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Genetic diversity in *Fusarium oxysporum* f.sp. *dianthi* and *Fusarium redolens* f.sp. *dianthi*

R.P. Baayen, F. van Dreven, M.C. Krijger and C. Waalwijk
Agricultural Research Department, Research Institute for Plant Protection, P.O. Box 9060, 6700 GW Wageningen, The Netherlands (Fax: 31.317.410113)

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Abstract

Pathogenic isolates were selected representing all known vegetative compatibility groups (VCGs) and races of *Fusarium oxysporum* sensu lato from *Dianthus* spp. On basis of differences in the internal transcribed spacer region of the ribosomal DNA, six VCGs were classified as *F. oxysporum* f.sp. *dianthi* and four as *F. redolens* f.sp. *dianthi*. All VCGs of *F. oxysporum* f.sp. *dianthi* were characterized by unique restriction fragment length polymorphisms (RFLPs), unique overall esterase profiles, and unique virulence spectra, supporting a clonal lineage concept. Two VCGs of *F. oxysporum* f.sp. *dianthi* nevertheless comprised more than one race, but races within the same VCG shared the same distinct overall virulence spectrum. VCGs belonging to *F. redolens* f.sp. *dianthi* also had unique RFLPs and unique virulence spectra, but had grossly identical esterase profiles. Three new races (9, 10 and 11) are described for *F. oxysporum* f.sp. *dianthi*, and four for *F. redolens* f.sp. *dianthi*. Two races previously considered lost were recovered; race 7 was identified as a member of VCG 0021 of *F. oxysporum* f.sp. *dianthi* while race 3 was identified as a distinct VCG and race of *F. redolens* f.sp. *dianthi*. A summary of races and VCGs in *F. oxysporum* f.sp. *dianthi* and *F. redolens* f.sp. *dianthi* is presented.

Introduction

Genetic and pathogenic diversity among isolates of *Fusarium oxysporum* sensu lato from *Dianthus* spp. has been extensively studied since 1975, when evidence was first presented for the existence of races within *F. oxysporum* f.sp. *dianthi* (Garibaldi, 1975). Eight races were eventually described (Garibaldi, 1981). Additional races were identified by Aloi and Baayen (1993) but could not be assigned race numbers, as two of the originally described races (3 and 7) that might possibly coincide with some of the novel races had been lost in Garibaldi's collection. Meanwhile, it remained unclear whether the fungal races under study belonged to *F. oxysporum* Schlecht.: Fr. sensu stricto or to the related species *F. redolens* Wollenw. Race 3 of the carnation wilt pathogen was initially described by Garibaldi (1979) as *F. oxysporum* var. *redolens* rather than *F. oxysporum* f.sp. *dianthi*, but the same race was later referred to as race 3 of *F. oxysporum* f.sp. *dianthi* (Garibaldi, 1981). A morphological study indicated that both species are conspecific, and that all isolates from carnation should hence be classified as *F. oxysporum* f.sp. *dianthi* (Baayen and Gams, 1988). Recent studies on the ITS region of the ribosomal DNA (rDNA) of large numbers of isolates belonging to various formae speciales as well as non-pathogenic isolates, however, have provided conclusive evidence that *F. oxysporum* sensu stricto and *F. redolens* are distinct species which both comprise isolates that are pathogenic to carnation (Waalwijk and Baayen, 1995; Waalwijk et al., 1996a, 1996b). Further support for the distinction between both species has been provided by sequence analysis of additional genomic sequences (K. O'Donnell, pers. comm.).

Baayen and Kleijn (1989) described five vegetative compatibility groups (VCGs) among isolates belonging to *F. oxysporum* f.sp. *dianthi* of which two should now be classified as *F. redolens*. The number of VCGs was extended by Aloi and Baayen (1993), who

demonstrated that races of *F. oxysporum* f.sp. *dianthi* sensu lato coincide with distinct VCGs. Support for the view that VCGs represent homogeneous genetic entities within *F. oxysporum* f.sp. *dianthi* sensu lato was presented by Manicom and Baayen (1993) using total DNA restriction fragment length polymorphisms (RFLPs) with a probe developed from race 2 of *F. oxysporum* f.sp. *dianthi* (Manicom et al., 1987). Similar results have been obtained more recently using RAPD analyses (Manulis et al., 1994; Kalc Wright et al., 1996; Migheli et al., 1996) and chromosome electrophoresis (Migheli et al., 1995). Further support at protein level for the notion that VCGs represent homogeneous genetic entities was provided for other formae speciales by isoenzyme profiling (Bosland and Williams, 1987; Elias and Schneider, 1992).

Changing views concerning the species identity of isolates, and increasing numbers of VCGs and possible new races (Aloi and Baayen, 1993; Kalc Wright et al., 1996; Manicom et al., 1990) necessitated a revision of the existing classification of isolates into species, VCGs and races. The present study was undertaken to present an overall scheme of genetic diversity in *F. oxysporum* f.sp. *dianthi* and *F. redolens* f.sp. *dianthi* at four different levels (vegetative compatibility, DNA fingerprints, isoenzyme fingerprints, and virulence), encompassing all VCGs and races described thus far. Esterases were selected for isoenzyme fingerprinting because of the relatively large differentiation that may be obtained with esterases compared with other enzymes. The recovery during this study of reference isolates of races 3 and 7 from the collection of dr F. Scala (Portici, Italy) further supported the desired revision of the status of races and VCGs in both fungi.

Materials and methods

Fungal isolates. The authors' collection of isolates of *F. oxysporum* f.sp. *dianthi* (Prill. & Delacr.) Snyder & Hansen and *F. redolens* f.sp. *dianthi* Gerlach was extended over a period of years by isolations from diseased *Dianthus* spp. from all over the world and by international exchange. Incoming isolates were screened for vegetative compatibility with tester mutants and/or pathogenicity to enlarge small VCGs, as well as to detect possible new pathogenic VCGs. Eventually, forty-nine isolates of *F. oxysporum* f.sp. *dianthi* and *F. redolens* f.sp. *dianthi* (Table 1) representing all pathogenic VCGs encountered were selected for analysis of molecular characteristics, esterase profiles and virulence. Geographic origin (Table 2) was not a selection criterion. We used reference isolates of VCGs 0020, 0021 and 0022 which have been studied extensively by Aloi and Baayen (1993) and Manicom and Baayen (1993). The selection thus included many isolates that have been described in previous studies, allowing the reader to compare present results with those published previously. While for at least three isolates per VCG and race (in so far as available) all characteristics were investigated, ten additional isolates were characterized only partially. Isolates were stored on PDA (Oxoid) or the low nutrient medium SNA (Nirenberg, 1976) supplemented with sterilized pieces of filter paper. Reference isolates were also stored in liquid nitrogen as well as lyophilized.

