Use of the RAPD Technique for Identification of *Fusarium oxysporum* f. sp. *dianthi* from Carnation

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ABSTRACT

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The random amplified polymorphic DNA (RAPD) method was used to distinguish among pathogenic and nonpathogenic isolates of *Fusarium oxysporum* recovered from carnation. Fifty-eight isolates, which were isolated in Israel from different cultivars of carnation, included 42 pathogenic isolates of race 2, one pathogenic isolate of race 4, and 15 nonpathogenic isolates. These isolates were examined for RAPD patterns with 30 arbitrary primers of 10 bases. The RAPD patterns generated by each of 22 primers enabled us to distinguish clearly between pathogenic and nonpathogenic isolates from carnation. The amplification patterns of race 2 isolates were identical for all the primers examined. Seventeen of 22 primers enabled differentiation between races 2 and 4. A 1.4-kb amplified DNA probe obtained from a pathogenic isolate of race 2 exclusively hybridized with DNA of race 2 isolates. Compared with other methods of identifying *F. o.* f. sp. *dianthi*, the RAPD procedure is simple, rapid, and reproducible.

Additional keywords: DNA fingerprinting, DNA polymorphisms.

Wilt disease, caused by *Fusarium oxysporum* Schlechtend.:Fr. f. sp. *dianthi* (Prill. & Delacr.) W.C. Snyder & H.N. Hans., is considered a major limiting factor in all areas of carnation (*Dianthus caryophyllus* L.) production (4). Eight physiological races have been reported within this forma specialis (3), but only race 2 is found worldwide (1). In Israel, among 66 isolates of *F. o. dianthi* isolated during four growing seasons, 65 were classified as race 2 and one as race 4 (2). Although the diseases caused by *F. oxysporum* are managed most effectively with resistant cultivars, indexing is routinely used in carnation culture to ensure the supply of cuttings is free of *F. o. dianthi*. Often, tissues of symptomless carnation cuttings yield *Fusarium* colonies on culture media. Although it is relatively easy to identify *Fusarium* to the species level, the confirmation of forma specialis and race determinations requires pathogenicity tests. In carnation, the pathogenicity test is relatively long (24–40 days) and requires extensive facilities if large numbers of samples are tested.

Several methods for detecting genetic variation in *F. oxysporum* have been used (5,6,9,10,12). In *F. o. dianthi*, two techniques for identification of pathogenic isolates, including vegetative compatibility groups (VCG) and restriction fragment length polymorphism (RFLP), were suggested (8,11). We report the use of the RAPD (random amplified polymorphic DNA) method (15,16) for generating amplification patterns specific to *F. o. dianthi*. This technique uses PCR (polymerase chain reaction) with a single short arbitrary primer to amplify a small number of fragments from genomic DNA. The purpose of this study was to develop a rapid procedure for identification of pathogenic *F. o. dianthi* isolated from carnation cuttings.

MATERIALS AND METHODS

Fungal isolates. The isolates of *F. oxysporum* used for this study are listed in Table 1. Isolations were taken from carnation plants showing typical disease symptoms or from symptomless cuttings. Isolates were recovered during 1988–1993 from 39 carnation cultivars collected at 22 sites in Israel. Race identification was done by inoculating differential cultivars as described previously (2). Of the 58 isolates listed in Table 1, 42 were race 2, one was race 4, and 15 were nonpathogenic. Single-spore isolates were made of each isolate, and the cultures were maintained on potato-dextrose agar. Isolates from other forma speciales used in this study were; *F. o. lycopersici* FOL-WOO54L, *F. o. radicis-*

lycopersici FORL-C63F, and *F. o. melonis* FOM-3009. These isolates were obtained from T. Katan (ARO, The Volcani Center, Bet Dagan, Israel).

DNA extraction. Cultures were grown in potato-dextrose broth in still culture for 4 days. The mycelium was filtered through cheesecloth, washed with sterile water, and transferred to filter paper for removal of excess water. Using a mortar and pestle, 0.5–1.0 g of wet mycelium was ground to a fine powder in liquid nitrogen. The ground mycelium was transferred to a 1.5-ml microcentrifuge tube and DNA was extracted according to Kistler et al (13).

