Vegetative-Compatibility Grouping of *Fusarium oxysporum* f. sp. *vasinfectum* from Tissue and the Rhizosphere of Cotton Plants

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ABSTRACT

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Nitrate-nonutilizing (*nit*) mutants were produced from 12 isolates of *Fusarium oxysporum* f.sp. *vasinfectum* race 3, collected from five sites in two regions in Israel. Complementation (heterokaryon) tests showed that all of the isolates belonged to a single vegetative-compatibility group (VCG), and two mutants were chosen as its testers. Additional isolates of *Fusarium* from root tissue and the rhizosphere of diseased susceptible (cv. Pima S-5) and healthy resistant (Pima-type cv. F-27) cotton plants (*Gossypium barbadense*), growing in soils naturally infested with *F. o. vasinfectum*, were analyzed for pathogenicity and vegetative compatibility with the testers. A total of 631 *Fusarium* isolates, obtained from three sites in two separate regions, were tested. All the *nit* mutants of all the pathogenic isolates formed heterokaryons with the testers, indicating that they belonged to the same VCG. None of the nonpathogenic isolates was vegetatively compatible with the testers. Pathogenesis occurred in 90–94% of isolates from tissues and 3–87% of isolates from the rhizosphere of susceptible plants. Pathogenesis occurred in 1% of isolates from tissues and 4% of isolates from the rhizosphere of the resistant Pima-type cultivar F-27. Population density of *F. o. vasinfectum* in the tissues of the susceptible plants ranged from 27,600 to 346,000 colony-forming units (cfu) per g of tissue, compared with 33–7,400 cfu per gram of soil in the rhizosphere of the same plants. The *nit* mutants retained pathogenicity to cotton. The *F. o. vasinfectum* testers were incompatible with testers of four other formae speciales of *F. oxysporum*.

Additional keyword: Fusarium wilt.

Fusarium wilt of cotton is a relatively new disease in Israel that was first detected in 1974 (8). The pathogen, identified as race 3 of *Fusarium oxysporum* f. sp. *vasinfectum*, is pathogenic to long-staple Pima-type cotton (*Gossypium barbadense*) plants, but not to the upland Acala-type cotton (*G. hirsutum*). Until the summer of 1986, this pathogen was restricted in Israel to one region, the Bet She'an Valley. In 1986, *F. o. vasinfectum* race 3 was found on wilted Pima plants in a second region, the Coastal Plain.

Pathogenicity tests were the primary means to distinguish different pathogenic *Fusarium* strains. However, such tests do not indicate whether various isolates of a given forma specialis or a physiologic race are genetically related. In 1985, Puhalla introduced a novel approach to demonstrate relatedness among *Fusarium* strains (11). By this method, the ability of two isolates to anastomose and form heterokaryons indicates that they are vegetatively compatible. Using nitrate-nonutilizing (*nit*) mutants, Puhalla showed that isolates in different formae speciales of *F. oxysporum* were in distinct vegetative-compatibility groups (VCG), based on the ability of complementary *nit* mutants to form wild-type heterokaryons. These results supported the theory that, in the absence of sexual cycle and meiotic recombination, gene sets that determine vegetative compatibility and genes for pathogenicity became fixed together through evolution, giving rise to distinct VCGs of *F. oxysporum* characterized by specific virulence.

The purposes of this study were: to test if vegetative compatibility exists among isolates of *F. o. vasinfectum* race 3 from different soils from two regions in Israel, and between *F. o. vasinfectum* race 3 and other formae speciales of *F. oxysporum*; to determine if there are nonpathogenic *F. oxysporum* isolates associated with cotton plants that are vegetatively compatible with *F. o. vasinfectum* race 3; and to determine the agreement between pathogenicity tests and the VCG technique in distinguishing between *F. o. vasinfectum* race 3 and nonpathogenic strains of *F. oxysporum* in mixed populations originating from plant tissue and rhizosphere.

