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Use of random amplified polymorphic DNA (RAPD) to identify races 1, 2, 4 and 8 of *Fusarium oxysporum* f. sp. *dianthi* in Italy

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Abstract

The RAPD fingerprinting procedure was used in combination with pathogenicity assays on differential cultivars to characterize a representative collection of 72 *Fusarium* spp. isolates of different geographic origin collected from diseased carnation. In *F. oxysporum* f. sp.*dianthi*, isolates were grouped according to the physiologic race: group 1 included isolates of race 4; group 2 was formed by isolates of race 2 and single representatives of races 5 and 6; group 3 included isolates of races 1 and 8. No correlation was found between RAPD data and geographic origin of the isolates tested: representatives of race 2 isolated in Italy, Israel and Japan had the same amplification profile. Three isolates which showed a low level of pathogenicity on all carnation cultivars tested shared an identical amplification pattern and are probably saprophytic *F. oxysporum*. Finally, two *F. redolens* isolates from Japan and seven non-pathogenic isolates of *F. proliferatum* collected from diseased carnation in Italy, Israel and The Netherlands were clearly distinguishable according to their RAPD fingerprint. The results are discussed in relation to previous studies on the genetic diversity of *F. oxysporum* f. sp. *dianthi* and to the development of *forma specialis*- and pathotype-specific diagnostic tools.

Introduction

Fusarium oxysporum Schlechtend.:Fr. f. sp. *dianthi* (Prill. *et* Delacr.) W.C. Snyder et H.N. Hans., incitant of vascular wilt on carnation (*Dianthus caryophyllus* L.), is the most important pathogen on this crop, causing severe losses in all growing areas (Baayen and Kleijn, 1989; Garibaldi and Gullino, 1987; Tramier et al., 1983). Physical and chemical disinfestation of soil and the use of systemic fungicides are not always suitable for disease control due to their high cost and sometimes limited effectiveness. To the authors' knowledge, Fusarium wilt resistance genes have not been cloned yet in carnation, but many resistant commercial cultivars are available and their use still provides the most effective solution to this problem. Indexing has been routinely adopted for obtaining pathogen-free propagative material (Garibaldi, 1983; Garibaldi and Gullino, 1987).

Eight physiological races or pathotypes of *F. oxysporum* f. sp. *dianthi* (*Fod*) have been reported in Italy on the basis of differences in virulence to differential cultivars of carnation (Garibaldi, 1975, 1981; Garibaldi et al., 1986b). Race 2, which probably originated from Europe, spread to most carnation growing countries through propagative material and is now found worldwide (Aloi and Baayen, 1993; Baayen et al., 1997). Races 1 and 8 apparently originated from the Italian Riviera, where they are specifically associated to the Mediterranean ecotypes (Garibaldi et al., 1986b). Only a few reports have documented the presence of these two pathotypes outside Italy, mainly in the French Riviera and in Spain (Aloi and Baayen, 1993; Baayen et al., 1997). Race 4 is associated with American carnation cultivars and has been reported in the United States (Aloi and Baayen, 1993; Baayen et al., 1997), Italy (Garibaldi 1983; Garibaldi et al., 1986b), Israel (Ben-Yephet et al., 1992), Spain and Colombia (Cevallos et al., 1990; Aloi and Baayen, 1993; Baayen et

al., 1997). Races 5, 6 and 7 have been reported from Great Britain, France and The Netherlands by Garibaldi (1983). However, only a few representatives of these pathotypes are currently available and long term storage could have modified their aggressiveness. Race 3 of *Fod* is presently classified *F. redolens* f. sp. *dianthi* race 3 (Baayen et al., 1997). Recently, the new races 9, 10 and 11 have been defined for classification of *Fod* in Australia (race 9; Kalc Wright et al., 1996) and the Netherlands (races 10 and 11; Baayen et al., 1997).

From a practical point of view, knowledge of the distribution of pathotypes is a basic prerequisite for the choice of suitable resistant varieties in specific growing areas (Garibaldi and Gullino, 1987, 1988). Distinction between saprophytic *Fusarium* spp. and *Fod* and among different races of *Fod* is based on pathogenicity tests (Garibaldi, 1975, 1981, 1983), which are time consuming (40-50 days) and require extensive facilities. Moreover, susceptibility of differential cultivars can be quantitatively influenced by many environmental and technical variables as differences in the defense reaction appear to be largely quantitative (Baayen, 1988; Baayen et al., 1988; Ben-Yephet et al., 1996; Demmink et al., 1987).

