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Vegetative compatibility grouping of *Verticillium dahliae* from pistachio in Iran

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Summary. Sixty-nine isolates of *Verticillium dahliae* were recovered from pistachio (*Pistacia vera*) trees in the Kerman province of Iran. They were analyzed using complementation tests with nitrate-nonutilizing (*nit*) mutants to identify their vegetative compatibility groups (VCGs) and were compared with the four internationally recognized VCGs. Based on their ability to form heterokaryons, three local VCGs were identified (using reference strains) as VCG2B (50 isolates), VCG4A (7 isolates) and VCG1 (2 isolates). Ten isolates could not be characterized with the reference strains. Thirty-four cotton isolates were also compared with the pistachio isolates using the reference strains; they were identified as VCG1 (10 isolates), VCG2B (11 isolates) and VCG4A (6 isolates). (A few isolates belonged to both VCG2A and VCG2B, and a few to both VCG4A and VCG4B.) The cotton defoliating isolates (D pathotype) belonged to VCG1, and the cotton non-defoliating isolates (ND pathotype) to VCG2 and VCG4. Greenhouse pathogenicity tests of 22 isolates on cotton and okra showed that all cotton and pistachio VCG1 isolates were highly virulent and were of the defoliating pathotype (D) in both hosts. On the other hand, the isolates of VCG2B and VCG4B ranged in virulence from weakly virulent to highly virulent irrespective of their host origin. The similarity in the VCG spectrum suggests that cotton was the most likely source of *Verticillium dahliae* in pistachio in Iran.

Key words: Verticillium wilt, VCG, *nit* mutants, chlorate-resistant medium, *Pistacia vera*.

Introduction

Verticillium dahliae is an economically important vascular wilt fungus widespread in temperate climates. It attacks over 160 species of plants, including pistachio, almond, pecan and walnut. In some countries, Verticillium wilt is a serious problem of pistachio (*Pistacia vera*) nut production. Verticillium wilt of pistachio is common and often severe in pistachio orchards where trees are planted on susceptible rootstocks, in soil with high levels of fungal inoculum, or in fields where susceptible crops have previously been grown (Tsror and Levin, 2003).

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Although the cultivation of pistachio has a long history in Iran, especially in saline soils with low precipitation, Verticillium wilt has not been a major problem until recent years (Aminaee and Ershad, 1999). Cotton (*Gossypium hirsutum*) is another crop with a long history in Iran, dating back more than 2000 years. Cotton cultivation occupies more than 200,000 ha in many parts of the country including Kerman province, which is also a major pistachio production area. *V. dahliae* is a limiting factor for cotton cultivation in most parts of Iran (Arabsalmani and Banihashemi, 2000).

Control of *V. dahliae* is difficult because of the lack of specificity of the host and the extreme variability of *V. dahliae* pathogenicity (Pegg, 2002). To control this disease it is necessary to know the structure and the genetic diversity of the pathogen

population (Chen, 1994). Vegetative compatibility, assumed to be controlled by multiple gene loci, is a useful marker to identify the races and genetic diversity of populations and variations in their pathogenicity (Leslie 1993, 1996; Rowe *et al*., 1997). Isolates of a fungus are vegetatively compatible if their hyphae are capable of fusion. The coupling of two different nuclei within a single cell (heterokaryosis) enables mutual complementation of the nutritional deficiencies brought by each nucleus, and possibly an exchange of genetic material via parasexuality. Therefore, in an imperfect fungus such as *V. dahliae*, the vegetative compatibility of two isolates of different origin indicates a degree of genetic relatedness (Leslie, 1993, 1996).

In a vegetative-compatibility analysis of 22 *V. dahliae* strains from diverse geographic and plant sources worldwide using nitrate–nonutilizing (*nit*) mutants, four vegetative compatibility groups (VCGs) were identified (Joaquim and Rowe, 1990; Strausbaugh, 1993). In a *V. dahliae* population associated with potato wilt in the USA, the dominant VCGs were VCG4 subgroups A and B, with only a rare occurrence of VCG1 and VCG2 (Joaquim and Rowe, 1990, 1991). While only one VCG occurs on potato, three VCGs have been found on cotton, although their distribution varied depending on the fungal strain and its geographic origin. It has been found that the cotton defoliating (D) and non-defoliating (ND) *V. dahliae* pathotypes belong to different VCGs (Puhalla, 1983; Joaquim and Rowe, 1990; Strausbaugh, 1993; Daayf *et al*., 1995; Rowe, 1995), and that the D strains all belong to one VCG, designated as VCG1 subgroup A, and the ND strains toVCG2 (Puhalla, 1983; Joaquim and Rowe, 1990; Strausbaugh, 1993; Daayf *et al*., 1995; Rowe, 1995; Elena and Paplomatas, 1998) and VCG4 (Joaquim and Rowe, 1990; Elena and Paplomatas, 1998; Korolev *et al*., 2001). A comprehensive study of the genetic diversity and phenotypic diversity of *V. dahliae* populations on cotton from Spain and Israel indicated that the D pathotype is pathologically and genetically homogeneous, whereas the ND pathotype is heterogeneous in its virulence, its VCG, and its molecular markers (Korolev *et al*., 2001). In an earlier study, a collection of 83 isolates of *V. dahliae* deriving from different plant species including cotton was isolated from different parts of Iran. Eight compatible groups were recognized using *nit* mutants. All defoliating isolates were assigned to VCG1 and most of the rest to VCG2 (Arabsalmani and Banihashemi, 2000). VCG diversity in *V. dahliae* has also been studied in many crops, and all isolates from some hosts were found to belong to between one and three VCGs (Daayf *et al*., 1995; Bao *et al*., 1998; Elena and Paplomatas, 1998). Although a few pistachio isolates have been included in previous vegetative compatibility studies (Joaquim and Rowe, 1990; Elena and Paplomatas, 1998; Rowe, 1995), the VCG diversity of *V. dahliae* populations infecting pistachio has not been extensively studied.