PCR conditions. PCR was carried out in 25 μl of a solution containing 50 ng of *F. oxysporum* genomic DNA; 2 mM $MgCl_2$; 0.4 μM of primer; 1.5 U of *Taq* DNA polymerase (Promega Corporation, Madison, WI); 100 μM each of dCTP, dGTP, dATP, and dTTP (Boehringer Mannheim, Germany) in 10 mM Tris-HCl, pH 9.0; 50 mM KCl; and 0.1% Triton X-100 (Union Carbide Chemicals and Plastics Co., Inc., Danbury, CT), under a drop of mineral oil. Amplification was performed in a thermal cycler (Hybaid Limited, Teddington, England) programmed for one cycle of 5 min at 94 C, 1 min at 35 C, and 2 min at 72 C, followed by 40 cycles of 1 min at 94 C, 1 min at 35 C, and 2 min at 72 C and a final incubation at 72 C for 5 min. After PCR, 12.5 μl of the products was electrophoresed in 1.2% agarose gel and visualized by ethidium bromide staining. Lambda DNA digested by *Pst*I was used as the molecular weight markers.

TABLE 1. Isolates of *Fusarium oxysporum* from carnation

Isolate number	Year of isolation	Host cultivar	Location in Israel	Race
1-88	1988	Eveline	Buregeta	2
2-88	1988	Yellow Palace	Buregeta	2
3-88	1988	Pink Candy	Buregeta	2
4-88	1988	Lior	Buregeta	2
8-88	1988	Lior	Hawwat Noy	2
9-88	1988	Tanga	Olesh	2
11-88	1988	Sarina	Ge'ullim	2
12-88	1988	Castellaro	Buregeta	2
21-88	1988	Orange Tony	Bet Ezra	2
23-88	1988	Cerise-Royallete	Bet Ezra	2
32-88	1988	Tanga	Mishmeret	2
34-88	1988	Lior	Kfar Ya'betz	2
2-89	1989	Delta	Kokhav Mikha'el	2
3-89	1989	Cerise-Royallete	Ge'a	2
6-89	1989	White-Royallete	Yad Natan	2
9-89	1989	Desio	Na'an	2
17-89	1989	Oren	Yanuv	2
18-89	1989	White-Royallete	Azri'el	2
22-89	1989	Shani	Hawwat Noy	2
24-89	1989	Rimon	Hawwat Noy	2
29-89	1989	Alicetta	Hawwat Noy	2
30-89	1989	1681	Hawwat Noy	2
33-89	1989	Smarty	Hawwat Noy	2
34-89	1989	Yael	Shilat	2
39-89	1989	Robina	Bet Dagan	2
40-89	1989	Galit	Bet Dagan	2
2-90	1990	Desio	Azri'el	2
32-90	1990	Sandrosa	Hawwat Noy	2
8-91	1991	F-541-121	Hawwat Noy	2
16-91	1991	Lara	Hawwat Noy	2
1-92	1992	Hermon	Bene Zion	2
2-92	1992	Fantasia	Bet Dagan	2
3-92	1992	Raggio-di-sole	Bet Dagan	2
4-92	1992	Aviv	Bet Dagan	2
5-92	1992	Galit	Bet Dagan	2
6-92	1992	Candy	Bet Dagan	2
7-92	1992	Lior	Bet Dagan	2
8-92	1992	Eveline	Bet Dagan	2
9-92	1992	Rony	Qalansuwa	2
52-93	1993	Bagatel	Qalansuwa	2
59-93	1993	Bagatel	Qalansuwa	2
67-93	1993	Bagatel	Qalansuwa	2
12-89	1989	Rosaline	Zofit	4
25-88	1988	Rosaline	Na'an	np[a]
45-88	1988	Sandrosa	Neta'im	np
3-90	1990	Miniderby	Azri'el	np
6-90	1990	Stefani	Tenuvot	np
F48	1992	Galatti	Tequma	np
R82a	1992	Francesco	Tequma	np
F83a	1992	Francesco	Tequma	np
W.Q.10	1992	White Queen	Hadar Am	np
W.Q.16	1992	White Queen	Hadar Am	np
F83b	1992	Francesco	Tequma	np
R82b	1992	Francesco	Tequma	np
62-93	1993	Bordeaux	Tequma	np
10-93	1993	Ruby Red	Zofit	np
11-93	1993	Ruby Red	Zofit	np
61-93	1993	Miniderby	Tequma	np

[a]np = nonpathogenic.