Vegetative compatibility tests. Vegetative compatibility tests were performed according to previously described methods (Aloi and Baayen, 1993) with *nit*1 and/or NitM tester mutants of the following isolates: DSM 62391, F100, F165, F276, F310, NAKS3, PD 89/1825, PD 90/291, PD 90/1882, WCS816 and WCS848, in the course of the study supplemented by isolates 70 and 621.

Esterase profiles. Seven-day-old cultures grown on a standard batch of PDA (Oxoid) in Petri dishes at 20 °C were used to prepare inoculum suspensions at 10^5 conidia per ml. One hundred microliter suspensions were spread on PDA plates covered by sterile cellophane. The same batch of PDA was used throughout all experiments. After 7 days of growth at 20 °C the mycelium was harvested, lyophilized in Eppendorf tubes (two replicates per Petri-dish culture) and stored at –20 °C. Mycelium was ground in liquid nitrogen and 10 μl extraction buffer containing 0.05 m Tris-HCl (pH 7.5), 5 mM EDTA and 1% w/v dithiothreitol was added per mg of mycelium. The mixture was centrifuged twice at 17500 $\times$ *g* at 4 °C and protein concentration in the supernatant was determined using bovine serum albumin as a standard.

Polyacrylamide gel electrophoresis (PAGE) was performed under high-pH, non-denaturing conditions on 10% (w/v) polyacrylamide gels following the method described by Laemmli (1970) on a Mini Protean II (Biorad). Samples for electrophoresis were prepared by adding 4 μl loading buffer (0.1% w/v bromophenol blue and 50% v/v glycerol) to 16 μl extract. Gels were stained for esterase activity in 50 ml 0.15

Table 1. Isolates of *Fusarium oxysporum* f.sp. *dianthi* and *F. redolens* f.sp. *dianthi* listed according to vegetative compatibility group (VCG), physiologic race, restriction fragment length polymorphism grouping (RFLP), esterase profile, source and geographic origin

Isolate	Race[1]	RFLP[2]	Esterase profile	Original host	Source[3]	Origin
				F. oxysporum* f.sp. *dianthi		
				VCG 0020		
F79	4	I	a	*Dianthus caryophyllus*	A.G.	Italy
F261	4	I	a	*D. caryophyllus*	A.G.	Italy
F310	4	I	a	*D. caryophyllus*	A.G.	Italy
F578	4	–[5]	a	*D. caryophyllus*	A.G.	Italy
F760	4	–	a	*D. caryophyllus*	A.G.	Italy
F761	4	–	a	*D. caryophyllus*	A.G.	Italy
F828	4	–	a	*D. caryophyllus*	A.G.	Italy
O–4	4	I	a	*D. caryophyllus*	T.K.	California, U.S.A.
				VCG 0021		
F75[4]	2	II	b	*D. caryophyllus*	A.G.	Italy
F107	2	II	b	*D. caryophyllus*	A.G.	Italy
FOD-E114	2	II	b	*D. caryophyllus*	T.K.	Israel
WCS816	2	II	b	*D. caryophyllus*	R.P.B.	The Netherlands
WCS848	2	II	b	*D. caryophyllus*	R.P.B.	The Netherlands
WCS851	2	–	b	*D. caryophyllus*	R.P.B.	France
F165	5	II	b	*D. caryophyllus*	A.G.	Italy
623	5	II	b	*D. caryophyllus*	F.S. ex A.G.	Italy
624	5	II	b	*D. caryophyllus*	F.S. ex A.G.	Italy
F256	6	II	b	*D. caryophyllus*	A.G.	Italy
625	6	II	b	*D. caryophyllus*	F.S. ex A.G.	Italy
626	7	II	b	*D. caryophyllus*	F.S. ex A.G.	Italy
95-13	–	II	b	*D. caryophyllus*	R.P.B.	The Netherlands
PD 89/1844	–	–	b	*D. caryophyllus*	R.P.B.	The Netherlands
106	–	–	b	*D. caryophyllus*	G.F.K.	Australia
				VCG 0022		
F1	1	III	c	*D. caryophyllus*	A.G.	Italy
F100	1	III	c	*D. caryophyllus*	A.G.	Italy
F101	1	III	c	*D. caryophyllus*	A.G.	Italy
F521	1	III	c	*D. caryophyllus*	A.G.	Italy
F1045	1	III	c	*D. caryophyllus*	A.G.	Italy
F276	8	III	c	*D. caryophyllus*	A.G.	Italy
F425	8	–	c	*D. caryophyllus*	A.G.	Italy
F639	8	III	c	*D. caryophyllus*	A.G.	Italy
F1068	8	III	c	*D. caryophyllus*	A.G.	Italy
				VCG 0025		
PD 90/291[6]	11	IV	d	*D. caryophyllus*	PD	The Netherlands
				VCG 0027		
WCS842	10	V	e	*D. caryophyllus*	R.P.B.	The Netherlands
NAKS3	10	V	e	*D. caryophyllus*	R.P.B.	The Netherlands
				VCG 0028		
68	9	VI	f	*D. caryophyllus*	G.F.K.	Australia
70	–	–	f	*D. caryophyllus*	G.F.K.	Australia
121	9	VI	f	*D. caryophyllus*	G.F.K.	Australia
B6D214/2	9	VI	f	*D. caryophyllus*	G.F.K.	Australia

Table 1. Continued

Isolate	Race[1]	RFLP[2]	Esterase profile	Original host	Source[3]	Origin
			F. redolens* f.sp. *dianthi			
			VCG 0001			
DSM 62393	1	VII	g	*Dianthus barbatus*	DSM	Germany
PD 89/1825	1	VII	g	*D. barbatus*	PD	The Netherlands
PD 92/1134	1	VII	g	*D. barbatus*	PD	The Netherlands
			VCG 0002			
PD 90/1882	2	VIII	g	*D. barbatus*	PD	The Netherlands
			VCG 0003			
621	3	IX	g	*D. caryophyllus*	F.S. ex A.G.	Italy
622	3	IX	g	*D. caryophyllus*	F.S. ex A.G.	Italy
			VCG 0004			
DSM 62390	4	X	g	*D. caryophyllus*	DSM	Germany
DSM 62391	4	X	g	*D. caryophyllus*	DSM	Germany
DSM 62392	4	X	g	*D. caryophyllus*	DSM	Germany

[1] Race classification of isolates based on original labels (collection A.G.), data from Aloi and Baayen (1993) for reference isolates of VCGs 0020, 0021 and 0022, and data from the present study for all other VCGs.
[2] Groupings by RFLP patterns with probe D4 as well as with probe F9 were identical.
[3] Abbreviations: CBS, Centraalbureau voor Schimmelcultures, Baarn, The Netherlands; DSM, Deutsche Sammlung von Mikroorganismen, Göttingen/Braunschweig, Germany; PD, Plantenziektenkundige Dienst, Wageningen, The Netherlands. Private collections: A.G., A. Garibaldi, Turin, Italy; F.S., F. Scala, Portici, Italy; G.F.K., G.F. Kalc Wright, Parkville, Australia; R.P.B., R.P. Baayen; T.K., T. Katan, Bet-Dagan, Israel.
[4] Self-incompatible isolate corresponding to VCG 0021 in all other properties.
[5] Not determined.
[6] A second isolate (PD 95/3729) with the same RFLP pattern was received in 1995, at the end of this study, and subsequently proved to originate from the same greenhouse from which isolate PD 90/291 had been obtained in 1990.

