Genetic Variation in *Fusarium oxysporum* from Cyclamen

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ABSTRACT

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Fusarium isolates were recovered from cyclamen plant material and from public culture collections. Isolates were identified to species, and the majority (79/90) were *F. oxysporum*, although isolates of *F. moniliforme*, *F. equiseti*, and *F. graminearum* also were recovered. The *F. oxysporum* isolates were subdivided further based on pathogenicity to young cyclamen plants in greenhouse tests, vegetative compatibility tests, hybridization with a repetitive DNA (fingerprint) probe, and organization of the ribosomal intergenic spacer (IGS) sequences. Fifty-three pathogenic and twenty-six nonpathogenic isolates were identified. The pathogenic isolates could be subdivided into three clonal lineages, each containing a single vegetative compatibility group (VCG), whose members had similar IGS organization and DNA fingerprints. The nonpathogenic isolates could be distinguished clearly from the pathogenic isolates, and usually one another, using either the VCG or the fingerprint criteria, but not necessarily the IGS criterion.

Additional keywords: *Fusarium oxysporum* var. *aurantiacum*.

Fusarium wilt of cyclamen (*Cyclamen persicum* Mill.) was first described by Wollenweber and Reinking (53) as *Fusarium oxysporum* Schlechtend.:Fr. var. *aurantiacum* (Link) Wr. A similar disease was described by Gerlach (19), and the causal agent was identified as *Fusarium oxysporum* Schlechtend.:Fr. f. sp. *cyclaminis* Gerlach. These two names were synonymized by Gordon in 1965 (22). The disease has been reported in many countries, including Australia (2), Belgium (47), Brazil (42), Bulgaria (14,30), France (47), Germany (19), Italy (6,17), Japan (35), the Netherlands (45,46), the United Kingdom (40), and the United States (50). Within *F. oxysporum* f. sp. *cyclaminis*, no races have been reported, and it is possible that all of these reports are of isolates that belong to one or a few clones dispersed with plants cultivated for ornamental purposes.

Of the 19 described cyclamen species, *C. persicum* is the major commercial ornamental species. Commercial cultivars of *C. persicum* are primarily diploid ($2n = 48$), but some tetraploid cultivars are available (49), and some plants with 72 and 136 chromosomes also have been observed (23). *C. persicum* is thought to have originated in the Middle East, although Algeria and Tunisia also are possible centers of origin (23). The first ornamental cyclamens are thought to have appeared in European gardens during the seventeenth century, and white-flowered plants were cultivated at Lille, France, in the 1730s (23). Major cultivation efforts to develop ornamental plants began in England and France in the 1860s (49) and in the Netherlands in the 1890s (23). F_1 hybrid cyclamen were commercialized in 1974, but open-pollinated diploid cultivars are still widely available (49).

F. oxysporum f. sp. *cyclaminis* may cause infected plants to die suddenly at all plant stages (50). The loss may be complete or limited to only a few of the plants in the nursery. The sudden, and often late, appearance of the symptoms makes control of the disease once symptoms have appeared difficult, if not impossible. Affected commercial nurseries usually lose most of their production and associated revenues and market share and may be unable to utilize contaminated growing areas for any crop until clean-up and decontamination procedures have been completed; they also must absorb large clean-up costs to disinfest contaminated growing areas and utensils.

Studies of *F. oxysporum* f. sp. *cyclaminis* have not been intensive, since effective control can be obtained using steamed soil and/or a benomyl soil drench as necessary (24). With the increased likelihood that benomyl will be removed from the market, further research is necessary to identify the populations that need to be controlled via biological control (18,35). Genetic characterizations of *Fusarium* populations are becoming more common. We used three approaches that have been used successfully with other fungi: analysis of vegetative compatibility groups (VCGs) (36), restriction fragment length polymorphism analysis of the in vitro amplified rDNA intergenic spacer (IGS) region (7,51), and DNA fingerprinting by probing Southern blots of total DNA with a randomly selected genomic clone (4,37,39). All of these characters assess variability at multiple loci and should reveal clonal structure within the population if it exists. If clonal structure is not detected, then depending on how the different approaches group the strains, it might be possible to determine how the pathogen population has evolved.

Our objective in this study was to evaluate *Fusarium* isolates from cyclamen for pathogenicity and genetic diversity using VCGs, DNA fingerprints, and ribosomal IGS sequence variability as measures. From these observations we expected to: (i) identify a genetic tool that could be used to identify isolates pathogenic to cyclamen without using a plant assay and (ii) to identify isolates that could be used to further resistance-breeding efforts against

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this destructive pathogen. A preliminary presentation of some of these results has been made (54).

MATERIALS AND METHODS

Culture conditions and strains. All fungal isolates used are listed in Table 1 (pathogenic isolates) or Table 2 (nonpathogenic isolates) and were collected from fresh plant material unless otherwise indicated. Plant material was placed on a peptone/*p*-chloronitrobenzene medium selective for *Fusarium* spp. (41). Individual isolates were purified through the subculture of a single conidium via micromanipulation (32) and preserved long-term as spore suspensions in 15% glycerol at –80°C as recommended by Yoder et al. (55). All strains used in this study have been deposited with the Fusarium Research Center, Department of Plant Pathology, The Pennsylvania State University, University Park.

Strains were grown on potato-dextrose agar (Difco Laboratories, Detroit), carnation leaf agar (15), and a modified Czapek-Dox medium with appropriate supplements (10) as necessary. Strains were incubated at 25°C with alternating 12-h periods of light and dark.

Pathogenicity tests. All fungal isolates were grown in 600-ml Erlenmeyer flasks containing 250 ml of Czapek-Dox liquid medium supplemented with 50 ppm of chloramphenicol inoculated with 2 ml of a spore suspension containing 10^5 to 10^6 spores per ml. Cultures were incubated statically at room temperature (20 to 24°C) for 7 days and aerated by bubbling sterile air through the culture. Each culture was filtered through cheesecloth, and the spore count in the filtrate was adjusted to 10^6 spores per ml based on spore counts in a hemacytometer.

Ten test cyclamen plants (3- to 5-cm shoots) were soaked in 100 ml of the fungal spore suspension for 10 min and transplanted into flats containing commercial potting mix (Jongkind, Aalsmeer, the Netherlands). Plants were maintained in a greenhouse with a constant temperature of 24°C. Supplemental light was provided for a total light period of 16 h. Plants were scored every other day beginning 14 days after planting and continuing through 28 days after planting. Pathogenic isolates were not retested for pathogenicity. Nonpathogenic isolates were tested for pathogenicity twice.

