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Vegetative compatibility of cotton-defoliating *Verticillium dahliae* in Israel and its pathogenicity to various crop plants

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Abstract *Verticillium dahliae* isolates recovered from a new focus of severe Verticillium wilt of cotton in the northeast of Israel were tested for vegetative compatibility using nitrate non-utilizing (*nit*) mutants and identified as VCG1, which is a new record in Israel. Other cotton isolates of *V. dahliae* from the northern and southern parts of the country were assigned to VCG2B and VCG4B, respectively. VCG1 isolates induced severe leaf symptoms, stunting and defoliation of cotton cv. Acala SJ-2, and thus were characterized as the cotton-defoliating (D) pathotype, whereas isolates of VCG2B and VCG4B were confirmed as the earlier described defoliating-like (DL) and non-defoliating (ND) pathotypes, respectively. This is the first record of the D-pathotype in Israel. The host range of representative isolates of each VCG-associated pathotype was investigated using a number of cultivated plants. Overall, the D isolates were more virulent than DL isolates on all tested host plants, but the order of hosts (from highly susceptible to resistant) was the same: okra (*Hibiscus esculentus* local cultivar), cotton (*Gossypium hirsutum* cv. Acala SJ2), watermelon (*Citrullus lanatus* cv. Crimson Sweet), safflower (*Carthamus tinctorius* cv. PI 251264), sunflower (*Helianthus annuum* cv. 2053), eggplant (*Solanum melongena* cv. Black Beauty), and tomato (*Lycopersicon esculentum* cv. Rehovot 13). The pattern of virulence of ND isolates differed from that of D and DL isolates, so that the former were highly virulent on eggplant but mildly virulent on cotton. Tomato was resistant to all cotton *V. dahliae* isolates tested. RAPD and specific PCR assays confirmed that the D isolates from Israel were similar to those originating from other countries.

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Introduction

Verticillium wilt, caused by *Verticillium dahliae*, is one of the most damaging diseases of cotton in most cotton-growing countries worldwide. Populations of the pathogen can be genetically characterised by means of vegetative, or heterokaryon, compatibility (Katan 2000; Korolev et al. 2000, 2001) 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 characterisation of local populations of *V. dahliae* into VCGs and phenotypic traits may help in the management of diseases they cause.

In a previous study of 565 isolates of *V. dahliae* from 13 host plants and soil in Israel, three distinct VCGs (VCG2A, VCG2B and VCG4B) were identified and characterised (Korolev et al. 2000). Most isolates formed two major geographic populations: VCG2B isolates were prevalent in the north whereas VCG4B were in the south of the country; in addition, VCG2A isolates, accounting for 3% to 8% of all isolates, were scattered throughout all regions. In 1997, a new focus of severe Verticillium wilt was detected in some cotton fields in the Hula Valley, northeastern Israel, with affected plants showing intense defoliation; a few isolates from that focus were identified as belonging to VCG1 (Korolev et al. 1999). Isolates of VCG1 from cotton of different geographical origins have been known to belong to the cotton-defoliating (D) pathotype (Bell 1994; Joaquim and Rowe 1990; Korolev et al. 2001; Puhalla 1979). So far, the D pathotype 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. 2001) and Turkey (Gore 2007), but not in Israel. 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 non-defoliating (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). Therefore, the proper characterisation of pathotypes in *V. dahliae* infecting cotton is of importance for disease-resistance breeding, efficient use of available tolerant cultivars, and proper implementation of crop rotation. In addition, the very limited studies carried out so far on phenotypic traits in D and ND *V. dahliae* from cotton have shown that isolates of these pathotypes may differ in optimum temperature for in vitro growth and pathogenicity or virulence to non-cotton hosts (Bejarano-Alcázar et al. 1996; Schnathorst and Mathre 1966).

We hypothesize that *V. dahliae* VCG1 might be widespread in the focus of the first detection in Israel, and that local isolates of the pathogen belonging to VCG1 may differ from those of other VCGs in phenotypic traits important for the management of the disease. Therefore, the objectives of the present study were to (1) determine the VCG of additional *V. dahliae* isolates from a range of cotton fields in the Hula Valley, as compared with isolates from other regions of Israel; (2) characterise VCG1 isolates with respect to high temperature and carbendazim sensitivity; (3) compare VCG1 isolates with other cotton-associated VCGs and with VCG1 isolates from Spain using pathogenicity tests on cotton and molecular markers; and (4) compare the host range and virulence of VCG1 isolates on additional host plants (eggplant, okra, safflower, sunflower, tomato and watermelon) which are actual or potential alternatives for crop rotation. A preliminary report of this study was presented at the Israeli Phytopthological Society meeting (Korolev et al. 1999), but was never published as a peer-reviewed article.

