Eur J Plant Pathol (2014) 140:689–699
DOI 10.1007/s10658-014-0500-z

ORIGINAL RESEARCH

VCG diversity and virulence of *Verticillium dahliae* from commercially available cotton seed lots in Turkey

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Accepted: 1 August 2014 / Published online: 8 August 2014

Abstract Samples of seeds collected from commercially available cotton seed lots in Turkey were assayed for *Verticillium dahliae*. *V. dahliae* was successfully isolated from 67 of 104 seed lots tested, a successful isolation rate of nearly 65 %. Vegetative compatibility of the isolates was assessed through complementation tests using nitrate non-utilizing mutants. Of the 188 isolates obtained, 105 were classified as VCG1A, 17 as VCG2A, 64 as VCG2B and two as VCG4B. All 50 of the isolates tested in the greenhouse on cotton cv. DP 15–21 and Acala SJ-1 were pathogenic on both cultivars. As a group, AUDPC values were significantly higher ($P<0.05$) for VCG1A than for VCG2 and VCG4B isolates. These data suggest that: (1) commercial cotton seed lots are commonly infected with *V. dahliae* and thus may serve as primary sources of the pathogen; (2) cotton isolates of *V. dahliae* belong to VCG1A, 2A, 2B and 4B and these strains are widely distributed via seed lots; and (3) VCG1 and VCG2 are distinct pathotypes of *V. dahliae* that vary in their virulence to cotton.

Keywords *Gossypium hirsutum* · Seed health · Verticillium wilt · Nit mutants · Virulence

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Introduction

In Turkey, about 488.500 ha of upland cotton (*Gossypium hirsutum* L.) is grown annually under irrigation in three main regions (Annonymous 2012). These include the Aegean, Mediterranean and Southeastern Anatolia. Verticillium wilt, caused by the soil inhabiting fungus *Verticillium dahliae* Kleb., is among the most serious diseases of cotton throughout Turkey causing substantial economic losses (Göre 2007). The pathogen infects roots, causing foliar wilt and defoliation. Disease severity depends on several factors, including the cultivar used, the developmental stage of the plant, environmental conditions, inoculum density in soil, and the virulence of *V. dahliae* strains (Göre et al. 2009). It was first reported in Turkey in 1941 (Iyriboz 1941), but was not identified as an important disease under field conditions until 1967 (Karaca et al. 1971). Since the mid 1990s, Verticillium wilt has progressively increased in many fields, and an

unusually high incidence of a severe wilt disease of cotton has been observed in Turkey (Göre et al. 2007). In 2004, epidemics of Verticillium wilt occurred in the Aegean region. A defoliating pathotype of *V. dahliae* was isolated from the diseased plants in some areas of Aydın, Denizli, İzmir, Manisa and Muğla provinces and later found to be widespread throughout the region later to the Mediterranean and Southeastern Anatolia regions (Derviş et al. 2008; Göre 2007; Göre et al. 2007). We do not know for sure how fields newly brought into cultivation became infested with this pathotype of *V. dahliae*, but it is possible that long-distance spread of the pathogen could be a result of infected seed. In most cases, certification standards for cotton seed in Turkey do not prescribe thresholds for infection by species of *Verticillium* nor is any widespread testing done of seed lots to assay for the presence of this pathogen. Results of our early studies showed that spread by infected seed was possible under greenhouse conditions that favour disease development (Göre et al. 2011). For this reason, understanding the population diversity within isolates of *V. dahliae* in commercial cotton seed lots is important for proper disease management programs. This information is a prerequisite for proper prevention of disease through seed treatment, setting tolerance levels for variety release, inspection of farmers seed production schemes, quarantine, germplasm management and exchange and optimization of storage conditions (Agarwal and Sinclair 1997).

Populations of the pathogen can be genetically characterized by means of vegetative or heterokaryon compatibility (Katan 2000; Korolev et al. 2000, 2008) and molecular markers (Pérez-Artés et al. 2000; Mercado-Blanco et al. 2003). Vegetative compatibility refers to the genetically controlled ability of individual fungal strains to undergo hyphal anastomosis and form viable heterokaryons; compatible isolates are placed in the same vegetative compatibility group (VCG). For strictly asexually-reproducing fungi, such as *V. dahliae*, isolates in different VCGs are thought to be genetically isolated populations that may differ in many traits, including those related to pathogenicity and virulence, adaptation to environments and sensitivity to fungicides (Katan 2000; Rowe 1995). Therefore, the characterization of local populations of *V. dahliae* into VCGs and phenotypic traits may help in the management of diseases they cause. The objectives of this study were to: (i) examine and compare VCG diversity and composition among *V. dahliae* isolates obtained from commercial seed lots in Turkey, and (ii) evaluate the virulence of different VCGs to cotton.