Vegetative compatibility tests (Puhalla, 1985; Leslie, 1993) have been used to identify races in *Fod* (Baayen et al., 1988; Katan et al., 1989), and the six known physiological races detected among Italian isolates (Garibaldi, 1981) were grouped into three vegetative compatibility groups (VCG): VCG 0020 coincides with race 4; VCG 0021 includes isolates of races 2, 5, 6 and 7; VCG 0022 comprises isolates of races 1 and 8. Moreover, VCGs 0025, 0027 and 0028 coincide with races 11, 10 and 9, respectively (Aloi and Baayen, 1993; Baayen et al., 1997). Data on DNA restriction fragment length polymorphisms (Manicom et al., 1990; Manicom and Baayen, 1993; Baayen et al., 1997), karyotype analysis by contour-clamped homogeneous electric field gel electrophoresis (Migheli et al., 1993, 1995), esterase profiles (Baayen et al., 1997) and sequence analysis of the ribosomal ITS1 and ITS2 regions (Waalwijk et al., 1996) have led to a similar classification.

Random amplification of polymorphic DNA by the use of arbitrarily chosen primers (Welsh and McClelland, 1990; Williams et al., 1990) has proven very effective for race differentiation in *F. oxysporum* formae speciales *ciceri* (Kelly et al., 1994), *cubense* (Bentley et al., 1994), *dianthi* (Manulis et al., 1994; Kalc Wright et al., 1996) *gladioli* (Mes et al., 1994), *phaseoli* (Woo et al., 1996), *pisi* (Grajal-Martìn et al., 1993) and *vasinfectum* (Assigbetse et al., 1994). In *Fod*, amplification patterns obtained with 17 primers of 10 bases enabled differentiation between 42 pathogenic isolates of race 2, one of race 4 and 15 nonpathogenic isolates collected in Israel (Manulis et al., 1994). A total of 34 pathogenic *Fod* and nonpathogenic *F. oxysporum* isolates collected from symptomless carnation cuttings in Australia were classified into three groups (nonpathogenic isolates, isolates of race 2 and isolates of the new race 9) on the basis of RAPD analysis with a single 10-mer primer (Kalc Wright et al., 1996).

Aim of this work was to test the hypothesis that different physiological races of *Fod* could be identified using RAPD analysis. We report here the use of this technique, coupled to pathogenicity assays on differential cultivars, to identify *Fod* races 1, 2 , 4 and 8 (VCGs 0020, 0021 and 0022) within a collection of 72 *Fusarium* spp. isolates of different geographic origin, collected from diseased carnation.

Materials and methods

Fungal isolates

A collection of 72 *Fusarium* spp. isolates, detected on diseased carnation in Italy, Israel, The Netherlands, Japan and France, was maintained on potato dextrose agar (PDA, Merck, Darmstadt, Germany) and carnation leaf agar (Fisher et al., 1982). Table 1 presents an enumeration of the isolates studied and their origin and identity, based on previous studies (Garibaldi, 1983; Garibaldi and Gullino, 1988; Aloi and Baayen, 1993; Baayen et al., 1997) as well as the results of RAPD and pathogenicity tests performed in the present work. Nonpathogenic *F. oxysporum*, *F. redolens* and *F. proliferatum* isolates were identified at the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands.

Pathogenicity tests

Pathogenicity assays were performed using the differential carnation cvs Indios, Sorriso, Manon, Faust, Selima and Nicol. All trials were carried out in a glasshouse (25–30 °C; relative humidity 50–90%; daily light conditions: 50–60 KLux per m^2) located at Albenga, a typical carnation growing area in the Liguria Region (Flower Riviera, north-western Italy).

Each isolate was grown in 250 ml Erlenmeyer flasks containing 100 ml of PDB (24 g l^{-1}) and yeast extract (5 g l^{-1}) with shaking (150 rpm) at 26 °C under con-

Table 1. *Fusarium* spp. isolates from diseased carnation, codes, geographic origin, and race determination based on RAPD analysis and pathogenicity on differential carnation cultivars. Previously published race determinations according to pathogenicity assay (a, b, c, d), vegetative compatibility test (c, d), analysis of esterase profiles (d), analysis of the ITS region of the ribosomal DNA (d) or analysis of restriction fragment length polymorphisms of total DNA (d) are listed on the right column