Materials and methods

Isolates of *V. dahliae*

Diseased plants were collected during 1997–1999 from seven cotton fields at four sites (Gadot,

Goshrim, Kfar Blum, and Shamir) in the Hula Valley (northeastern Israel), as well as from six sites in the north and two sites in the south of the country. A total number of 152 *V. dahliae* isolates was gathered as previously described (Korolev and Katan 1997, 1999). Ten additional isolates originated from cotton gin waste. One monoconidial culture was obtained from each source and stored on Czapek–Dox agar (CDA) at 4°C to 6°C. Nine *V. dahliae* isolates from Israel and three from Spain, previously characterised by VCG, pathogenicity and molecular markers, were used as representatives of known *V. dahliae* pathotypes from cotton (Korolev et al. 2000, 2001; Pérez-Artés et al. 2000). The isolates are listed in Table 1.

VCG analysis

Nitrate nonutilizing (*nit*) mutants were generated from all *V. dahliae* isolates on water–agar–chlorate (WAC) medium (2% agar, 0.02% glucose, and 5% potassium chlorate; Korolev and Katan 1997). The mutants were partially phenotyped (Correll et al. 1987) and then used in complementation tests as previously described (Korolev et al. 2000); *nit* mutants derived from reference strain T9, kindly provided by R. Rowe, were used as testers to identify VCG1. Israeli *nit* testers were used to identify VCG2A, VCG2B, and VCG4B. These latter testers were previously developed from local isolates and correlated with the international OARDC reference strains of *V. dahliae* VCGs (Korolev et al. 2000; Rowe 1995).

Effect of temperature on mycelial growth and survival

The response of *V. dahliae* isolates to temperature was determined with respect to (a) rate of radial growth and (b) survival of mycelial plugs at temperatures that halt radial growth. The rate of radial growth was determined at eight temperatures (10°C, 15°C, 18°C, 21°C, 24°C, 27°C, 30°C and 33°C) as previously described (Korolev et al. 2000). The area under the growth curve (AUGC) was calculated as indicated for area under disease progress curve (AUDPC) by Campbell and Madden (1990) for a 21-day period and optimum temperatures were determined; the development of colony melanisation was visually assessed. To compare survival at high temperature, 10 mycelial plugs of each isolate taken from colonies grown at 24°C were placed on a 9 cm diam CDA plate and incubated at 33°C and 35°C for 10, 15 and 20 days. The plate was then returned to 24°C for an additional 2 week incubation period. Plugs that formed colonies were counted and the percentage of survival was calculated for each isolate. Two types of inoculum were used: non-pigmented mycelial plugs from the edge of young, actively-growing colonies (10 to 14 days-old), and heavily pigmented plugs with abundant microsclerotia from old colonies (3 to 4 weeks-old). For each VCG, five isolates differing in place or year of recovery were used (VCG1: cot200, cot242, cot308, cot356 and cot357; VCG2B: cot63, cot112, cot117, cot149 and cot299; VCG4B: cot12, cot23, cot24, cot175 and cot263) (Table 1; Korolev et al. 2000). The experiment was run in triplicate and repeated once.

Effect of carbendazim on growth and survival

Fungicide-amended media were prepared by adding an aqueous suspension of carbendazim (MBC; Delsene 50DF, E.I.Du Pont de Nemours & Co., Wilmington, DE, USA) to CDA before autoclaving. To compare mycelial growth rate, mycelial plugs (4 mm diam) from the margins of actively-growing colonies were placed in the centre of CDA plates (9 cm diam) amended with carbendazim (0, 0.01, 0.1, 0.2, 0.4, 0.5, 0.7, 0.9, and 1.0 μg ml^{-1}) and cultures were incubated at 24°C. The diameter of the growing colonies was recorded after 7, 14, and 21 days of incubation, and the AUGC was calculated as indicated for AUDPC by Campbell and Madden (1990). Effective concentrations reducing radial growth by 50% (EC_{50} values) were calculated from curves fitted to the growth rate (AUGC) *v. carbendazim* concentration. To compare survival of mycelial plugs at carbendazim concentrations that inhibited mycelial growth, four concentrations (1.0, 5.0, 10.0, and 75.0 μg ml^{-1}) were tested. Mycelial plugs were taken from 1 month-old colonies with abundant microsclerotia grown on CDA at 24°C. For each combination of isolate-carbendazim concentration, 10 plugs were placed on a 9 cm diam CDA plate amended with carbendazim and incubated for 2 weeks. Then, the plugs were transferred to fungicide-free CDA plates and incubated for an additional 10 days. Plugs yielding colonies were counted and the percentage of survival was calculated for each combination of isolate–carbendazim concentration. Five isolates of