Material and methods

Collection of seed lots and isolation of *V. dahliae*

Samples from 104 acid-delinted and fungicide treated seed lots belonging to twenty-nine different cultivars were obtained in November 2007, from commercial seed companies and research centres in Turkey (Table 1). These cultivars comprised approximately 98 % of cotton plantings in Turkey in 2007. One hundred seeds were selected randomly from each cultivar seed lot, then washed, surface sterilized in 1.2 % NaOCI for 5 min, rinsed thoroughly, dried, and placed onto Petri dishes containing Sorenson's NP-10 semi-selective media (Kabir et al. 2004; Sorenson et al. 1991). The Petri dishes were incubated at 24 °C with 12 h:12 h L:D cycle (near-UV and cool-white fluorescent light by day) for 14 days (du Toit et al. 2005). Agar sections containing *V. dahliae* grown from tissue were transferred to water agar for monoconidial isolation. The 188 *V. dahliae* isolates were subcultured to 1 % potato dextrose agar (PDA) consisting of 10.0 g of PDA, 15.0 g of Bacto agar, and 0.1 g of streptomycin sulphate in 1 l of distilled water, and incubated in the dark at 24 °C for 7 days. Monoconidial isolates were obtained by micromanipulation of conidia streaked to Czapek Dox agar (CDA) as described by Nitzan et al. 2002.

Generation and characterization of *nit* mutants

nit mutants were generated on water agar – chlorate (WAC) medium consisting of 2 % agar, 0.02 % glucose and 3 % potassium chlorate (Korolev et al. 2008). Mycelial plugs were placed on WAC medium at five or six points in Petri dishes (9-cm-diam) and incubated at 24 °C. Chlorate-resistant sectors, evident after 10–14 days, were transferred to CDA (5-cm-diam plates). Sectors that grew on CDA as colonies with a thin, expansive mycelium were considered *nit* mutants. CDA amended with sodium nitrite (0.5 g l^{-1}) or hypoxanthine (0.2 g l^{-1}) was used for partial phenotyping of the *nit* mutants (Correll et al. 1987).

Table 1 Detection and vegetative compatibility characterization of *Verticillium dahliae* isolates from commercially available cotton seed lots in Turkey

Cultivar	Company[a]	Number of seed lots tested	Number of seed lots from which *V. dahliae* was isolated	VCG distribution of isolates			
				VCG1A	VCG2A	VCG2B	VCG4B
Candia	Bayer	6	3	5	1	2	
Carmen	Bayer	10	7	7	1	7	
Celia	Bayer	3	2	4			
Flora	Bayer	3	3	5	1	3	
Julia	Bayer	3	0				
Çukurova 1518	ÇARI	2	2	3	1	4	
Sayar 314	ÇARI	2	2	4	1		
Erşan 92	KARI	3	2	3		1	
Maraş 92	KARI	2	1			1	
ST-373	May Çukonar	7	0				
ST-453	May Çukonar	4	1	1			
ST-468	May Çukonar	3	3	9	2	5	
ST-488	May Çukonar	3	3	5	2	4	
DD- 493	Monsanto	4	1		1	2	
Delta Opal	Monsanto	3	2	2	1	1	
DP-388	Monsanto	3	3	10	3	5	1
DP-419	Monsanto	3	3	4		2	
SG-125	Monsanto	7	5	5	1	3	
M-503	NCRI	4	4	5	1	3	
Nazilli 84-S	NCRI	4	3	3		4	
Aksel	Özbuğday	2	1	1		2	
BA-119	Özbuğday	2	2	2		3	
BA-151	Özbuğday	2	2	5			
BA-308	Özbuğday	2	1	1			
BA-525	Özbuğday	2	2	7		1	1
BA-Gold	Özbuğday	7	3	1		3	
Flaş	Özbuğday	2	2	6	1	4	
Şahin 2000	Özbuğday	5	3	4		2	
Tex	Özbuğday	1	1	3		2	
	Total	104	67	105	17	64	2

