

# Genetic Diversity of Pathogenic and Nonpathogenic Populations of *Fusarium oxysporum* Isolated from Carnation Fields in Argentina

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## ABSTRACT

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In order to elucidate the origin of *Fusarium oxysporum* f. sp. *dianthi* in Argentina, the genetic diversity among pathogenic isolates together with co-occurring nonpathogenic isolates on carnation was investigated. In all, 151 isolates of *F. oxysporum* were obtained from soils and carnation plants from several horticultural farms in Argentina. The isolates were characterized using vegetative compatibility group (VCG), intergenic spacer (IGS) typing, and pathogenicity tests on carnation. Seven reference

strains of *F. oxysporum* f. sp. *dianthi* also were analyzed and assigned to six different IGS types and six VCGs. Twenty-two Argentinean isolates were pathogenic on carnation, had the same IGS type (50), and belonged to a single VCG (0021). The 129 remaining isolates were nonpathogenic on carnation and sorted into 23 IGS types and 97 VCGs. The same VCG never occurred in different IGS types. Our results suggest that the pathogen did not originate in the local populations of *F. oxysporum* but, rather, that it was introduced into Argentina. Given the genetic homogeneity within Argentinean isolates of *F. oxysporum* f. sp. *dianthi*, either IGS type or VCG can be used for the identification of the forma specialis *dianthi* currently in Argentina.

The fungal species *Fusarium oxysporum* Schlechtend. emend. W. C. Snyder & H. N. Hans. is ubiquitous in soils all over the world. However, based on many different criteria, this species is highly diverse. From a plant pathology point of view, this species includes nonpathogenic strains that survive saprophytically in soil and pathogenic strains that induce crown and root rots on several plant species, but the most damaging strains induce tracheomyces in many crops of economical importance (28). These wilt-inducing pathogens have a narrow host specificity, which led to the concept of forma specialis to designate strains pathogenic on a single plant species (4). From a genetic point of view, *F. oxysporum* also is highly diverse. Vegetative compatibility was one of the first methods used to evaluate genetic diversity in pathogenic populations of *F. oxysporum* and has been used to assign pathogenic isolates to vegetative compatibility groups (VCGs) (17,18,20,24,31). The clonal lineage concept generally is supported for VCGs, but there is significant genetic divergence between VCGs within several formae speciales (6,19,22). Indeed, phylogenetic studies have not confirmed initial hypotheses that formae speciales are homogeneous subspecies (5,19,30). Instead, the polyphyletic structure of many formae speciales now provides evidence for the multiple evolutionary origin of pathogenicity.

*F. oxysporum* f. sp. *dianthi* is the causal agent of vascular wilt of carnation (*Dianthus caryophyllus* L.), the most devastating disease of carnation worldwide (15,35). Six VCGs have been identified within *F. oxysporum* f. sp. *dianthi* (6). Based on restriction fragment length polymorphism (RFLP) analyses of total DNA, VCGs represent homogeneous genetic entities within *F. oxysporum* f. sp. *dianthi* (6,26); however, based on multiple gene

genealogies, this forma specialis is polyphyletic (5). VCG 0021 is phylogenetically distinct from the remaining five VCGs, which cluster close together in the same clade. These five VCGs have restricted geographic distribution, but the more cosmopolitan VCG 0021 has been recovered from France, Spain, Italy, Greece, Israel, The Netherlands, and Australia (1,6,13). In Argentina, carnation growing has expanded rapidly during the last 20 years. *Fusarium* wilt caused by *F. oxysporum* f. sp. *dianthi* is the most devastating disease of carnation in Argentina, especially in intensive crops in the La Plata region. However, nothing is known about the populations of *F. oxysporum* f. sp. *dianthi* in this region. Pathogenic isolates could have been introduced into the country, possibly through infested cuttings, or these isolates could have evolved in the local population from their soilborne relatives and recently have been selected for when a suitable host became available.

Despite their widespread occurrence and abundance, soil populations of *F. oxysporum* have received less attention than pathogenic populations. The studies that have been conducted found that these populations can be highly diverse, even within a single soil (2,3,11,12,16). These populations are an important pool of genetic diversity that has not been explored in any detail. Some pathogenic isolates of *F. oxysporum* are genetically more similar to other soilborne isolates than to other pathogenic isolates from the same forma specialis (3,33,37). Thus, studies of both pathogenic and nonpathogenic populations of *F. oxysporum* are needed to understand the origin of pathogenic strains.

In order to elucidate the origin of the forma specialis *dianthi* in Argentina, we have investigated the genetic diversity among pathogenic isolates together with co-occurring isolates nonpathogenic on carnation. Genetic similarity between these two groups of isolates may indicate that pathogenic strains could be recent derivatives from local nonpathogenic populations. Alternatively, genetic similarity between Argentinean pathogenic isolates and pathogenic isolates from a foreign source would suggest a recent introduction of the forma specialis *dianthi* in Argentina. The diversity of a large collection of *F. oxysporum* strains originating

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TABLE 1. Isolates of *Fusarium oxysporum* analyzed in this study