Vegetative compatibility tests. Vegetative compatibility tests were made using nitrate nonutilizing (*nit*) mutants generated according to standard protocols (10). *nit* mutants were subdivided into *nit1*, *nit3*, and NitM classes based on their ability to differentially utilize NO_2^- and hypoxanthine as sole nitrogen sources (10). Mutants in different classes were paired to determine vegetative compatibility; *nit1* and NitM mutants were used wherever possible. Each pairing was made in a reciprocal manner on a minimal medium containing KNO_3 as the sole nitrogen source, and a *nit1* and NitM mutant from strain one was paired with a NitM and *nit1* mutant from strain two, respectively. Pairings were initially made in 96-well microtiter dishes. Negatives were confirmed in 24-well hybridoma plates (33); positives were confirmed in standard 60-mm plastic petri dishes. Strains with mutants that complemented to give prototrophic growth were said to be vegetatively compatible and to belong to the same VCG. Strains that did not complement were said to be vegetatively incompatible and to belong to different VCGs.

Mutants in different phenotypic classes originating from a common parent were tested for complementation. If these mutants complemented, then the parental strain was heterokaryon self-compatible. When such complementation did not occur, pairings were still made with mutants derived from other strains, but if no complementation occurred, then these strains were designated as HSI (heterokaryon self-incompatible), and no conclusions about vegetative compatibility could be drawn (11), although these strains could be associated with a VCG on the basis of other characters.

Based on the system of Puhalla (44), we have used 015 (the next available number since 013 has been used for *Fusarium oxy-*

TABLE 1. *Fusarium oxysporum* strains pathogenic to cyclamen used in this study

No.	Country of origin[a]	VCG[b]	DNA fingerprint[c]	IGS pattern[d]	Other accession no.[e]
1	Neth.	0151	1	1/3	FRC O-1797
2	Neth.	0151	1	...	FRC O-1798
3	UK	0151	1	...	FRC O-1799
8	UK	0152	2	2	FRC O-1800
9	UK	0152	2	2	FRC O-1801
11	Japan	0151	1	...	FRC O-1802
19	Neth.	0151	1	1/3	FRC O-1803
28	UK	0151	1	1/3	FRC O-1804
34	Neth.	0151	1	1/3	FRC O-1805
36	Neth.	0151	1	1/3	FRC O-1806
45	Neth.	0151	1	1/3	FRC O-1807
52	Neth.	0151	1	1/3	FRC O-1808
54	Neth.	0151	1	..	FRC O-1809
56	Neth.	0151	1	...	FRC O-1810
58	Neth.	0151	1	...	FRC O-1811
60	Neth.	0151	1	...	FRC O-1812
62	Neth.	0151	1	...	FRC O-1813
64	Neth.	0151	1	...	FRC O-1814
66	Neth.	0151	1	1/3	PBN90-4; FRC O-1815
67	Neth.	0151	1	1/3	PBN90-5; FRC O-1816
68	Neth.	0151	1	1/3	PBN92-1; FRC O-1817
70	Neth.	0151	1	1/3	PBNV1[f]; FRC O-1818
71	Neth.	0151	1	1/3	PBN-NAKS-60; FRC O-1819
72	Neth.	0151I	1	1/3	FRC O-1820
73	Neth.	0151	1	1/3	FRC O-1821
74	Aust.	0151	1	1/3	FRC O-1099
75	Aust.	0151	1	1/3	FRC O-1100
76	Aust.	0152	2	2	FRC O-1205
77	U.S.	0152	2	2	FRC O-1239
78	U.S.	0152	2	2	FRC O-1234
82	Neth.	0151	1	1/3	FRC O-1822
86	Neth.	0151	1	1/3	FRC O-1823
89	Neth.	0151	1	...	FRC O-1824
90	Ger.	0153I	3	1/3	CBS 159.57; FRC O-1825
91	Neth.	0151	1	...	FRC O-1826
96	Neth.	0151	1	1/3	FRC O-1827
97	Neth.	0151	1	...	FRC O-1828
98	Neth.	0151	1	...	FRC O-1829
99	Fr.	0153I	3	1/3	ATCC 34371; FRC O-1830
100	Ger.	0153I	3	1/3	ATCC 16061; FRC O-1831
107	Neth.	0151	1	1/3	FRC O-1832
114	Neth.	0151I	1	1/3	FRC O-1833
115	Neth.	0153I	3	1/3	FRC O-1834
116	Neth.	0151I	1	...	FRC O-1835
124	Neth.	0151	1	...	FRC O-1836
126	Neth.	0151	1	...	FRC O-1837
128	Neth.	0151	1	...	FRC O-1838
129	Neth.	I	1	...	FRC O-1839
131	Neth.	0151	1	...	FRC O-1840
132	Neth.	0151	1	1/3	FRC O-1841
139	Neth.	0152	2	2	FRC O-1842
140	Neth.	0152	2	2	FRC O-1843
141	Neth.	0152	2	2	FRC O-1844

[a] Neth. = the Netherlands; UK = the United Kingdom; Aust. = Australia; U.S. = the United States; Ger. = Germany; and Fr. = France.

[b] VCG = vegetative compatibility group; 0151–0153 = multiple occurrence VCGs within *F. oxysporum* f. sp. *cyclaminis*; and I = heterokaryon self-incompatible, which may be assigned to a multiple occurrence VCG, if not, then no conclusions on vegetative compatibility can be drawn.

[c] Figure 1 shows representatives of the different fingerprint patterns.

[d] IGS = intergenic spacer sequence. Figure 2 shows representatives of the different IGS organizational patterns. ... = no data.

[e] Sources: ATCC = American Type Culture Collection, Rockville, MD; CBS = Centraal Bureau voor Schimmelcultures, Baarn, the Netherlands; FRC = Fusarium Research Center, Department of Plant Pathology, The Pennsylvania State University, University Park; and PBN = Proefstation voor de Bloemisterij in Nederland, Aalsmeer, the Netherlands.