[a] ÇARI Çukurova Agricultural Research Institute, KARI Kahramanmaraş Agricultural Research Institute, NARI Nazilli Cotton Research Institute

Vegetative compatibility grouping

Complementation between phenotypically distinct *nit* mutants was tested on CDA. Each plate (5-cm diam) was inoculated with three mutants, 1–1.5 cm apart in a triangular pattern, and incubated at 24 °C. Plates were scored for prototrophic growth 14 to 28 days after inoculation. Complementation was evident by the formation of a dense, aerial growth where mycelia from two mutants had met and formed a prototrophic heterokaryon. When mutants of two different strains formed a heterokaryon, their parents were assigned to the same VCG. Each pairing was performed at least twice. Sixteen reference strains (Joaquim and Rowe 1990; Korolev et al. 2000, 2008) used in this study (Table 2) were kindly provided by R.C. Rowe and N. Korolev.

Tests for pathogenicity and virulence

Fifty *V. dahliae* isolates representing the multimember four VCGs were tested in two independent experiments (I and II) on cotton the stem-injection method (Bejarano-Alcázar et al. 1996). Twenty-six of these isolates belonged to VCG1A, seventeen to VCG2B, five to VCG2A and two to VCG4B. Pathogenicity of *V. dahliae* isolates was evaluated using two cotton cultivars, *Gossypium hirsutum* cvs. Acala SJ-1 and DP 15–21, which are moderately susceptible and highly susceptible to Verticillium wilt, respectively (Mert et al. 2005; Schnathorst and Mathre 1966). *V. dahliae* isolates cot200 and cot274, previously characterized as defoliating and non-defoliating isolates, respectively (Korolev et al. 2008), were used as reference isolates.

The stem-injection inoculation, disinfested (1 % NaOCl for 2.5 min) germinated seeds were sown in 15-cm-diam pots (one plant per pot) filled with a sterilized potting mixture (sand : clay loam : peat; 1:1:1, vol:vol). Plants were grown in a growth chamber under fluorescent illumination of 216–270 μEm−2 s−1, 14:10 L:D. Temperature and relative humidity, respectively, were 24–27 °C and 50–70 % during the light period, and 18–22 °C and 60–80 % during the dark period. Plants were watered as required and fertilized every 2 weeks with a watersoluble fertilizer (20–10–20, N:P:K). Six-week-old plants were inoculated with 6 μl of a 4×10^6 conidia ml^{-1} suspension in sterile distilled water. Control plants were treated similarly with sterile distilled water (Bejarano-Alcázar et al. 1996). Disease severity in individual plants was rated daily on a scale of 0–4 according to the percentage of foliage affected by chlorotic, necrotic and wilt symptoms and/or defoliation, in an acropetal progression (0=no symptoms; 1= 1–33 % foliage affected; 2=34–66 % foliage affected; 3=67–100 % foliage affected; 4=dead plants). To determine virulence for each isolate, area under the disease progress curve (AUDPC) was calculated for each individual plant based on foliar symptom ratings during the 5–14 day period following inoculation (Campbell and Madden 1990).

Statistical analysis

Experiments I and II consisted of a factorial treatment design (*V. dahliae* isolates×cultivars) with 10 replicates, each one consisting of a single potted plant, in a complete randomized design. The experiments were repeated once. Recorded values were averaged across plants within each experimental unit for further data analysis. Results across replicated experiments were consistent. Therefore, disease severity data from all experiments were pooled and the combined data set analyzed as a two-way factorial using nonparametric methods (Brunner et al. 2002; Shah and Madden 2004). The overall effect of Verticillium isolates and VCGs on disease reaction on each inoculated cultivar was determined by the analysis of variance type statistic of ranked data using the PROC Mixed procedure in SAS (version 9.0; SAS Institute, Cary, NC) to generate relative effects (REs), and the LD_CI macro to generate 95 % confidence intervals (Brunner et al. 2002; Shah and Madden 2004). RE values are generated by the equation: RE=(R – 0.5)/N; where R is the mean treatment ranking and N is the total number of observations in the analysis. Replications of experiments and blocks within experiments were considered random effects in the analysis, while isolate and cultivar were considered fixed effects. Linear single-degree-of-freedom contrasts were computed to test the effect of selected experimental treatment combinations (Gómez and Gómez 1984).