The objectives of the present study were: i) to determine the VCG diversity of *V. dahliae* isolates collected from naturally infected pistachio trees grown throughout the Kerman province of Iran; ii) to correlate the results with internationally recognized VCGs, and iii) to investigate the probable origin of pistachio isolates of *V. dahliae* in Iran.

Materials and methods

Fungal isolates

Two groups of *V. dahliae* isolates were used in this study. Group I comprised isolates from diseased pistachio trees and were obtained during 1999–2001 from different areas of Kerman province, the main pistachio-growing region of Iran. To collect these isolates, symptomatic branches of pistachio trees were surface-sterilized with 96% ethyl alcohol and flamed. After removing the bark, chips (0.5–1 cm) of discolored wood were placed on Petri dishes containing potato dextrose agar (PDA) amended with streptomycin sulphate (100 mg l^{-1}) and rifampin (10 mg l^{-1}), or on a *Verticillium* selective medium (Ausher *et al*., 1975). Dishes were incubated at 25°C for up to four weeks until colonies with verticillately branched conidiophores developed around the wood chips. One isolate was retained from each tree.

Group II consisted of 34 single-spore *V. dahliae* isolates from cotton deposited in the culture collection of the Soil-borne Diseases Laboratory, Department of Plant Protection, Shiraz University, Shiraz, Iran. They had been isolated in different parts of Iran. The cotton isolates had previously been characterized as D (15 isolates) or ND (19 isolates) by pathogenicity tests using the stem-injection inoculation method. Single-spore cultures were prepared from all the isolates in both groups (Arabsalmani, 1999).

Generation and characterization of *nit* mutants

Nit mutants were generated following the method of Puhalla (1983), adapted for *V. dahliae* by Joaquim and Rowe (1990) and used with some modifications. All the isolates were grown on PDA at 25°C for two to three weeks. Mycelial plugs (3–6 per dish) from the margins of growing colonies were transferred to dishes containing a chlorate medium.

In a preliminary experiment, a selection of mutants from colonies grown on corn meal agar (CMA) with 0.02% glucose (CMC) (Correll *et al*., 1988) or on water agar (2%) with 0.02% glucose (WAC) amended with 3% chlorate (Korolev and Katan, 1997) yielded only a small number of *nit* mutants with low stability, while the majority of colonies obtained with 30 g l^{-1} of KClO3 developed a dense mycelium all around the periphery of the colony. The chlorate concentration was increased to 5% in WAC and to 7% in CMC. Five dishes of each chlorate medium were used for each isolate. Plates were incubated at 25°C in the dark for up to one month and monitored for the growth of fast-growing chlorate-resistant sectors. Using a bacteriological loop, conidia from the surface of each selected sector were streaked on minimal medium (MM). Colonies with thin and expansive growth with little or no sporulation and aerial mycelium on MM medium were considered *nit* mutants. These mutants were identified as *nit1, nit3* and *Nit*M based on their growth on a nitrate, nitrite and hypoxantine medium (Correll *et al*., 1987) respectively.

Complementation tests

The self-compatibility of each isolate was determined by pairing different complementary *nit* mutants (*nit*1 with *nit*3, *nit*3 with *Nit*M and *nit*1 with *Nit*M). The one pair (sometimes two pairs) of complementary and compatible mutants that was the most efficient in stable heterokaryon formation at the contact zone between the colonies was chosen as representative of that isolate. Representative *nit*1 and *Nit*M mutants of each isolate were paired in all possible inter-isolate combinations. Sometimes reciprocal pairing was done and the procedure was repeated twice. A group of 30 pistachio isolates was selected for the initial tests. Complementation between *nit* mutants was tested in 9-cm diameter Petri dishes containing MM. Three mutants were placed in a triangular pattern, 1–1.5 cm apart, and with three triangles on each dish. Heterokaryon formation was examined after 10 and 17 days. Complementation was evident if there was formation of a dense, aerial growth of mycelia from the two mutants which had met and formed a prototrophic heterokaryon. Reactions were categorized as strong (+), weak or uncertain (+/-) or no reaction (-) by the extent of prototrophic growth at the contact zone between the colonies. Isolates were assigned to a given VCG when their mutants formed a strong heterokaryon with a specific tester strain.

Assignment of isolates to standard VCGs

Representative mutants of the remaining isolate, and selected isolates from local VCGs, were paired with reference testers, supplied by R.C. Row, Ohio Agricultural Research and Development Center, Ohio State University, Wooster, OH, USA (Joaquim and Rowe, 1990).The *nit* mutants of the following tester strains were used for each VCG: T_9 (VCG1), PH (VCG2A), 115 (VCG2B), PCW (VCG3), BB (VCG4A) and S39 (VCG4B). Pairing was done by placing the mycelial plug of a *nit* mutant of unknown VCG in the center of a Petri dish containing MM, surrounded by five mycelial plugs from known tester strains. *nit*1 and *Nit*M mutants of each tester strain were paired against each unknown *nit* mutant. All combinations were tested at least twice.