MATERIALS AND METHODS

Media. Potato-dextrose agar (PDA; Difco) was used to maintain cultures and to grow inoculum for pathogenicity tests. *Fusarium*-selective medium (10), acidified with 1 ml/L of 90% lactic acid to suppress bacterial contamination, was used for isolation of *Fusarium* from plant tissue and the rhizosphere. Puhalla's *Fusarium*-minimal medium (FMM) (11) is a sucrose-salt medium containing nitrate as the nitrogen source. FMM was used to recognize *nit* mutants and for complementation (heterokaryon) tests. *Neurospora*-minimal medium (NMM) (2) was used to compare growth of *nit* mutants in the presence of ammonium nitrogen. Nitrite and hypoxanthine media were used for partial phenotypic characterization of *nit* mutants (3). Chlorate media, based on FMM or potato-sucrose agar (11), were used to generate *nit* mutants. FMM acidified with 1 ml/L of 90% lactic acid was used to reisolate *nit* mutants from inoculated diseased plants.

Pathogens. The following pathogenic strains of *Fusarium* were used: *F. oxysporum* Schlecht. f. sp. *vasinfectum* (Atk.) Snyd. & Hans., *F. oxysporum* Schlecht. f.sp. *niveum* (E. F. Smith) Snyd. & Hans., *F. oxysporum* Schlecht. f. sp. *melonis* Snyd. & Hans., *F. oxysporum* Schlecht. f.sp. *dianthi* (Prill & Del.) Snyd. & Hans., and *F. oxysporum* Schlecht. f.sp. *lycopersici* (Sacc.) Snyd. & Hans.

Isolates of *F. o. vasinfectum* and pathogenicity tests. All isolates of the pathogen were obtained from cotton plants showing typical disease symptoms (wilt and xylem discoloration). They were identified as belonging to race 3 by their pathogenicity to Pima S-5 cultivar (*G. barbadense* L.), but not to cultivar SJ-2 (*G. hirsutum* L.) or to the resistant F-27 Pima cultivar. Only pathogenic isolates of *F. oxysporum* were classified as *F. o. vasinfectum*. Diseased plants were collected from four fields in the Bet She'an Valley and

from one field in the Coastal Plain (Table 1). The fungus was isolated by plating plant tissues (surface-disinfested with 1% sodium hypochlorite for 2 min) on PDA and incubating them at 27 C for 5 days. A pathogenicity test of the *Fusarium* isolates was carried out in the greenhouse by inoculating each isolate on seven cotton seedlings of cultivar S-5 using the root-dip technique (7). Symptoms were visible on the inoculated seedlings in 7–10 days. Noninoculated Pima S-5 seedlings, and Acala and Pima F-27 seedlings inoculated with a total of about 100 isolates from different sources and maintained as controls under the same conditions, remained healthy throughout the pathogenicity tests.

Isolation of *nit* mutants. Plates (9 cm in diameter) of chlorate media were inoculated at four points with small mycelial transfers of FMM cultures (one isolate per plate) and incubated at 27 C. Fast-growing sectors that emerged from the restricted colonies were transferred to FMM plates (6 cm in diameter) and examined after a 3-day incubation. Colonies with an expanding thin mycelium were considered *nit* mutants (11). All *nit* mutants showed wild-type growth on NMM and PDA.

Complementation tests. Complementation between *nit* mutants was tested on FMM plates (6 cm in diameter). Usually, three mutants were inoculated on each plate, forming a triangle, and the plates were incubated at 27 C. Complementation was evident by the formation of a dense aerial wild-type mycelium where two mutants had met and formed a heterokaryon (Fig. 1). Absence of wild-type growth at the contact zone between two *nit* mutants of the same parent isolate indicated allelic, overlapping, or otherwise noncomplementary mutations (Fig. 1). On the other hand, absence of wild-type growth at the contact zone of *nit* mutants from different parent isolates could be caused not only by noncomplementarity but also by vegetative incompatibility, which prevents heterokaryon formation. Heterokaryons were usually evident within 7 days. Some pairs of mutants reacted faster (4–6 days), whereas few required up to 14 days to form visible heterokaryons.