Species and f. sp.	Race	RAPD group	Isolate	Origin	Reaction on *Dianthus caryophyllus*[1]						References[2]
					Sorriso	Indios	Manon	Faust	Selima	Nicol	
Fod[3]	4	1	310	Italy	4.0 b	4.0 b	4.0 b	4.0 b	0.0 a	4.0 b	a,b,c,d
	4	1	28	Italy	4.0 b	4.0 b	4.0 b	4.0 b	0.0 a	4.0 b	
	4	1	209	Italy	4.0 b	4.0 b	4.0 b	4.0 b	0.0 a	4.0 b	
	4	1	245	Italy	4.0 b	4.0 b	4.0 b	4.0 b	0.0 a	4.0 b	
	4	1	327	Italy	4.0 b	4.0 b	4.0 b	4.0 b	0.0 a	4.0 b	
	4	1	435	Italy	1.6 b	4.0 c	3.4 c	4.0 c	0.0 a	4.0 b	
	4	1	468	Italy	4.0 b	4.0 b	4.0 b	4.0 b	0.0 a	4.0 b	
	4	1	493	Italy	4.0 b	4.0 b	4.0 b	4.0 b	0.0 a	4.0 b	
	4	1	510	Italy	4.0 b	4.0 b	4.0 b	4.0 b	0.0 a	4.0 b	
	4	1	523	Italy	3.6 b	4.0 b	4.0 b	4.0 b	0.0 a	4.0 b	b
	4	1	578	Italy	4.0 b	4.0 b	4.0 b	4.0 b	0.0 a	4.0 b	b,c,d
	4	1	606	Italy	3.8 b	4.0 b	3.7 b	4.0 b	0.0 a	4.0 b	
	4	1	738	Italy	2.9 b	4.0 d	3.4 c	4.0 d	0.0 a	4.0 d	
	4	1	752	Italy	2.0 b	4.0 c	3.5 d	4.0 d	0.0 a	4.0 d	
	4	1	757	Italy	2.5 b	4.0 c	4.0 c	4.0 c	0.0 a	4.0 c	
	4	1	758	Italy	1.6 b	4.0 c	4.0 c	4.0 c	0.0 a	4.0 c	
	4	1	775	Italy	4.0 b	4.0 b	4.0 b	4.0 b	0.0 a	4.0 b	
	4	1	814	Italy	4.0 b	4.0 b	4.0 b	4.0 b	0.0 a	4.0 b	
	4	1	828	Italy	4.0 b	4.0 b	4.0 b	4.0 b	0.0 a	4.0 b	b,c,d
	2	2	75	Italy	3.8 b	4.0 b	4.0 b	4.0 b	4.0 b	0.0 a	a,b,c,d
	2	2	218	Italy	4.0 b	4.0 b	4.0 b	4.0 b	4.0 b	0.0 a	
	2	2	451	Italy	4.0 b	4.0 b	4.0 b	4.0 b	4.0 b	0.0 a	b
	2	2	593	Italy	4.0 b	4.0 b	4.0 b	4.0 b	4.0 b	0.0 a	b
	2	2	598	Italy	4.0 b	4.0 b	4.0 b	4.0 b	4.0 b	0.0 a	b
	2	2	1024	Italy	3.4 b	4.0 c	4.0 c	4.0 c	4.0 c	0.0 a	
	2	2	1027	Italy	4.0 c	3.7 b	4.0 c	4.0 c	4.0 c	0.0 a	
	2	2	1041	Italy	3.7 b	4.0 c	4.0 c	4.0 c	4.0 c	0.0 a	
	2	2	1121	Italy	4.0 b	4.0 b	4.0 b	4.0 b	4.0 b	0.0 a	
	2	2	1171	Israel	4.0 b	4.0 b	4.0 b	4.0 b	4.0 b	0.0 a	
	2	2	1172	Israel	4.0 b	4.0 b	4.0 b	4.0 b	4.0 b	0.0 a	
	2	2	1178	Israel	4.0 b	4.0 b	4.0 b	4.0 b	4.0 b	0.0 a	
	2	2	1198	Japan	3.0 b	3.9 c	4.0 c	3.6 c	4.0 c	0.0 a	
	2	2	1227	Israel	4.0 b	4.0 b	4.0 b	4.0 b	4.0 b	0.0 a	
	5	2	165	France	0.8 a	4.0 b	4.0 b	4.0 b	N.T.	N.T.	a,c,d
	6	2	256	The Netherlands	2.5 a	4.0 b	4.0 b	4.0 b	N.T.	N.T.	a,c,d
	1	3	1	Italy	0.0 a	4.0 b	0.0 a	0.0 a	N.T.	N.T.	a,b,c,d
	1	3	572	Italy	0.0 a	4.0 b	1.6 b	0.0 a	N.T.	N.T.	
	1	3	625	Italy	0.0 a	4.0 b	0.4 a	1.0 a	N.T.	N.T.	
	1	3	676	Italy	0.0 a	4.0 b	0.0 a	0.0 a	N.T.	N.T.	
	1	3	707	Italy	0.4 a	4.0 b	1.2 a	1.0 a	N.T.	N.T.	
	1	3	708	Italy	0.2 a	4.0 b	0.0 a	0.4 a	N.T.	N.T.	
	1	3	718	Italy	0.0 a	4.0 b	0.0 a	0.0 a	N.T.	N.T.	
	1	3	732	Italy	0.8 a	4.0 b	0.0 a	0.0 a	N.T.	N.T.	
	1	3	746	Italy	0.4 a	4.0 b	0.0 a	0.0 a	N.T.	N.T.	b
	1	3	774	Italy	0.4 a	4.0 b	0.0 a	0.7 a	N.T.	N.T.	
	1	3	805	Italy	0.0 a	4.0 b	0.0 a	0.0 a	N.T.	N.T.	b
	1	3	964	Italy	0.0 a	4.0 b	0.0 a	0.0 a	N.T.	N.T.	