Results

Collection of *V. dahliae* isolates and assignment to VCG

V. dahliae was isolated from 67 of 104 seed lots belonging to 29 cultivars grown in Turkey, a successful isolation rate of nearly 65 % (Table 1). With regard to cultivars in tested seed lots, DP-388 was the cultivar from which *V. dahliae* was predominantly isolated (10.1 % of the total number of isolates) (Table 1). Cultivar ST-468 was second in importance regarding the number of *V. dahliae* isolates collected (8.5 % of the total). Cultivar Carmen was the third in the ranking from which pathogen isolates were sampled (7.9 %). When grown on chlorate-amended medium, mycelial growth of all isolates was restricted due to chlorate sensitivity. Sectoring frequency on chlorate-containing medium and phenotype ratio of *nit* mutants varied among isolates. Four to ten *nit* mutants were obtained from each isolate of *V. dahliae*. A total of 1217 *nit* mutants were obtained from the 188

Table 2 *nit* mutant tester strains of *Verticillium dahliae* previously assigned to vegetative compatibility groups

Isolate	VCG and Ref. nos.	Mutant phenotype	Host of origin	Geographical origin
T9	1A Joaquim and Rowe 1990, 1991	*nit*1 and NitM	Cotton	USA
cot200	1A Korolev et al. 2008	*nit*1 and NitM	Cotton	Israel
9.6	1B Chen 1994	*nit*1and NitM	Yellowwood	USA
1990.1	1B Chen 1994	NitM	Japanese maple	USA
PH	2A Joaquim and Rowe 1990, 1991	*nit*1 and NitM	Pistachio	USA
pt72	2A Korolev et al. 2000	*nit*1	Potato	Israel
ep8	2A Korolev et al. 2000	NitM	Eggplant	Israel
cot11	2B Korolev et al. 2000	NitM	Cotton	Israel
cot274	2B Korolev et al. 2008	*nit*1	Cotton	Israel
115	2B Joaquim and Rowe 1990, 1991	*nit*1 and NitM	Cotton	Syria
70–21	3 Joaquim and Rowe 1991	*nit*1 and NitM	Pepper	USA
PCW	3 Joaquim and Rowe 1990, 1991	*nit*1 and NitM	Pepper	USA
BB	4A Joaquim and Rowe 1990, 1991	*nit*1 and NitM	Potato	USA
S39	4B Joaquim and Rowe 1991	*nit*1 and NitM	Potato	USA
pn4	4B Korolev et al. 2000	NitM	Peanut	Israel
Tom53	4B Korolev et al. 2000	*nit1*	Tomato	Israel

isolates. The *nit*1 phenotype was recovered most frequently (89 %), followed by NitM (11 %). Several *nit*1 and NitM mutants from each isolate were selected for complementation tests. No self-incompatibility was observed between complementary *nit* mutants recovered from the same isolate of *V. dahliae*. Based on positive complementation reactions with reference testers, four VCGs were found: 105 isolates were assigned to VCG1A, 64 isolates to VCG2B, 17 isolates to VCG2A, and 2 to VCG4B (Table 1). In this study, tested isolates failed to establish compatible heterokaryons with VCG1B tester strains.

Prevalence of VCG1A and disease incidence caused by isolates of this group was higher than those found for VCG2A and VCG2B in most (19 out of 29) of the cultivars inspected (Table 1). Only in Çukurova-1518, Maraş 92, DD-493, Nazilli-84-S, Aksel, BA-119 and BA-Gold cultivars was the prevalence of VCG1A lower than that of VCG2A or VCG2B. VCG2A was detected in thirteen cultivars, with the highest pathogen prevalence and disease incidence in DP-388. Isolates of VCG4B showed the lowest prevalence value and were detected only in the cultivars DP-388 and BA-525. In 20 of 29 cultivars, both VCG1A and VCG2B isolates were recovered from the same seed lot. Simultaneous presence of VCG1A, VCG2A, VCG2B and VCG4B was only detected in DP-388 (Table 1).