Table 1 Isolates of *V. dahliae* from cotton used in this study

VCG	Isolate[a]	Geographic origin			Tested by[b]		Reference
		Country	Region	Site	Mol	Path	
1	cot308	Israel	Hula Valley	Goshrim	+	+	This study
	9 isolates	Israel	Hula Valley	Goshrim	…	…	This study
	cot350	Israel	Hula Valley	Kfar Blum	+	…	This study
	cot351	Israel	Hula Valley	Kfar Blum	+	…	This study
	cot352	Israel	Hula Valley	Kfar Blum	+	…	This study
	cot354	Israel	Hula Valley	Kfar Blum	+	…	This study
	cot356	Israel	Hula Valley	Kfar Blum	+	+	This study
	2 isolates	Israel	Hula Valley	Kfar Blum	…	…	This study
	cot200	Israel	Hula Valley	Shamir	+	+	This study
	cot213	Israel	Hula Valley	Shamir	+	+	This study
	cot228	Israel	Hula Valley	Shamir	+	…	This study
	cot229	Israel	Hula Valley	Shamir	+	…	This study
	cot239	Israel	Hula Valley	Shamir	+	…	This study
	cot242	Israel	Hula Valley	Shamir	+	+	This study
	cot357	Israel	Hula Valley	Shamir	+	…	This study
	11 isolates	Israel	Hula Valley	Shamir	…	…	This study
	V138I	Spain	Guadalquivir Valley	El Carpio	+	+	Pérez-Artés et al. (2000); Korolev et al. (2001)
	V117I	Spain	Guadalquivir Valley	Lebrija	+	+	Pérez-Artés et al. (2000); Korolev et al. (2001)
2A	V1761	Spain	Guadalquivir Valley	Los Palasios	+	+	Pérez-Artés et al. (2000); Korolev et al. (2001)
2B	3 isolates	Israel	Central Region	Meitav	…	…	This study
	cot299	Israel	Galilee Hills	Balfuria	+	+	This study
	9 isolates	Israel	Galilee Hills	Balfuria	…	…	This study
	cot112	Israel	Galilee Hills	Kfar Hahoresh	+	+	Korolev et al. (2000); Korolev et al. (2001)
	cot215	Israel	Hula Valley	Gadot	+	…	This study
	3 isolates	Israel	Hula Valley	Gadot	…	…	This study
	12 isolates	Israel	Hula Valley	Shamir	…	…	This study
	cot117	Israel	Northern Coastal Plain	Ein Shemer	+	+	Korolev et al. (2000); Korolev et al. (2001)
	cot232	Israel	Northern Coastal Plain	Ein Shemer	+	…	This study
	56 isolates	Israel	Northern Coastal Plain	Ein Shemer	…	…	This study
	5 isolates	Israel	Northern Coastal Plain	Ein Shemer	…	…	This study
	cot149	Israel	Northern Coastal Plain	Gan Shmuel	+	+	Korolev et al. (2000)
	ps4	Israel	Yisre'el Valley	Galed	…	…	This study
	cot63	Israel	Yisre'el Valley	Ramat David	+	+	Korolev et al. (2000)
	cot222	Israel	Yisre'el Valley	Ramat David	+		This study
	14 isolates	Israel	Yisre'el Valley	Ramat David	…	…	This study
4B	cot175	Israel	Negev	Be'ery	+	+	Korolev et al. (2000)
	cot24	Israel	Negev	Be'ery	+	+	Korolev et al. (2000); Korolev et al. (2001)
	cot85	Israel	Negev	Bet Kama	+	+	Korolev et al. (2000)
	cot129	Israel	Negev	Ein Hashlosha	+	+	Korolev et al. (2000); Korolev et al. (2001)
	cot93	Israel	Negev	Kfar Azza	+	+	Korolev et al. (2000)
	5 isolates	Israel	Negev	Yad Mordehai	…	…	This study
	cot223	Israel	Judean Hills	Zorrah	+	…	This study
	14 isolates	Israel	Judean Hills	Zorrah	…	…	This study

