A Restriction Fragment Length Polymorphism Probe Relating Vegetative Compatibility Groups and Pathogenicity in *Fusarium oxysporum* f. sp. *dianthi*

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We thank T. Katan and R. Cohen, The Volcani Centre, for discussions and access to material, respectively.
Accepted for publication 11 September 1989 (submitted for electronic processing).

ABSTRACT

Manicom, B. Q., Bar-Joseph, M., Kotze, J. M., and Becker, M. M. 1990. A restriction fragment length polymorphism probe relating vegetative compatibility groups and pathogenicity in *Fusarium oxysporum* f. sp. *dianthi*. Phytopathology 80:336-339.

Forty-six isolates of *Fusarium* from diseased carnations were examined for DNA restriction fragment length polymorphisms (RFLPs) and vegetative compatibility groups (VCGs). Both RFLPs and VCGs identified two major coincident groups. RFLPs identified three minor groups which were not assigned to VCGs. Testing of subsets of the groups showed the two major groups to be pathogenic. The two methodologies give equivalent results for the genetic separation of populations, and it is suggested that once the groundwork is laid for a host/pathogen complex, these methods can replace differential hosts for the determination of taxonomic units.

Fusarium oxysporum f. sp. *dianthi* (Prill. & Del.) Snyd. & Hans. is a fungal pathogen of cultivated species of carnations (*Dianthus caryophyllus* L.), causing a serious wilt disease. Eight races have been reported within this forma specialis by Garibaldi (12), all but one of which are currently believed to be confined to the French and Italian Riviera. Race 2, to which only partial resistance exists, is found worldwide and is the only race currently known in Israel.

Isolation of *F. oxysporum* from diseased carnation is usually regarded as presumptive evidence for assignment of the isolate to *F. o. dianthi*, although this is not necessarily true. While it is relatively easy to identify *Fusarium* to species level, the confirmation of forma specialis requires pathogenicity tests, which are time consuming, and, if large numbers are involved, requires extensive facilities. Race determinations further increase the experimental requirements due to the need for differential hosts.

Methods for the rapid identification of pathogenic isolates would be an advantage for plant disease clinics or in applying phytosanitary regulations. Two techniques with powerful resolving ability for the rapid classification of fungal isolates are the use of restriction fragment length polymorphisms (RFLPs) (4,17,19) and vegetative compatibility groups (VCGs) (7,24).

We report here on the use of VCGs and RFLPs for the determination of genetic groups and their relation to pathogenicity for a collection of isolates of *F. oxysporum* obtained from diseased carnations.

MATERIALS AND METHODS

Fungal isolates. Forty-six *Fusarium* isolates from diseased carnation plants were supplied under code by Dr. Ruth Cohen of the Plant Disease Clinic, ARO, Bet Dagan. Most of these were *F. oxysporum*, but no particular effort was made to exclude other Fusaria recovered from the plants, as we wished to gain an idea of the specificity of the technique. Single spore isolates were made of each strain and the cultures were maintained on carnation leaf agar (10). Sources and codes are given in Table 1.

DNA extraction. The previously described CTAB method (19) was used for the first part of this study. Later, the method of Raeder and Broda (26), which is faster and more convenient and which gave identical RFLPs, was adopted as the standard, with the addition of a final precipitation step using 0.25 volumes of 7.5% ammonium acetate and 2 volumes of ethanol. This enhanced the digestibility of DNA from many isolates.

RFLP probe. Cloning of the random probe used in this study (probe D4) has been described previously (19). The probe consisted of an approximately 3,400 base pair (bp) random, *Hin*dIII restriction fragment, from a partial digest of a total DNA extract of *F. o. dianthi*, cloned into pBR322.

Restriction, blotting, and hybridization. Methods based on those of Meinkoth and Wahl (20) were used as previously described (19). Total DNA was digested with *Hin*dIII according to manufacturer's instructions and the restriction digests were electrophoretically resolved in 1% agarose gels. The DNA was blotted to nitrocellulose or nylon membranes, probed with nick-translated probe D4 and autoradiographed, all as previously described.

Pathogenicity tests. A subset of isolates was randomly drawn from each of the major RFLP groups and the remaining groups for pathogenicity testing (see Table 2). Fungi to be tested were grown for 8 days on commercial (Merck) potato-dextrose agar petri plates. The mycelium was then homogenized in 200 ml of water and counts of spores and pieces of mycelium containing intact cells were made with a hemacytometer. The concentration was adjusted to approximately 7×10^6 propagules per milliliter.