M phosphate buffer (pH 7.2) containing 15 mg fast blue RR in 5 ml phosphate buffer and 20 mg α-naphtyl acetate in 0.5 ml acetone. After 45 min of staining, gels were washed with water, soaked in 10% glycerol and dried for 5 h at 40 °C in between two sheets of cellophane.

Reproducible esterase profiles were obtained by extracting and processing the lyophilized mycelium all within a day. Eight samples of lyophilized mycelium were investigated per isolate, representing two repetitions in time of two cultures sampled in duplicate. Extraction and analysis was performed systematically in such a manner that culturing series effects, processing series effects, Petri-dish effects, and electrophoresis condition effects, if any, could be retrieved. Esterase profiles of isolates were transformed to a binary code for presence or absence of bands. Profile similarity dendrograms were constructed with the computer programme GENSTAT (Payne et al., 1987) using the procedures FSIMILARITY with Euclidean distances and HCLUSTER with average linkage.

Extraction of DNA. Isolates were inoculated onto PDA (Oxoid) plates covered by sterile cellophane and cultured for seven days at 20 °C. Mycelium was harvested, lyophilized and processed according to the methods described by Raeder and Broda (1985). Alternatively, DNA was extracted from liquid cultures grown for five days in potato dextrose broth at 27 °C.

Analysis of the ITS region of the ribosomal DNA. PCR reactions were performed with the primers ITS1 and ITS4 (White et al., 1990) for at least three isolates per VCG. Amplifications were done using 10 ng of template DNA in a volume of 100 μl containing 0.6 μM of both primers, 60 μM of each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 50 mM KCl, 20 mM Tris-HCl (pH 8.3), 0.05% detergent W-1 and 2 U of Taq DNA polymerase (Life Technologies). Cycling conditions were 94 °C for 1 min, 56 °C for 1 min and 72 °C for 1.5 min (35 cycles), followed by an extension at 72 °C for 10 min. Amplification was checked on 1.0% agarose gels. Isolates were identified as *F.*

Table 2. Geographic distribution of vegetative compatibility groups (VCGs) of *Fusarium oxysporum* f.sp. *dianthi* and *F. redolens* f.sp. *dianthi* in the collection of the authors

Taxon and VCG	Origin and numbers of isolates
F. oxysporum f.sp. *dianthi*	
VCG 0020	Italy (10), Spain (3), Israel (1), Colombia (2), U.S.A.(3)
VCG 0021	Poland (1), Czechia (2), The Netherlands (48), U.K. (8), France (23), Spain (10), Italy (12), Greece (6), Israel (15), Morocco (1), Australia (3), Japan (13), Argentina (4), Colombia (6)
VCG 0022	Italy (19), Spain (1), France (1), The Netherlands (1)
VCG 0025	The Netherlands (2)
VCG 0027	The Netherlands (2)
VCG 0028	Australia (4)
F. redolens f.sp. *dianthi*	
VCG 0001	The Netherlands (2), Germany (1)
VCG 0002	The Netherlands (1)
VCG 0003	Italy (2)
VCG 0004	Germany (3)

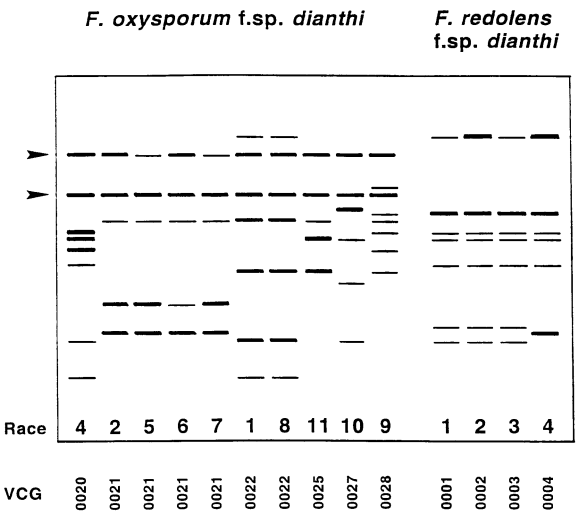

Figure 1. Schematic presentation of the esterase profiles observed among the races of *Fusarium oxysporum* f.sp. *dianthi* and *F. redolens* f.sp. *dianthi*, exemplified by a single representative of each race. The arrowheads indicate two bands which differentiated *F. oxysporum* f.sp. *dianthi* from *F. redolens* f.sp. *dianthi*. Thick bands represent proteins with strong esterase activity.

oxysporum or *F. redolens* by digesting the amplified products with the discriminating restriction enzymes *Hha*I and/or *Hinf*I and analysing the digestion products on agarose gels as described previously (Waalwijk et al., 1996a).

Restriction fragment length polymorphisms of total DNA. DNA preparations restricted with *Hin*dIII were electrophoretically resolved on agarose gels, blotted to nylon membranes and hybridized with probe D4 (Manicom et al., 1987) or probe F9, a 0.5 kb PCR product obtained after amplification of total DNA of an isolate of race 1 with RAPD primer CTGTTGCTAC. The amplification product is specific for pathogenic isolates of *F. oxysporum* f.sp. *dianthi* (Kalc Wright et al., 1996). Probe F9 was used additional to probe D4 to check whether the correspondence of RFLP fingerprint grouping to VCG grouping has a more general basis. RFLP similarity dendrograms were constructed as for esterase profiles.