VCG vegetative compatibility group, *ellipses* not tested in this study

[a] Isolates from Israel were obtained during 1994 to 1999; isolates from Spain were obtained from 1984 to 1994.

[b] Isolates were tested (+) by molecular analysis (Mol) and/or pathogenicity assays (Path). All isolates were tested by vegetative compatibility.

each VCG were used (isolates are listed in the previous section).The experiment was run in triplicate and repeated twice.

Pathogenicity tests

Pathogenicity and virulence (i.e., amount of disease caused in a host genotype) of *V. dahliae* isolates were determined by artificial inoculation experiments carried out at two laboratories using different methods: the Instituto de Agricultura Sostenible, Cordoba, Spain, and the Department of Plant Pathology, ARO The Volcani Centre, Bet-Dagan, Israel.

In Spain, plants were inoculated by the stem-injection method (Bejarano-Alcázar et al. 1996) using cotton cvs Acala SJ-2 and Crema 111. Disease reaction of 'Acala SJ-2' to *V. dahliae* pathotypes from cotton have been studied in previous work (Bejarano-Alcázar et al. 1996; Korolev et al. 2001). 'Crema 111' is a cultivar recently developed in Spain for which a degree of tolerance to *V. dahliae* is claimed. Seeds were disinfested (1% NaOCl for 2 min), germinated, and sown in 12 cm diam plastic pots (one plant per pot) filled with a non-sterilised potting mixture (sand/clay loam/peat; 2:1:2, vol/vol/vol) fertilised with a slow-release fertiliser (14–9–15, N–P–K, plus Mg_2O and trace elements; Plantacote® plus 4M Aglukon, Düsseldorf, Germany) at a rate of 200 g/100 kg. Plants were grown in a greenhouse at 18–24°C. Inoculum was obtained by flooding 7- to 10 day-old monoconidial cultures grown on PDA at 25°C with sterile distilled water (SDW) and filtering the suspension through sterile cheesecloth. The conidial concentration in the suspension was adjusted to 3×10^6 conidia per milliliter for each isolate with SDW. Six week-old plants were inoculated with 5 µl of the inoculum suspension at each of two opposite points at the base of the first and second stem internodes. Control plants were treated similarly with SDW. Disease severity in individual plants was rated on a scale of 0–4 (0 = no symptoms, 4 = plant dead) 2, 3 and 4 weeks after inoculation (Bejarano-Alcázar et al. 1996; Korolev et al. 2001). At the termination of the experiment, plants were excised above the cotyledon node for fresh weight determination. Experiments consisted of a two factor (*V. dahliae* isolates × cotton cultivars) treatment design with eight replicates (pots) in randomized complete blocks.

In Israel, plants were inoculated by the root-dip method using cotton cv. Acala SJ-2, eggplant cv. Black Beauty, okra (local cultivar), safflower cv. PI251264, sunflower cv. 2053, watermelon cv. Crimson Sweet and tomato cv. Rehovot 13 (susceptible to Verticillium wilt, not possessing the *Ve* gene) as hosts. Conidia from 10- to 14 day-old colonies grown on PDA were washed off the medium, diluted to 10^6 conidia ml^{-1}, and used as inoculum. Seedlings at the cotyledon stage were uprooted from the substrate, their roots washed in tap water, trimmed, and dipped in the inoculum for 3 min. Non-inoculated control seedlings were dipped in water. Seedlings were then transplanted (five per pot) to 700 ml pots filled with sandy soil (3.4% clay, 96.2% sand, and 0.4% organic matter; pH 6.9) and maintained in a greenhouse at 24°C to 27°C and a 12 h photoperiod for 4 weeks. Fifteen seedlings of each host were inoculated with each isolate and the experiment was repeated once with each host. Mean disease severity index (DSI) was assessed 1, 2, 3 and 4 weeks after inoculation on a 0–4 rating scale (0 = no visible symptoms, 1 = leaf-wilt symptoms without apparent vascular discolouration; 2 = vascular discolouration with leaf-wilt symptoms on ≤50% of leaves; 3 = vascular discolouration with leaf-wilt symptoms on >50% of leaves; 4 = plant dead) using the formula: $\mathrm{DSI} = [\sum (R \times N)] \times 100/H \times T$, where R = disease rating; N = number of plants with this rating; H = the highest rating, i.e., 4; T = the total number of plants counted. Percentage of dead plants was also recorded 4 weeks after inoculation.