Rooted cuttings of the carnation cultivars Barbi, White Royalette, Tony, and Red Baron were obtained from a commercial nursery. These are all of the "Miniature" group. Red Baron is resistant to race 1 of *F. o. dianthi* and susceptible to race 2; all the other cultivars are susceptible to both races (11). Eight plants of each cultivar were planted in a row in 40- × 30- × 15-cm containers in a sterile commercial potting mix (peat/sponge/volcanic tuf, 3:1:1). After planting, 200 ml of inoculum was poured into shallow trenches between the rows of cuttings. The plants were maintained in a glasshouse at 27–30 C, and container positions were randomized every few days. Plants were scored weekly from the third week after planting according to the disease index scale of Baayen and De Maat (3).

After the final score at 42 days, reisolations were made from the stem just above the crown onto potato-dextrose agar containing 250 mg/L of chloramphenicol. RFLP patterns of these reisolated fungi were subsequently determined according to the methods described above.

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Vegetative compatibility grouping. The methods of Puhalla (24) were used for the determination of VCGs. Many isolates were relatively insensitive to chlorate and in these cases the concentration of $KClO_3$ was raised to 3.5 or 6%.

Initially, unspecified mutants from each of the isolates were tested against each other to find strongly complementing pairs. These were then used in all combinations with pairs, if found, or unspecified mutants from the other isolates to determine VCGs. Two major groups emerged at this stage. The phenotype of a selection of mutants was identified and *nitM*, *nit1*, and *nit3* tester strains were designated for each of the two major groups (7). These were used for complementation tests with all available mutants of the unassigned isolates. Six isolates of *F. oxysporum* remained unclassified. As *nitM* mutants were not obtained, these could not be definitively grouped. The possibility of self-incompatibility was not further investigated (7,15).

TABLE 1. Fusarium isolates, restriction fragment length polymorphism (RFLP), and vegetative compatibility groups (VCG), cultivar of isolation, and species determination

Isolate no.	Host cultivar[a]	RFLP pattern	VCG group	*Fusarium* species[b]
1	P	D4-14	0021	Fox
3	CR	D4-14	0021	Fox
4	CR	D4-14	0021	Fox
8	M	D4-14	0021	Fox
10	TA	D4-14	0021	Fox
11	M	D4-14	0021	Fox
12	M	D4-14	0021	Fox
13	TA	D4-14	0021	Fox
14	TA	D4-14	0021	Fox
15	T	D4-14	0021	Fox
16	T	D4-14	0021	Fox
17	L	D4-14	0021	Fox
18	R	D4-14	0021	Fox
19	P	D4-14	0021	Fox
20	P	D4-14	0021	Fox
22	P	D4-14	0021	Fox
25	NA	D4-14	0021	Fox
26	NA	D4-14	0021	Fox
28	NA	D4-14	0021	Fox
30	NA	D4-14	0021	Fox
31	CR	D4-14	0021	Fox
33	CR	D4-14	0021	Fox
34	CR	D4-14	0021	Fox
35	CR	D4-14	0021	Fox
36	CR	D4-14	0021	Fox
37	CR	D4-14	0021	Fox
46	CR	D4-14	0021	Fox
49	CR	D4-14	0021	Fox
51	NA	D4-14	0021	Fox
5	WR	D4-01	0022	Fox
6	P	D4-01	0022	Fox
7	R	D4-01	0022	Fox
21	P	D4-01	0022	Fox
38	R	D4-01	0022	Fox
40	CR	D4-01	0022	Fox
42	CR	D4-01	0022	Fox
44	CR	D4-01	0022	Fox
45	CR	D4-01	0022	Fox
23	NA	Two		Fox
32	CR	1 + 1		Fox
2	CR	Nil		Fox
39	R	Nil		Fox
48	CR	Nil		Fox
50	CR	Nil		Fox
24	NA	Nil		Mon
47	CR	Nil		Mon

[a]P = Pepito, CR = Cerise Royalette, WR = White Royalette, R = Rony, T = Tony, TA = Tanga, M = Ministar, NA = Not available.
[b]Fox = *Fusarium oxysporum*, Mon = *Fusarium moniliforme*. All collected in 1985 and 1986.

Species identification. The methods and identification keys of Nelson et al (21) were used to identify isolates to species.