Table 1. Continued

Species and f. sp.	Race	RAPD group	Isolate	Origin	Reaction on *Dianthus caryophyllus*[1]						References[2]
					Sorriso	Indios	Manon	Faust	Selima	Nicol	
	1	3	1031	Italy	0.0 a	4.0 b	0.0 a	0.0 a	N.T.	N.T.	
	1	3	1180	Italy	0.0 a	4.0 b	0.0 a	0.0 a	N.T.	N.T.	
	8	3	276	Italy	4.0 b	4.0 b	0.0 a	4.0 b	N.T.	N.T.	a,d
	8	3	325	Italy	3.8 b	4.0 b	0.0 a	4.0 b	0.0 a	0.0 a	
	8	3	617	Italy	4.0 b	4.0 b	0.0 a	4.0 b	N.T.	N.T.	
	8	3	640	Italy	4.0 b	4.0 b	0.0 a	4.0 b	N.T.	N.T.	b
	8	3	684	Italy	4.0 b	4.0 b	0.0 a	4.0 b	N.T.	N.T.	
	8	3	788	Italy	4.0 b	4.0 b	0.0 a	4.0 b	N.T.	N.T.	
	8	3	821	Italy	4.0 b	4.0 b	1.2 a	4.0 b	N.T.	N.T.	
	8	3	834	Italy	4.0 b	4.0 b	0.0 a	4.0 b	N.T.	N.T.	b,c
	8	3	882	Italy	3.6 c	3.1 bc	0.1 a	2.8 b	N.T.	N.T.	
	8	3	895	Italy	4.0 b	4.0 b	0.0 a	4.0 b	N.T.	N.T.	
	8	3	902	Italy	2.8 b	2.9 b	0.4 a	3.5 b	N.T.	N.T.	
Fo[4]	–	4	264	Italy	0.0 a	0.0 a	0.0 a	0.6 a	0.0 a	0.0 a	
	–	4	789	Italy	0.0 a	0.4 a	0.8 a	0.2 a	N.T.	N.T.	
	–	4	812	Italy	0.0 a	0.0 a	0.2 a	0.0 a	N.T.	N.T.	
Fr[5]	N.T.[7]	5	1200	Japan	1.3 ab	0.5 a	0.4 a	2.1 b	0.0 a	0.0 a	
	N.T.	5	1202	Japan	3.0 c	2.2 b	0.0 a	0.4 a	0.0 a	0.0 a	
Fp[6]	–	6	316	Italy	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	
	–	6	1035	Italy	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	
	–	6	1215	Israel	0.8 a	0.0 a	0.1 a	0.0 a	0.0 a	0.0 a	
	–	6	1223	The Netherlands	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	
	–	6	1225	The Netherlands	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	
	–	6	1226	The Netherlands	0.0 a	0.0 a	1.0 b	0.3 a	0.0 a	0.0 a	
	–	6	1228	The Netherlands	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	

[1] Disease index ranged from 0 to 4 (0 = healthy plant; 1 = initial symptoms of leaf chlorosis and internal browning of the lower vessels; 2 = initial symptoms of wilting and internal browning of the upper vessels; 3 = severe symptoms of wilting and initial symptoms of leaf necrosis; 4 = plant totally wilted, leaves completely necrotic). Values in the same row followed by the same letter do not differ significantly according to the Student-Newman-Keuls test ($P < 0.05$); [2] a: Garibaldi, 1983; b: Garibaldi and Gullino, 1988; c: Aloi and Baayen, 1993; d: Baayen et al., 1997; [3] *Fod*: *F. oxysporum* f. sp. *dianthi*;[4] *Fo*: *F. oxysporum*; [5] *Fr*: *F. redolens*; [6] *Fp*: *F. proliferatum*; [7] N.T.: not tested.