Fusarium counts in rhizosphere soil and in tissue. Cotton seeds of the indicated cultivars were sown in 12-cm pots filled with naturally infested soils brought from the fields. The pots were maintained in the greenhouse for 25 days. Diseased Pima S-5 or healthy F-27 plants were then uprooted, in three replicates, and soil particles adhering to the roots were collected in a sterile vial by shaking. The remaining soil, tightly adhering to the roots (less than 5% of the total amount of the rhizosphere soil), was collected by shaking in sterile 0.1% water agar for 30 min. The two soil fractions were then combined to constitute the rhizosphere soil sample. After removal of the soil, *Fusarium* populations in plant tissues were determined essentially as described previously (7). The roots were washed thoroughly, blotted on filter paper, weighed, surface-disinfested, and macerated in sterile 0.1 % water agar for 40 sec in a high-speed homogenizer (Ultra Turax, W. Germany). Aliquots (0.2 ml) of serial dilutions of the rhizosphere soil and the tissue suspensions were spread over *Fusarium*-selective medium. Total *Fusarium* sp. populations were counted after a 5-day incubation at 27 C. Results were expressed as colony-forming units per gram of dry soil or fresh tissue. Randomly chosen colonies with morphological characteristics of *F. oxysporum*, thus obtained from tissues and rhizosphere soils, were transferred individually to PDA plates. After a 7-day incubation at 27 C, the isolates were tested for pathogenicity, and the percentages of the pathogenic strains were calculated.

TABLE 1. List of sites, isolates of *Fusarium oxysporum* f. sp. *vasinfectum* race 3, and numbers of *nit* mutants and *nit*-complementation groups obtained from each isolate

Site	Isolate	Number of *nit* mutants	Number of complementation groups
En Harold[a]	FOV-EH	9	4
Nir Dawid[a]	FOV-ND	159	≥4[b]
	FOV-ND I	7	2
	FOV-ND II	5	3
Ma'oz Hayyim[a]	FOV-MH-2	5	2
	FOV-MH-3	4	3
	FOV-MH-4	2	1
Hefzi bah[a]	FOV-FIL-61	3	3
	FOV-FIL-64	1	1
	FOV-FIL-66	2	1
	FOV-FIL-69	3	2
HaHoterim[c]	FOV-348	8	4

[a] Located in the Bet She'an Valley.
[b] Not all possible combinations of *nit* mutants were tested.
[c] Located in the Coastal Plain.

RESULTS

Selection of *nit* testers. Varying numbers of chlorate-resistant sectors and proportions of *nit* mutants were obtained from the different *F. o. vasinfectum* isolates. The mutants of each isolate were first paired among themselves on FMM to reveal complementation within the isolate (Table 1, Fig. 1). Several mutants of each isolate, representing all of its *nit* complementation groups, were then paired on FMM with mutants of the other isolates in all possible combinations. All the mutants were able to anastomose with at least some of the other mutants and produce heterokaryons with wild-type growth where colonies had met. The pattern of heterokaryon formation between the mutants of the different isolates indicated that all *F. o. vasinfectum* isolates belonged to a single VCG. Based on their ability to form heterokaryons with many of the mutants, six mutants were chosen and compared further for their ability to form (with the collection of mutants mentioned) clearly defined heterokaryons within the shortest time. Since heterokaryons with mutations at the same locus do not show wild-type growth, at least two complementary mutants are needed as testers of a given VCG. Two of the mutants were finally chosen as testers of the VCG of *F. o. vasinfectum* race 3, based on their fast reaction (4–6 days) with the other mutants. Most of the mutants in the collection formed wild-type heterokaryons with both testers, whereas only a few reacted with either one. The testers were designated FOV-51 and FOV-210 and were used to identify the VCG in populations of *Fusarium* sp. isolated from cotton. Both *nit* testers were characterized as carrying mutations of the *nit* M group, as indicated by their mutant phenotype on nitrate and hypoxanthine media and wild-type growth on NMM and nitrite media (3).