RAPD and specific PCR assays

Twenty-seven *V. dahliae* isolates from cotton in Israel were studied. These isolates belonged to VCG 1 (13 isolates), VCG 2B (eight isolates) and VCG 4B (six isolates; Table 1). *Verticillium dahliae* isolates V138I and V176I from cotton in Spain, previously characterised, respectively, as D or ND pathotype by means of molecular, pathogenicity, and VCG assays (Bejarano-Alcázar et al. 1996; Korolev et al. 2001; Pérez-Artés et al. 2000), were used as reference strains. Mycelia were grown, lyophilised and ground as previously described (Pérez-Artés et al. 2000). DNA was extracted from ground mycelium according to Raeder and Broda (1985), and used for RAPD and specific PCR assays as described by Pérez-Artés et al. (2000). RAPD assays were performed using

random primers OPH04, OPH19 and OPH20 (Operon Technology, Alameda, CA, USA) and primer KS, that amplify D- and ND-specific DNA fragments from *V. dahliae* DNA (Pérez-Artés et al. 2000). Specific PCR assays were carried out using primer pairs D-1/D-2 and ND-1/ND-2, that amplify single bands of 0.55 or 1.5 kb from DNAs of D and ND *V. dahliae*, respectively (Pérez-Artés et al. 2000), and primer set DB19/DB22/espdef01 that amplifies either a single 523 bp band typical for ND isolates, or a 539 bp band together with a 334 bp amplicon from cotton and olive D *V. dahliae* isolates (Mercado-Blanco et al. 2003). Amplifications were performed in a Perkin-Elmer 9600 thermal cycler (Perkin-Elmer, Norwalk, CT, USA). Reactions were repeated four times for VCG2B and VCG4B isolates and twice for VCG1 isolates. Reactions always included positive (DNA from V138I and V176I isolates) and negative (no template DNA) controls.

Data analysis

AUGC data of the temperature and carbendazim experiments were subjected to analysis of covariance, using temperature or carbendazim concentration as linear and quadratic covariates. Pairs of means were compared thereafter by contrast *t* tests. Disease ratings were plotted over time to obtain disease progress curves. AUDPC calculated by the trapezoidal integration method (Campbell and Madden 1990) was expressed as percentage of the maximum possible area for a 4-week period. Data of the pathogenicity experiments in Israel (AUDPC, DSI, mortality) were subjected to contrast *t* tests. Data of pathogenicity experiments in Spain (AUDPC) were arcsin-transformed and subjected to analysis of variance; mean plant fresh weight, and mean disease rating were compared using the Fisher's protected least significant difference test. All tests were performed at $P \leq 0.05$ using JMP 5.0 software (Statistical Analysis System, SAS Institute Inc., Cary, NC, USA).

Results

Regional distribution of cotton-associated VCG

At least one *nit* mutant was recovered from each of the 162 *V. dahliae* isolates from Israel. Most mutants (94%) were classified as *nit1* and 6% were classified as NitM. Based on positive complementation reactions with reference testers, three VCGs were found: 35 isolates were assigned to VCG1, 107 isolates to VCG2B, and 20 to VCG4B (Table 1). VCG1 isolates were recovered from three sites (Goshrim, Kfar Blum, and Shamir) in the Hula Valley. In Goshrim and Kfar Blum, all isolates were of VCG1. Both VCG1 and VCG2B isolates were recovered from two fields in Shamir, whereas only VCG2B was found in a third field at this site. Only VCG2B isolates were recovered from Gadot (Hula Valley) as well as from additional sites in the northern part of the country outside the Hula Valley, and only VCG4B isolates were recovered from two sites in the southern part of Israel, in accordance with a previous study (Korolev et al. 2000).