Virulence tests. The virulence of isolates was characterized using a differential set of seven carnation cultivars: White Royalette, Early Sam, Raggio di Sole, Izmir, Lena, Pallas and Elsy, rooted cuttings of which were obtained from commercial supplies and from the DLO Centre for Plant Breeding and Reproduction Research, Wageningen. Plants grown from cuttings in four weeks time were inoculated following stem inoculation procedure III described by Baayen and Schrama (1990). Experiments were performed in a climatized greenhouse compartment at 25 °C with additional artificial light during winter. Development of disease symptoms was monitored for maximally 3 months using a disease index from 0 (healthy) to 5 (dead) as described previously (Baayen and Van der Plas, 1992). The frequency distributions of disease indices for all plants of each cultivar – isolate combination were examined using the non-parametric Wilcoxon's test (Sokal & Rohlf, 1969).

Results

Identification of vegetative compatibility groups. Ten vegetative compatibility groups were present among isolates able to induce wilt disease in *Dianthus* species (Tables 1 and 2). These were classified as *F. oxysporum* f.sp. *dianthi* (six VCGs) or *F. redolens* f.sp. *dianthi* (four VCGs) on basis of the ITS region of the rDNA, a character that differentiates unambiguously between both species (Waalwijk et al., 1996a, 1996b).

We propose to apply a similar four-digit code as used for VCGs in *F. oxysporum* to *F. redolens*, with

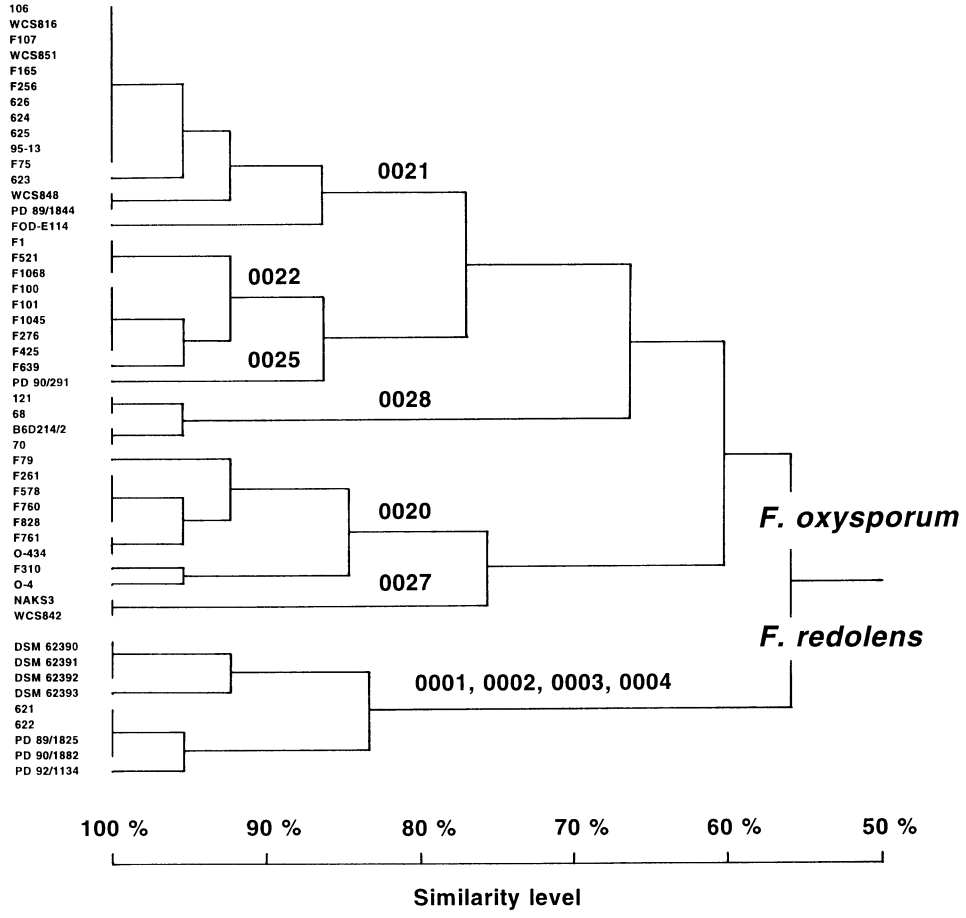

Figure 2. Dendrogram of the esterase profiles encountered among isolates of *Fusarium oxysporum* f.sp. *dianthi* and *F. redolens* f.sp. *dianthi*. Branches coincide with VCGs (indicated by four-digit codes) only in *F. oxysporum* f.sp. *dianthi*. Profile diversity within VCGs is large compared to the diversity observed between VCGs. The four isolates of VCG 0021 shown in Figure 1 fall in the uppermost branch of the dendrogram for this VCG. Minor deviations among isolates of *F. redolens* f.sp. *dianthi* as illustrated in Figure 1 do not coincide with VCGs.

VCGs 0001 to 0009 to be allocated to isolates pathogenic to *Dianthus* species. Following previous conclusions by Waalwijk et al. (1996a), the former VCGs 0023 and 0024 of *F. oxysporum* f.sp. *dianthi* (Aloi and Baayen, 1993) are presently reclassified formally as *F. redolens* f.sp. *dianthi* VCG 0004 and VCG 0001, respectively. VCG 0003 of *F. redolens* f.sp. *dianthi* comprises two rediscovered isolates of race 3, in line with Garibaldi's (1979) original description of this race as belonging to *F. oxysporum* var. *redolens* rather than to *F. oxysporum* f.sp. *dianthi*. These isolates are classified here as *F. redolens* f.sp. *dianthi* race 3 (Table 1).

In addition to the former VCGs 0023 and 0024 of *F. oxysporum* f.sp. *dianthi*, single-member VCG 0026 has also been abandoned (Table 1). Renewed testing of the sole member of this VCG, isolate WCS842, proved that it is actually compatible with the single member of VCG 0027, isolate NAKS3. The differential RFLP pattern for VCG 0026 described previously (Manicom and Baayen, 1993) was found to be due to contamination with a non-pathogenic isolate. The new VCG 0028 consists of pathogenic isolates from Australia described as VCG B by Kalc Wright et al. (1996). Isolates of VCG C of the latter authors were compatible with present VCG 0021, as were reference isolates of the single VCG reported for Greece by Elena and Tjamos (1995) and the single VCG encountered in Argentina (G.A. Lori, La Plata, Argentina, pers. comm.). All isolates from Japan belonged to VCG 0021 (Table 2), as did most of the isolates in The Netherlands, France and Israel (Table 2), in agree-

ment with previous reports (Aloi and Baayen, 1993; Manulis et al., 1994). Using reference isolates from our collection, the single VCG in Hungary was also identified as VCG 0021 (Pomázi et al., 1991). Outside of Italy and neighbouring countries, isolates belonging to VCGs 0020 and 0022 were quite rare (Table 2). Colombian isolates of VCG 0020 classified as race 4 were received from E. Garcés de Granada (Bogotá, Colombia); isolates of VCG 0020 (O-434 and O-1110) originating from Pennsylvania, U.S.A. were received from P.E. Nelson.

