f. sp. *phaseoli*, since there are numerous VCGs and races and no apparent direct correspondence was found between the characters. Similar complexity has been observed within *F. oxysporum* f. sp. *melonis* (20,21,22,23), *F. oxysporum* f. sp. *lycopersici* (13,14), *F. oxysporum* f. sp. *dianthi* (1,32), and *F. oxysporum* f. sp. *cubense* (40). In contrast, a direct VCG-race relationship has been observed in other formae speciales such as *F. oxysporum* f. sp. *elaeidis*, F. oxysporum f. sp. *niveum*, *F. oxysporum* f. sp. *conglutinans*, and *F. oxysporum* f. sp. *apii*, in which one VCG corresponded to one race (7,11,15,25,29).

Using methods to determine pathogenic races, VCGs, RFLPs, and RAPD markers, we were able to distinguish and characterize different *F. oxysporum* f. sp. *phaseoli* isolates. The molecular markers corresponded to VCGs, but not to races; therefore, they cannot be used to determine the pathogenic types. It was also possible to characterize isolates that were nonpathogenic, self-incompatible, or both by use of molecular techniques, which was not possible by use of traditional methods from greenhouse and lab culture studies, to aid in the investigation of the complex relationships among the isolates. The RFLP and RAPD analyses suggest that there may be genetic diversity between isolates of the forma specialis and isolates of the nonpathogenic group. However, further investigations are required to determine if the polymorphisms that differentiate the pathogenic from the nonpathogenic isolates hold true with the addition of more isolates.

Our search of the world fungal collections has revealed that there were few isolates of *F. oxysporum* f. sp. *phaseoli* available for the present work. Although the use of a small number of isolates limited many of our conclusions, the results obtained using isolates from eight countries represent a significant contribution to the study of this forma specialis. Future studies should include a larger sample size of isolates from the present sources, as well as from additional diverse geographic locations to better understand the biodiversity within this forma specialis.

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