Pathogenicity test

The pathogenicity of twenty VCG-characterized isolates from cotton, pistachio, tomato (tom-1) and sesame (se4) was tested by the root-dipping method using the cotton cv. Bakhtegan (less susceptible) and the okra cv. Shadegan (susceptible) as differential hosts. Isolates were grown on CMA at 25°C in the dark for 10 days. Conidia were washed off the agar surface with 30–50 ml of tap water. Inoculum concentration was determined with a haemacytometer and adjusted to $1–5\times10^7$ conidia per ml. Seedlings at the cotyledon stage were uprooted from the substrate, and their roots were washed in tap water, trimmed and dipped in the inoculum suspension for 5 min. Non-inoculated control seedlings were dipped in sterile distilled water (SDW). Each isolate was inoculated on 45 seedlings of each host plant. Seedlings were then transferred to 700-ml pots filled with non-sterilized potting mixture (clay loam/peat; 2:1, v:v) (five seedlings per pot) and maintained in a greenhouse at 24 to 28°C and a 12-h day for 56 days. Seedlings were inspected daily for foliar symptoms and defoliation. The mean disease

severity index was assessed 2, 3, 4 and 8 weeks after inoculation on a scale of 0–4 (0, no symptoms; 4, dead plant) (Arabsalmani, 1999). The area under disease progress curve (AUDPC) was calculated by the trapezoidal integration method (Campbell and Madden, 1990) and disease was expressed as the percentage of the maximum possible area for the eight week-period of the experiment. Inoculation tests were repeated twice. The experiment was regarded as a factorial treatment design (*V. dahliae* isolates × host) with 9 replicates (pots) in a randomized complete block design.

Results

Isolates of *V. dahliae*

A total of 69 isolates of *V. dahliae* were recovered from the pistachio trees. Morphological observations revealed that all the isolates had the characteristic features of *V. dahliae*. Isolates are listed in Table 3.

Generation of *nit* mutants

On CMC amended with 7% and on WAC amended with 5% chlorate, all isolates of *V. dahliae* except two (VP45 and CS-33) produced numerous

Table 1. Vegetative compatibility grouping of a collection of 30 *Verticillium dahliae* isolates from pistachio [a].

	VP26	VP25	VP28	VP32	VP1	VP3	VP5	VP24	VP31	VP34	VP12	VP14	VP21	VP23	VP42	VP36	VP47	VP62	VP50	VP5	VP44	VP70	VP69	VP52	VP15	VP12	VP2	VP10	VP20	VP29
VP26	+																													
VP25	+	+	-																											
VP28	+	-	+	+																										
VP32	+	-	+	+	+																									
VP1	+	+	+/-	+	+	-																								
VP3	+	+	-	-	-	+	-																							
VP5	-	+	+	+	-	-	+	-																						
VP24	+	+	-	-	-	+	+	+																						
VP31	+	-	+	-	+/-	-	-	-	+	-																				
VP34	+	+/-	+	+	-	+	+	-	-	+																				
VP30	-	+	+	-	+	+/-	-	-	-	-	+																			
VP14	+	-	-	+	+	+	-	-	-	-	-	+	-																	
VP21	-	-	-	-	+	-	-	+/-	+/-	-	-	-	+																	
VP23	+	+	+	+/-	-	+/-	+	+/-	-	-	-	-	-	+																
VP42	+/-	+	+				+/-	-	-	-	-	-			+															
VP36	-	+	+	+		+				+						+														
VP47	-	+	+	+		+	-	-	-	-				+		+	+													
VP62	-	-	-	-	-	-	+	-	-	-	-	+	-	+	-	-	-	+												
VP50	+/-	+/-	+/-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+											
VP65	-	-	-	-	-	-	-	-	-	-	+	+	-	-	+	+	+	+	+/-	+										
VP44	-	-	-	-	-	-	+/-	-	-	+/-		+/-	-	-	+	+	-	-	+	-	+									
VP70	-	-	-	-	+/-	-	+/-	-	-	+/-	-	-	-	-	+/-	+/-	+	+	+	+		+								
VP69	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-							
VP52	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+/-	-	-	-						
VP15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+					
VP12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+				
VP2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+/-	+
VP10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+/-
VP20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+/-	+	+	+
VP29	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+/-	+	+

[a] Intensity of prototrophic growth of the heterokaryon: +, strong reaction; +/-, weak reaction; -, no reaction; blank space, no data.

chlorate-resistant sectors, which produced *nit* mutants with high stability. Wild-type isolates varied in the frequency of sectoring. *nit* mutants were generated from all isolates except VP6, VP51, VP59 (from pistachio) and D11 (from cotton). Among the 1740 chlorate-resistant sectors obtained from 103 isolates, 1387 (80%) were *nit* mutants. Of these mutants, 747 (53.7%) were *nit*1, 473 (34%) *Nit*M, 43 (3.2%) *nit*3, and 9% reverted back to the wild-type colony morphology. In addition, 46 mutants could be either *nit*1 or *nit*3 because they did not grow on nitrite medium but had wild type growth on hypoxanthine medium. The remaining 25 mutants showed the *nit* phenotype on both nitrite and hypoxanthin, so that they could not be characterized as *nit*3 or *Nit*M. Both *Nit*M and *nit*1 mutants were recovered from about 95% of the isolates. Isolates VP69 and VP52 (from pistachio), CS45, D8 and D12 (from cotton) did not form intra-isolate heterokaryons and were considered self-incompatible.