Esterase profiles. Isolates from different VCGs of *F. oxysporum* f.sp. *dianthi* all produced unique esterase profiles whereas within a VCG all isolates had the same overall pattern (Figure 1; Table 1). On the other hand, isolates of *F. redolens* f.sp. *dianthi* all had very similar esterase profiles, with only marginal variation between isolates of different VCGs. Specific esterase bands present in all VCGs of *F. oxysporum* and absent in VCGs of *F. redolens* were observed (arrowheads in Figure 1). Cluster analyses performed on the complete set of data also separated both species from one another (Figure 2). Given the diversity in esterase profile encountered within VCGs of *F. oxysporum* f.sp. *dianthi* compared to the diversity between VCGs (Figure 2), the discriminative ability of esterase profiling for identification of unknown isolates was relatively poor.

Restriction fragment length polymorphisms. Each of the VCGs in *F. oxysporum* f.sp. *dianthi* as well as all VCGs in *F. redolens* f.sp. *dianthi* had distinct RFLP patterns when probed with either D4 or with the new probe F9 (Table 1). Representative examples of the patterns obtained with these probes in the various VCGs in both taxa are shown in Figure 3. No bands specific to either species were observed and cluster analyses did not separate the two species from one another (not shown). Analyses were consequently performed for both species individually (Figure 4). Compared to esterase profiles, only minor diversity in RFLP pattern was observed within some VCGs, particularly within VCG 0021 of *F. oxysporum* f.sp. *dianthi* (Figure 4). Moreover, in many VCGs the RFLP pattern was identical for all isolates. The discriminative ability of RFLPs for identification of unknown isolates was significantly better than that of esterase profiling (compare Figures 2 and 4). Indeed, at the end of this study an unknown isolate (PD 95/3729) with the RFLP pattern of VCG 0025 was received that subsequently could be traced

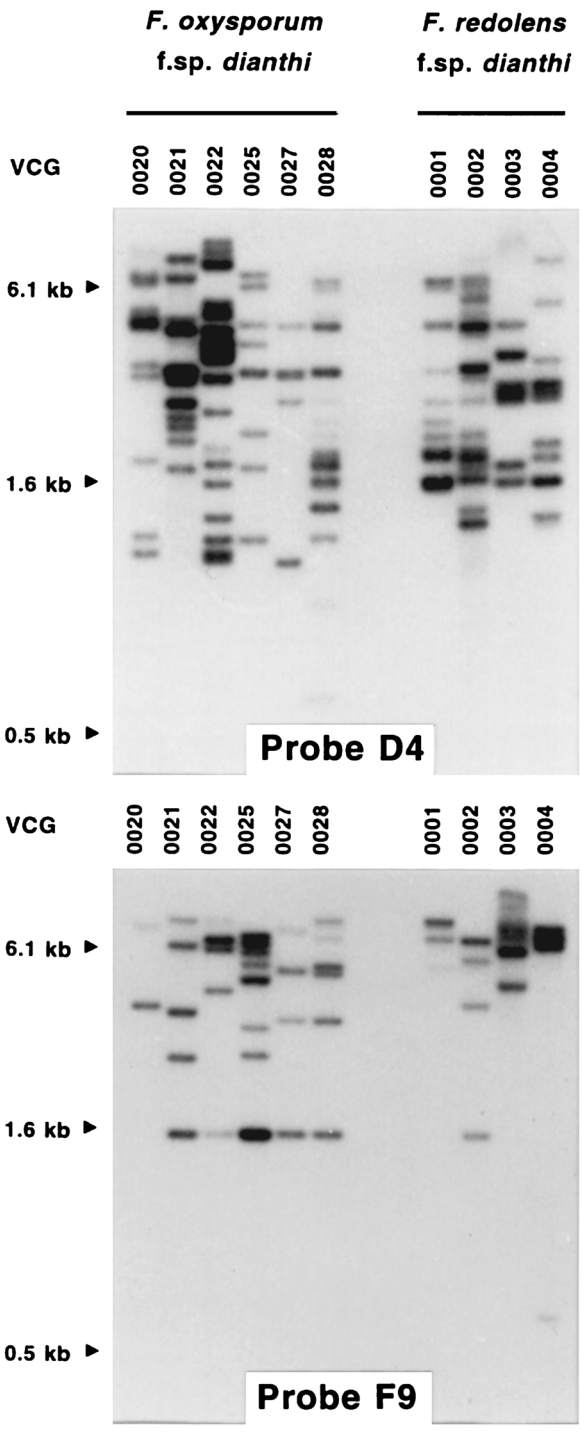

Figure 3. Restriction fragment length polymorphisms observed for the vegetative compatibility groups within *Fusarium oxysporum* f.sp. *dianthi* and *F. redolens* f.sp. *dianthi*, all exemplified by single representatives. Southern blots of total DNA were restricted with *Hin*dIII and probed with D4 and F9.

down to the same greenhouse from which the type isolate of this VCG, PD 90/291, had been obtained five years before.

Virulence patterns. The virulence patterns of the reference isolates of races 1, 2, 4 and 8 of *F. oxysporum* f.sp. *dianthi* (Table 3) corresponded to those reported previously (Demmink et al., 1989; Baayen et al., 1991; Baayen and Van der Plas, 1992; Aloi and Baayen, 1993). All races were highly virulent both to cv. Early Sam and to cv. White Royalette (not shown), in agreement with previous reports (Aloi and Baayen, 1993). Race 1 was virulent on cvs. White Royalette and Early Sam, and slightly virulent on cv. Elsy. Race 8, also belonging to VCG 0022, was distinguished from race 1 by its virulence to cvs. Pallas and Raggio di Sole. These cultivars had been deliberately included in the trials because they enable for discrimination between race 1 and race 8 (Garibaldi, 1983). As expected race 4 was more virulent to cv. Pallas than to cv. Lena (Demmink et al., 1989; Aloi and Baayen, 1993), thus providing differentiation from race 2. Attempts to differentiate races 5, 6 and 7 from race 2 (all belong to VCG 0021) were not possible: the reference isolates of races 5, 6 and 7 had a much reduced aggressiveness due to degeneration and virulence was not adequately restored upon passage through susceptible cv. Early Sam.

In repeated trials, the Australian isolates 68, 121 and B6D214/2 (VCG 0028) were highly virulent to cvs. White Royalette and Early Sam, and consistently were fairly virulent to the remaining five cultivars (Table 4). No other race being equally virulent to cvs. Raggio di Sole, Izmir, Lena, Pallas and Elsy, these isolates are classified as race 9 of *F. oxysporum* f.sp. *dianthi*.