RESULTS

Of the 46 isolates investigated, 29 gave a complex pattern of bands (D4-14) when a *Hind*III digest of total DNA was probed with D4. These included the intense band at approximately 3,000 bp and a faint band at approximately 300 bp (not reproduced in Fig. 1 to avoid overexposing the other bands), corresponding to the probe itself. Nine isolates gave a single band pattern (D4-01). This heavy band, centered at about 4,000 bp appeared to be composed of three closely spaced bands but was not clearly resolved, even on extended runs. Six isolates did not hybridize with the probe at all. Two of these, isolates 24 and 47, were later found to be *F. moniliforme*. Isolate 32 gave two closely spaced bands, the lower of which was of the same size as one of the D4-01 bands. Additional faint, higher and lower molecular weight bands were present. Isolate 23 gave a unique two-band pattern (Fig. 1 and Table 1).

All the Fusaria with a D4-14 pattern belonged to a single VCG, as did all with the D4-01 pattern (Table 1). In line with Puhalla's (24) coding system, these are temporarily labeled VCG 0021 and VCG 0022, respectively. Among the six unassigned isolates of *F. oxysporum*, no *nitM* mutants were produced despite repeated attempts. *Nit1* and *nit3* mutants were obtained from all except isolate 32, for which only the latter was found. They could thus be excluded from VCGs 0021 and 0022 by complementation with the tester strains. No complementation was found among all possible pairings of the available mutants, and these isolates remain unassigned.

The four representative isolates from each of the RFLP groups D4-14 and D4-01 were pathogenic. One of the two isolates of *F. moniliforme* was tested and, as expected, found to be nonpathogenic. Two of the four isolates not reacting with the probe and one of the two isolates with unique RFLP patterns were also nonpathogenic. Isolate 50 produced some mild leaf yellowing, but symptoms did not progress beyond this (Table 2).

Pathogenicity data was analyzed with the Friedman test, nonpathogenic isolates being excluded from the analysis (6). Cultivars differed significantly in susceptibility, as did isolates in aggressiveness (Table 2). However, overall the two major VCGs did not differ in pathogenicity. There was no separation of groups by locality or nursery. Although disease outbreaks normally

TABLE 2. Results of pathogenicity tests on four carnation cultivars with isolates from various RFLP groups[a]

RFLP group	Isolate code	Cultivar				
		B[b]	WR	T	RB	Mean
D4-12	51	3.0	3.8	3.1	3.0	3.2 c[c]
	3	2.4	3.8	3.3	3.8	3.3 c
	16	1.4	3.1	1.9	2.6	2.3 b
	46	1.5	3.6	2.6	1.8	2.4 b
D4-01	21	0.4	1.9	0.3	0.8	0.8 a
	40	1.4	2.6	3.5	2.4	2.5 b
	42	2.0	4.0	2.9	3.6	3.1 c
	44	1.3	3.0	2.3	2.6	2.3 b
Cultivar mean		1.7 a[b]	3.2 c	2.5 b	2.6 b	
Nil	2	0.0	0.0	0.0	0.0	
	24	0.0	0.0	0.0	0.0	
	50	0.0	0.4	0.1	0.3	
Two	23	0.0	0.0	0.0	0.0	
Control		0.0	0.0	0.0	0.0	

[a]Mean disease score of eight plants on a scale of 0 = healthy, 4 = dead after 42 days.
[b]B = Barbi, WR = White Royalette, T = Tony, RB = Red Baron.
[c]Means with the same letter do not differ significantly at the 5% level (Friedman test). Nonpathogenic isolates were excluded from the analysis.

comprised a single RFLP group, in one case both groups were isolated from the same batch of plants submitted to the clinic by a nursery.

All isolates in groups D4-14 and D4-01 were recovered from inoculated plants and proved to have the same RFLP pattern on retesting. Those isolates that did not react with the probe and were nonpathogenic could not be recovered from the plants. Isolate 23, which had a rare RFLP pattern but was not pathogenic, was recovered.

DISCUSSION

The current usage of formae speciales and races in nomenclature of *F. oxysporum* is of considerable practical value to plant pathologists. Armstrong and Armstrong (1) have, however, reviewed some of the difficulties in assigning a designation to an isolate based on pathogenicity tests. Among others, some races have a wide host range, host cultivars with the same name can have differing resistance genes, and environmental conditions, host age, and inoculating methodology can affect pathogenicity (13,18,23). For example, the reaction of isolate 21 in this study could be seen as defining a new race (Table 2).

There is thus a strong argument for the development of objective techniques as indicators of genetically isolated groups. One such technique is that of RFLP analysis. RFLPs can in theory, by suitable choice of a probe, be set to any particular taxonomic level, or used as a markers for a trait, such as pathogenicity. The random probe used here delimited two major groups among the isolates. These were subsequently found to be both pathogenic and separate VCGs. The probe did not react with *F. moniliforme* and nonpathogenic isolates of *F. oxysporum*, with two exceptions.