Isolates <sup>a</sup>	Geographic origin	Sample location <sup>b</sup>	Host/substrat <sup>c</sup>	Year	IGS type <sup>d</sup>	VCG <sup>e</sup>	Pathogenicity <sup>f</sup>
1	Argentina/La Plata	M0	Soil	2000	14	sm	Nonpathogen
124	Argentina/La Plata	M0	Soil	2000	51	sm	Nonpathogen
2	Argentina/La Plata	M1	Soil	2000	52	20	Nd
66	Argentina/La Plata	M1	Soil	2000	51	11	Nd
3	Argentina/La Plata	M2	Soil	2000	53	sm	Nonpathogen
67	Argentina/La Plata	M2	Soil	2000	3	sm	Nonpathogen
4	Argentina/La Plata	M3	Soil	2000	22	sm	Nonpathogen
126	Argentina/La Plata	M3	Soil	2000	14	sm	Nonpathogen
5	Argentina/La Plata	M4	Soil	2000	3	sm	Nonpathogen
69	Argentina/La Plata	M4	Soil	2000	53	sm	Nonpathogen
6	Argentina/La Plata	M5	Soil	2000	21	sm	Nonpathogen
70	Argentina/La Plata	M5	Soil	2000	51	sm	Nonpathogen
7	Argentina/La Plata	M6	Soil	2000	53	sm	Nonpathogen
71	Argentina/La Plata	M6	Soil	2000	58	sm	Nonpathogen
8	Argentina/La Plata	M7	Soil	2000	54	sm	Nonpathogen
72	Argentina/La Plata	M7	Soil	2000	59	sm	Nonpathogen
9	Argentina/La Plata	M8	Soil	2000	51	sm	Nonpathogen
73	Argentina/La Plata	M8	Soil	2000	53	sm	Nonpathogen
10, 74	Argentina/La Plata	M9	Soil	2000	51	sm	Nonpathogen
11	Argentina/La Plata	M10	Soil	2000	14	sm	Nonpathogen
12, 159	Argentina/La Plata	M11	Soil	2000	52	20	Nd
13	Argentina/La Plata	M12	Soil	2000	51	sm	Nonpathogen
129	Argentina/La Plata	M12	Soil	2000	14	sm	Nonpathogen
14	Argentina/La Plata	M13	Soil	2000	51	sm	Nonpathogen
181	Argentina/La Plata	M13	Soil	2000	33	91	Nonpathogen
15, 130	Argentina/La Plata	M14	Soil	2000	55	sm	Nonpathogen
16	Argentina/La Plata	M15	Soil	2000	33	sm	Nonpathogen
131	Argentina/La Plata	M15	Soil	2000	61	sm	Nonpathogen
17	Argentina/La Plata	M18	Soil	2000	58	sm	Nonpathogen
80	Argentina/La Plata	M18	Soil	2000	61	sm	Nonpathogen
201	Argentina/La Plata	M	Soil	1998	50	0021	Pathogen
18	Argentina/La Plata	I1	Soil	1999	20	76	Nonpathogen
81	Argentina/La Plata	I1	Soil	1999	60	sm	Nonpathogen
19	Argentina/La Plata	I5	Soil	1999	52	20	Nonpathogen
132	Argentina/La Plata	I5	Soil	1999	20	sm	Nonpathogen
20, 82	Argentina/La Plata	I6	Soil	1999	20	76	Nonpathogen
21	Argentina/La Plata	I7	Soil	1999	20	76	Nonpathogen
135	Argentina/La Plata	I7	Soil	1999	52	20	Nonpathogen
22	Argentina/La Plata	I8	Soil	1999	51	sm	Nonpathogen
134	Argentina/La Plata	I8	Soil	1999	20	76	Nd
83	Argentina/La Plata	I9	Soil	1999	53	sm	Nonpathogen
84	Argentina/La Plata	I9	Soil	1999	22	sm	Nonpathogen
232, 251	Argentina/La Plata	I	Soil	1999	50	0021	Pathogen
24	Argentina/La Plata	F1	Soil	2000	52	sm	Nonpathogen
85	Argentina/La Plata	F1	Soil	2000	14	sm	Nonpathogen
25	Argentina/La Plata	F2	Soil	2000	14	65	Nonpathogen
86	Argentina/La Plata	F2	Soil	2000	52	20	Nd
26	Argentina/La Plata	F3	Soil	2000	52	sm	Nonpathogen
138	Argentina/La Plata	F3	Soil	2000	42	sm	Nonpathogen
27	Argentina/La Plata	F4	Soil	2000	52	20	Nd
139	Argentina/La Plata	F4	Soil	2000	14	sm	Nonpathogen
28	Argentina/La Plata	F5	Soil	2000	52	20	Nd
89	Argentina/La Plata	F5	Soil	2000	52	sm	Nonpathogen
29	Argentina/La Plata	F6	Soil	2000	42	sm	Nonpathogen
141	Argentina/La Plata	F6	Soil	2000	52	20	Nd
30	Argentina/La Plata	F7	Soil	2000	52	20	Nonpathogen
91	Argentina/La Plata	F7	Soil	2000	52	20	Nd
31	Argentina/La Plata	F8	Soil	2000	14	sm	Nonpathogen
92	Argentina/La Plata	F8	Soil	2000	52	sm	Nonpathogen
32	Argentina/La Plata	F9	Soil	2000	56	sm	Nonpathogen
144	Argentina/La Plata	F9	Soil	2000	51	sm	Nonpathogen
33	Argentina/La Plata	F10	Soil	2000	20	sm	Nonpathogen
145	Argentina/La Plata	F10	Soil	2000	56	sm	Nonpathogen
34	Argentina/La Plata	F11	Soil	2000	52	20	Nd
146	Argentina/La Plata	F11	Soil	2000	32	sm	Nonpathogen
35, 147	Argentina/La Plata	F12	Soil	2000	42	sm	Nonpathogen

(continued on next page)

<sup>a</sup> Isolates followed by an asterisk were provided by R. Baayen (1,6).<sup>b</sup> Letters designate different commercial fields of carnation in La Plata (Argentina) and numbers designate different greenhouses in each field.<sup>c</sup> Race of the reference strains of *F. oxysporum* f. sp. *dianthi* is indicated in parentheses.<sup>d</sup> Intergenic spacer (IGS) types are defined in Table 3.<sup>e</sup> Isolates with the same number belong to the same vegetative compatibility group (VCG); sm designates single-member VCGs.<sup>f</sup> Nd indicates not determined.