stant light. After 7 d, fungal cultures were aseptically filtered through 4 layers cheese cloth and conidia were brought to a final cell density of 10^5 CFU ml^{-1}. The inoculum was applied to the plant roots by dipping rooted cuttings in the conidial suspensions at transplanting. A set of new differential cultivars was determined in this work as old differentials described by Garibaldi (1981, 1983, Garibaldi and Gullino, 1988) were no longer available among Italian growers. Thus, cvs Indios, Sorriso, Faust and Manon were inoculated with all isolates; cvs. Selima and Nicol only with isolates which had been attributed to races 2 and 4 in previous experiments (Garibaldi, 1981, 1983; Garibaldi and Gullino, 1988; Aloi and Baayen, 1993; Manicom and Baayen, 1993; Baayen et al., 1997) or after RAPD analysis. The differential response of the cultivars used in the pathogenicity tests to races 1, 2, 4, 5, 6 and 8 is given in Table 2. Differentials for races 5 and 6 were no longer available and could not be included. A mock treatment was added by dipping rooted cuttings in sterile distilled H_2O at transplanting. Plastic pots (15 l vol.) were filled with a steam-disinfected potting mixture (pH 5.5) containing two parts soil (pH 6.9, P 352, K 1700, Ca 1500, mg 415, Zn 29, Mn 8.7, Fe 130 mg ml^{-1}) and one part peat moss (v/v). Ten plants of each cultivar per isolate were transplanted in each pot. The pots were watered daily and a N (total nitrogen 20%; 7% NH_4^+, 13% NO_3^-): K_2O (10%): P_2O_5 (10%) liquid fertilizer was distributed in the irrigation water at a concentration of 0.08%.

Disease incidence was determined at weekly intervals by counting wilted plants. At the end of the experiment (approximately six weeks after transplanting) each plant was uprooted and cut lengthwise to evaluate

Table 2. Summarized response[a] of six carnation cultivars tested to races 1, 2, 4, 5, 6 and 8 of *Fusarium oxysporum* f. sp. *dianthi*

Cultivar	Race 1	Race 2	Race 4	Race 5	Race 6	Race 8
Sorriso	R	S	S	I	I	S
Indios	S	S	S	S	S	S
Manon	R	S	S	S	S	R
Faust	R	S	S	S	S	S
Selima	N.T.	S	R	N.T.	N.T.	R
Nicol	N.T.	R	S	N.T.	N.T.	R

[a] Abbreviations: R, highly resistant; I, moderately resistant; S, susceptible; N.T., not tested.

the presence of mild symptoms of Fusarium wilt. The disease index used throughout the experiments ranged from 0 to 4 (0 = healthy plant; 1 = initial symptoms of leaf chlorosis and internal browning of the lower vessels; 2 = initial symptoms of wilting and internal browning of the upper vessels; 3 = severe symptoms of wilting and initial symptoms of leaf necrosis; 4 = plant totally wilted, leaves completely necrotic). The experiments were repeated twice. An analysis of variance was performed on the disease indices and the arcsin-transformed mean values of ten plants for each cultivar/isolate combination were examined using the Student-Newman-Keuls test, using the computer program package SPSS-Win (Norusis, 1992).

Genomic DNA isolation

Fusarium spp. genomic DNA was purified from lyophilized mycelium by a miniprep method described previously (Migheli et al., 1996). Briefly, 50 mg of ground mycelium was suspended in 1 ml of 50 mm EDTA, 0.2% SDS (pH 8.5), 100 μg proteinase K, and incubated 15 min at 70 °C. After incubation, 100 μl of 5.0 m potassium acetate was added and the mixture kept in an ice bath for 30 min. After centrifuging at 3000 x *g* for 15 min, the supernatant was extracted at least three times with equal volumes of phenol-chloroform and once with chloroform. Nucleic acids were then precipitated with isopropanol, rinsed with ethanol, adjusted to a final concentration of 100 ng per microliter in TE, pH 8.0 (Maniatis et al., 1982) and stored at 4 °C.

PCR conditions

PCR was carried out in 15 μl of reaction mix containing 10 mm Tris-HCl, pH 9.0, 1.5 mm $MgCl_2$, 50 mm KCl, 0.1% Triton X-100, 0.01% (w/v) gelatin, 60 μm each of dATP, dCTP, dGTP, and dTTP, 5 pM of primer, about 0.2 ng of template DNA, and 0.75 U of Super-Taq DNA polymerase (Stehelin and Cie AG, Basel, Switzerland; now available from HT Biotechnology, Cambridge, UK). Ten 10-mer oligonucleotides (Operon Technologies Inc., Alameda, CA, USA; Table 3) were tested as primer sequences. Reagents (without DNA) were combined into a premixture and aliquots were pipetted into 0.5-ml Eppendorf tubes before the addition of template DNA to minimize the risk of cross-contamination (Kwok and Higuchi, 1989).