The isolates in VCG 0025 (PD 90/291) and VCG 0027 (NAKS3 and WCS842) of *F. oxysporum* f.sp. *dianthi* differed throughout the trials from races 1 and 8 in their virulence to cv. Izmir. Moreover, they differed from race 4 in their reciprocal virulence to cvs. Izmir and Pallas (Table 3). Their virulence to the cultivars used in this study resembled that of race 2 (VCG 0021) to some extent. However, isolates NAKS3 and WCS842 have been discriminated by Aloi and Baayen (1993) from race 2 by their lack of virulence to cv. Golia (Table 6) and are here classified as race 10. Isolate PD 90/291 was identified as a new race by Aloi and Baayen (1993) because of its virulence to cv. Handy, which is resistant to the races in VCG 0021 (Table 6). It is presently classified as race 11. Unfortunately the latter two cultivars proved no longer available.

The four VCGs in *F. redolens* f.sp. *dianthi* coincided with groups of isolates with distinct virulence spectra (Table 5 and Table 6) and are hence classified as four distinct races. The isolates belonging to VCGs 0001 (race 1) and 0002 (race 2) originate from sweet william (*Dianthus barbatus*), and were virulent to only few cultivars of carnation (*D. caryophyllus*). Race 1 was virulent only to cv. White Royalette. The same pattern shown for isolates DSM 62393 and PD 92/1134 was found for PD 89/1825 (not shown). Race 2 was virulent to cvs. White Royalette and Early Sam (Table 5). The single isolate belonging to race 2, PD 90/1882, was also included in a trial with *F. oxysporum* f.sp. *dianthi*, where disease indices of 4.8 and 1.6 were obtained for cvs. White Royalette and Early Sam, respectively. Its avirulence to cvs. Izmir and Raggio di Sole was already reported by Aloi and Baayen (1993).

The isolates belonging to VCGs 0003 (race 3) and 0004 (race 4) were all taken from carnation, and had a wider range of virulence to this host. Race 3 was weakly virulent to cvs. White Royalette, Early Sam and Raggio di Sole (Table 5), several of the plants showing distinct but weak disease symptoms. Prolonged storage on PDA in culture collections has likely resulted in reduced aggressiveness of these two isolates. Results shown in Table 5 for isolates 621 and 622 pertain to strains that had already been subjected to passage through a susceptible cultivar. Race 4 was virulent to all cultivars studied except Elsy (Table 5).

The virulence of all races of *F. oxysporum* f.sp. *dianthi* and *F. redolens* f.sp. *dianthi* to the cultivars used in this study and to cvs. Golia, Handy and Jordan is surveyed in Table 6.

Discussion

Classification of VCGs and races of isolates belonging to *F. oxysporum* sensu lato from carnation has been hampered by controversies concerning the distinction of *F. redolens* from *F. oxysporum* (Gerlach and Pag, 1961; Gerlach and Nirenberg, 1982; Nelson et al., 1983; Baayen and Gams, 1988). Recent studies by Waalwijk and Baayen (1995) and Waalwijk et al. (1996a, 1996b) have demonstrated that *F. oxysporum* and *F. redolens* can be distinguished unambiguously on the basis of sequence differences in the ITS2 region of the rDNA; they are probably not even sister species (K. O'Donnell, person. comm.). Using the ITS criterion, six of the VCGs associated with *Fusarium* wilt of carnation could be identified as belonging to

Table 3. Disease levels incited by isolates belonging to five vegetative compatibility groups of *Fusarium oxysporum* f.sp. *dianthi* on carnation cvs. Early Sam, Raggio di Sole, Izmir, Lena, Pallas, and Elsy, assessed after 13 weeks on a scale from 0 (healthy) to 5 (dead)[1]

VCG	Isolate	Race	Cultivar					
			Early Sam	Raggio di Sole	Izmir	Lena	Pallas	Elsy
0022	F1	1	4.8 a	0.1 c	0.1 c	0.0 c	0.2 c	1.8 b
0022	F639	8	3.4 ab	2.0 bc	0.4 de	0.0 e	4.2 a	1.4 cd
0020	F310	4	3.3 a	0.8 b	1.3 b	1.6 b	2.8 a	0.0 c
0021	WCS816	2	3.8 a	2.8 b	2.8 ab	1.9 b	0.3 c	0.5 c
0027	WCS842	10	3.4 a	2.6 ab	3.0 a	1.5 bc	1.4 c	1.5 c
0027	NAKS3	10	2.3 abc	2.2 ab	2.8 a	2.3 ab	1.5 bc	1.1 c
0025	PD 90/291	11	1.9 b	2.5 ab	3.2 a	0.8 cd	0.3 d	1.8 bc

[1] Values on the same line followed by the same letter are not significantly different according to Wilcoxon's test ($P = 0.01$).

Table 4. Disease levels incited by three isolates belonging to vegetative compatibility group 0028 of *Fusarium oxysporum* f.sp. *dianthi* on carnation cvs. White Royalette, Early Sam, Raggio di Sole, Izmir, Lena, Pallas, and Elsy, assessed after $6\frac{1}{3}$ weeks on a scale from 0 (healthy) to 5 (dead)[1]

Isolate	Cultivar						
	White Royalette	Early Sam	Raggio di Sole	Izmir	Lena	Pallas	Elsy
68	4.5 a	4.9 a	1.7 c	3.1 b	3.0 b	2.8 bc	3.2 b
121	4.2 a	4.8 a	1.8 b	2.8 b	3.0 b	2.6 b	2.9 b
B6D214/2	3.9 a	4.9 a	2.8 b	3.1 b	2.5 b	2.6 b	3.3 b

[1] Values on the same line followed by the same letter are not significantly different according to Wilcoxon's test ($P = 0.01$).