Vegetative-compatibility tests with testers of other formae speciales of *F. oxysporum*. Four *nit* mutants of the present study (including the two testers) were paired on FMM with 11 *nit* testers of the following four formae speciales (T. Katan and J. Katan,

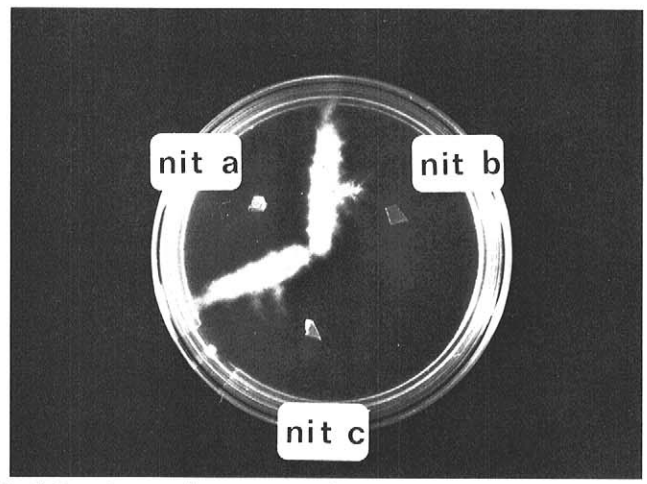

Fig. 1. Complementation test among three *nit* mutants (marked *nit* a, *nit* b, and *nit* c) of a single isolate of *Fusarium oxysporum* f. sp. *vasinfectum* race 3. Complementation between mutant *nit* a and mutants *nit* b or *nit* c is manifested by wild-type growth of the heterokaryon formed where the mutant colonies met, whereas mutants *nit* b and *nit* c are noncomplementary with each other.

unpublished): *niveum* (2 testers), *melonis* (5 testers), *lycopersici* (2 testers, race 1 and race 2), and *dianthi* (2 testers). The *F. o. vasinfectum* race 3 testers, FOV-51 and FOV-210, also were paired with Puhalla's testers 0-1078/A and B (f. sp. *lycopersici* race 2) and 0-4/A (f. sp. *dianthi*) (11). No heterokaryon formed between the *F. o. vasinfectum* testers and any of the other formae speciales.

Vegetative compatibility and pathogenicity of *Fusarium* sp. from cotton. *Fusarium* populations in three sites where the soil had been naturally infested with *F. o. vasinfectum* race 3 were tested for pathogenicity to Pima cotton and for VCG using the testers. *Fusarium* was isolated from the tissue and rhizosphere of cotton plants growing in the soils to be analyzed, and each isolate was subjected to the two tests. From each isolate, 1–3 *nit* mutants were generated and paired with the testers. The results of these tests are presented in Table 2. Of 631 isolates tested, 283 were from tissue and 348 were from rhizosphere. Without exception, all of the pathogenic isolates and none of the nonpathogenic isolates produced visible heterokaryons with the testers (Fig. 2). Between 90–94% of the *Fusarium* isolates from tissue of a susceptible cultivar was pathogenic (Table 2). The proportions of pathogenic isolates from rhizospheres ranged between 3–87% in the three soils tested. When a susceptible cultivar and a resistant cultivar were planted separately in the Nir Dawid soil, 94% of isolates recovered from tissues of the susceptible cultivar and 4% (one isolate) of isolates recovered from the resistant cultivar was pathogenic. The percentages of pathogenic isolates from the rhizospheres of the susceptible and the resistant plants were 87 and 1, respectively. Population density of *F. o. vasinfectum* in the tissues of the susceptible S-5 plants was much higher (10–836 times) than in the rhizospheres of these plants. In comparison with the susceptible plants, populations of the pathogen in tissue and rhizosphere of the resistant F-27 plants were very low (Table 2). Density of nonpathogenic *Fusarium* population in the tissues of the susceptible plants was 3–28 times higher than in their rhizospheres.

Twenty-five *nit* mutants of various complementation groups, originating from nonpathogenic isolates from tissues, were paired in various combinations in an attempt to reveal common VCGs among nonpathogenic root colonizers from different populations (4). Only three isolates, originating from two sites, could be assigned to one VCG, whereas none of the other combinations resulted in heterokaryon formation. Similarly, no heterokaryons were observed between mutants of nonpathogenic tissue and rhizosphere colonizers originating from the same soil. Although some isolates of these tests were represented by two or three complementary mutants, not all possible combinations were tried. Therefore, the presence of VCGs among the nonpathogens has not been ruled out conclusively.