Pathogenicity and virulence

In Turkey, disease reaction of cotton cv. DP 15–21 and Acala SJ-1 varied with the VCG of tested isolates. VCG1A isolates induced severe foliar symptoms, stunting with epinasty followed by chlorosis, then necrosis and finally defoliation. Accordingly, isolates of VCG1A were assigned to the previously described cotton-defoliating (D) pathotype (Bell 1994; Bejarano-Alcázar et al. 1996; Schnathorst and Mathre 1966). Disease progressed rapidly and reached a peak 9–12 days after inoculation, with 90–100 % dead plants at the end of the experiments. VCG2B as a group was less virulent than VCG1A and induced no defoliation or only partial defoliation. However, some of the VCG2B isolates did not differ significantly from VCG1A isolates based on AUDPC or mortality scored 2 weeks after inoculation ($P<0.05$); VCG2B isolates were confirmed as the cotton defoliating-like (DL) pathotype (Table 3). VCG2A and VCG4B isolates induced mild to moderate symptoms without defoliation, slow disease progress, and no plant mortality 2 weeks after inoculation, and were designated as the cotton non-defoliating (ND) pathotype (Korolev et al. 2000).

Table 3 Median (M), mean area under the disease progress curve (AUDPC), mean rank (*R*) and relative treatment effects (*REs*) calculated for the severity of Verticillium wilt symptoms on Acala SJ-1 and DP 15–21 caused by isolates of *Verticillium dahliae* from commercially available cotton seed lots[a]

VCG	Isolate	Acala SJ-1				Deltapine 15–21			
		M	AUDPC	*R*	*REs*[b]	M	AUDPC	*R*	*REs*
1A	119/3	3.50	436.50	37.75	0.71	3.95	446.90	38.42	0.80
	125/8	3.00	400.50	37.00	0.52	3.10	357.95	37.15	0.47
	151/5	2.40	330.20	32.50	0.30	2.65	342.20	34.37	0.33
	308/1	3.25	474.75	37.37	0.61	3.85	464.45	38.27	0.77
	388/4	3.00	413.00	37.00	0.50	3.25	382.25	37.37	0.53
	388/7	3.90	455.30	38.35	0.85	4.00	391.90	38.50	0.82
	419/6	3.05	408.60	37.07	0.54	3.65	367.05	37.97	0.69
	453/1	3.75	498.25	38.12	0.80	3.45	467.15	37.67	0.61
	468/8	3.35	480.95	37.52	0.65	3.65	509.60	37.97	0.69
	488/1	3.50	372.75	37.75	0.71	3.70	387.65	38.05	0.71
	503/1	3.30	404.10	37.45	0.63	3.55	432.10	37.82	0.65
	525/9	3.45	465.90	37.67	0.69	3.80	441.35	38.20	0.75
	Ak/1	3.25	383.50	37.37	0.61	3.25	433.50	37.37	0.53
	Ck/1	3.50	465.25	37.75	0.71	3.75	490.75	38.12	0.73
	Cl/4	3.40	486.55	37.60	0.67	3.55	515.85	37.82	0.65
	Cn/3	3.40	530.95	37.60	0.67	3.80	583.30	38.20	0.75
	Cr/15	3.45	482.80	37.67	0.69	3.65	500.85	37.97	0.69
	Er/3	3.75	505.75	37.97	0.80	4.00	498.15	38.50	0.82
	Fl/10	3.30	454.10	37.45	0.63	3.00	418.00	37.00	0.43
	Fl/8	4.00	490.00	38.50	0.89	4.00	526.90	38.50	0.82
	Go/1	3.30	469.10	37.45	0.63	3.80	465.10	38.20	0.75
	Nz/7	3.75	433.25	38.12	0.80	3.20	459.80	37.30	0.51
	Op/2	3.10	372.15	37.15	0.56	3.80	388.25	38.20	0.75
	Sh/2	2.35	405.80	32.12	0.29	2.70	429.10	34.75	0.34
	Sy/4	3.45	439.65	37.67	0.69	3.60	460.25	37.90	0.67
	Tx/3	2.35	389.50	32.12	0.29	2.55	388.20	33.62	0.30
	Cot200	2.90	387.00	36.25	0.49	3.45	455.00	37.07	0.62
2A	388/5	2.45	204.00	32.87	0.32	1.90	202.55	28.25	0.11
	468/16	2.30	206.40	31.75	0.27	2.35	189.55	32.12	0.23
	488/9	2.15	226.95	30.62	0.21	2.60	226.55	34.00	0.31
	Cn/4	2.35	264.55	32.12	0.29	2.75	278.50	35.12	0.36
	Cr/10	2.30	251.40	31.75	0.27	2.10	245.05	30.25	0.16
2B	119/4	2.80	355.40	35.50	0.45	2.80	347.90	35.50	0.37
	125/3	2.55	344.65	33.62	0.36	3.25	407.25	37.37	0.53
	388/17	1.70	157.65	25.75	0.12	1.90	173.85	28.00	0.12
	419/4	3.25	407.25	37.37	0.61	3.80	421.35	38.20	0.75
	468/12	3.20	461.65	37.30	0.60	3.30	462.85	37.45	0.55
	488/10	3.30	391.60	37.45	0.63	3.35	390.95	37.52	0.57
	493/2	2.80	307.90	35.50	0.45	2.40	276.45	32.50	0.25
	503/3	2.25	253.25	31.37	0.25	3.00	390.50	37.00	0.44
	Ak/2	3.00	398.00	37.00	0.52	3.50	407.75	37.75	0.63