Primers. Ten-base oligonucleotide primers were purchased from Operon Technologies, Inc., Alameda, CA; kits A, B, C, D, and E were used. The primers were selected randomly from these kits.

Southern blot analysis of RAPD products. DNA samples after amplification were electrophoresed in 1.2% agarose gels, alkali denatured, transferred to nylon membranes (Amersham International Corp., Birmingham, England) by the capillary transfer method, and fixed to the membrane by UV-irradiation (13). Two randomly selected amplification products obtained with template DNA from isolate 32-90 were recovered from the gel, purified with a Geneclean kit (Bio 101 Inc., San Diego, CA), and labeled with a digoxigenin-dUTP kit (Boehringer Mannheim). For dot blot hybridization, 10 μg of total DNA from pathogenic and nonpathogenic isolates was spotted on nylon membranes. Prehybridization and hybridization were performed at 42 C as recommended by the DNA-labeling manufacturer.

RESULTS

To select primers that generate informative arrays of PCR products, we tested 30 primers with DNA of *F. o. dianthi* race 2 (isolate 29-89). Representative results obtained with seven primers are given in Figure 1. The RAPD patterns differed with each of the primers. Some of the primers generated more DNA fragments than did others. The size of the products was within the range of 0.5 to 2.5 kb. Among the 30 primers examined, 22 gave reproducible PCR patterns, whereas eight produced either

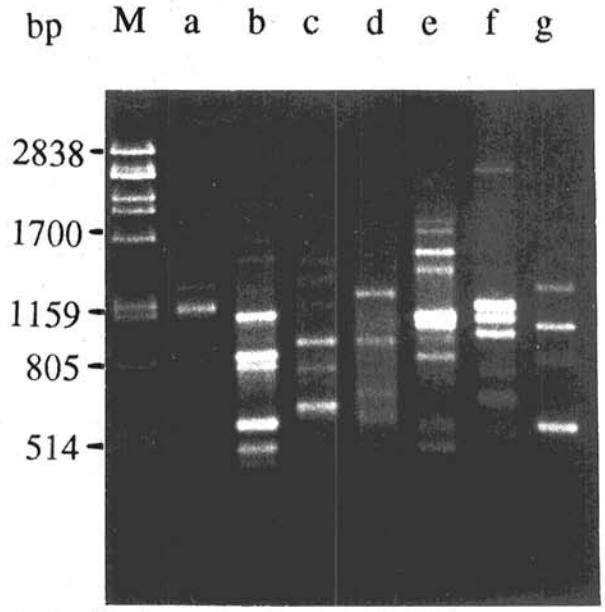

Fig. 1. Amplification patterns generated with *Fusarium oxysporum* f. sp. *dianthi* (isolate 29-89) with seven primers. Lane M contains molecular weight markers of lambda DNA digested by *Pst*I. Lanes a–g contain polymerase chain reactions with primers ACCGCGAAGG, GTCGCCGTCA, TCTGGTGAGG, GTGTGCCCCA, GAGAGCCAAC, CAGGCCCTTC, and CCGCATCTAC, respectively.

no amplification or only very weak amplification products. Only major amplification products were considered.

PCRs were carried out with each of the 22 primers and DNA from 61 *F. oxysporum* isolates. Representative results are given in Figure 2. All 42 *F. o. dianthi* race 2 isolates produced identical RAPD patterns with all the primers. The patterns were distinguishable from those produced by nonpathogenic isolates recovered from carnation and from other forma speciales. The *F. o. dianthi* race 4 isolate differed from race 2 in its RAPD pattern when tested with 17 primers, whereas five primers produced identical patterns. Representative results are given in Figure 3, in which primers h, i, b, and j produced similar patterns and primers k, f, and g produced different patterns.