VCGs in *V. dahliae* from pistachio

Complementary *nit* mutants from different isolates were paired in 912 inter-isolate combinations (870 *nit*1×*Nit*M pairings, 23 *Nit*M×*nit*3 pairings, and 19 *nit*3×*nit*1 pairings). When one or more mutants from a given isolate formed complementary heterokaryons with one or more mutants from another isolate, the isolates were considered compatible and assigned to the same VCG. Based on their complementation, the isolates were grouped into three local VCGs: 22 isolates in group 1 (VCGA), 4 isolates in group 2 (VCGB) and 2 isolates (VP12 and VP15) in the third group (VCGC) (Table 1). Isolates VP69 and VP52 were self-incompatible and did not form heterokaryons with any other *nit* mutants used in this test.

Based on the formation of strong and stable complementary heterokaryons, seven *Nit*M mutants were chosen as tester strains of the three local VCGs (VP26, VP50 and VP28 from VCGA; VP2 and VP10 from VCGB; VP12 and VP15 from VCGC) and paired with *nit*1 mutants from the 40 remaining pistachio isolates (Table 2). All the isolates assigned to a given VCG reacted strongly with at least one of the testers or with the other isolates of the same VCG.

In a few cases, a weak positive reaction was seen between isolates coming from different VCGs. Moreover, some isolates showed strong complementation with one tester, but a weak reaction with another tester of the same VCG. Such weak and often unreliable complementation was seen in 20–25% of the pairings (Table 2). Hence only strong positive reactions were regarded as confirming vegetative compatibility.

Assignment of isolates to VCGs

Based on complementation between *nit* mutants, 103 isolates from pistachio and cotton were unevenly assigned to the three international VCGs. Sixty two isolates (59% of total) belonged to VCG2B, 13 isolates (12%) to VCG4A and 12 isolates (11%) to VCG1 (Table 3 and 4). None of the isolates was compatible with the VCG3 testers. Eleven isolates (VP6, VP51, VP59, VP45, VP69, VP52, D11, CS33, CS45, D8 and D12) were not assigned to any known VCG because they either did not produce sectors or *nit* mutants, or were self-incompatible and did not complement with any other isolate or with the testers. VP61, VP62, VP66 and D5 were self-compatible, but their mutants reacted negatively with the American and Iranian testers so that their VCGs were not determined. All the VCG2B isolates were compatible with reference isolate 115, but a few also showed a weak complementation reaction with T_9 (VCG1), and some others with the BB tester of VCG4A. Strong complementation was seen between local VCGA with VCG2B, and VCGB with VCG4A, and VCGC with VCG1 (Table 3). *Nit* mutants of isolate D10 did not form heterokaryons with any testers but this isolate was compatible with other isolates in VCG1, so that it was regarded as belonging to VCG1 (Table 4).

Strong complementation was observed between the mutants of the local isolates and the international testers (Table 3). The local VCG2B isolates almost always reacted strongly with the testers except for VP63, VP47 and VP57, which reacted variably with other testers of VCG2A. Some isolates showed sparse and limited colony formation with the testers of VCG4A, VCG4B and VCG1. VP3 from VCG2B showed strong compatibility with the testers of both VCG2B and VCG4B.

The ten defoliating isolates all belonged to VCG1, while the isolates of the ND pathotype belonged to VCG2B (9 isolates) and VCG4A (4 isolates); two ND isolates (CS-57 and CS56) were moderately to strongly compatible with both the VCG4A and the VCG4B testers and regarded as VCG4(A/B). Howe-

Table 2. Complementation test between *nit*1 mutants from 42 pistachio isolates and seven *Nit*M mutant testers from three local vegetative compatibility groups (VCGs) of *Verticillium dahliae*.

VCG	Isolate (*nit*)	*Nit*M tester strains						
		VCGA		VCGC			VCGB	
		26VP	28VP	50VP	2VP	10VP	15VP	12VP
I	VP1	[a] +	+	+	-	-	+/-	-
	VP3	-	-	-	-	-	-	-
	VP4	+	+	+/-	-	-	+/-	-
	VP7	+	+	+	-	-	-	-
	VP8	+	+/-	+	-	-	-	-
	VP9	+	+	+	-	-	-	-
	VP11	+	+	+/-	-	-	-	-
	VP13	+	+/-	+/-	-	-	-	-
	VP14	+	+	+	-	-	-	-
	VP17	+	+/-	+	+/-	-	-	-
	VP18	+	+	+	-	-	-	-
	VP19	+	+	+/-	-	-	-	-
	VP22	+	+	+	+/-	+/-	-	-
	VP31	+/-	+	+	-	-	-	-
	VP33	+	+	+	+/-	-	-	-
	VP35	+	+	+	-	-	-	-
	VP37	+	+/-	+/-	-	-	-	-
	VP38	+	+	+	-	-	-	-
	VP39	+/-	+	+	-	-	+/-	-
	VP40	+	+	+	-	-	-	-
	VP41	+	+	+	-	-	-	+/-
	VP43	+	+/-	+	-	+/-	-	-
	VP46	+	+	+	-	+	-	-
	VP48	+	+/-	+/-	-	+	-	-
	VP49	+/-	+/-	+	-	+	-	-
	VP53	+	-	-	-	+/-	-	-
	VP54	+	+	+	-	+/-	-	-
	VP55	+	+/-	-	-	-	-	-
	VP56	+	+	+	-	-	-	-
	VP57	+	+/-	+	-	-	-	-
	VP60	+	+	+	+/-	-	-	-
	VP61	-	-	-	-	-	-	-
	VP63	+/-	+	+	-	-	-	-
	VP64	+	-	+	+/-	-	-	-
	VP66	+	+	+	-	-	+/-	-
	VP67	+	+	+	-	-	-	-
	VP68	+	+/-	+	-	-	-	-
II	VP16	-	-	-	+	+	-	-
	VP27	-	-	-	+	+/-	-	-
	VP58	+/-	-	-	+	+	-	-
III	VP15	-	-	-	-	-	+	+
	VP12	+/-	-	-	-	-	+	+