[f] Isolated from nutrient solution used in greenhouse culture of cyclamen.

sporum f. sp. *melonis* [25] and 014 has been used for *Fusarium oxysporum* f. sp. *elaeidis* [16]) as a prefix to all of our VCG numbers for the *cyclaminis* forma specialis. Thus, our VCG numbers are composed of the three-figure prefix to indicate the forma specialis and then an additional digit to indicate the VCG within the forma specialis.

Construction of pFON10. Plasmid pFON10 was randomly selected after transformation of *Escherichia coli* XL-1 Blue cells (Stratagene, La Jolla, CA). *Eco*RV-digested (Pharmacia, Milwaukee, WI), calf intestine phosphatase-treated (Boehringer GmbH, Mannheim, Germany) pBluescriptSK+ (Stratagene) vector (100 ng) was ligated overnight at 15°C to 50 ng of *Eco*RV-digested total DNA from *F. oxysporum* f. sp. *niveum* race 1, with 1 unit of T4 DNA ligase (Pharmacia) in 10 µl of a polyethylene glycol-containing ligation buffer (31). The entire ligation mixture was used for transformation.

DNA isolation, hybridization, and amplification. Mycelium was grown, as described above, for the pathogenicity tests. Mycelium was harvested by vacuum filtration through a 56-µm mesh filter, frozen, and stored at –70°C until needed. Frozen mycelium (300 mg) was ground to a fine powder with liquid N_2 using a mortar and pestle. The powder was transferred to a 2-ml microfuge tube containing 600 µl of extraction buffer (1.5% cetyltrimethylammoniumbromide [CTAB], 75 mM Tris-HCl [pH 8.0], 15 mM EDTA, and 1.5 M NaCl) and incubated in a 65°C water bath for 30 min. After incubation an equal volume of $CHCl_3$/isoamyl alcohol (24:1, vol/vol) was added and vortexed to form an emulsion. The sample was centrifuged at 10,000 × *g* for 10 min at room temperature. The upper aqueous phase was transferred to a fresh tube and then reextracted. The final aqueous phase was mixed with an equal volume of 1% CTAB, 50 mM Tris-HCl (pH 8.0), and 10 mM EDTA and incubated at 4°C for 1 h to precipitate the DNA. DNA was pelleted at 2,500 × *g* for 10 min and dissolved in 0.3 ml of Tris-EDTA (TE) (48) + 1 M NaCl. Two volumes of 95% ethanol were added, and the mixture was incubated at –20°C for 1 h. DNA was pelleted by centrifugation at 10,000 × *g* for 10 min, washed once with 70% ethanol, and dried under vacuum before being redissolved in 100 µl of sterile TE.

For Southern blots and hybridizations, approximately 10 µg of DNA was digested with 60 units of an appropriate restriction enzyme for 3 h and added to a well in a 0.8% agarose gel. Fragments were separated by electrophoresis in 1× Tris-borate-EDTA (48) at 2.8 V/cm for 17 h. Gels were incubated in 0.25 N HCl for 15 min at room temperature and the DNA was denatured twice in 0.6 M NaCl and 0.4 N NaOH for 15 min. DNA was transferred from the gel to Hybond N^+ (Amersham, Buckinghamshire, England) using the denaturation buffer and a vacuum transfer device. DNA was cross-linked to the membrane by exposure to UV light (254 nm) using a Stratalinker 1800 (Stratagene) in the auto crosslink mode. Membranes were prehybridized in 30 ml of an aqueous solution (4× SSC [1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate, pH 7.0], 5× Denhardt's solution, and 0.5% sodium dodecyl sulfate [SDS]) (48) for 30 min at 65°C. The prehybridization solution was removed, and the membrane was further prehybridized for 45 min in the same solution containing 0.1 mg of sonicated salmon sperm DNA per ml before addition of the probe. Plasmid probes were labeled by random priming using α^{32}P-dCTP with the Oligolabelling kit (Pharmacia). Membranes were hybridized with the probe for at least 16 h at 65°C. Blots were washed twice with 2× SSC and 0.1% SDS for 5 min at room temperature and twice with 0.2× SSC and 0.1% SDS at 65°C for 15 min. Hybridized membranes were exposed to X-ray film (Kodak X-Omat-AR, Eastman Kodak Co., Rochester, NY) at –70°C with intensifying screens.

PCR was carried out in 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM each of dATP, dCTP, dTTP, and dGTP, 1 µM each of primers ZUP114 and ZUP115, 10 to 40 ng of genomic DNA, and 1 unit of Ampli*Taq* polymerase (Perkin-Elmer-Roche, Branchburg, NJ) in a final volume of 50 µl. The primer sequences were: ZUP114: GGCTG CTGGC ACCAG ACTTG, and ZUP115: CTTCG ATGTC GGCTC TTCCTA, which are slightly modified versions of primers H (8) and NS2 (52). Reactions were performed in a Perkin-Elmer 9600 thermal cycler, beginning with a 1-min denaturation step at 94°C and followed by 30 cycles of 0.5 min at 94°C, 1.5 min at 65°C, and 1.5 min at 72°C. The 72°C segment was lengthened progressively by 2 s after each cycle and by an additional 5 min after the last cycle. Amplified products were digested with restriction enzymes (Pharmacia) as indicated, and the restricted fragments were separated in 1.8% Metaphor agarose gels (FMC Bioproducts, Rockland, MD). Gels were stained with ethidium bromide and irradiated with UV light to view the DNA bands (48).

TABLE 2. *Fusarium oxysporum* strains not pathogenic to cyclamen used in this study

No.	Country of origin[a]	VCG[b]	DNA fingerprint[c]	IGS pattern[d]	Other accession no.[e]
4	Neth.	N	0	...	FRC O-1845
6	Neth.	N	U	1/3	FRC O-1846
7	Neth.	N	U	...	FRC O-1847
12	Neth.	4	4	4	FRC O-1848
14	Neth.	5	5	5	FRC O-1849
18	Neth.	4	4	...	FRC O-1850
20	Neth.	N	0	U	FRC O-1851
21	Neth.	N	U	1/3	FRC O-1852
22	Neth.	N	U	...	FRC O-1853
23	Neth.	N	U	U	FRC O-1854
27	Neth.	4[f]	4	4	FRC O-1855
29	Neth.	5	5	5	FRC O-1856
30	Neth.	N	U	8	FRC O-1857
31	Neth.	5	5	5	FRC O-1858
32	Neth.	5	5	5	FRC O-1859
33	Neth.	N	U	U	FRC O-1860
40	Neth.	4[f]	4	4	FRC O-1861
41	Neth.	4[f]	4	4	FRC O-1862
42	Neth.	N	6	6	FRC O-1863
43	Neth.	N	6	5	FRC O-1864
44	Neth.	7	7	1/3	FRC O-1865
47	Neth.	N	U	U	FRC O-1866
48	Neth.	N	U	8	FRC O-1867
51	Neth.	7	7	1/3	FRC O-1868
69	Neth.	N	U	1/3	PBN V3[g]; FRC O-1869
137	Neth.	N	U	...	FRC O-1870