Table 3 (continued)

VCG	Isolate	Acala SJ-1				Deltapine 15–21			
		M	AUDPC	*R*	*REs*[b]	M	AUDPC	*R*	*REs*
	Ck/6	2.90	345.45	36.25	0.49	2.45	295.85	32.87	0.27
	Cn/7	2.25	235.75	31.37	0.25	2.60	261.55	34.00	0.31
	Cr/8	3.35	343.45	37.52	0.65	3.65	393.30	37.97	0.69
	Fl/4	1.70	176.40	25.75	0.12	1.90	163.80	28.25	0.11
	Fl/5	2.70	314.10	34.75	0.41	2.95	379.80	36.32	0.42
	Go/3	2.30	282.65	31.75	0.27	2.60	337.80	34.00	0.31
	Nz/2	2.80	364.15	35.50	0.45	3.10	325.45	37.15	0.47
	Sh/6	3.75	475.85	37.97	0.80	3.40	410.35	37.60	0.59
	Cot274	1.75	185	25.87	0.15	1.65	218.00	24.87	0.10
4B	388/19	1.50	162.75	23.25	0.09	1.90	156.30	28.25	0.11
	525/7	1.10	111.70	18.25	0.04	1.30	129.10	20.75	0.05
Contrast (*P*)[c]			F value	Pr > F			F value	Pr > F	
	VCG1A vs VCG2A		32.77	<.0001			38.37	<.0001	
	VCG1A vs VCG2B		8.32	0.0061			10.17	0.0027	
	VCG1A vs VCG4B		136.05	<.0001			101.88	<.0001	
	VCG2A vs VCG2B		6.52	0.0144			10.30	0.0025	
	VCG2A vs VCG4B		47.38	<.0001			23.53	<.0001	
	VCG2B vs VCG4B		75.78	<.0001			53.28	<.001	

[a] For median disease rating (M), severity of Verticillium wilt symptoms was assessed visually according to the percentage of foliar tissue affected (foliar symptoms including chlorosis, defoliation, stunting, and wilting) using an ordinal 0–4 rating scale in which 0=no symptoms; 1=1–33, 2=34–66, and 3=67–100 % of foliar tissue affected; and 4=dead plant

[b] Estimated relative effects (*REs*) in experiments based on the analysis of variance-type statistics of ranked data using the PROC Mixed procedure in SAS for the severity of Verticillium wilt symptoms on the cultivars Acala SJ-1 and DP 15–21 caused by *Verticillium dahliae* isolates of vegetative compatibility groups (VCGs), 95 % confidence intervals

[c] Linear single-degree-of-freedom contrast computed to test the effect of selected treatment combinations. Probability for the *t* statistic of linear single-degree-of-freedom contrasts, significance level $P<0.05$

Median, AUDPC, mean rankings and estimated relative effects for the severity of symptoms caused by isolates on the cultivars DP 15–21 and Acala SJ-1 in experiments, as well as results of linear single-degree of-freedom contrasts computed to test the effect of selected treatment combinations, are shown in Table 3. Significant differences ($P<0.05$) in mean symptom severity rankings were observed between cultivars and VCGs of isolates. In experiment I and II, all 50 isolates from cotton seed lots were pathogenic to cotton, as their AUDPC values were significantly greater than the uninoculated control. Examination of the distribution of individual strains of each VCG within selected ranges of AUDPC values illustrated that the majority of strains in VCG1A were more virulent than strains in VCG2 and especially those in VCG4B ($P<0.05$). In experiment I and II, 21 of 26 and 18 of 26 strains assigned to VCG1A, respectively, resulted in AUDPC values above 400 (Table 3). Of strains assigned to VCG2B, only 3 of 17 in experiment I and 5 of 17 in experiment II had AUDPC values >400. In experiment I and II, no strains assigned to VCG2A and VCG4B had AUDPC values >400.