Table 3. Code and sequence of the 10 primers which were selected as the most informative markers in the identification of *Fusarium oxysporum* f. sp. *dianthi* isolates, with total number of polymorphic DNA amplimers obtained with each primer in random amplified polymorphic DNA (RAPD) experiments

Code	Sequence (5′ to 3′)	Polymorphic amplimers	Size range of amplimers (kb)
OPB-05	TGCGCCCTTC	6	0.6–2.0
OPB-06	TGCTCTGCCC	7	1.0–3.0
OPB-15	GGAGGGTGTT	6	0.4–1.8
OPB-16	TTTGCCCGGA	12	0.4–3.0
OPE-05	TCAGGGAGGT	6	1.3–2.1
OPE-08	TCACCACGGT	6	0.8–2.2
OPE-09	CTTCACCCGA	3	0.8–2.5
OPE-11	GAGTCTCAGG	13	0.5–3.0
OPE-13	CCCGATTCGG	5	0.6–4.0
OPE-16	GGTGACTGTG	8	0.3–2.8

Amplification was performed in a Perkin-Elmer (Norwalk, CT) Cetus Gene Amp PCR System 9600 programmed for one cycle of 2.5 min at 94 °C, 45 cycles of 30 s at 94 °C, 1 min at 36 °C, 2 min at 72 °C, with no ramping, followed by one cycle of 5 min at 72 °C. Amplification was repeated at least 2 times for each sample.

Analysis of RAPD products

Half of the reaction product was loaded in 1% SeaKem LE agarose (FMC BioProducts, Rockland, ME, USA) gels containing 0.5 μg ml^{-1} ethidium bromide. Forty-two amplified samples and three molecular weight markers (1 Kb DNA Ladder, Life Technologies, Gaithersburg, MD, USA) were usually analyzed in each gel run. Electrophoresis was performed for 15 h at 0.8 V cm^{-1} in 1 x TAE (Maniatis et al., 1982) as running buffer and amplimers were directly viewed over a UV light source.

Comparison of each profile for each of the primers was based on the presence (1) versus absence (0) of RAPD amplimers that migrated to the same position in the gel. Bands of the same size obtained by the same primer were scored as identical and only bands repeatable in at least two experiments with the same primer at different times were evaluated.

Results

Pathogenicity assay on differential carnation cultivars

Six weeks after transplanting, control plants had not developed any symptoms, while plants of the sensitive carnation cultivars inoculated with pathogenic *Fod* isolates had wilted completely. Pathogenic isolates belonging to the physiologic race 1 induced wilt symptoms only on cv Indios. Isolates of race 2 were pathogenic on cvs Sorriso, Indios, Manon, Faust and Selima, but not on Nicol. Isolates of race 4 were pathogenic on cvs Sorriso, Indios, Manon, Faust and Nicol, but not on Selima. Isolates 165 of race 5 and 256 of race 6 induced wilting on cvs Indios, Manon and Faust, but were moderately aggressive (disease indices 0.8 and 2.5, respectively) on cv Sorriso. Isolates of race 8 were pathogenic on cvs Sorriso, Indios and Faust, but not on Manon (Table 1). The virulence of the various races to the cultivars tested is summarized in table 2. *F. proliferatum* isolates 1035, 1223, 1225 and 1228 were nonpathogenic. *F. proliferatum* isolates 1215 and 1226 induced some restricted rotting and death of a few leaves close to the stem base, but not typical Fusarium wilt symptoms. *F. redolens* isolates 1200 and 1202, and *F. oxysporum* isolates 264, 789 and 812 had no or very low pathogenicity on all carnation cultivars tested (Table 1).

RAPD-PCR analysis

RAPD patterns were obtained for the 72 isolates of *Fusarium* spp. with 10 primers, and a total of 72 polymorphic bands was selected as the most informative markers in the identification of *Fod* isolates (Table 3). The molecular size of amplimers ranged from 0.3 to 4.0 kb and all the primers tested revealed at least one polymorphic band which could be used to define homogeneous groups among the different isolates. All the isolates belonging to the same RAPD group shared the same markers with all the tested primers, leading to a similarity level of 100% within the same RAPD group: for this reason, cluster analysis is not shown here. Representative results are given in Figure 1. The 60 *Fod* isolates tested in this study formed three RAPD groups which were clearly distinguishable from those produced by nonpathogenic *F. oxysporum*, *F. redolens* and *F. proliferatum* isolates. RAPD group 1 included all representatives of race 4; isolates of race 2 and the two single representatives of races 5 and 6 were included in RAPD group 2; group 3 was formed by isolates of races 1 and 8 (Table 1; Figure 1). Three non-pathogenic *F. oxysporum* isolates were clustered in RAPD group 4 (Table 1). Finally, RAPD groups 5 and 6 were formed by isolates of *F. redolens* and *F. proliferatum*, respectively (Table 1; Figure 1).