[a] Neth. = the Netherlands.
[b] VCG = vegetative compatibility group; 4–5 = multiple occurrence VCG but not within *F. oxysporum* f. sp. *cyclaminis*; and N = heterokaryon self-compatible but not in either of the two multiple occurrence VCGs.
[c] Figure 1 shows representatives of the different fingerprint patterns. Numbers indicate multiisolate fingerprints, except for 0, which indicates that no hybridization was observed with the probe. U indicates that a unique fingerprint was detected for that strain.
[d] IGS = intergenic spacer sequence. Figure 2 shows representatives of the different IGS organizational patterns. Numbers indicate multiisolate organizational pattern.; U indicates a unique organizational pattern. ... = no data.
[e] Sources: ATCC = American Type Culture Collection, Rockville, MD; CBS = Centraal Bureau voor Schimmelcultures, Baarn, the Netherlands; FRC = Fusarium Research Center, Department of Plant Pathology, The Pennsylvania State University, University Park; and PBN = Proefstation voor de Bloemisterij in Nederland, Aalsmeer, the Netherlands.
[f] No observable chlamydospores.
[g] Isolated from nutrient solution used in greenhouse culture of cyclamen.

RESULTS

Over several years we accumulated 79 strains of *F. oxysporum* that originated from cyclamen plants (Tables 1 and 2). During this process, we also identified strains of *F. moniliforme*, *F. graminearum*, and *F. equiseti*, although none of these strains was pathogenic to cyclamen in our greenhouse tests. The 79 *F. oxysporum* strains we examined include strains we isolated from fresh plant material and strains we obtained from culture collections in the

Netherlands and the United States. Isolates from the Netherlands dominate our sample, but isolates from Australia, France, Germany, Japan, the United Kingdom, and the United States also are included. We tested these isolates for their pathogenicity to young cyclamen plants in a greenhouse assay and for their genetic relatedness using VCGs and two molecular markers.

***F. oxysporum* f. sp. *cyclaminis* pathogenicity to cyclamen.** *F. oxysporum* f. sp. *cyclaminis* may attack both young and old cyclamen plants. Young plants were used for artificial inoculation and greenhouse/growth-chamber studies. The primary symptoms in these plants are wilting and retarded growth. Plants may wilt before clear stunting symptoms can be noticed. In young inoculated plants, the first symptoms usually appear in the second- to fourth-leaf stage as the older leaves begin to yellow. Yellowing develops from the stem base and progresses into the leaf. A leaf spot then develops and grows out to the edges of the leaf. As the leaf ages, the center of the spot becomes necrotic. Yellowing may occur at multiple sites per leaf, and as the yellowing progresses, these spots may coalesce to form a single large lesion. Wilting develops quickly, and most plants are completely wilted within 3 weeks of inoculation. The yellow leaf symptoms can result from causes other than *Fusarium* infection (e.g., leaf damage and drought stress), but in these cases, yellowing is not necessarily associated with wilting. In addition to yellowing, some infected plants have a brown-black discoloration of the veins in the young tuber. This discoloration may result from fungal infection or be due to normal pigmentation in some genotypically dark-colored varieties.

Under nursery or commercial production conditions, symptoms usually become visible in older plants (3-6 months) and may be triggered by water stress or excessive heat. The infected plant usually starts to wilt on one side, and the leaves turn yellow. Wilting and yellowing symptoms are similar to those seen in young plants. Conidia are commonly formed on these plants either on the tuber or at the site of wilting. The spores initially appear grayish but, unlike *Botrytis* spp. spores, become pinkish-white as they age. The spores form a mat of fungal tissue close to the tuber or stem that is less fluffy than similar colonies formed by *Botrytis* spp. Eventually the entire plant wilts and dies. Transverse sections of the tuber show an orange-brown one-sided discoloration of the veins. Wilting alone is insufficient to implicate *F. oxysporum* f. sp. *cyclaminis* as the cause of death since these symptoms also may be caused by pests (e.g., cutworms), other fungi (e.g., *Botrytis* spp.), bacteria (e.g., *Erwinia* spp.), and poor plant management (e.g., overwatering).

All of our isolates were tested for pathogenicity in a young plant assay. *Fusarium* cultures were obtained from culture collections and recovered from cyclamen; a pure culture of each isolate was started from a single microconidium. These isolates were reinoculated into young plants to confirm that they were pathogenic and should be designated as *F. oxysporum* f. sp. *cyclaminis*. Approximately one-third (26/79) of the isolates that we recovered were not pathogenic when they were reinoculated into the young plants. DNA fingerprints were determined for most of the recovered isolates that were pathogenic. In all cases, the DNA fingerprint of the recovered isolate was the same as that of the isolates used to inoculate the plant. In our experiments, most of the pathogenic isolates killed the plants within 18 days of inoculation, although two isolates, 3 and 90 (Table 1), took 22 and 29 days, respectively, to kill the plants in one trial and 18 and 28 days to kill the plants in a second trial. None of the plants inoculated with 25/26 strains designated as nonpathogenic (Table 2) showed any disease symptoms. The other strain, 137, killed 1 to 2 of the 10 inoculated plants per trial in each of the three pathogenicity trials with this strain.

Vegetative compatibility. *nit* mutants were generated for all of the strains listed in Tables 1 and 2. Initially, *nit1* and NitM mutants of 23 of these strains were paired, and four multimember VCGs (0151, 0152, 4, and 5) and seven single-member VCGs were detected. VCGs 0151 and 0152 were associated with the pathogenic strains in this set, and the remaining nine VCGs were associated with the nonpathogenic strains. *nit1* mutants from the remaining isolates were paired with testers representing VCGs 0151 and 0152. Of the 53 total pathogenic isolates examined (Table 1), 37 clearly belonged to VCG 0151. Five strains clearly belonged to VCG 0152, and three additional strains could be associated with this VCG on the basis of weak complementation reactions (Table 3). Analyses of DNA fingerprints and IGS sequences confirmed the assignment of these strains to the same clonal lineage.