Pathogenicity of *nit* mutants. Thirty-five *nit* mutants, originating from 10 pathogenic cultures, were tested for pathogenicity. All the mutants were pathogenic on Pima cotton,

TABLE 2. Pathogenicity[a] and vegetative compatibility[b] of *Fusarium* isolates from tissue and the rhizosphere of cotton plants in soils naturally infested with *F. oxysporum* f. sp. *vasinfectum* race 3

		Numbers of isolates								Pathogenic isolates			
		Tissue				Rhizosphere				cfu/g[d]		(%)	
		P		NP		P		NP					
Site	Cultivar[c]	(+)	(−)	(+)	(−)	(+)	(−)	(+)	(−)	T[e]	R[e]	T	R
HaHoterim	S-5	90	0	0	8	63	0	0	24	346,000	3,000	92	72
Ma'oz Hayyim	S-5	60	0	0	7	3	0	0	87	27,600	33	90	3
Nir Dawid	S-5	85	0	0	5	71	0	0	11	71,400	7,400	94	87
Nir Dawid	F-27	1	0	0	27	1	0	0	88	24	12	4	1
Total		236	0	0	47	138	0	0	210				

[a] Pathogenicity tested on Pima S-5 cotton. P, pathogenic; NP, nonpathogenic.
[b] Vegetative compatibility was tested by heterokaryon formation with tester strains of *F. o. vasinfectum* race 3. (+), compatible; (−), incompatible.
[c] S-5, susceptible; F-27, resistant to Fusarium wilt.
[d] Colony-forming units per gram of fresh tissue (T) or dry rhizosphere soil (R).
[e] T, tissue; R, rhizosphere.