TABLE 1. (continued from preceding page)

Isolates <sup>a</sup>	Geographic origin	Sample location <sup>b</sup>	Host/substrat <sup>c</sup>	Year	IGS type <sup>d</sup>	VCG <sup>e</sup>	Pathogenicity <sup>f</sup>
224, 225	Argentina/La Plata	F	Soil	2000	50	0021	Pathogen
36, 97	Argentina/La Plata	Y1	Soil	2000	20	76	Nd
37	Argentina/La Plata	Y2	Soil	2000	52	20	Nd
98	Argentina/La Plata	Y2	Soil	2000	52	sm	Nonpathogen
38	Argentina/La Plata	Y3	Soil	2000	20	sm	Nonpathogen
39	Argentina/La Plata	Y4	Soil	2000	52	20	Nd
100	Argentina/La Plata	Y4	Soil	2000	52	sm	Nonpathogen
151	Argentina/La Plata	Y5	Soil	2000	52	20	Nd
41, 152	Argentina/La Plata	Y6	Soil	2000	20	76	Nd
42	Argentina/La Plata	Y7	Soil	2000	51	sm	Nonpathogen
153	Argentina/La Plata	Y7	Soil	2000	62	sm	Nonpathogen
43	Argentina/La Plata	Y8	Soil	2000	52	sm	Nonpathogen
154	Argentina/La Plata	Y8	Soil	2000	63	sm	Nonpathogen
44, 105	Argentina/La Plata	Y10	Soil	2000	20	sm	Nonpathogen
45	Argentina/La Plata	Y11	Soil	2000	20	76	Nd
106	Argentina/La Plata	Y11	Soil	2000	20	sm	Nonpathogen
46	Argentina/La Plata	Y12	Soil	2000	3	sm	Nonpathogen
156	Argentina/La Plata	Y12	Soil	2000	22	sm	Nonpathogen
47	Argentina/La Plata	Y13	Soil	2000	51	sm	Nonpathogen
108	Argentina/La Plata	Y13	Soil	2000	20	sm	Nonpathogen
48	Argentina/La Plata	Y13b	Soil	2000	33	sm	Nonpathogen
191	Argentina/La Plata	Y13b	Soil	2000	52	20	Nd
49	Argentina/La Plata	Y14	Soil	2000	51	sm	Nonpathogen
110	Argentina/La Plata	Y14	Soil	2000	14	sm	Nonpathogen
50	Argentina/La Plata	Y15	Soil	2000	52	20	Nd
111	Argentina/La Plata	Y15	Soil	2000	52	sm	Nonpathogen
51, 196	Argentina/La Plata	Y16	Soil	2000	57	sm	Nonpathogen
99	Argentina/La Plata	Y3	Soil	2000	50	0021	Pathogen
52	Argentina/La Plata	T1	Soil	2000	5	sm	Nonpathogen
113	Argentina/La Plata	T1	Soil	2000	23	sm	Nonpathogen
53	Argentina/La Plata	T2	Soil	2000	33	91	Nd
114	Argentina/La Plata	T2	Soil	2000	3	sm	Nonpathogen
54	Argentina/La Plata	T3	Soil	2000	58	sm	Nonpathogen
162	Argentina/La Plata	T3	Soil	2000	51	11	Nonpathogen
55	Argentina/La Plata	T4	Soil	2000	23	sm	Nonpathogen
116	Argentina/La Plata	T4	Soil	2000	51	sm	Nonpathogen
56	Argentina/La Plata	Z1	Soil	2000	52	sm	Nonpathogen
164	Argentina/La Plata	Z1	Soil	2000	42	sm	Nonpathogen
57	Argentina/La Plata	Z2	Soil	2000	59	sm	Nonpathogen
167	Argentina/La Plata	Z2	Soil	2000	14	65	Nd
58	Argentina/La Plata	Z3	Soil	2000	21	sm	Nonpathogen
119	Argentina/La Plata	Z3	Soil	2000	55	sm	Nonpathogen
59	Argentina/La Plata	U1	Soil	2000	52	sm	Nonpathogen
170	Argentina/La Plata	U1	Soil	2000	51	sm	Nonpathogen
171	Argentina/La Plata	U2	Soil	2000	61	sm	Nonpathogen
61	Argentina/La Plata	X1	Soil	2000	51	sm	Nonpathogen
172	Argentina/La Plata	X1	Soil	2000	14	sm	Nonpathogen
175	Argentina/La Plata	X2	Soil	2000	52	20	Nd
177	Argentina/La Plata	X2	Soil	2000	52	sm	Nonpathogen
122	Argentina/La Plata	D1	Soil	2000	52	20	Nonpathogen
178	Argentina/La Plata	D1	Soil	2000	51	sm	Nonpathogen
64	Argentina/La Plata	P1	Soil	2000	51	sm	Nonpathogen
180	Argentina/La Plata	P1	Soil	2000	14	sm	Nonpathogen
1001 32/99, 1003 33/99	Argentina/La Plata	S	Carnation	1993	20	76	Nonpathogen
1005 34/99, 1008 36/99	Argentina/La Plata	Y	Carnation	1997	51	sm	Nonpathogen
1007 35/99	Argentina/La Plata	Q	Carnation	1997	51	sm	Nonpathogen
218, 249, 250,	Argentina/La Plata	F	Carnation	1999	50	0021	Pathogen
227	Argentina/La Plata	I	Carnation	1998	50	0021	Pathogen
143, 152, 200, 231, 260	Argentina/La Plata	M	Carnation	1999	50	0021	Pathogen
136	Argentina/Fcio Varela	A	Carnation	1995	50	0021	Pathogen
137	Argentina/Berazategui	B	Carnation	1993	50	0021	Pathogen
245	Argentina/La Plata	C	Carnation	1997	50	0021	Pathogen
138	Argentina/Berazategui	D	Carnation	1994	50	0021	Pathogen
74	Argentina/Marcos Paz	G	Carnation	1992	50	0021	Pathogen
R1	Argentina/Rosario	R	Carnation	1993	50	0021	Pathogen
67	Argentina/La Plata	T	Carnation	1992	50	0021	Pathogen
F140*	Italy	...	Carnation (4)	Unknown	56	0020	Pathogen
WCS850*	The Netherlands	...	Carnation (2)	Unknown	50	0021	Pathogen
WCS816*	The Netherlands	...	Carnation (2)	Unknown	50	0021	Pathogen
F100*	Italy	...	Carnation (1)	Unknown	12	0022	Pathogen
PD 90/291*	The Netherlands	...	Carnation (11)	Unknown	51	0025	Pathogen
NAKS3*	The Netherlands	...	Carnation (10)	Unknown	64	0027	Pathogen
B6D214/2*	Australia	...	Carnation (9)	Unknown	61	0028	Pathogen