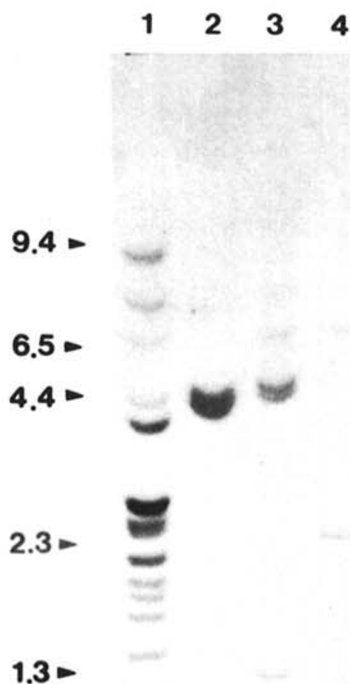

Fig. 1. Patterns of representative isolates of the four restriction fragment length polymorphism groups. Total DNA digested with *Hin*dIII and probed with D4. 1 = D4-14 pattern, VCG 0021 (isolate 30), 2 = D4-01 pattern, VCG 0022 (isolate 42), 3 = isolate 32, 4 = isolate 23. DNA size markers in kilobases are indicated on the left.

Isolate 23 did not produce disease symptoms but was reisolated from inoculated plants, implying that at least it is adapted to penetrating and growing in carnations. Isolate 32 was not tested biologically.

Little is known of the random probe used. It is derived from the nuclear DNA and a 387-bp portion giving the multiband pattern has been sequenced, but a computer search of the EMBL and GENBANK data bases has not shown it to be related to any currently known sequence (25). The patterns obtained imply a repetitive sequence, but there were no particularly unusual features that might give a clue to its function (unpublished observations).

A second objective technique is that of VCG determination. A VCG is defined as a group of isolates that are able to anastomose and form heterokaryons among one another, but not with isolates outside the group, this being controlled by multiple incompatibility loci (7). It was originally suspected that VCGs may correspond with formae speciales (24). Subsequent studies found that for *F. o. apii*, VCGs corresponded to races (8,14), as was the situation with *F. o. vasinfectum* (16). However, more than one race, as determined by differential hosts, can occur within a VCG (5,9,15) and a race can comprise more than one VCG (15,22).

Here, too, the results imply that one race has two VCGs. Only race 2 of *F. o. dianthi* is known in Israel, and the reaction of Red Baron confirms that race 1 was not among the test set. However, it cannot be discounted that a complete set of differential hosts, or a host yet to be found, may not resolve the groups into races. In general, therefore, VCGs, although grouping isolates into genetically homogeneous strains, appear to cut across race designations.

Thus, two separate methodologies have independently produced evidence for genetically separate groups within *F. o. dianthi* and provided a basis for the identification of pathogenic isolates. Only a subset of isolates were tested for pathogenicity. However, the correlation between pathogenicity and the two major VCG/RFLP groups leads us to believe that all members of these two groups are pathogenic. Similarly, those Fusaria not reacting with the probe are expected to be nonpathogenic, assuming that they are isolated from carnations, as it is known that the probe also reacts with *F. o. gladioli* and *F. o. lycopersici* (19). Of the two isolates producing rare RFLP patterns, only isolate 23 was tested biologically and this also proved to be nonpathogenic.

The use of techniques such as these should go a long way toward consolidating and rationalizing the more than 120 formae speciales and races of *F. oxysporum* described by Armstrong and Armstrong (2). Bosland and Williams (5) have laid the groundwork with their studies on *F. oxysporum* from crucifers.

LITERATURE CITED

1. Armstrong, G. M., and Armstrong, J. K. 1975. Reflections on the wilt Fusaria. Annu. Rev. Phytopathol. 13:95-103.
2. Armstrong, G. M., and Armstrong, J. K. 1981. Formae speciales and races of *Fusarium oxysporum* causing wilt diseases. Pages 391-399 in: Fusarium, Diseases, Biology and Taxonomy. P. E. Nelson, T. A. Toussoun, and R. J. Cook, eds. The Pennsylvania State University Press, University Park.
3. Baayen, R. P., and De Maat, A. L. 1987. Passive transport of microconidia of *Fusarium oxysporum* f. sp. *dianthi* in carnation after root inoculation. Neth. J. Plant Pathol. 93:3-13.
4. Beckmann, J. S., and Soller, M. 1986. Restriction fragment length polymorphisms in plant genetic improvement. Pages 197-250 in: Oxford Surveys of Plant Molecular and Cell Biology. B. J. Miflin, ed. Oxford University Press, Oxford.
5. Bosland, P. W., and Williams, P. H. 1987. An evaluation of *Fusarium oxysporum* from crucifers based on pathogenicity, isozyme polymorphism, vegetative compatibility, and geographic origin. Can. J. Bot. 65:2067-2073.
6. Conover, W. J. 1980. Practical Nonparametric Statistics. John Wiley & Sons, New York.
7. Correll, J. C., Puhalla, J. E., Schneider, R. W., and Kraft, J. M. 1985. Differentiating races of *Fusarium oxysporum* f. sp. *pisi* based