[a] +, strong reaction; +/-, weak reaction; -, no reaction.

Table 3. Vegetative compatibility groups (VCGs) of *Verticillium dahliae* isolates from pistachio in Iran using international tester strains.

VCG group	Isolate	Geographic origin/ source[b]	Nit mutants of reference strains (OARDC)[a]							
			VCG1	VCG2A	VCG2B	VCG4A		VCG4B		VCG3
			T9	PH	115	BB	P103	MT	S39	PCW
VCG2B	VP1	Ker / 2	+/-	-	+	-	-	-	-	-
	VP4	Ker / 2	+/-	-	+	-	-	-	-	-
	VP5	Ker / 2	-	-	+	-	-	-	-	-
	VP7	Ker / 2	-	-	+	-	-	-	-	-
	VP8	Ker / 2	-	-	+	-	-	-	-	-
	VP9	Ker / 2	-	-	+	-	-	-	-	-
	VP11	Ker / 5	-	+/-	+	-	-	-	-	-
	VP13	Ker./ 5	-	-	+	-	-	-	-	-
	VP14	Ker / 5	-	-	+	-	-	-	-	-
	VP17	Ker / 1	+/-	-	+	-	+/-	-	-	-
	VP18	Ker / 1	-	-	+	-	-	-	-	-
	VP19	Ker / 2	-	-	+	-	-	-	-	-
	VP21	Ker / 2	-	-	+	-	-	-	-	-
	VP22	Raf / 3	-	-	+	+/-	-	-	-	-
	VP23	Raf / 3	-	-	+	-	-	-	-	-
	VP24	Raf / 4	-	+/-	+	-	-	-	-	-
	VP25	Raf / 4	-	-	+	-	-	-	-	-
	VP26	Raf / 3	-	-	+	-	-	-	-	-
	VP28	Raf / 5	-	-	+	-	-	-	-	-
	VP30	Raf / 5	-	-	+	-	+/-	-	-	-
	VP31	Raf / 5	-	-	+	-	-	-	-	-
	VP32	Raf / 5	-	-	+	+/-	+/-	-	-	-
	VP33	Raf / 3	-	-	+	-	-	-	-	-
	VP34	Raf / 3	-	-	+	-	-	-	-	-
	VP35	Raf / 3	-	-	+	-	-	-	-	-
	VP36	Raf / 3	-	-	+	-	-	-	-	-
	VP38	Ker / 1	-	+/-	+	-	-	-	-	-
	VP39	Ker / 1	-	-	+	-	-	-	-	-
	VP40	Ker / 1	-	-	+	-	-	-	-	-
	VP41	Ker / 1	+/-	-	+	-	-	-	-	-
	VP42	Ker / 1	-	-	+	-	-	-	-	-
	VP43	Ker / 1	-	-	+	-	-	-	+/-	-
	VP44	Ker / 1	-	+/-	+	-	-	-	-	-
	VP46	Ker / 5	-	+	+	-	-	-	-	-

(*continued on the next page*)

(Table 3 continued)