from soils and carnation plants from several horticultural farms in Argentina together with reference strains of *F. oxysporum* f. sp. *dianthi* was assessed using VCG analysis, RFLP analysis of the ribosomal intergenic spacer (IGS), and pathogenicity tests on carnation. An additional objective was to seek for specific traits enabling the identification of the pathogenic form of *F. oxysporum*

on carnation in Argentina. Part of this work was already published as a disease note (25).

## MATERIALS AND METHODS

**Fungal isolates.** In all, 151 isolates of *F. oxysporum* were recovered from soil and carnation plants in Argentina (Table 1). Of these, 130 isolates were sampled from soil from 64 greenhouses on 10 different horticultural farms with Fusarium wilt in the major carnation-producing area of Argentina (Green belt of La Plata, Province of Buenos Aires). The different horticultural farms were separated from each other by 1 to 8 km. To avoid pathogenic isolates of *F. oxysporum* and to sample isolates representative of the indigenous soilborne populations, samples were taken from soil adjacent to the carnation greenhouses. Each soil sample was a composite of five subsamples of approximately 100 g each. Twenty-one isolates of *F. oxysporum* were collected from diseased and symptomless plants of both American and Mediterranean carnation types, originating from different producing areas in Argentina. Seven reference strains representative of all known VCGs within *F. oxysporum* f. sp. *dianthi* also were included in this study (Table 1).

For fungal isolation, soil samples were air dried for 10 days at room temperature, mixed manually, and crushed with a mortar to pass through a 4-mm sieve. Soil (5 g) was introduced into a glass beaker, suspended in 45 ml of sterile distilled water, and shaken for 20 min using a magnetic shaker. Then, 10-fold dilutions were made up to  $10^{-4}$  and 1 ml of each dilution was plated on Komada's selective medium (23) with five replications. The plates were incubated at 23 to 25°C for 5 to 7 days. Fungal isolation was performed from plates containing individual colonies corresponding to the dilution  $10^{-2}$ ,  $10^{-3}$ , or  $10^{-4}$ , depending on the soil sample. Five to eight arbitrarily chosen colonies with cultural and morphological features of *F. oxysporum* were transferred to potato dextrose agar (PDA) (29). For complementary taxonomic identification, the isolates were transferred to carnation leaf agar (29), incubated under fluorescent lights at 22°C for 7 to 14 days, and identified morphologically according to the criteria of Nelson et al. (29). Two isolates of *F. oxysporum* per soil sample were retained. For fungal isolation from plants, roots were washed under tap water, split in half, and surface sterilized in 0.5% NaOCl for 2 min. Roots were rinsed in sterile distilled water and placed on PDA. After 5 to 7 days of incubation at 23 to 25°C, colonies of *F. oxysporum* were identified as above. Single-spore cultures were made for the 151 isolates of *F. oxysporum* and were stored in mineral oil or silica gel at 4°C (36).

**Genotypic characterization.** DNA was extracted from the 151 isolates of *F. oxysporum* collected from soil and plants and the seven reference strains by using a rapid minipreparation procedure (12). All the isolates were characterized by RFLP analysis of the ribosomal IGS (10). A fragment of the IGS was amplified by PCR with oligonucleotide primers PNFo (5'-CCCGCCTGGCTGCGTCCGACTC-3') and PN22 (5'-CAAGCATATGACTACTGGC-3') and digested with seven restriction enzymes: *AluI*, *HaeIII*, *HinfI*, *MspI*, *RsaI*, *ScrFI*, and *XhoI*. Each isolate was assigned to an IGS type defined by the combination of the restriction patterns obtained with the seven enzymes. Map locations of the restriction sites in the IGS region were determined from the restriction patterns and previously published data (10–12). The pairwise restriction site differences between IGS types were represented as a dendrogram with the numerical taxonomy and multivariate analysis system (NTSYS) computer program (version 2.0; State University of New York, Stony Brook).

**Vegetative compatibility testing.** VCGs were determined through the complementation of nitrate-nonutilizing (*nit*) mutants as a visual indicator of heterokaryon formation (31). Mutants were generated for each strain on potato-sucrose chlorate medium (KPS) and minimal chlorate medium (KMM) with 1.5% potas-

TABLE 2. Restriction patterns of polymerase chain reaction-amplified intergenic spacer fragments of *Fusarium oxysporum* isolates<sup>a</sup>