on vegetative compatibility. (Abstr.) Phytopathology 75:1347.
8. Correll, J. C., Klittich, C. J. R., and Leslie, J. F. 1987. Nitrate nonutilizing mutants of *Fusarium oxysporum* and their use in vegetative compatibility tests. Phytopathology 77:1640-1646.
9. Correll, J. C., Puhalla, J. E., and Schneider, R. W. 1986. Identification of *Fusarium oxysporum* f. sp. *apii* on the basis of colony size, virulence, and vegetative compatibility. Phytopathology 76:396-400.
10. Fisher, N. L., Burgess, L. W., Toussoun, T. A., and Nelson, P. E. 1982. Carnation leaves as a substrate and for preserving cultures of *Fusarium* species. Phytopathology 72:151-153.
11. Garibaldi, A. 1977. Race differentiation in *Fusarium oxysporum* f. sp. *dianthi* and varietal susceptibility. Acta Hortic. 71:97-101
12. Garibaldi, A. 1983. Resistenza di cultivar di garofano nei confronti di otto patotipi di *Fusarium oxysporum* f. sp. *dianthi* (Prill. et Del.) Snyd. et Hans. Riv. Ortoflorofrutti. Ital. 67:261-270.
13. Hart, L. P., and Endo, R. M. 1981. The effect of time of exposure to inoculum, plant age, root development, and root wounding on Fusarium yellows of celery. Phytopathology 71:77-79.
14. Ireland, K. F., and Lacey, M. L. 1986. Laboratory identification of pathogenic isolates of *Fusarium oxysporum* f. sp. *apii* race 2. (Abstr.) Phytopathology 76:956.
15. Jacobson, D. J., and Gordon, T. R. 1988. Vegetative compatibility and self-incompatibility within *Fusarium oxysporum* f. sp. *melonis*. Phytopathology 78:668-672.
16. Katan, T., and Katan, J. 1988. Vegetative-compatibility grouping of *Fusarium oxysporum* f. sp. *vasinfectum* from tissue and rhizosphere of cotton plants. Phytopathology 78:852-855.
17. Kistler, H. C., Bosland, P. W., Benny, U., Leong, S., and Williams, P. H. 1987. Relatedness of strains of *Fusarium oxysporum* from crucifers measured by examination of mitochondrial and ribosomal DNA. Phytopathology 77:1289-1293.
18. Kraft, J. M., and Haglund, W. A. 1978. A reappraisal of the race classification of *Fusarium oxysporum* f. sp. *pisi*. Phytopathology 68:273-275.
19. Manicom, B. Q., Bar-Joseph, M., Rosner, A., Vigodsky-Haas, H., and Kotze, J. M. 1987. Potential applications of random DNA probes and restriction fragment length polymorphisms in the taxonomy of the Fusaria. Phytopathology 77:669-672.
20. Meinkoth, J., and Wahl, G. 1984. Hybridization of nucleic acids immobilized on solid supports. Anal. Biochem. 138:267-284.
21. Nelson, P. E., Tousson, T. A., and Marasas, W. F. O. 1983. Fusarium Species. An Illustrated Manual for Identification. The Pennsylvania State University Press, University Park.
22. Ploetz, R. C., and Correll, J. C. 1988. Vegetative compatibility among races of *Fusarium oxysporum* f. sp. *cubense*. Plant Dis. 72:325-328.
23. Pound, G. S., and Fowler, D. L. 1953. Fusarium wilt of radish in Wisconsin. Phytopathology 43:277-280.
24. Puhalla, J. E. 1985. Classification of strains of *Fusarium oxysporum* on the basis of vegetative compatability. Can. J. Bot. 63:179-183.
25. Queen, C., and Korn, L. J. 1984. A comprehensive sequence analysis program for the IBM personal computer. Nucleic Acids Res. 12:581-599.
26. Raeder, U., and Broda, P. 1985. Rapid preparation of DNA from filamentous fungi. Lett. Appl. Microbiol. 1:17-20.