Effect of temperature on mycelial growth and survival

Growth of VCG1 and VCG4B isolates was optimal at 25°C (with VCG1 isolates forming colonies with larger diameter), whereas the optimum for VCG2B isolates was 20°C (Fig. 1a). Temperatures in the range of 28°C to 30°C reduced melanisation of microsclerotia in VCG1 isolates and completely suppressed it in VCG4B isolates, but did not substantially reduce radial growth in either VCG. In contrast, that temperature range induced scant growth along with intensive melanisation in VCG2B isolates. Unlike VCG1 and VCG4B isolates, growth of VCG2B colonies was often unstable, with mycelial stunting and subsequent sectoring, which resulted in irregular colony shape (Fig. 2). None of the tested *V. dahliae* isolates grew at 33°C, but all of them survived at 33°C for 3 weeks. Likewise, 10-day exposure to 35°C was not lethal to any isolate. However, differences among VCGs were evident following longer exposure of isolates to 35°C. Isolates of VCG1 and VCG4B did not differ significantly and were more tolerant of 35°C than were VCG2B isolates. Also, old, melanised plugs were more tolerant of 35°C than were young ones (Fig. 1b,c).

Effect of carbendazim on growth and survival

Growth of VCG1, VCG2B, and VCG4B was equally affected by carbendazim, resulting in the same EC_{50} values (0.2 mg l^{-1}). A low concentration of carben-

dazim (0.1 mg l^{-1}) sometimes stimulated radial growth, especially of VCG2B isolates. Growth of all isolates was temporarily inhibited by carbendazim at 0.9 mg l^{-1}, but this effect was only fungistatic since growth was resumed when plugs were returned to fresh CDA without carbendazim. At 5 and 10 mg l^{-1} of carbendazim, VCG1 and VCG4B isolates survived

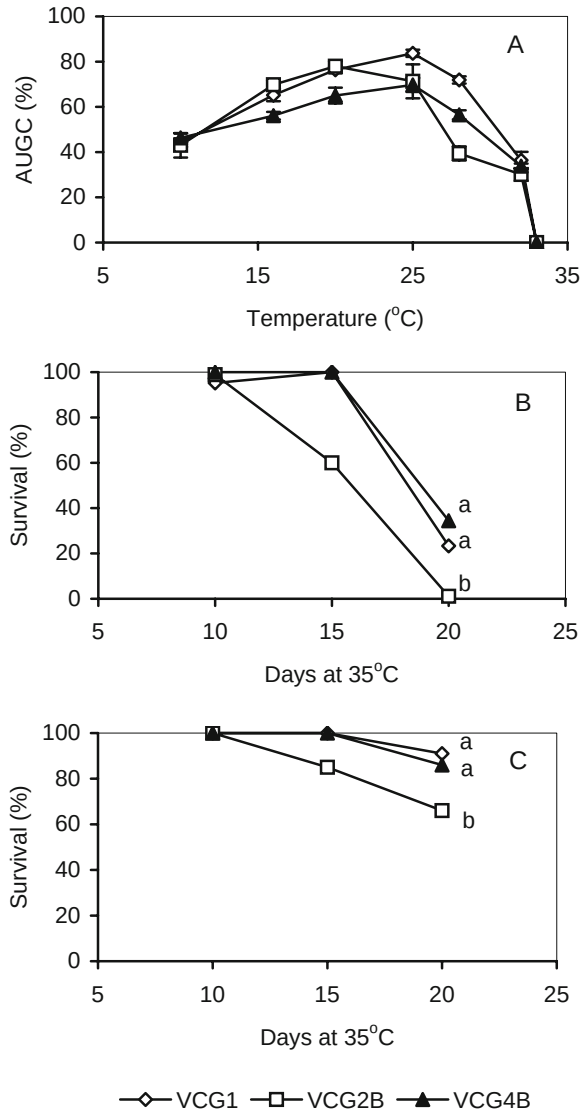

Fig. 1 Effect of temperature on growth and survival of three *V. dahliae* VCGs. **a** Radial growth. Bars represent standard deviation. *AUGC* area under growth curve (Czapek–Dox agar, 3 weeks). *VCG* vegetative compatibility group. **b** Survival at 35°C of mycelial plugs derived from young mycelium. **c** Survival at 35°C of mycelial plugs derived from old mycelium. Mean data for five isolates in each VCG. Means with the same letter were not significantly different according to contrast *t* tests ($P \leq 0.05$)