Enzymes	Fragments (bp)
<i>AluI</i>	
1	850, 500, 270, 85
2	540, 500, 320, 270, 85
3	740, 500, 390, 85
6	740, 500, 235, 145, 85
20	1115, 500, 85
<i>HaeIII</i>	
1	460, 145, 130, 115*, 100, 95*, 85, 80, 60, 55*
2	460, 175, 145, 130, 100, 95*, 85, 80, 70, 60, 55
4	500, 175, 145, 130, 100, 95*, 85, 80, 70, 60, 55
6	460, 145, 115*, 100*, 95*, 85, 80, 60, 55*
8	460, 145, 130, 115, 100, 95*, 85, 80, 70, 60, 55*
11	480, 145, 130, 115*, 100, 95*, 85, 80, 60, 55*
19	460, 175, 145, 130, 115, 100, 95*, 85, 80, 60, 55
21	460, 195, 145, 130, 115, 100, 95*, 85, 60, 55*
25	270, 190, 175, 145, 130, 115, 100, 95*, 85, 60, 55*
27	460, 150, 145, 115*, 100, 95*, 85, 80, 60, 55*
28	460, 145, 130, 115*, 100, 95, 85, 60, 55*
29	600, 145, 130, 115, 100, 95*, 85, 80, 60, 55*
<i>HinfI</i>	
1	700, 550, 210, 195
2	700, 550, 210, 120, 80
5	700, 580, 210, 120, 80
19	700, 580, 210, 195
20	700, 550, 210, 130, 80
25	700, 330, 210, 200, 120, 80
<i>MspI</i>	
1	560, 275, 200, 105, 95, 85, 75, 60*, 50
4	420, 275, 200, 135, 105, 95, 85, 75, 60*, 50
6	560, 275, 200, 105, 95, 75, 60*, 50
11	560, 275, 200, 115, 105, 85, 75, 60*, 50
19	590, 275, 200, 105, 95, 85, 75, 60*, 50
22	470, 275, 200, 105*, 95, 85, 75, 60*, 50
23	385, 275, 200, 185, 105, 95, 85, 75, 60*, 50
24	560, 275, 200, 105*, 95, 85, 60*, 50
<i>RsaI</i>	
1	610, 560, 400, 90
3	1200, 400, 90
4	650, 560, 400, 90
9	650, 540, 400, 90
<i>ScrFI</i>	
1	460, 215, 180, 170, 135, 110, 90, 85, 60
2	460, 305, 180, 170, 135, 110, 90, 60
11	460, 215, 180, 170, 135, 110*, 85, 60
12	460, 215, 180, 170, 135, 90, 85*, 60
12b	460, 215, 180, 170, 135, 90, 85, 75, 60
13	470, 215, 180, 170, 135, 120, 90, 85, 60
19	460, 305, 180, 170, 135, 110, 90, 60, 55
20	460, 265, 215, 180, 135, 110, 90, 60
<i>XhoI</i>	
1	1300, 370
2	1670
3	650*, 370

<sup>a</sup> For each restriction enzyme, the various patterns are indicated by numbers in the first column, and the sizes in base pairs (bp) of the corresponding restriction fragments are indicated in the second column. Estimates of fragment sizes were determined by electrophoresis in 4 to 6% Nusieve 3:1 agarose (FMC, Rockland, ME) and by comparison with the molecular weight marker VIII (Roche Diagnostic, Meylan, France) with measurements rounded to the nearest 5 bp. This was done for purposes of comparison among isolates; the values do not reflect absolute base pair fragment sizes. Restriction fragments of <50 bp were not taken into consideration because they were not clearly resolved by electrophoresis. Asterisks indicate two restriction fragments of the same size (doublet). Numbers corresponding to the different restriction patterns follow those previously described (12).

sium chlorate (31). Because many isolates failed to form mutants on these media, chlorate concentration often was increased to 3%, and in some cases to 4.5%. The fast-growing chlorate-resistant sectors originating from the initially restricted colony were transferred to minimal medium (MM) (31) containing nitrate as the sole nitrogen source. Colonies that appeared thin and expansive without any aerial mycelium were considered to be *nit* mutants, and *nit* mutants were classified as *nit1*, *nit3*, or NitM based on their phenotype on media containing one of three different nitrogen sources (nitrate, nitrite, and hypoxanthine) (7). At least one *nit1* or *nit3* mutant and one NitM mutant were obtained from each isolate and used for complementation tests. Pairings were made on MM in 9-cm petri dishes. Three mutants were inoculated on each plate, forming a triangle configuration, and the plates were incubated at 23 to 25°C in the dark and scored for complementation 7 and 14 days after incubation. Different mutants derived from the same strain were paired with one another to test for self-incompatibility (8). Vegetative compatibility was determined by pairing complementary *nit* mutants derived from all 151 isolates in all pairwise combinations. When two mutants formed a visible and robust heterokaryon indicated by the presence of dense aerial mycelium, the corresponding strains were placed in the same VCG. Once the VCGs were identified, representative isolates of each group were tested for compatibility with the reference strains of *F. oxysporum* f. sp. *dianthi* (Table 1). All pairings were repeated at least twice.

**Pathogenicity tests.** In all, 127 isolates of *F. oxysporum* were tested for pathogenicity on carnation cv. Pink Francesco and cv. Rendez-vous, both susceptible to all races of *F. oxysporum* f. sp. *dianthi*. The 127 isolates tested included the 21 isolates collected from plants and 106 soil isolates representing at least one isolate per VCG. Isolates were grown on PDA and a 5-mm plug was transferred to petri dishes, 16 cm in diameter, containing moistened (30 to 40%), autoclaved polished rice. The dishes were

incubated in darkness for 10 days and the colonized rice was dried and powdered. The number of propagules per gram of rice was determined by serial dilutions on PDA plates. The inoculum of *F. oxysporum* was added to autoclaved soil at a concentration of 10<sup>4</sup> propagules per g of dry soil. For each fungal isolate tested, 16 rooted carnation cuttings were planted individually in pots of 1 liter. The 16 pots were distributed in four blocks, each block containing four pots per isolate tested. Controls consisted of rooted cuttings planted in autoclaved soil mixed with sterile powdered polished rice. Plants were grown in a greenhouse with only natural daylight. Initial Fusarium wilt symptoms were recorded 50 days after inoculation. Symptoms were assessed weekly for 90 additional days using a wilt-severity index corresponding to a scale from 0 (no disease symptoms) to 5 (complete wilt and death). Noninoculated plants maintained as controls under the same conditions remained healthy throughout the pathogenicity test.