Discussion

Segregation of *V. dahliae* isolates originating from cotton seed lots into VCGs in Turkey is reported for the first time. Sixty-seven out of the 104 seed lots tested (nearly 65 %) had seeds that were infected with *V. dahliae.* Isolate incidence varied among cultivars,

and reached the highest values in DP-388 and ST-468 cultivars. In contrast, the pathogen was not present in seeds of the cultivars ST-373 or Julia. Considering that only 100 seeds per seed lot were tested, it is likely that more seed lots were infected at lower rates that would not be detected by our methods. Based on these findings, it is clear that commercial seed lots grown in Turkey commonly contain seeds infected by *V. dahliae,* and that this is a primary method by which the pathogen may be introduced into production fields.

In the present study, two phenotypic classes of Nit mutants were identified among 1217 mutants; 89 % of these isolates were identified as *nit*1/*nit*3, and 11 % as NitM. Although others have reported similar results (Brooker et al. 1991; Göre 2007; Korolev and Katan 1997; Korolev et al. 2008; Derviş et al. 2008), the ratio of *nit*1 to NitM mutants varies widely among studies (as high as 49:51, and as low as 6:94). This could be attributed to a number of factors, e.g., source, condition, and age of isolates, as well as media type and components. *nit*3 mutants were rarely produced in this study and the vast majority of mutants were of the *nit*1 phenotype mutants. Some *nit*3 mutants could not be distinguished from nit1 because they did not grow on nitrite medium. Similar results were also reported by several researchers (Chen 1994; Daayf et al. 1995; Strausbaugh 1993; Korolev and Katan 1997; Korolev et al. 2000). In these studies, such mutants in *V. dahliae* were phenotyped as *nit*1, because they complemented NitM mutants but did not complement *nit*1. Therefore, we also considered this kind of mutant as nit1. Overall, four multimember VCGs (VCG1A, VCG2A, VCG2B, VCG4B) were identified among the 188 isolates. Remarkably, VCG1A was the most prevalent (55.9 %) VCG in seed lots, followed by VCG2B (34.0 %), VCG2A (9 %) and VCG4B (1.1 %). In previous studies, similar VCG diversity in *V. dahliae* populations obtained from cotton was identified. In the first study of the diversity of *V. dahliae* isolates from cotton in Turkey, Derviş and Biçiçi (2005) determined VCGs of 70 isolates from the east Mediterranean region of Turkey. Based on complementarity of *nit* mutants, 55.7 % of the isolates were assigned to VCG2B, 27.1 % to VCG2A, and 4.2 % to VCG4B. Göre (2007) used 101 isolates of *V. dahliae* from cotton grown at different locations in the Aegean region of Turkey to determine VCGs; 46 % of the isolates were identified as VCG1A, 33 % as VCG2B, 12 % as VCG2A and 4 % as VCG4B. Recently, Derviş et al. (2008) reported that out of 100 isolates of *V. dahliae* from cotton in the Southeastern Anatolia and East Mediterranean regions, 49 % were classified as VCG1A, 39 % as VCG2B, 9 % as VCG2A and 3 % as VCG4B. The frequency of VCGs of *V. dahliae* from cotton differed in some other countries. In China, most of 114 isolates were assigned to VCG2 (90.4 %), 9.6 % were classified as VCG1A (Zhengjun et al. 1998). In Greece, 64.8 % of the isolates were assigned to VCG2, 2.8 % to VCG4, 1.4 % to VCG1A and the remaining 31 % of the isolates could not be grouped to any of VCGs tested (Elena 1999). In Israel, VCG2B was the largest group and included 107 isolates (66 % of all the isolates). VCG1A and VCG4B included 21.6 and 12.4 % of the isolates, respectively (Korolev et al. 2008). In our study, *V. dahliae* isolates were obtained from a wide range of cotton cultivars grown in Turkey. About half of the isolates were VCG1A; the rest were VCG2B, VCG2A and a few were VCG4B.