Table 5. Disease levels incited by isolates belonging to four vegetative compatibility groups of *Fusarium redolens* f.sp. *dianthi* on carnation cvs. White Royalette, Early Sam, Raggio di Sole, Izmir, Lena, Pallas, and Elsy, assessed after 8 weeks on a scale from 0 (healthy) to 5 (dead)[1]

VCG	Isolate	Race	Cultivar						
			White Royalette	Early Sam	Raggio di Sole	Izmir	Lena	Pallas	Elsy
0001	DSM 62393	1	4.6 a	0.0 b	0.1 b	0.0 b	0.1 b	0.0 b	0.3 b
0001	PD 92/1134	1	5.0 a	0.0 b	0.0 b	0.0 b	0.0 b	0.0 b	0.0 b
0002	PD 90/1882	2	1.8 a	1.3 ab	0.0 c	0.0 c	0.0 c	0.0 c	0.1 b
0003	621	3	0.5 ab	0.4 ab	0.9 a	0.0 b	0.0 b	0.0 b	0.0 b
0003	622	3	0.8 a	0.5 ab	1.7 a	0.1 b	0.1 b	0.0 b	0.0 b
0004	DSM 62390	4	3.1 a	3.1 a	4.1 a	1.4 b	2.0 b	0.8 bc	0.1 c
0004	DSM 62391	4	4.3 a	1.8 ab	2.2 a	0.8 bc	0.8 bc	0.6 bc	0.0 c
0004	DSM 62392	4	2.8 ab	4.1 a	3.1 a	1.6 b	1.2 bc	0.6 c	0.0 c

[1] Values on the same line followed by the same letter are not significantly different according to Wilcoxon's test ($P = 0.01$).

Table 6. Survey of vegetative compatibility groups (VCGs), RFLP groups, esterase profiles and virulence patterns in *Fusarium oxysporum* f.sp. *dianthi* and *F. redolens* f.sp. *dianthi*

Taxon (ITS type)	*Fusarium oxysporum* f.sp. *dianthi* (I)							*F. redolens* f.sp. *dianthi* (II)			
VCG	0020	0021	0022	0022	0025	0027	0028	0001[1]	0002[1]	0003[2]	0004[2]
RFLP group[3]	I	II	III	III	IV	V	VI	VII	VIII	IX	X
Esterase profile	a	b	c	c	d	e	f	g	g	g	g
Race	4	2,5,6,7	1	8	11	10	9	1	2	3	4
Virulence[4] on cvs.											
White Royalette	+++	+++	+++	+++	+++	+++	+++	+++	++	±	+++
Early Sam	+++	+++	+++	+++	+++	+++	+++	–	+	±	+++
Raggio di Sole	+	++	–	+	+++	+++	++	–	–	+	+++
Izmir	+	++	–	–	+++	+++	++	–	–	–	+
Lena	++	++	–	–	+	+	++	–	–	–	+
Pallas	+++	–	–	+++	±	+	++	–	–	–	±
Elsy	–	–	+	+	+	+	++	–	–	–	–
Golia	+++	+++	+++	+++	+++	–		–	–		–
Handy	–	–	–	–	+++	–		–	–		–
Jordan	–	+++	–	–	+++	+++		–	–		–

[1] From *Dianthus barbatus*.
[2] From *D. caryophyllus*.
[3] Groupings by RFLP patterns with probe D4 as well as with probe F9 were identical.
[4] From highly virulent (+++) to avirulent (–). Data summarize the results of all bioassays (including those shown in Tables 3 to 5). Data for cvs. Golia, Handy and Jordan were derived from Aloi and Baayen (1993) because these cultivars could no longer be obtained. Data for VCG 0021 pertain only to race 2 of *F. oxysporum* f.sp. *dianthi*.

F. oxysporum f.sp. *dianthi* while four belonged to *F. redolens* f.sp. *dianthi*. Esterase profiles of both taxa also were clearly different. Indications that such is the case had previously been obtained in a preliminary study on esterase profiles (A. Kerssies and R.P. Baayen, unpubl.). A third esterase profile is common to isolates of *F. proliferatum* from foot-rot affected carnations (R.P. Baayen, unpubl.), confirming the potential of esterase profiling for species identification. Isoenzyme patterns of esterase also have potential for the detection of *F. oxysporum* in planta (Kerssies et al., 1994).

From the present study and the previous ones can be concluded that isolates within individual VCGs of *F. oxysporum* f.sp. *dianthi* are highly similar to one another in DNA fingerprints, karyotype, esterase profile and virulence (see also Manicom and Baayen, 1993; Manulis et al., 1994; Kalc Wright et al., 1996; Migheli et al., 1995, 1996). The correspondence probably also extends to the IGS region of the rDNA and to the mitochondrial and plasmid-like DNA as shown for other formae speciales (Kistler et al., 1987; Woudt et al., 1995). Given the large differences in virulence spectra, RFLPs and esterase profiles found in this study between the various VCGs in *F. oxysporum* f.sp. *dianthi* and in *F. redolens* f.sp. *dianthi*, it is difficult to conceive that pathogenic VCGs within the same taxon (for instance within *F. oxysporum* f.sp. *dianthi*) have a common ancestor. Rather, such distinct VCGs should be considered the clonal progeny of different individuals that each have independently acquired pathogenicity to carnation. The forma specialis concept is thus likely to be polyphyletic, at least in the case of *F. oxysporum* f.sp. *dianthi*.

Our results further substantiate that VCGs have a predictive value for races. Where virulence assays are tedious, time-consuming and often poorly reproducible, esterase profiling and DNA fingerprints provide promising alternatives. Not all methods are equally useful: in this study, the discriminative ability of esterase profiling was distinctly less than that of DNA fingerprinting. While tests with predictive value at race level can be derived from this study, this is not the case for tests at forma specialis level. If indeed formae speciales are not a natural but rather an artificial (although practical) concept, the possibility should