Eight pathogenic strains were HSI (11). Of these eight strains, three (72, 114, and 116) could be assigned to VCG 0151 on the basis of weak complementation with some other strains within this VCG. One strain (129) was HSI and did not visibly complement with any of the strains in VCG 0151 but is probably closely related to it since it has the same DNA fingerprint as do the strains in VCG 0151 (discussed below). The remaining four pathogenic strains (90, 99, 100, and 115) could not be assigned to either VCG 0151 or 0152. *nit1* and NitM mutants from these strains were paired with each other, but pairing interactions were generally weak, including the *nit1* × NitM self-controls. These strains were not vegetatively compatible with any of the nonpathogenic strains examined. Based on the weak pairing data and support from fingerprint and IGS analyses (discussed below), these strains represent a third clonal lineage of pathogenic strains that contains a VCG designated 0153. Since 15% of the pathogenic isolates we observed were HSI, we think it unlikely that the HSI determinants have any effect on pathogenicity, which would be expected if *vic* genes are a part of the host-pathogen interaction process as has been suggested by Kroon and Elgersma (34).

nit1 mutants derived from the remaining 16 nonpathogenic strains were paired with NitM mutants from the five known multimember VCGs to determine whether any of these strains were members of one of these VCGs. None of the nonpathogenic strains were HSI. No additional members of these first five VCGs were identified. Isolates 44 and 51 had a similar DNA fingerprint pattern (Table 2) and were vegetatively compatible; isolates 42 and 43 also shared a fingerprint pattern but were vegetatively incompatible. No further VCG tests were made with these nonpathogenic isolates.

DNA fingerprint analyses. Southern blots with *Eco*RV-digested DNA of all strains listed in Tables 1 and 2 were hybridized with pFON10, a plasmid that carries a 6.0-kb *Eco*RV DNA fragment from *F. oxysporum* f. sp. *niveum*. This probe yielded informative fingerprints of *F. oxysporum* strains pathogenic to several plant hosts (L. P. Woudt, A. Sikkema, M. Q. J. M. van Grinsven, W. A. J. de Milliano, *unpublished data*). These patterns could be used to subdivide the strains into a series of fingerprint groups (FPGs). Hybridization patterns for representative strains with at least two strongly hybridizing bands are shown in Figure 1. For all of the *F. oxysporum* isolates that we examined, both patho-

TABLE 3. Pattern of nitrate nonutilizing (*nit*) mutant pairing between *Fusarium oxysporum* f. sp. *cyclaminis* strains in vegetative compatibility group 0152[a]

	NitM							
nit1	8 (1)	9 (2)	139 (2)	78 (1)	77 (2)	76 (3)	140 (5)	141 (2)
8 (2)	+	+	w	w	w	w	w	w/–
9 (1)	+	+	+	+	w	–	w	w/–
139 (7)	+	+	+	+	+	–	w	–
78 (13)	+	+	+	+	+	–	w	v
77 (4)	w	w	+	+	+	w	w	w
76 (13)	w	w/–	–	w	w	w	w	w
140 (5)	w	w	v	+	v	w	+	w
141 (8)	w	w/–	–	–	–	w	w	+

[a] Numbers in parentheses indicate the number of different *nit* mutants of this type from this strain that were used to arrive at this conclusion. + = strong complementation; – = no complementation; w = weak complementation; and v = very weak complementation.

genic and nonpathogenic, the hybridization patterns of strains within a FPG were identical or virtually identical; different FPGs shared very few, if any, common bands. For example, two or three hybridizing fragments (1.7, 2.2, and 14 kb) migrated at the same position for fingerprints of FPG1 and FPG3, two (1.7 and 10.5 kb) for fingerprints of FPG1 and FPG2, and one (1.7 kb) for fingerprints of FPG2 and FPG3; a 1.7-kb fragment also was found in some nonpathogenic strains. We have no information to suggest that these similarly sized bands are allelic, and it is possible that these similarities in position are simply coincidental.

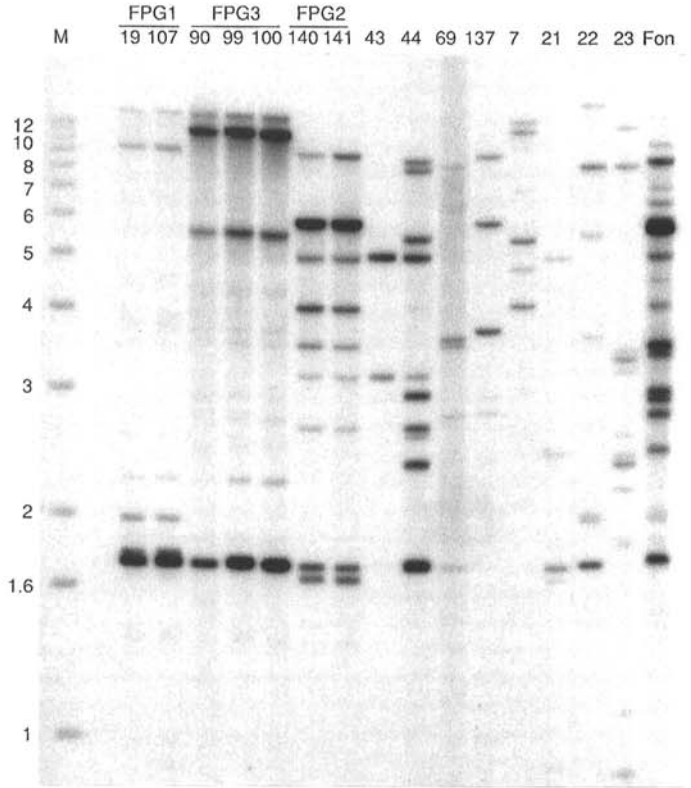

Fig. 1. DNA fingerprint patterns detected using the pFON10 probe. Numbers indicate strains (Tables 1 and 2). Lane M, 1-kb ladder (Gibco-BRL, Gaithersburg, MD); fragment sizes are given in kilobase pairs. Lane Fon, *Fusarium oxysporum* f. sp. *niveum* race 1, from which pFON10 was derived. Fingerprint groups (FPG) are indicated for strains of *F. oxysporum* f. sp. *cyclaminis*.