Overall, virulence of *V. dahliae* isolates on DP 15–21 and Acala SJ-1 cotton correlated with their VCG: isolates of VCG1A and VCG4B were the most and least virulent, respectively. The first symptoms developed 5 to 7 days after inoculation. The plants inoculated with isolates of VCG1A showed severe symptoms causing defoliation at the 4–6 true leaf stage. In those plants, disease symptoms developed earlier and were more severe, and plants also died earlier than those inoculated with the isolates in the other VCGs. In previous VCG studies, VCG1B isolates were proved to be of the ND pathotype in virulence assays on cotton (Bell 1994; Chen 1994; Jiménez-Díaz et al. 2011), but not observed in this work. Inoculations with isolates in VCG2B resulted in mildly virulent reactions similar to those with VCG2A, although some isolates in VCG2B were as virulent as isolates in VCG1A. These results are in agreement with results from previous studies on *V. dahliae*, which have demonstrated some correlations between VCGs and virulence on certain hosts (Bhat and Subbarao 1999; Daayf et al. 1995; Göre 2009; Korolev 1998; Tsror et al. 2001). So far the D pathotype from cotton has been reported from several locations in the Americas (Schnathorst and Mathre 1966), China (Zhengjun et al. 1998), Central Asia (Daayf et al. 1995), Spain (Bejarano-Alcázar et al. 1996; Korolev et al. 2008), Turkey (Göre 2007) and Israel (Korolev et al. 2008). Epidemics caused by the D pathotype develop earlier, more rapidly, and result in a greater reduction of cotton yield compared with the losses caused by the ND pathotype (Bejarano-Alcázar et al.

1995, 1997). Furthermore, the D pathotype overcomes valuable tolerance to the ND pathotype in certain cotton cultivars (Bell 1994; Schnathorst and Mathre 1966). In North America, VCG4A isolates were more aggressive on potato than VCG4B, 4AB, and 2B isolates (Joaquim and Rowe 1991; Strausbaugh 1993). Moreover, VCG4A isolates interacted synergistically with the root lesion nematode *Pratylenchus penetrans*, causing reduced tuber yield (Botseas and Rowe 1994). In Israel, VCG4B isolates were the most aggressive to potato, and VCG2A isolates were the most aggressive to tomato (Tsror, (Lahkim) et al. 2001); VCG2B isolates were the least aggressive to both potato and tomato (Tsror, (Lahkim) et al. 2001). In Turkey, VCG1A isolates were the most aggressive to both chrysanthemum and olive, and VCG2B isolates were the most aggressive to eggplant (Göre 2009; Derviş et al. 2009, 2010). Differences in virulence among isolates within a VCG also have been observed in *Fusarium oxysporum* (Bosland and Williams 1987; Correll et al. 1987; Elmer and Stepheus 1989; Puhalla and Hummel 1983), *Colletotrichum* spp. (Brooker et al. 1991; Nitzan et al. 2006), *Aspergillus* spp. (Wicklow and Horn 2007) and *Neurospora crassa* (Tomsett and Garret 1980). Variation in virulence among isolates in the same VCG is viewed by some workers as an indication of the presence of a continuum of virulence rather than distinct pathotypes (Ashworth 1983; Grogan et al. 1979). However, data presented here strongly suggest that symptoms of cotton wilt are more pronounced on plants infected with VCG1A and VCG2B isolates, as compared with VCG2A and 4B.

Our findings have broad implications for the Turkish cotton industry. Extensive infection of cotton seed lots with *V. dahliae* certainly explains the widespread distribution of VCG1A isolates across Turkish cotton production regions. It also brings into question the importance of seedborne as compared with soilborne inoculum of *V. dahliae*, the latter being the target of most current management practices. Considerable resources are expended annually in some production regions to fumigate fields prior to planting cotton for control of Verticillium wilt. The importance of seedborne inoculum in the development of Verticillium wilt following fumigation warrants investigation. The use of partially resistant cultivars is the most practical disease management strategy (Bölek et al. 2005; Göre et al. 2009, 2011; Heale 1988) and efforts are ongoing to develop cultivars with improved resistance to Verticillium wilt and high market acceptance. Better understanding of the genetic diversity that exists among populations of *V. dahliae* that affect cotton and the use by breeders of the most aggressive strains, i.e., VCG1A, in screening germplasm will be essential to accomplish this goal.

Acknowledgments Our thanks are expressed to Mr. Ercan Gül for technical assistance. We thank William E. Fry for helpful comments on the manuscript prior to submission.

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