	VP47	Ker / 5	-	+	+	-	-	-	-	-
	VP48	Ker / 5	-	+/-	+	-	-	-	-	-
	VP49	Ker / 5	-	-	+	-	-	-	-	-
	VP50	Ker / 5	-	-	+	-	-	-	-	-
	VP53	Ker / 1	-	-	+	-	-	+/-	-	-
	VP54	Ker / 2	-	+/-	+	-	-	-	-	-
	VP55	Ker / 1	-	-	+	-	-	-	-	-
	VP56	Ker / 5	-	-	+	-	-	-	-	-
	VP57	Ker / 5	-	+	+	-	-	-	-	-
	VP60	Raf / 5		-	+	-	-	-	-	-
	VP63	Raf / 4	-	+	+	-	-	-	-	-
	VP64	Raf / 4	-	-	+	+/-	-	+/-	-	-
	VP65	Raf / 4	-	-	+	-	-	-	+/-	-
	VP67	Raf / 4	-	-	+	-	-	-	-	-
	VP68	Raf / 4	-	+/-	+	-	-	-	+/-	-
	VP70	Raf / 4	-	-	+	-	-	-	-	-
VCG4A	VP2	Ker / 2	-	-	-	+	-	-	-	-
	VP10	Ker / 2	-	-	-	+	+	-	-	-
	VP16	Ker / 2	-	-	-	+	+/-	-	-	-
	VP20	Ker / 2	-	-	-	+	+/-	-	-	-
	VP29	Raf / 3	-	-	+/-	+/-	+	-	-	-
	VP58	Ker / 5	-	-	+/-	-	+	-	-	-
	VP27	Raf / 3	-	-	-	+	+	-	-	-
	VP12	Ker / 2	+	-	-	-	-	-	-	-
	VP15	Ker / 2	+	-	-	-	-	-	-	-
Not characterized	VP3	Ker / 2	-	+/-	+	-	-	-	+	-
	VP62[d]	Raf / 4	-	-	-	-	-	-	-	-
	VP66[d]	Raf / 4	-	-	-	-	-	+/-	-	-
	VP69[c]	Raf / 4	-	-	-	-	-	-	-	-
	VP52[c]	Ker / 1	-	-	-	-	-	-	-	-
	VP61[d]	Raf / 4	-	-	-	-	-	-	-	-
	VP6[e]	Ker / 2	-	-	-	-	-	-	-	-
	VP51[e]	Ker / 1	-	-	-	-	-	-	-	-
	VP59[e]	Ker / 5	-	-	-	-	-	-	-	-
	VP45[f]	Ker / 1	-	-	-	-	-	-	-	-

[a] +, strong reaction; +/-, weak reaction; -, no reaction.
[b] Ker, Kerman; Raf, Rafsanjan. 1, B. Hossani; 2, B. Nazari; 3, B. Beegezadeh; 4, Lahijan; 5, Shiraz University collection
[c] Self incompatible
[d] No reaction with any VCG.
[e] No *nit* mutants obtained.
[f] No sectors generated.

Table 4. Vegetative compatibility groups (VCGs) of *Verticillium dahliae* isolates from cotton using international tester strains.

VCG group	Isolate	*Nit* mutants of reference strains (OARDC)[a]								Pathotype[b]
		VCG1	VCG2A	VCG2B	VCG4A		VCG4B		VCG3	
		T9	PH	115	BB	P103	MT	S39	PCW	
VCG2B	CS-7	[1]-	-	+	-	-	-	-	-	ND
	CS-19	-	-	+	+/-	-	-	-	-	ND
	CS-61	-	-	+	-	-	-	-	-	ND
	CS-9	+/-	-	+	-	-	-	-	-	ND
	CS-45	-	-	+	-	-	-	-	-	ND
	CS-1	-	-	+	-	-	-	-	-	ND
	CS-4	-	-	+	-	-	-	-	-	ND
	CS-35	-	-	+	-	-	-	-	-	ND
	SS-4	+/-	+	+	+/-	-	-	-	-	ND
	Ok-8	-	+	+	-	-	+/-	-	-	ND
	D-46	-	-	+	-	-	-	+/-	-	ND
VCG4A	CS-3	-	-	-	+	+	+/-	+/-	-	ND
	CS-16	-	-	-	+	+/-	-	-	-	ND
	CS-39	-	-	+/-	+	-	-	-	-	ND
	CS-57	-	-	-	-	+	-	+	-	ND
	CS-56	-	-	-	+/-	+	+	-	-	ND
	C-7	-	-	-	-	+	-	+/-	-	ND
VCG1	CV-42	+	-	-	-	-	-	-	-	ND
	D-1	+	-	+/-	-	-	-	-	-	D
	D-4	+	-	-	-	-	-	-	-	D
	D-3	+	-	-	-	-	-	-	-	D
	D-6	+	-	-	-	-	+/-	-	-	D
	D-7	+	-	-	-	-	+/-	+/-	-	D
	D-15	+	-	+/-	-	-	-	-	-	D
	D-14	+	+/-	+/-	-	-	-	-	-	D
	D-43	+	-	-	-	-	+/-	-	-	D
	D-74	+	-	-	-	-	-	-	-	D
Not characterized	D-10	-	-	-	-	-	-	-	-	D
	CS-45[a]	-	-	-	-	-	-	-	-	ND
	D-8[a]	-	-	-	-	-	-	-	-	D
	D-12[a]	-	-	-	-	-	-	-	-	D
	D-5[b]	-	-	-	-	-	-	-	-	D
	D-11[c]	-	-	-	-	-	-	-	-	D
	CS-33[d]	-	-	-	-	-	-	-	-	ND

[a] +, +/-, - ; see Table 1.
[b] ND, cotton nondefoliating; D, cotton defoliating.
[c, d, e, f] See Table 2.

ver, isolates Ok8 and SS4 complemented strongly with tester PH of VCG2A and tester 115 of VCG2B (VCG2 [A/B]) (Table 4).

Pathogenicity test

All isolates were pathogenic to cotton and okra as their AUDPC values were significantly (P=0.01) greater than those of the uninoculated control. Irrespective of the host origin, the isolates tested were slightly to highly virulent on cotton and mildly to highly virulent on okra. The mean AUDPC values were calculated for each VCG, and VCG1 isolates were found to be significantly (P=0.01) more aggressive than VCG2B and especially VCG4A isolates.