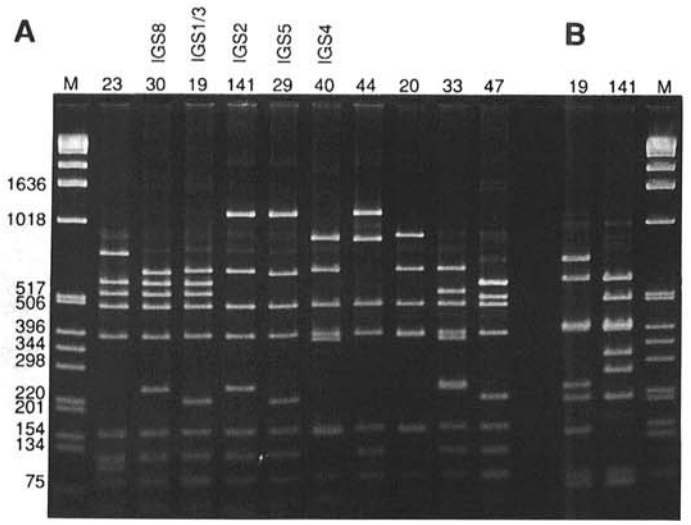

Fig. 2. Ribosomal intergenic spacer (IGS) sequence variability in *Fusarium oxysporum* recovered from cyclamen. **A,** *Rsa*I digests. Numbers indicate strains (Tables 1 and 2). Representative patterns for multiple strains also are designated by IGS-type numbers. **B,** *Alu*I digests of strains of vegetative compatibility group (VCG) 0151 (19) and VCG 0152 (141). Lane M, 1-kb ladder (Gibco-BRL, Gaithersburg, MD); fragment sizes are given in base pairs.

Fingerprints of the *F. oxysporum* f. sp. *cyclaminis* isolates generally were less complex than the fingerprint of the *F. oxysporum* f. sp. *niveum* strain from which pFON10 was derived (Fig. 1). Fingerprints of the pathogenic strains fell into one of three distinct groups (FPG1, FPG2, or FPG3) with 41, 8, and 4 members, respectively, and corresponded with the groupings made using the VCG character. Within a fingerprint group, variation was recorded only twice and, in each case, was limited to a single fragment, e.g., isolate 90 from FPG3 (Fig. 1).

The fingerprint patterns (Fig. 1) associated with the nonpathogenic isolates were more diverse than those associated with the pathogenic strains (Table 2). For two isolates (4 and 20), no hybridization with pFON10 was detected. As with the pathogenic isolates, nonpathogenic isolates from multimember VCGs 4 and 5 had uniform fingerprints within the VCG; these strains are designated as FPG4 and FPG5, respectively. The remaining 15 isolates had from 2 to 10 strongly hybridizing fragments (Fig. 1). In two cases, a pair of isolates had a similar fingerprint, designated FPG6 (isolates 42 and 43) and FPG7 (isolates 44 and 51). The remaining 11 isolates all had fingerprints that were quite different from one another. Based on the variability we observed with respect to fingerprints, the nonpathogenic isolates appeared to be more genetically diverse than the pathogenic isolates. In no instance did pathogenic and nonpathogenic strains have the same pFON10 fingerprint, thus pathogenic strains could be readily differentiated from nonpathogenic strains on the basis of their DNA fingerprints.

Ribosomal IGS sequence analyses. As a third measure of differences and similarities between these isolates, we compared restriction digests of polymerase chain reaction (PCR)-amplified ribosomal IGS sequences (Fig. 2). We first amplified IGS fragments from five members of VCG 0151, five members of VCG 0152, and all four members of VCG 0153 and then digested these fragments with *Hae*III, *Rsa*I, and *Alu*I. No polymorphism was observed with *Hae*III. Isolates from VCGs 0151 and 0153 had the same IGS 1/3 banding pattern in the *Rsa*I and *Alu*I digests and differed from the patterns seen in digests of VCG 0152 in a manner that required a minimum of at least two changes in the IGS DNA sequence for each enzyme (Fig. 2). Subsequently, we used *Rsa*I digests of the IGS region from an additional 10 isolates from VCG 0151, the remaining three isolates from VCG 0152, and 21 of the 26 nonpathogenic isolates to further characterize this cyclamen *Fusarium* population. The additional isolates from VCG 0151 all had the IGS 1/3 banding pattern, and the isolates from VCG 0152 all had the IGS 2 banding pattern. Thus, the IGS sequence restriction patterns were consistent with the hypothesis that these three VCGs/FPGs represent clonal lineages.

Among the 21 nonpathogenic isolates, we identified eight restriction patterns that differed from any of the patterns seen in the pathogenic isolates (Fig. 2A). Unlike the fingerprint hybridization patterns, however, five of the nonpathogenic strains (6, 21, 44, 51, and 69) had the same IGS restriction pattern as the pathogenic isolates in VCGs 0151 and 0153. The two nonpathogenic strains (44 and 51) that shared DNA fingerprint 7 both had IGS pattern 1/3. Five other nonpathogenic isolates (14, 29, 31, 32, and 43), including all four members of VCG 5, had an IGS pattern that was similar to the IGS 2 pattern. Thus, the ribosomal IGS sequence was not a reliable method for distinguishing *F. oxysporum* f. sp. *cyclaminis* strains from nonpathogenic *F. oxysporum* strains also found in cyclamen.

DISCUSSION

F. oxysporum f. sp. *cyclaminis* can be latently present in plants (45), leading to disease when plants mature or when growth conditions favor the pathogen. This latency emphasizes the need to

maintain a growth environment that is free of *F. oxysporum* f. sp. *cyclaminis*. To maintain such an environment, methods for detecting this pathogen are essential. At present, large-scale screening of (symptomless) plant parts, growth substrates, and/or recirculated nutrient solutions for pathogenic strains is not feasible since nonpathogenic strains of *F. oxysporum* frequently are found in association with cyclamen plants. These nonpathogenic strains are morphologically indistinguishable from the pathogenic strains. Pathogenicity assays using young cyclamen plants are cumbersome, expensive, and time-consuming and are not suitable for screening large numbers of isolates as part of a greenhouse management or resistance-breeding program.