Discussion

The RAPD fingerprinting procedure was used in this study to identify races of *Fod* found on diseased carnation. As a diagnostic tool, this technique appears easier and more promising for practical application compared to other characterization methods based on vegetative compatibility groups (VCG, Aloi and Baayen, 1993; Baayen and Kleijn, 1989; Katan et al., 1989), DNA restriction fragment length polymorphism (RFLP, Baayen et al., 1997; Manicom et al., 1990; Manicom and Baayen, 1993), electrophoretic karyotype (EK) variability (Migheli et al., 1993, 1995), esterase profiles (Baayen et al., 1997) and sequence analysis of the ribosomal ITS1 and ITS2 regions (Waalwijk et al., 1996). Very little amounts of template DNA are required and results can be obtained rapidly, enabling a large number of isolates to be analyzed at the same time (Foster et al., 1993). RAPD profiles obtained with a fast protocol for DNA extraction directly from cultures growing on PDA (Migheli and Cavallarin, 1994) allowed the complete analysis (i.e., from DNA extraction to U.V. visualization of amplimers in the agarose gel) in less than 8 h and were comparable to those obtained in this study from minipreparations of DNA. Some difficulties in reproducing minor amplification products could be encountered by changing the batch of thermostable DNA polymerase, as described in an extensive study where 13 different polymerases were tested under the same reaction conditions (Schierwater and Ender, 1993). For this reason, only reproducible amplimers were considered in the RAPD analysis of *Fusarium* spp. isolates.

In the present study, we focused on the *Fod* races in VCGs 0020, 0021 and 0022, since only these