All isolates belonging to VCG1 were highly aggressive to cotton and okra (AUDPC>50). These isolates were previously designated as the D pathotype (based on the disease severity index [DSI] and defoliation) (Correll *et al*., 1987), and induced severe stunting and foliar wilting, complete defoliation and the death of the test plants. The VCG2B isolates were more heterogeneous and more varied in their pathogenicity pattern. All the VCG4A isolates and 8 of the VCG2B isolates were of the ND pathotype and induced mild to moderate wilt on cotton (AUDPC=10–50) and moderate to severe wilt on okra (AUDPC>50). The remaining three VCG2B isolates (VP42, D46 and CS9) were highly

Table 5. Virulence of Verticillium *dahliae isolates* from each vegetative compatibiliy group (VCG) to cotton and okra.

VCG group	Isolate	% AUDPC[a]					
		Cotton			Okra		
VCG2B	VP-26	fe[b]	21.6	D[c]	bcd	63.8	ABC
	VP-8	bcd	48.8	BC	abcd	81.4	AB
	VP-67	fe	19.53	D	cd	52.9	BC
	VP-42	bc	60.3	BC	ab	71.9	AB
	VP-34	f	26.6	D	abc	75.3	AB
	VP-63	feg	15.4	DE	cd	40.8	BC
	CS-35	fe	18.2	DE	bcd	59.12	ABC
	D-46	bc	56.7	BC	a	80.7	AB
	CS-61	fe	20.8	D	abc	76.8	AB
	CS-9	bc	69.9	ABC	abc	95.8	A
	tom-1	feg	17.6	DE	bcd	50.4	ABC
VCG4A	VP-10	g	13.79	E	bcd	47.3	BC
	VP-20	fe	19.63	D	ab	86.4	A
	C-7	f	27.6	D	bcd	72.2	AB
	CS-39	f	23.2	D	ab	87.8	A
	D-1	ab	79.6	A	a	94.7	A
	D-15	a	89.2	A	a	91.3	A
VCG1	D-4	abc	64.8	ABC	ab	87.1	A
	D-10	abc	68	ABC	ab	86.8	A
	VP-15	bc	58.2	ABC	abc	83.7	AB
	Se-4	ab	79.8	A	abcd	74.2	AB
	control	h	2	F	h	1.7	F

[a] Area under disease progress curve (AUDPC), expressed as a percentage of the maximum possible area for a 56-day period.
[b] In each column, values with different lower-case letters are significantly different according to the pairwise t-test with the Bonferroni correction (P=0.05).
[c] For each isolate (horizontal line), upper-case letters refer to differences between hosts.

virulent on cotton, inducing severe foliar symptoms, stunting and sometimes the death of the plant, but with only partial defoliation (lower to middle leaves of the plant). Based on these features the isolates were identified as PD (partial defoliation) isolates (Korolev *et al*. 2001).

Discussion

One hundred and three isolates of *V. dahliae* obtained from pistachio and cotton were assessed for their vegetative compatibility. A sample of 69 pistachio isolates is relatively large compared to sample sizes from other woody plants tested in the literature. It represents a considerable number of isolates from pistachio trees. This is the first study of the VCG diversity of *V. dahliae* infecting pistachio. The sample size from other studies was too small to make an accurate assessment (Joaquim and Rowe, 1990; Rowe, 1995; Elena and Paplomatas, 1998). The data in the present study suggest that VCG diversity is limited, with an uneven distribution of isolates in the *V. dahliae* population from pistachio in Iran. Three local VCGs were identified as VCG2B (50 isolates), VCG4A (7 isolates) and VCG1 (2 isolates). That only a small number of VCGs exist within the *V. dahliae* population has been reported by a number of authors (Daayf *et al*., 1995; Elena and Paplomatas, 1998). Chen (1994), Daayf *et al*. (1995), and Elena and Paplomatas (1998) also classified 30 to 40 isolates of *V. dahliae* in 3 VCGs. In most of these studies, one to three VCGs were found among isolates from any given host. Even though the *V. dahliae* isolates tested in this study came from different sites, they all belonged to the same VCG, suggesting that there was no relation between the VCG and the geographic origin of the isolates. This is consistent with the results of other studies (Correll *et al*., 1988; Jaoquim and Rowe, 1990, 1991; Daayf *et al*., 1995; Rowe *et al*., 1997).

About 74.3% of the isolates belonged to a single VCG. The two isolates of VCG1 were found in only one orchard in Kerman, while VCG2B and VCG4A isolates occurred throughout the many regions of Kerman and Rafsanjan. This demonstrates the wide distribution of VCG2B on pistachio. Because of the lack of a sexual stage, the only means to exchange genetic material between two strains of *Verticillium* is by anastomosis and heterokaryosis. Isolates within the same VCG may share a common gene pool, and they are isolated from other strains or VCGs within the species by an incompatibility mechanism (Leslie, 1993; Rowe *et al*., 1997).

The number of times a VCG is recovered from a given geographic region depends not only on its frequency in the *V. dahliae* population and on the rate of reproduction of isolates from this VCG, but also on the number and spectrum of the samples examined (Leslie, 1996; Katan, 2000). The low detection of VCG1 did not necessarily imply that it was rare. The isolates of this VCG may have occurred at low population levels, escaping detection.