To examine the reproducibility of the RAPD patterns, we used DNA preparations of eight isolates from separate cultures as well as different single spores from the same cultures. Identical DNA patterns were obtained with all the tested primers (results not shown). The RAPD profiles were obtained with *Taq* DNA polymerase from Promega. A few experiments carried out with Pyrostase (thermostable DNA polymerase from Molecular Genetic Resources Inc., Tampa, Florida) also resulted in a reproducible pattern of RAPD products, although the intensity of the bands was somewhat different. To test the homology among the amplification products produced by DNA of race 2 isolates, two 1.4- and 0.5-kb DNA fragments were purified from agarose gel,

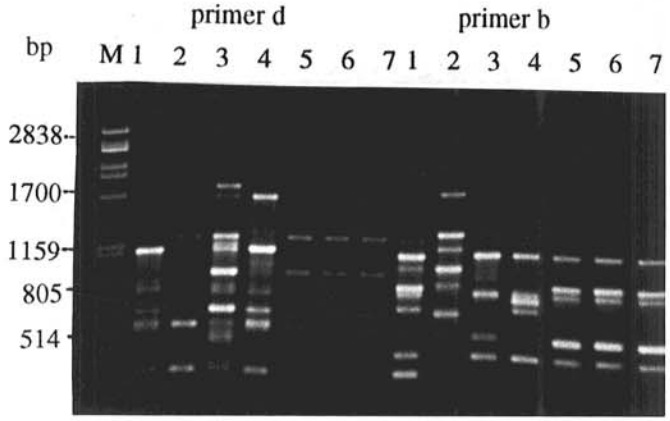

Fig. 2. Comparison of the random amplified polymorphic DNA patterns between pathogenic and nonpathogenic *Fusarium oxysporum* isolates. Polymerase chain reactions were carried out with primers b and d (the sequences are given in Fig. 1). Lanes 1–7 contain different isolates. 1–4 are nonpathogenic; 5–7 are pathogenic; 1 is F83a; 2 is W.Q.1O; 3 is R82a; 4 is R82b; 5 is 24-89; 6 is 33-89; and 7 is 34-89. Lane M contains the molecular weight markers of lambda DNA digested by *Pst*I.

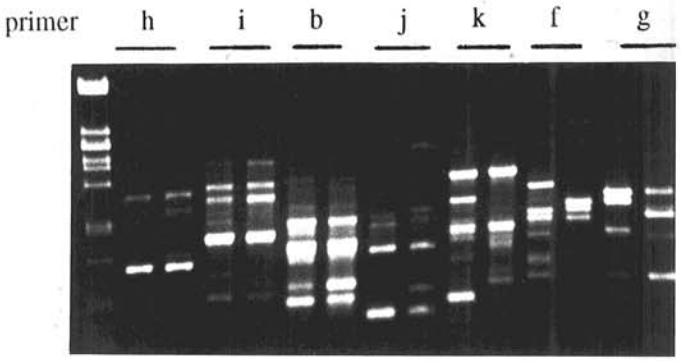

Fig. 3. Comparison of the random amplified polymorphic DNA patterns between pathogenic isolates of races 4 and 2 of *Fusarium oxysporum* f. sp. *dianthi*. Each primer was examined with isolates 12-89 (race 4) and 29-89 (race 2). Lanes h–g contain polymerase chain reactions with primers GGTGCGGGAA, AGGGGTCTTG, GTCGCCGTCA, CTACCGTGCT, CCAGATGCAC, CAGGCCCTTC, and CCGCATCTAC, respectively. Primers in lanes h, i, b, and j gave identical patterns; primers in lanes k, f, and g gave different patterns.

labeled with digoxigenin, and used as probes. A Southern blot of RAPD products hybridized with the 1.4-kb probe is illustrated in Figure 4. Results indicated that comigrating DNA fragments generated with a single primer from different *F. o. dianthi* race 2 isolates were homologous, whereas no hybridization occurred with RAPD products of race 4 or other *F. oxysporum* forma speciales. Similar results were obtained with the 0.5-kb probe (results not shown). In dot blot experiments, the 1.4-kb probe did not hybridize with total DNA of the nonpathogenic isolates.