## RESULTS

**Genotypic characterization.** Oligonucleotide primers PNFO and PN22 permitted amplification of a single DNA fragment of ≈1.7 kb for each of the 158 isolates. The PCR products were digested with each of the seven restriction enzymes. Depending on the enzyme, 3 to 12 different restriction patterns were obtained among the 158 isolates of *F. oxysporum* (Table 2). In all, 26 combinations of patterns, representing 26 IGS types, were identified among the 158 isolates (Table 3). The seven reference strains of *F. oxysporum* f. sp. *dianthi* had one of six IGS types. The 151 isolates recovered from soil and carnation plants in Argentina were distributed in 24 IGS types, including 5 IGS types common to the reference strains (Table 1). IGS types were represented by 1 to 30 isolates (Table 4). In all, 80 restriction sites, including 39 polymorphic sites, were identified among the 26 IGS types. The

TABLE 3. Intergenic spacer (IGS) types and restriction patterns of *Fusarium oxysporum* isolates revealed by restriction fragment length polymorphism analysis of polymerase chain reaction-amplified IGS sequences

IGS type <sup>c</sup>	Restriction patterns of amplified IGS fragments digested with enzymes <sup>a</sup>							Representative isolates <sup>b</sup>	
	<i>AluI</i>	<i>HaeIII</i>	<i>HinfI</i>	<i>MspI</i>	<i>RsaI</i>	<i>ScrFI</i>	<i>XhoI</i>	Nonpathogenic	Pathogenic
3	1	1	2	1	1	1	2	5	...
5	1	1	2	1	1	2	2	52	...
12	1	6	2	4	1	1	2	...	F100
14	1	4	5	1	3	1	1	1	...
20	2	1	2	1	3	1	2	18	...
21	1	8	2	6	1	1	1	6	...
22	1	11	2	11	4	11	2	4	...
23	1	1	2	1	3	1	2	55	...
32	2	1	2	1	3	12	2	146	...
33	2	1	2	1	3	12b	2	16	...
42	1	8	2	1	1	1	1	35	...
50	3	2	1	22	1	2	1	...	99, WCS850
51	1	21	5	19	1	1	2	9	PD 90/291
52	1	1	2	23	1	1	1	2	...
53	1	27	2	19	1	1	2	3	...
54	6	28	19	6	3	19	1	8	...
55	1	21	25	24	1	20	2	15	...
56	1	25	20	6	9	12	1	32	F140
57	20	1	5	1	1	13	2	51	...
58	2	6	2	1	3	12b	2	54	...
59	1	19	2	1	1	1	2	57	...
60	1	29	2	1	3	13	2	81	...
61	1	1	2	1	4	1	1	80	B6D214/2
62	1	1	2	19	1	1	2	153	...
63	1	8	2	23	1	1	1	154	...
64	1	8	2	6	1	1	3	...	NAKS3

<sup>a</sup> Numbers designate the various patterns obtained for each restriction enzyme and follow those previously described (12). The restriction patterns are described in Table 2.

<sup>b</sup> Isolates are described in Table 1; ... indicates that no pathogenic or nonpathogenic isolate was found in the IGS type.

<sup>c</sup> IGS types represent the combination of patterns obtained with seven restriction enzymes. The numbers assigned to IGS types 3 to 42 follow those previously described (12).

restriction site differences between IGS types are illustrated in Figure 1.

**Vegetative compatibility grouping.** During VCG testing, there was no evidence of self-incompatibility, and all of the compatibility reactions were easily discernible. The 151 isolates of *F. oxysporum* collected in Argentina were assigned to 98 VCGs (Table 1). Twenty-two isolates recovered from soil or diseased plants from different locations all were compatible with reference strains WCS816 and WCS850 and were formally classified as VCG 0021. All isolates from diseased plants were in this group. The 129 remaining isolates were from soil and symptomless plants and sorted into 97 VCGs. VCG 20 included 19 isolates, VCG 76 had 12 isolates, and VCGs 11, 65, and 91 each had two isolates (Table 4). The 92 remaining VCGs all contained only a single isolate. Isolates in the same VCG also had the same IGS type.

**Pathogenicity tests.** Disease reactions for the 127 isolates of *F. oxysporum* tested were clear cut. Either severe symptoms were induced on carnation plants within 50 to 90 days (disease index 5) or no disease symptoms at all (disease index 0) were observed. Twenty-two isolates were pathogenic and assigned to the forma specialis *dianthi*. These 22 pathogenic isolates were the ones that belonged to VCG 0021, as did two reference strains of *F. oxysporum* f. sp. *dianthi* (Table 1). The other 105 isolates of *F. oxysporum* were nonpathogenic on carnation.

**Correspondence between pathogenicity, VCG, IGS types, and the origin of the isolates.** The 22 pathogenic Argentinean isolates all had IGS type 50 and VCG 0021, as did the two reference strains of *F. oxysporum* f. sp. *dianthi* race 2 (Table 1). The other five reference strains of *F. oxysporum* f. sp. *dianthi* in this study represent five other VCGs and were assigned to five other IGS types. Thus, IGS type 50 was specific for VCG 0021. The 22 Argentinean isolates identified as *F. oxysporum* f. sp. *dianthi* in-

cluded the 16 isolates recovered from diseased plants and six soil isolates. The remaining isolates were nonpathogenic toward carnation and had 23 IGS types and 97 VCGs that were different from those of the pathogenic strains. The maximum frequency (*F*) with which nonpathogenic isolates of VCG 0021 could be occurring and not have been detected with 95% certainty was calculated from the following formula:  $(1 - F)^N = 0.05$ , where *N* is the number of isolates. With our sample of 151 isolates analyzed, this maximum frequency was found to be 2%.

Each IGS type included 1 to 21 VCGs (Table 4); however, most VCGs were single-member. Only two VCGs included more than two isolates. VCG 76 included 12 soil isolates from three locations and VCG 20 included 19 soil isolates from six different locations. Genotypic diversity was found within the populations sampled from soil. For each of the six locations represented by at least six isolates of *F. oxysporum* (M, I, F, Y, T, and Z), 6 to 13 IGS types were detected. Finally, 3 IGS types (51, 56, and 61) of the 23 detected were found in both isolates of *F. oxysporum* that were nonpathogenic toward carnation and the reference strains of *F. oxysporum* f. sp. *dianthi* (Table 3), but the nonpathogenic and pathogenic isolates were in different VCGs.