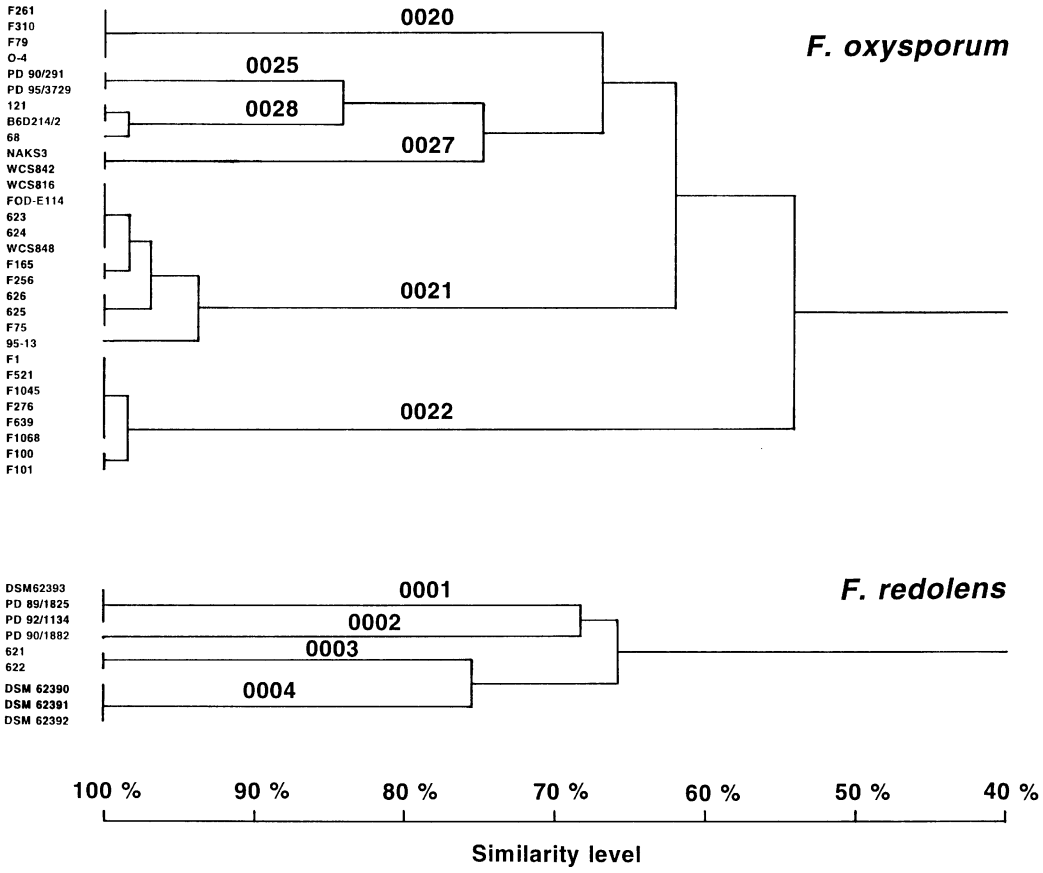

Figure 4. Dendrogram of restriction fragment length polymorphisms obtained with probe D4 for isolates of *Fusarium oxysporum* f.sp. *dianthi* and *F. redolens* f.sp. *dianthi*. Branches coincide with VCGs (indicated by four-digit codes) in both species. RFLP pattern diversity within VCGs is small compared to the diversity observed between VCGs.

in fact be discarded that molecular diagnostics can be developed at forma specialis level.

A certain degree of pathogenic and genetic diversity was encountered within individual VCGs of *F. oxysporum* f.sp. *dianthi*. Pathogenic diversity within VCG 0022 involves two races (1 and 8) that can only be distinguished from one another on few cvs. such as Pallas and Raggio di Sole [Garibaldi, 1983] and genetically are best classified as near-isogenic lines (present data; Migheli et al. (1996); C. Waalwijk, unpubl.). Both races also induce the same unusual wilt symptoms (Baayen et al., 1988). Differential resistance in these two cultivars to races 1 and 8 may be due to one or more resistance genes in these cultivars which are triggered by specific elicitors produced by isolates of race 1 and not by isolates of race 8. Race 8 may have arisen from race 1 by adaptation to resistance in cv. Pallas or one of its sports (colour mutants), which are traditionally widely grown because of their relative resistance to race 2. Pathogenic diversity within VCG 0021 involves four races (2, 5, 6 and 7), but we were not able to verify the distinction between these races. If at all races 2, 5, 6 and 7 are really distinct from one another, isolates previously classified as race 2 and/or VCG 0021 (Aloi and Baayen, 1993; Elena and Tjamos, 1995; Katan et al., 1989 and others) may equally well belong to race 5, 6 or 7. Minor variations in RFLP patterns and esterase profiles were observed particularly within VCG 0021, but could not be related to race designations under which the isolates were received. Because neither *F. oxysporum* nor *F. redolens* has a known sexual cycle, mechanisms to introduce pathogenic and genetic diversity within VCGs are largely restricted to mutations and transpositions carried on within clonal lineages. The relatively large diversity in RFLP pattern observed within the cosmopolitan VCG

0021 may therefore reflect a longer evolutionary history.

The origin of VCG 0021 and therefore of race 2 is most likely to be Europe, the gene center of *Dianthus caryophyllus* and the origin of carnation breeding. VCG 0021 has probably been imported to many carnation-growing countries with propagative material from Europe. Most other VCGs have a restricted geographic distribution. VCG 0028 (race 9) is restricted to Australia, where it is of similar importance as VCG 0021 (Kalc Wright et al., 1996). This VCG has probably evolved within Australia from unknown origin in the recent past, upon the introduction of carnation into Australia from Europe. VCG 0022 (races 1 and 8) seems to originate from the Italian Flower Riviera, one of the traditional centers of carnation breeding. Within the province of Liguria, races 1 and 8 are dominant in specific areas where the so-called Mediterranean cultivars have been grown for several decades (Garibaldi et al., 1986). Races 1 and 8 have been encountered only incidentally outside of Italy, mainly in the French part of the Flower Riviera and in Spain. Races 1 and 8 are avirulent to the so-called American-type carnations, developed in the United States since 1852 (Garibaldi, 1983; Kalc Wright, 1993). The origin of VCG 0020 (race 4) is debatable. Race 4 is associated with the American-type carnations (Garibaldi et al., 1986), and indeed the isolates of *F. oxysporum* f.sp. *dianthi* that we obtained from the United States (O–4 from California, and O-434 and O-1110 from Pennsylvania) all belong to VCG 0020. One of these (O-434) was already isolated in 1966 (P.E. Nelson, pers. comm.). On the other hand, race 4 has been frequently encountered in Italy since 1978 (Garibaldi, 1983; Garibaldi et al., 1986), and has now also been found in Israel (present data; see also reports by Ben-Yephet et al. (1992) and Manulis et al. (1994)), Spain (present data and J.L. Andrés Ares, pers. comm.) and Colombia (present data).

Correspondence between esterase profiles and VCGs as presently described for *F. oxysporum* f.sp. *dianthi* has previously been reported for three VCGs among isolates of *F. oxysporum* from crucifers (Bosland and Williams, 1987). Elias and Schneider (1992) were able to discriminate VCG 0031 of *F. oxysporum* f.sp. *lycopersici* from VCGs 0030 and 0032 by means of isozyme analysis. The latter two VCGs were indistinguishable by isozyme polymorphisms (Elias and Schneider, 1992) and RFLPs (Elias et al., 1993), and indeed have recently been found to be compatible with one another, although heterokaryon formation is slower than usual (J.J. Mes, pers. comm.). The aforementioned two studies involved the use of several enzymes including esterase, but only a limited number of esterase bands was distinguished. The present study is restricted to esterase profiles, but with a considerably higher resolution than reported by Bosland and Williams (1987) and Elias and Schneider (1992). Judged from esterase profiles, the genetic diversity in *F. redolens* f.sp. *dianthi* appeared rather small, suggesting a relatively young history of this species. The occurrence of VCGs in other formae speciales belonging to *F. redolens* instead of *F. oxysporum* remains to be investigated.

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