DISCUSSION

Since its recent introduction (15,16), the RAPD technique has been widely used to detect genetic polymorphisms in various organisms, including fungi (7,14). Our results indicate that an array of race 2 isolates recovered over a 5-yr period from various carnation cultivars and sites in Israel produced identical RAPD patterns with all the arbitrary primers used. Thus, all the race 2 isolates examined are identical for a large number of RAPD loci. The uniformity of the *F. o. dianthi* found in Israel has been previously suggested on the basis of VCG (8), although race identification was not determined. Another study, using the RFLP technique, showed that two major coincident groups of VCG and RFLP exist in the population of *F. o. dianthi* in Israel (11). Our results stress the uniformity of *F. o. dianthi* race 2 isolates in Israel. This uniformity may be explained by the clonal structure of race 2 all over the world or by the fact that all the Israeli isolates may be clonal derivatives of a single isolate introduced into the country, which subsequently proliferated and was distributed. However, because numerous carnation cultivars have been introduced into Israel, it is less likely that *F. o. dianthi* evolved from a single source. It would be worthwhile to compare the RAPD pattern of race 2 isolates from Israel with those from other countries.

Our results may imply that with use of the RAPD procedure it is possible to distinguish between different races. Although only one isolate of race 4 was examined, it did show DNA polymorphism between the two races with most of the primers that were employed. However, additional isolates of race 4 should be examined to confirm the latter assumption. The exclusive hybridization of amplified DNA probes derived from RAPD products of race 2 with DNA of the same race might be manipu-

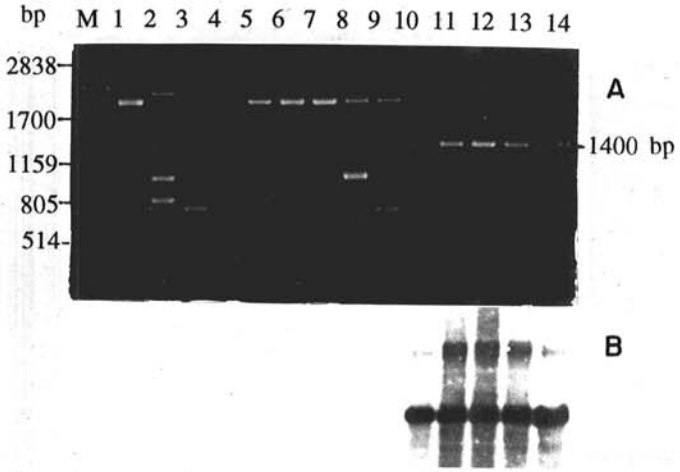

Fig. 4. Representative Southern blot test of homology with random amplified polymorphic DNA polymerase chain reaction products. **A,** Gel electrophoresis of fragments generated from pathogenic and nonpathogenic isolates of *Fusarium oxysporum* with the primer TTTCCCACGG. Lanes 1–5 contain nonpathogenic isolates 45-88, 25-88, W.2.16, F83b, and R82a, respectively; 6–8 contain *F. o. radicis-lycopersici*, *F. o. lycopersici*, and *F. o. melonis*, respectively; 9 contains race 4; and 10–14 contain pathogenic isolates 3-88, 32-90, 8-91, 2-90, and 29-89, respectively. Lane M contains the molecular weight markers of lambda DNA digested by *Pst*I. **B,** Southern blot of DNA probed with the 1.4-kb fragment generated from DNA of isolate 32-90.

lated to obtain DNA-specific probes for practical diagnosis of *F. o. dianthi* race 2 with the dot blot assay.

The RAPD procedure may be superior to RFLP and VCG in identifying *F. oxysporum* from carnation, because it is technically simple, rapid, requires only small amounts of DNA, and involves no radioactivity. In carnation, it might be adapted for use in a large-scale screening of pathogenic isolates. Its potential for differentiating races of *F. o. dianthi* is promising and should be thoroughly examined.

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