We used three primary traits to characterize strains of *Fusarium* recovered from cyclamen in addition to pathogenicity: VCG, hybridization pattern with the fingerprint probe pFON10, and rDNA IGS sequence restriction pattern. We found some variation within all of these traits but have grouped strains that appear to be related for discussion purposes (Table 4). We observed one strain (137) that gave mixed results in our pathogenicity studies and two strains that differed from the standard of their fingerprint group by the position of a single band. There were several IGS patterns that were as closely related as the fingerprint patterns (e.g., IGSs 2 and 5), but the strains in these groups differed in VCG and fingerprint pattern, suggesting that strains with the same IGS pattern might be fortuitously similar rather than similar due to a clonal relationship. Thus, the VCG and the fingerprint data provide a more meaningful grouping of these isolates than do the IGS data.

The VCG data were subject to two types of variability. In one case, the variation was due to HSI strains that were unable to pair with any other member of the VCG or with themselves. These strains were assigned to a clonal lineage that contains a single VCG on the basis of their DNA fingerprints. The second type of variation can be seen in the pairing relationships within VCG 0152 (Table 3). These strains were united by their weak pairing with one or several of the other strains in the VCG. The relationship was confirmed by the uniformity of these strains with respect to pFON10 hybridization patterns. This pairing pattern would be difficult to observe in many cases since usually one (or at most a few) strain is selected as a standard to represent the entire VCG. Strains of unknown VCG are paired with the standards, and negatives are excluded from the VCG. Under such a scenario, strain 76 probably would have been identified as HSI, and strains 140 and 141 would have been assigned to separate, unique VCGs. The cause of this variegated pairing reaction pattern is unknown but has been observed in other fungi, including *Aspergillus* (9), *Cryphonectria* (1), and *Verticillium* (26). Weak pairing reactions could result from two causes: the inability to form a heterokaryon or the inability to maintain a stable heterokaryon once it has been formed.

It is possible that some strains carry mutations in loci that affect the ability to form a heterokaryon in which complementation or *vic* allele interaction can occur. These mutations would result in an epistatic block to *vic* gene interactions and give an HSI phenotype. From previous work with *F. moniliforme* (11), it is known that the HSI phenotype is continuous if the number of fused cells counted microscopically is used as the phenotype criterion rather than the presence or absence of prototrophic growth along the interaction line. Strains that fuse asexually, but poorly, would be expected to be more dependent on the partner strain for heterokaryon formation (11,25) and, consequently, to interact weakly with some members of the group, strongly with others, and not at all with the remainder. If the reduction in hyphal fusion/heterokaryon formation ability occurred after the establishment of the pathogenic clones, then the VCG phenotype would be masked, but these strains should be the same as the others in their clonal lineage for other polymorphic traits, e.g., fingerprint and IGS patterns. This pattern would be similar to that described by Joaquim and Rowe (26) in *V. dahliae* and to what we have observed in *F. oxysporum* f. sp. *cyclaminis*.

An alternative hypothesis is that the strains in VCG 0152 are able to form heterokaryons but are unable to maintain them. Under this hypothesis, members of this VCG might differ at one or perhaps two *vic* loci with respect to a standard strain. If these strains can still form weak, short-lived heterokaryons, such as those seen in *Aspergillus* (9) or *Cryphonectria* (1), then weak complementation reactions, such as we observed, could result. Although mutations that change or inactivate *vic* loci are known in some fungi (e.g., *Podospora anserina* [3]), we have not observed spontaneous changes in cultures from one VCG to another under laboratory conditions and expect these mutations to be rarer than those that would result in the HSI phenotype. The uniformity of the strains in our proposed VCG 0152 for both fingerprint and IGS patterns also is consistent with the hypothesis that these strains all belong to a single VCG, since VCG and fingerprint pattern are correlated in all of the other VCGs/FPGs that we observed. Thus, we believe that some factor other than heterozygous *vic* loci is responsible for the weak complementation we observed.

Diagnostics. We examined three genetic traits that have been correlated with pathogenicity in other *F. oxysporum* forma speciales: vegetative compatibility, DNA fingerprinting, and ribosomal IGS sequences. Using any of these traits, it might be possible to develop a diagnostic assay that may be of use in either breeding or greenhouse management programs. These assays detect more variability within *F. oxysporum* f. sp. *cyclaminis* than was reported by Kerssies et al. (29) using α-esterase and β-D-glucosidase. However, it is possible that the strains examined by these researchers belonged only to a single clonal lineage and that this test would show more variability if different strains were used. Since the isozyme activity might be directly related to pathogenicity (29), the results obtained using strain 137, a consistent but weak pathogen, would be particularly interesting.

VCG grouping has been developed as a diagnostic method for research purposes for numerous formae speciales of *F. oxysporum* (reviewed by Leslie [36]). Usually, implementation of this method requires induction of complementary auxotrophic mutants that are used to force heterokaryons under selective conditions. In a situation such as that in *F. oxysporum* f. sp. *cyclaminis*, only a few VCGs need to be detected, so it should be possible to develop tester strains that carry two markers (36) that would eliminate the need to induce *nit* mutations in every recovered isolate. The development of such strains could result in an assay system in which a large number of isolates could be examined quickly and eco-

TABLE 4. Interrelationship between genetic markers scored in *Fusarium oxysporum* recovered from cyclamen

Clonal lineage[a]	No.of strains	Pathogenic to cyclamen	VCG[b]	HSI[c]	FPG[d]	IGS[d]
1	41	Yes	0151	Yes	1	1/3
2	8	Yes	0152	Yes	2	2
3	4	Yes	0153	Yes	3	1/3
4	5	No	4	No	4	4
5	4	No	5	No	5	5
6	1	No	...	No	6	6
7	2	No	7	No	7	1/3
8	1	No	...	No	6	5
9–11	3	No	...	No	U	1/3
12–13	2	No	...	No	U	8
14–21	8	No	...	No	U	U

[a] Entries 1–8 represent clones in so far as they can be distinguished from the data presented. Entries 9–21 represent 13 single member lineages that have been grouped as much as the data permit.

[b] VCG = vegetative compatibility group.

[c] Indicates whether heterokaryon self-incompatible (HSI) strains were assigned to this lineage.