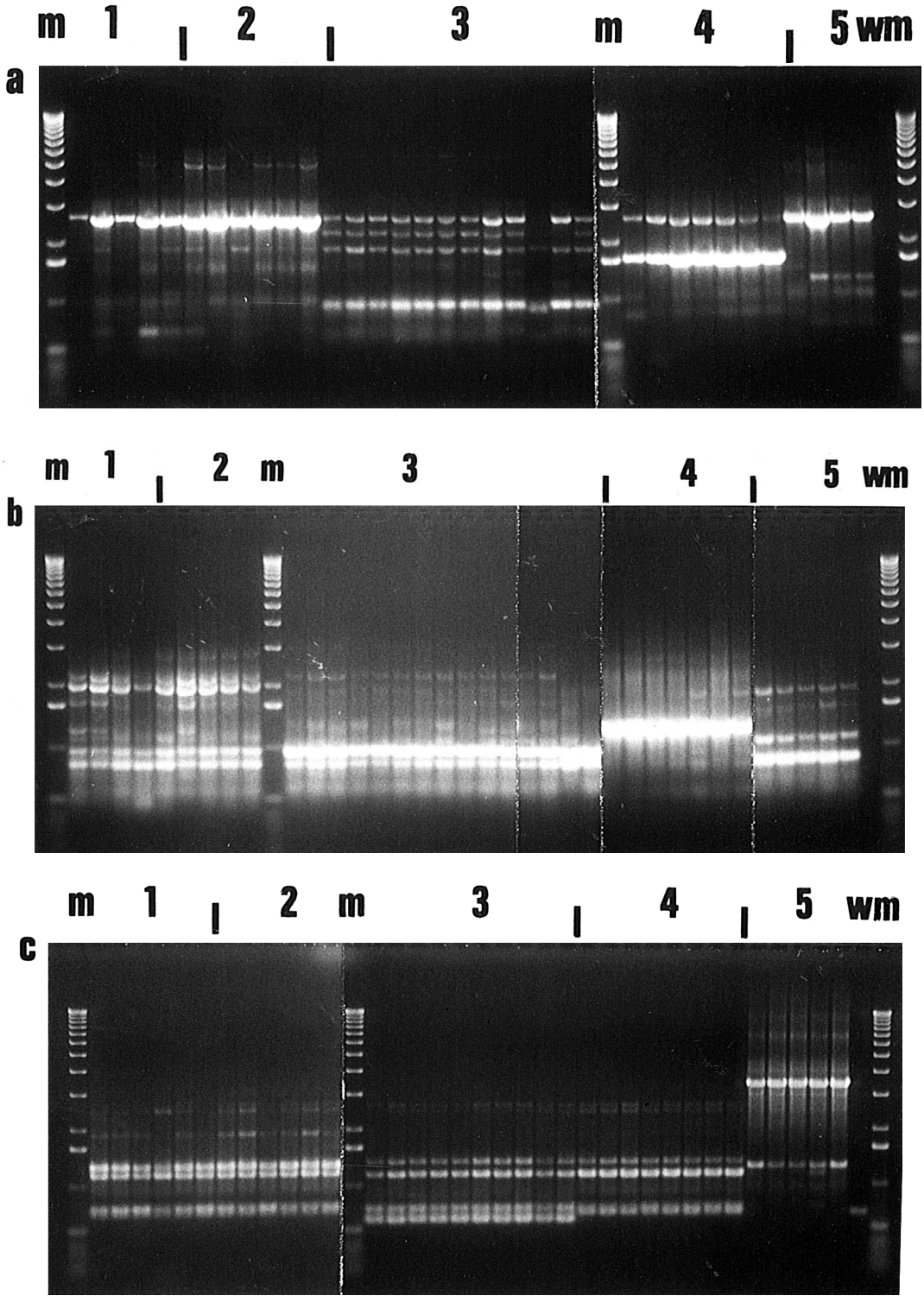

Figure 1. Random amplified DNA polymorphisms of different *Fusarium* spp. isolates from diseased carnation obtained with primer: **a**: OPE05 (5′-TCAGGGAGGT-3′); **b**: OPE08 (5′- TCACCACGGT-3′); **c**: OPE13 (5′-CCCGATTCGG-3′). Isolates belonging to: **1**: *Fod* race 1; **2**: *Fod* race 8; **3**: *Fod* race 2; **4**: *Fod* race 4; **5**:*F. proliferatum*; **w**: water control; **m**: DNA molecular weight marker (1 Kb DNA Ladder).

three VCGs are widespread in their occurrence and commonly found in Italy. Among the *Fod* isolates, those assigned to pathotype 4 formed an homogeneous cluster, which corresponds to VCG 0020 (Aloi and Baayen, 1993). Members of races 2, 5 and 6 shared the same RAPD profile, in accordance to VCG (Aloi and Baayen, 1993), RFLP (Manicom et al., 1990; Manicom and Baayen, 1993) and esterase (Baayen et al., 1997) grouping. *Fod* race1 and *Fod* race8 were clustered within the same RAPD group: these two races were previously assigned to the same VCG (Aloi and Baayen, 1993) and RFLP groups (Manicom et al., 1990; Manicom and Baayen, 1993) and presented similar electrophoretic karyotypes (Migheli et al., 1995), esterase profiles (Baayen et al., 1997) and sequences of the ribosomal ITS1 and ITS2 regions (Waalwijk et al., 1996). This further confirms that races 1 and 8 are closely related, although differing from each other in virulence (Aloi and Baayen, 1993; Garibaldi, 1981, 1983), and can be considered as near-isogenic (Baayen et al., 1997). The fact that races 1 and 8 were reported only in the Italian and French Riviera, on cultivars of the Mediterranean ecotype, supports the hypothesis that race 8 may have arisen from race 1 by adaptation to resistant cultivars, only differing by one or a few avirulence genes involved in specific recognition, present in race 1 and absent in race 8. A total of 100 10-mer random primers (Operon series A, B, E, M, T) and the two15-bp oligonucleotides $(CAT)_5$ and $(TCC)_5$ were tested to attempt differentiation of 10 *Fod* isolates of races 1 and 8, but all primers generated overlapping amplification profiles (data not shown).

Preliminary data on the distribution of the transposable element *Fot*1 (Daboussi et al., 1992; Daboussi and Langin, 1994) in the genome of *Fod* provide further support for the present grouping of race 1 together with race 8, and races 5 and 6 together with race 2, while race 4 belongs to a third main group (Migheli et al., 1997).

The low level of pathogenicity of isolates 264, 789 and 812 on the tested cultivars was confirmed by their different RAPD profiles. The three isolates, which were recovered in different years from diseased carnations grown in different locations, shared identical amplification patterns and are probably saprophytic *F. oxysporum*, frequently associated with the carnation rhizosphere, playing a role in the biological control of Fusarium wilt (Garibaldi et al., 1986a).

Two *F. redolens* isolates from Japan and seven non-pathogenic isolates of *F. proliferatum* collected from diseased carnation in Italy, Israel and The Netherlands were clearly recognized according on their RAPD fingerprint.

The data presented here confirm that RAPD fingerprinting represents a powerful diagnostic tool for the identification of all major races of *Fod* present in Italy, as already suggested for races 2, 4 and 9 by Manulis et al. (1994) and Kalc Wright et al. (1996). The fact that all isolates within the same VCG generated identical amplification profiles with each of the random primers tested indicates that RAPDs are indeed very faithful and suggests that RAPD-derived diagnostics are a realistic goal. The purification, cloning and sequencing of selected amplimers is now being performed and will allow the development of *forma specialis*- and/or race-specific probes and primers to be used in dot-blot or PCR-based diagnostic tools for a fast and reliable screening of pathogenic isolates.

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