TABLE 4. Intergenic spacer (IGS) types, vegetative compatibility groups (VCGs) and origin of 151 isolates of *Fusarium oxysporum* collected in Argentina

IGS type <sup>a</sup>	VCGs <sup>b</sup>	No. of isolates	Commercial field <sup>c</sup>
3	4 VCG sm	4	M,Y,T
5	1 VCG sm	1	T
14	VCG 65	2	F,Z
20	10 VCG sm	10	M,F,Y,X,P
	VCG 76	12	I,Y,S
21	7 VCG sm	7	I,F,Y
22	2 VCG sm	2	M,Z
23	3 VCG sm	3	M,I,Y
32	2 VCG sm	2	T
33	1 VCG sm	1	F
42	VCG 91	2	M,T
	2 VCG sm	2	M,Y
50	5 VCG sm	5	F,Z
51	VCG 0021	22	M,I,F,Y
51	VCG 11	2	M,T
52	20 VCG sm	20	M,I,F,Y,T,U,X,D,P
	VCG 20	19	M,I,F,Y,X,D
	11 VCG sm	11	F,Y,Z,U,X
53	5 VCG sm	5	M,I
54	1 VCG sm	1	M
55	3 VCG sm	3	M,Z
56	2 VCG sm	2	F
57	2 VCG sm	2	Y
58	3 VCG sm	3	M,T
59	2 VCG sm	2	M,Z
60	1 VCG sm	1	I
61	3 VCG sm	3	M,U
62	1 VCG sm	1	Y
63	1 VCG sm	1	M,Z

<sup>a</sup> IGS types are defined in Table 3.

<sup>b</sup> Single-member VCGs designated by "sm".

<sup>c</sup> Letters designate different commercial fields of carnation in La Plata (Argentina).

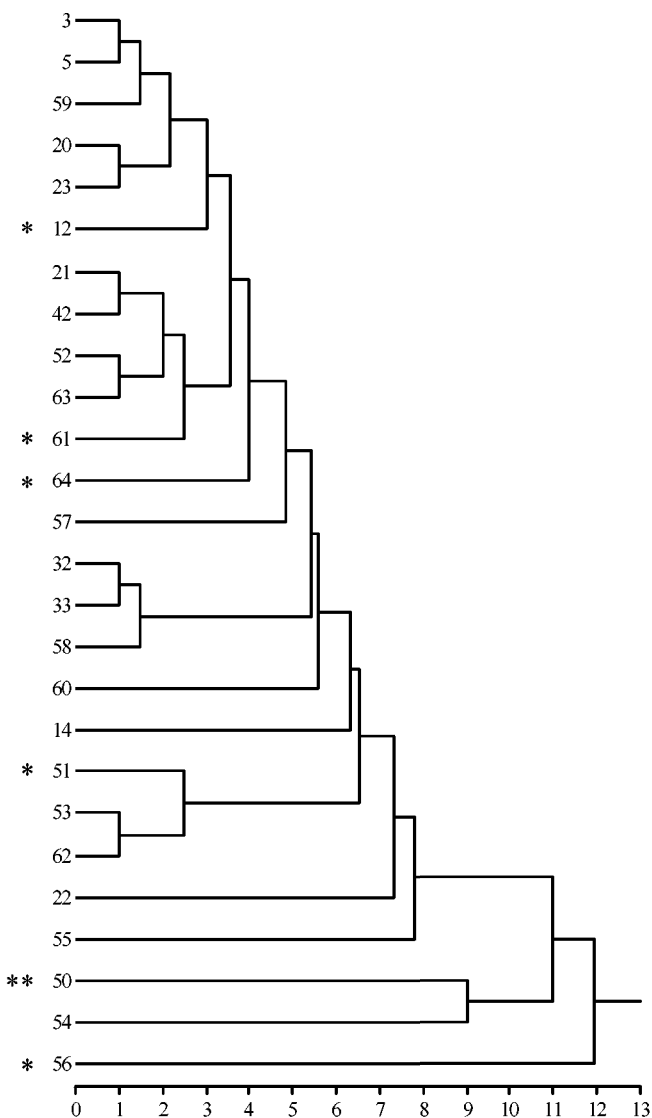


Fig. 1. Dendrogram showing the restriction site differences in the intergenic spacer (IGS) region of *Fusarium oxysporum* between the 26 IGS types defined in Table 3. The IGS types marked by an asterisk include isolates of *F. oxysporum* f. sp. *dianthi*. The IGS type marked by two asterisks corresponds to *F. oxysporum* f. sp. *dianthi* in Argentina. The scale indicates the number of restriction site differences.

## DISCUSSION

In Argentina, Fusarium wilt of carnation became a very severe disease when the cropping of this flower expanded rapidly in the 1970s. A common explanation for this problem is that the pathogen was introduced accidentally, probably along with imported cuttings. Alternatively, pathogenic strains could have been selected from the indigenous populations of soilborne *F. oxysporum*. We tested these hypotheses by analyzing genetic diversity in both pathogenic and nonpathogenic populations of *F. oxysporum* with VCG analyses and IGS typing. All isolates in the same VCG also had the same IGS type. This result is consistent with previous studies that suggested that VCGs have a clonal origin and may be considered as the population unit in *F. oxysporum* (21,34).