In some cases it was difficult to assign an isolate to a VCG because complementation between the *nit* mutants of *V. dahliae* is complex, and not all isolates within a VCG complement one another. Furthermore, weak complementation reactions sometimes occurred in pairings between isolates of different VCGs. This may be because some isolates lacked a strong affinity to anastomose and form heterokaryons. These quantitative differences in heterokaryon intensity can be attributed to the nature of individual isolates and to differences between phenotypically similar mutants of an isolate. Thirteen isolates were not compatible with any international reference strain, and sometimes positive interactions with these testers were difficult to confirm. This problem may have been due to the selection of reference strains which were suited for VCG assessment of *V. dahliae* collections in the USA, but may not have been the best choice in other situations (Katan, 2000; Pegg, 2002). For accurate VCG analysis, dependable tester strains must be chosen on a local or regional basis if they are to be effective for routine population analysis. After the local VCGs were precisely assigned, the interaction between its local testers and the international references was examined. On the basis of this last test, three local VCGs were found and designated as Group I, Group II and Group III, corresponding to VCG2B, VCG4A and VCG1 respectively. Several isolates that were not compatible with any international testers were compatible with some isolates belonging to one of the local Iranian VCGs. Finally, four isolates did not complement with any of the reference strains or the Iranian testers. This may indicate the existence of a new VCG. According to Katan (2000) such a single-member VCG should be tested more carefully, or should wait until additional members are discovered that have the same

compatibility characteristics. Since some isolates of VCG2B were strongly compatible with tester strains while others were not, it appears that a greater genetic variation may exist in VCG2.

VCG analysis of a representative collection of cotton isolates previously pathotyped by virulence (Arabsalmani, 1999), placed all D isolates in VCG1 and all ND isolates in VCG2 or VCG4, the two major VCGs including the cotton non-defoliating isolates. This grouping has been corroborated with cotton defoliating strains from America, Central Asia, Spain and China, (Puhalla, 1983; Jaoquim and Rowe, 1990; Strausbaugh, 1993; Bell, 1994; Daayf *et al*.,1995; Korolev *et al*., 2001) which further confirmed the status of the VCG1/D pathotype as a distinct subspecific population within *V. dahliae*. Non-defoliating strains from cotton-growing regions around the world have been found in both VCG2 (Puhalla 1983; Jaoquim and Rowe, 1990; Strausbaugh, 1993; Daayf *et al*., 1995; Elena and Paplomatas ,1998; Korolev *et al*., 2001) and VCG4 (Elena and Paplomatas, 1998; Korolev *et al*., 2001).

Pathogenicity tests showed that all VCG1 isolates were highly virulent causing defoliation, stunting and death to both pistachio and cotton hosts, and they were characterized as the D pathotype. Conversely, VCG4A isolates exhibited weak to moderate virulence. Isolates that belonged to VCG2B, irrespective of their host origin, were not of uniform virulence, and the deviation of a few isolates from the average virulence of this group created a partial overlap or continuity with isolates from other VCGs and induced moderate to severe virulence on both hosts. However, since distinguishing between complete or partial defoliation was difficult, the pistachio and cotton isolates of VCG4A were classified as the ND, and those of VCG2B as ND or PD.

The varying degrees of virulence among *V. dahliae* isolates of cotton from Iran agree with previous reports from the USA, Spain and Israel, where isolates with a virulence level intermediate between the D and ND isolates have caused severe Verticillium wilt irrespective of whether they induce partial defoliation (Schnathorst and Mathre, 1966; Ashworth, 1983; Korolev *et al*., 2001) or not (Bejarano-Alcazar *et al*., 1996). In the past, strain 115 from Syria showed the same type of pathogenicity, and was stated to be intermediate between the D and the ND pathotype (Schnathorst, 1973). This isolate was later characterized as belonging to VCG2B (Joaquim and Rowe, 1990). In the present study, all the pistachio and cotton isolates of VCG2B were compatible with this tester strain, further illustrating the high variability noted in this VCG. Some of the ND isolates were assigned to VCG4A. VCG4A is a major component of the *V. dahliae* population in potato fields of North America (Joaquim and Rowe, 1990, 1991; Strausbaugh, 1993). This could suggest that VCG4A in Iranian cotton isolates originated in potato fields.

One pistachio isolate from VCG1 (VP12) induced severe disease in both hosts, but more especially on cotton, and was designated as belonging to the D pathotype, although further pathogenicity tests with more isolates are needed to generalize this finding. In another study, the interaction between pistachio cultivars and different pathotypes of *V. dahliae* isolates was assessed by the DSI and stem colonization (SC) (Hadizadeh and Banihashemi, 2004). The isolates caused different degrees of pathogenicity on pistachio cultivars. The D strain showed high DSI and SC on the susceptible, less susceptible, and tolerant cultivars. But the ND strain induced very mild symptoms on tolerant cultivars and moderate symptoms on less susceptible cultivars.

The origin of VCG2B in the pistachio orchards in the Kerman province of Iran is not known. Cotton was grown in some parts of this province before pistachio cultivation began. Most of the pistachio and cotton isolates of VCG2B in Iran are also widespread in cotton fields in the country. The common VCG group and the pathogenicity data suggest that there is a general uniformity within the *V. dahliae* population infecting cotton and pistachio in Iran. We speculate that the VCG2B found on pistachio may have originated in cotton fields.

In conclusion, we are of the opinion that the vegetative compatibility genes which control the formation of VCGs are dependent on the geographical origin of the strains.

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