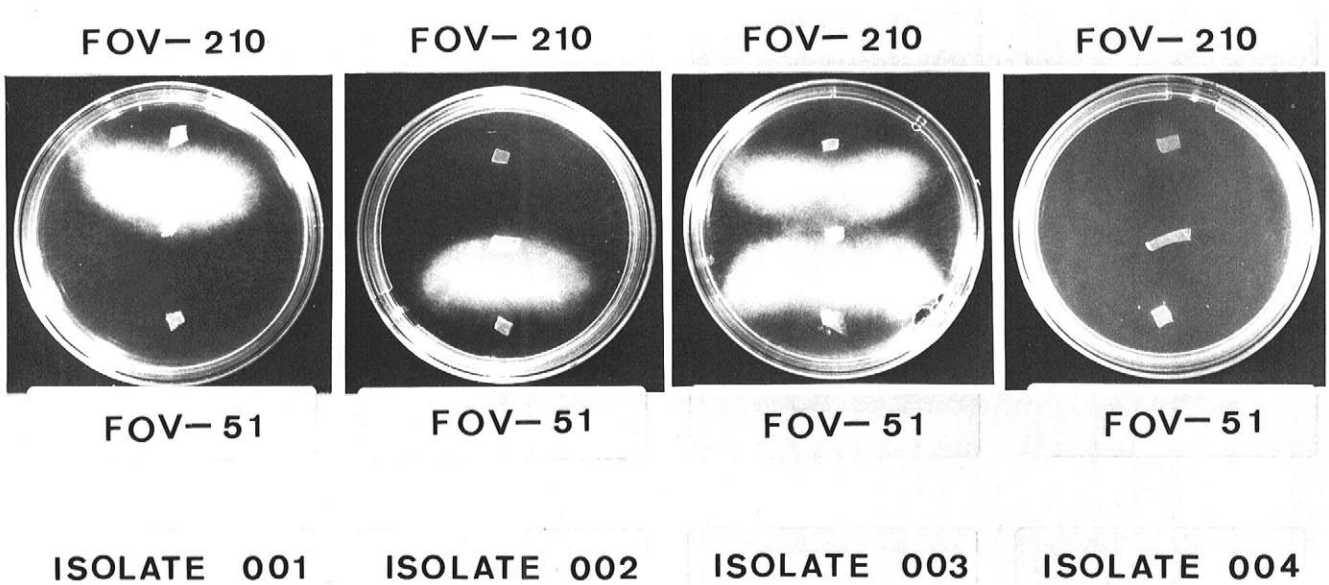

Fig. 2. Four possible types of reaction in vegetative-compatibility grouping (VCG) tests between *nit* mutants of *Fusarium* isolates (numbered 001–004) and two testers of *F. oxysporum* f.sp. *vasinfectum* race 3. One *nit* mutant of each isolate was placed in the center of an FMM plate and the testers (FOV-51 and FOV-210) were placed at opposite edges. Isolates 001, 002, and 003 formed visible heterokaryons with either one or both testers (VCG reaction: +), whereas isolate 004 failed to form such heterokaryons (VCG reaction: −).

and no difference was observed between them and wild-type cultures when both were included in routine pathogenicity tests. Reisolation of the inoculated mutants from diseased plants, and their recognition, could be achieved in one step by using either chlorate media, on which they grew rapidly, or acidified FMM, on which they acquired the typical *nit* character. After isolation, mutants carrying different *nit* markers could be identified by their ability to complement with either one or both testers (Fig. 2).

DISCUSSION

In vegetative-compatibility tests of 386 isolates of *F. o. vasinfectum* race 3 obtained from tissue and rhizosphere of cotton plants from five sites in two separate regions in Israel, it was shown that all the isolates belonged to a single VCG. This VCG differed from the VCGs of four other formae speciales of *F. oxysporum*, as indicated by the lack of interaction between *F. o. vasinfectum* race 3 and these pathogens. None of 257 nonpathogenic isolates, colonizing tissue and rhizosphere of the same plants, was vegetatively compatible with the pathogenic strain. Thus, *F. o. vasinfectum* race 3 appears to constitute a distinct genetic population within the *F. oxysporum* complex. Puhalla (11) used one isolate of *F. o. vasinfectum* and found that it was vegetatively incompatible with isolates of 11 formae speciales. We do not know if Puhalla's isolate and our strains belong to the same VCG.

The VCG test was as accurate as a pathogenicity test and could distinguish between pathogenic and nonpathogenic isolates in mixed *Fusarium* populations associated with cotton roots. Similarly, the VCG technique provided a reliable test to distinguish between *F. o. apii* race 2 and nonpathogenic *Fusarium* strains associated with celery roots (5).

Worldwide, the races of *F. o. vasinfectum* are quite distinctly separated geographically, and race 3 was, for a long time, restricted to Egypt (6). In two cases, it was perhaps also recorded in the USSR (9) and the Sudan (12). In this study, we show that race 3 in two separate regions in Israel belongs to a single VCG. Its emergence in the two regions could have resulted either from a common source or through independent evolution. The vegetative compatibility of the Israeli strains with race 3 from Egypt has yet to be determined.

Nonpathogenic isolates of *Fusarium* colonized roots of both susceptible and resistant cotton cultivars. However, unlike the situation reported with isolates from celery (4), we could not find distinct VCGs among nonpathogenic tissue and rhizosphere colonizers. Although many *Fusarium* strains are able to colonize roots of various plants (7), only the pathogenic strain can produce the disease syndrome in its respective host. The population level of *F. o. vasinfectum* in the roots of the resistant cultivar was much lower than its level in the roots of the susceptible cultivar (Table 2). A similar phenomenon was found with *F.o.lycopersici* in tomatoes (1). The rhizosphere of the resistant cultivar did not support the proliferation of the pathogen (Table 2). This finding may explain the lower Fusarium-wilt incidence observed in susceptible cotton planted in a soil previously cropped to a resistant cotton cultivar (8).

The *nit* mutants of *F. o. vasinfectum* race 3 retained their pathogenicity to cotton. Consequently, they are suitable for ecological studies in soil where "marked" strains are needed. Their chlorate resistance, combined with their distinct morphology on acidified FMM, facilitates their reisolation and identification.

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