[d] FPG = fingerprint group; IGS = intergenic spacer sequence. Classes represented by two or more strains were assigned a number. All classes that were represented only once are designated by a U; for example, strains in lineages 9, 10, and 11 all had IGS phenotype 1/3, but each had a unique restriction fragment length polymorphism pattern.

nomically. Based on our present work, however, such an assay would be expected to give a high number of false negatives, i.e., to not detect *F. oxysporum* f. sp. *cyclaminis* when it is present in the test population. These false negatives have several sources. First, the number of HSI strains in the population is relatively high at 15% (8/53), and the number of isolates that pair poorly is even higher. These isolates include many of those associated with VCGs 0152 and 0153. For commercial purposes, this high level of false negatives probably is unacceptable, and a means of reducing this problem to a more acceptable level needs to be found.

DNA fingerprinting with a probe that hybridizes to multiple sites in the target organism has been used in numerous fungi to track different clonal lineages. Our pFON10 probe provides us with a fingerprint that effectively distinguishes *F. oxysporum* f. sp. *cyclaminis* from other strains of *F. oxysporum* that are associated with cyclamen. This assay yielded neither false negatives nor false positives based on the strains in our sample and is potentially useful as a diagnostic assay. DNA fingerprinting is a relatively time- and technology-intensive process, however, since enough DNA must be recovered from each test isolate for a Southern blot. Such an assay is feasible in a research setting (e.g., a resistance-breeding program) but would not be usable for a general greenhouse management program, where the number of isolates to be examined is much higher and the cost per sample examined needs to be much lower.

Ribosomal IGS spacer sequences potentially are most useful for diagnostics since they can be examined via PCR amplification using readily available primers. This process could be adapted to handle the volume of strains necessary for a greenhouse management program at an economically feasible cost. Unfortunately, we did not detect sufficient specificity in these sequences to distinguish pathogenic and nonpathogenic strains from each other, since five nonpathogenic strains would have been grouped with the 45 pathogenic strains associated with VCGs 0151 and 0153 (Table 4). Thus, this assay would yield a relatively high rate (10% [5/50]) of false positives (i.e., identifying nonpathogenic strains as pathogens) and would lead to the unnecessary destruction of innocuous material in a greenhouse management program and to the misidentification of resistant plants in a resistance-breeding program.

Population structure. We used three genetic characters to examine the population composition of *F. oxysporum* that was associated with cyclamen. Two of these phenotypes, VCGs and DNA fingerprinting, could be used to distinguish pathogenic and nonpathogenic isolates from each other, similar to the results of Mes et al. (38) for *Fusarium oxysporum* f. sp. *gladioli*. Since both pathogenic and nonpathogenic isolates are associated with cyclamen plants, this distinction is important. Such associations also have been seen with *F. oxysporum* from melons (20,21,25), carnations (27,28), and celery (12,13). In both the previous work and in our study, however, the level of variability observed within the pathogenic population was less than that observed within the nonpathogenic population (12,13,21,27,28).

Pathogenic and nonpathogenic lineages need not be completely distinct from one another, however. Gordon and Okamoto (20,21) found some overlap between pathogenic strains when they used mitochondrial DNA polymorphism to detect differences between pathogenic and nonpathogenic strains of *F. oxysporum*. They also found that strains in different VCGs could share a common mitochondrial DNA haplotype. They explained their results by assuming that strains in different VCGs could form rare heterokaryons that survived long enough for some cytoplasmic exchange to occur.

Our ribosomal IGS data support a more complicated evolutionary relationship between some of the nonpathogenic and pathogenic strains, especially for those strains in the clonal lineages associated with VCGs 0151 and 0153. Strains with the 1/3 IGS phenotype could be pathogenic or nonpathogenic and belonged to at least four VCGs. They also carried six hybridization patterns with our fingerprint probe. Without additional data, it is impossible to determine the relationship between these strains or to create a phylogenetic tree. This group contains pathogenic strains from widely dispersed locations (Australia, France, Germany, Japan, the Netherlands, and the United Kingdom), a pattern that suggests that this pathogen may have accompanied apparently healthy plants being moved around the world. Such a pattern resembles that seen with *Fusarium oxysporum* f. sp. *cubense*, the causal agent of Panama disease of bananas, in which clear lineages can be seen within VCGs (5,43). The strains in VCGs 0152 and 5 all appeared to be closely related to one another and to strain 42, especially since strains 43 and 42 shared a common DNA fingerprint pattern. The differences between these strains at the IGS level were relatively minor and could require as little as a single change to account for most of the observed differences.

Within the IGS types, the subgroups are usually discordant for multiple characters, since pathogenicity is correlated with both VCG and DNA fingerprint probe hybridization pattern. If the subgroups are related by mutation, then multiple changes must occur fairly closely in time to one another, since we have no examples of strain subgroups differing by only a single character. An alternative explanation is recombination, but the characters used are insufficient to determine if there are types that could give rise to the different combinations we observed. It is not clear whether pathogenicity is lost by pathogenic strains to give rise to their nonpathogenic relatives or whether pathogenicity is an acquired trait selected for by the cultivation and selected breeding of cyclamens. The differences in the IGS patterns suggest there are at least two distinct lineages of pathogens (IGSs 1/3 and 2), however, which suggests that pathogenicity has arisen separately on at least two occasions. Further sampling is warranted to determine whether the pathogenic strain types have the same geographic distribution or whether some of the differences we observed in strain origin are merely the result of small sample size from locations outside the Netherlands.

Further work with *Fusarium* from cyclamen should include studies with increased sample size and more geographic diversity. Samples intensively collected from infested and uninfested greenhouses could be used to identify biocontrol strains and to determine relatedness of pathogenic and nonpathogenic populations in a "local setting." Samples from a broader geographic area also could be useful in determining whether both of the two major pathogenic strain types are found worldwide or whether quarantine or other regulatory action needs to be taken to prevent the spread of either of these strain types. Finally, the variation seen within identifiable subgroups suggests that additional variation remains to be described. Studies similar to those conducted with *F. oxysporum* f. sp. *cubense* could provide valuable data on the origin and spread of *F. oxysporum* f. sp. *cyclaminis* and might suggest novel control methods. Studies of *Fusarium* collected from the suspected center of origin of cyclamen could be of particular interest.

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