Both VCG and IGS typing revealed the absence of diversity among strains of *F. oxysporum* pathogenic to carnation in Argentina, which all had the same IGS type (type 50) and belonged to the same VCG (0021). On the contrary, the nonpathogenic isolates on carnation were very diverse, with 129 isolates divided into 23 IGS types and 97 VCGs. VCG 0021 was not detected among nonpathogenic isolates on carnation. The maximum frequency with which nonpathogenic isolates of VCG 0021 could be occurring and not have been detected with 95% certainty is 0.02 with our sample size. IGS typing also clearly differentiated the Argentinean nonpathogenic and pathogenic isolates on carnation. These results indicate that the pathogen on carnation and the co-occurring soilborne populations of *F. oxysporum* in Argentina have distinct origins. Moreover, the IGS type and VCG specific of the pathogenic isolates in Argentina also were shared by the reference strains of *F. oxysporum* f. sp. *dianthi* that belong to the most widely distributed VCG (0021) in the world. All together, these results do not support the hypothesis that the pathogen is a recent selection from the local populations of *F. oxysporum*, but rather that it was introduced into Argentina. In addition, IGS type 50 also was found to be specific to VCG 0021 of *F. oxysporum* f. sp. *dianthi*. Indeed, this IGS type never has been detected in populations of *F. oxysporum* previously analyzed (10–12). Given the genetic homogeneity within Argentinean isolates of *F. oxysporum* f. sp. *dianthi* and the specificity of their VCG and IGS type, either tool could be used for the identification of the forma specialis *dianthi* in Argentina, instead of the time- and space-consuming experiments of inoculation of the host plant usually performed to confirm the pathogenicity of isolates. However, because of the similarity of IGS types between strains of *F. oxysporum* f. sp. *dianthi* and soilborne isolates in Argentina, the possibility of the future emergence of a locally derived pathogen can not be excluded.

Similar studies have been conducted for other formae speciales of *F. oxysporum* to try to understand the origin of a pathogen in a given area. Results vary, and both hypotheses, introduction from a foreign source and development of pathogens within a preexisting population, have been supported. For example, Mouyna et al. (27) found that the strains of *F. oxysporum* f. sp. *elaeidis* responsible for Fusarium wilt in newly planted oil palm groves in South America were genetically similar to pathogenic strains from Western Africa. By analyzing genetic variation in *F. oxysporum* f. sp. *radicis-lycopersici* in Florida, Rosewich et al. (32) identified a founder population closely related to isolates from Europe. In both cases, the authors suggest a recent introduction or migration of the pathogenic strain from foreign countries where the populations preexisted. In contrast, Gordon and Okamoto (16) found that some strains of *F. oxysporum* f. sp. *melonis* from California had the same mitochondrial genotype as some co-occurring soilborne isolates. Similarly, by using multilocus DNA sequences, Skovgaard et al. (33) failed to clearly distinguish pathogenic strains of *F. oxysporum* colonizing the roots of pea from soilborne populations in Denmark. Both authors

concluded that the pathogen could have been recently derived from the preexisting soilborne populations of *F. oxysporum* or that the nonpathogenic populations could be composed of pathogenic strains having lost their pathogenicity.

This limited survey of the literature dealing with the diversity affecting populations of *F. oxysporum* shows that there probably is no general rule that applies to the origin of the pathogenic populations of all formae speciales. Some pathotypes probably have a clonal origin and may have spread worldwide without significant genetic exchange, whereas other pathotypes are closely related to local soilborne populations, from which they may have evolved. It must be stressed that only a few studies have considered these other soilborne populations of *F. oxysporum*, which may harbor the majority of the genetic diversity within the species. More studies of these populations of *F. oxysporum* are needed to get a more accurate view of the relationships between populations of *F. oxysporum* pathogenic and nonpathogenic on a specific host.

The reference strains of *F. oxysporum* f. sp. *dianthi* used in this study had been characterized already for several criteria but not for IGS type (1,5,6). They are representative of the six VCGs already identified within the forma specialis, each VCG corresponding to a different IGS type. The two reference strains that belonged to the same VCG also had the same IGS type. There was no significant clustering of the pathogenic strains based on IGS type. These results are consistent with those of Baayen et al. (5,6), who reported that the genetic divergence between isolates of *F. oxysporum* f. sp. *dianthi* from the same VCG was low, but was high between VCGs. These authors suggest a multiple evolutionary origin for the pathogenicity to carnation in *F. oxysporum* that yields the *dianthi* forma specialis.

VCG typing allowed further discrimination of the isolates nonpathogenic on carnation within the IGS types. The 129 isolates of *F. oxysporum* were distributed into 97 VCGs, which is much higher than the number of VCGs found in most pathogenic populations (17,18). Previous attempts to classify nonpathogenic isolates of *F. oxysporum* based on vegetative compatibility also have resulted in a large number of VCGs (9,14,16,34). In our study, 71% of the isolates were in single-member VCGs. However, two VCGs included 12 and 19 isolates, and were found to be common to three and six soils, respectively. These VCGs may represent ancient indigenous populations well established in these soils. Alternatively, they may be favored or distributed by a cultural practice common to carnation growers. Until now, the few studies of VCGs in soilborne populations of *F. oxysporum* usually have paired isolates originating from the same soil. But some particular VCGs may have a widespread distribution in soils from different parts of the world. To answer this question, many isolates from different soils need to be tested. If the relationship between VCG and IGS type that we detected holds, then the IGS type could be used to make initial groups, and only isolates with the same IGS type would need to be tested for vegetative compatibility. Such studies could give us a new picture of the VCG diversity in soilborne populations of *F. oxysporum*.

Previously, most diversity studies of *F. oxysporum* were conducted with strains from a single forma specialis, which strengthened the idea that formae speciales were homogeneous entities. Our results show the value of evaluating populations of *F. oxysporum* that are both pathogenic and nonpathogenic on the host under study and the necessity of conducting similar studies at a larger scale. Recently, the genetic and evolutionary relationships within *F. oxysporum* were investigated for several formae speciales (5). Studies expanded to include both the pathogenic and the nonpathogenic diversity of *F. oxysporum* at a broad geographic scale might change current perceptions of *F. oxysporum* and may increase our understanding of the origin of the pathogenic diversity in this complex fungal species.

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