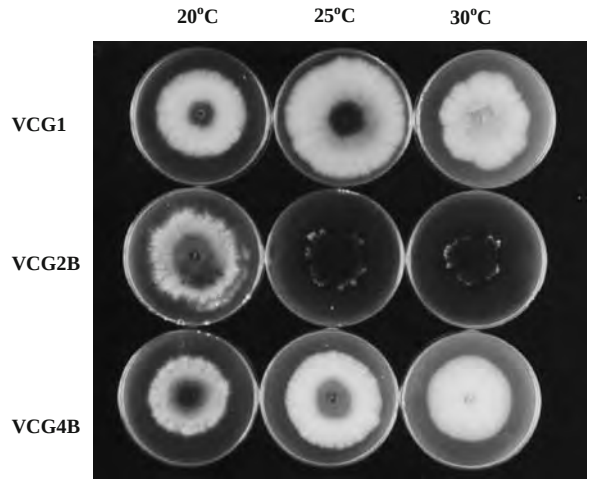

Fig. 2 Variation in colony growth and pigmentation on Czapek–Dox agar among *V. dahliae* isolates of three vegetative compatibility groups (VCG) at three temperatures. *Top row* VCG1 isolate cot200; *middle row* VCG2B isolate cot117; *bottom row* VCG4B isolate cot24. Three weeks in the dark; 9 cm plates

better than VCG2B isolates, while no isolate survived exposure to carbendazim at 75 mg l^{-1} (Fig. 3).

Pathogenicity of *V. dahliae* isolates on cotton

In Israel, disease reaction of cotton cv. Acala SJ-2 varied with the VCG of tested isolates. VCG1 isolates induced severe foliar symptoms, stunting with epinasty followed by chlorosis, then necrosis and finally defoliation. Accordingly, isolates of VCG1 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 plateau 2 to 3 weeks after inoculation, with 90% to 100% dead plants at the end of the experiment. VCG2B as a group was less virulent than VCG1 and induced no defoliation or only partial defoliation. However, some of the VCG2B isolates did not differ significantly from VCG1 isolates based on AUDPC or mortality scored 4 weeks after inoculation; VCG2B isolates were confirmed as the cotton-defoliating-like (DL) pathotype. VCG4B isolates induced mild to moderate symptoms without defoliation, slow disease progress, and 20% or less plant mortality by 4 weeks after inoculation, and were confirmed as the cotton ND pathotype (Korolev et al. 2000; Tables 2 and 3; Fig. 4).

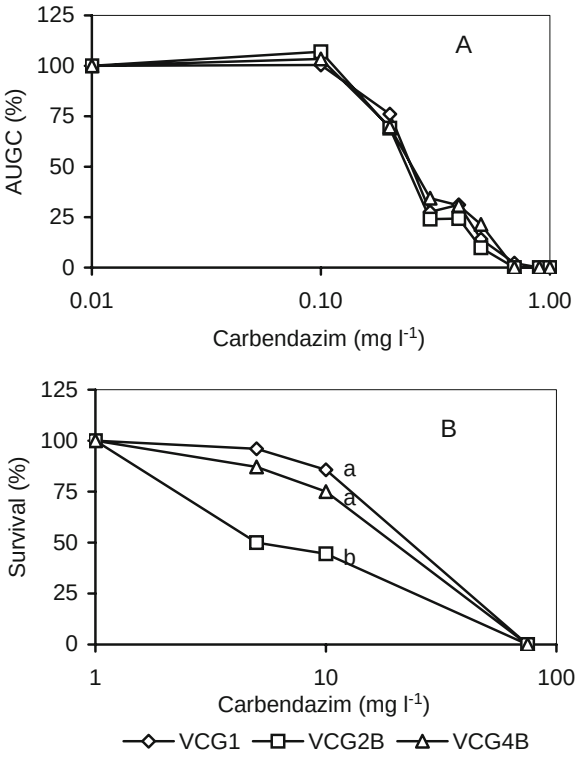

Fig. 3 Effect of carbendazim on growth (**a**) and survival (**b**) of three VCGs of *V. dahliae*. *VCG* vegetative compatibility group. Mean data for five isolates in each VCG. Means with the same letter were not significantly different according to the contrast *t* tests ($P \leq 0.05$)

In Spain, disease reactions varied with *V. dahliae* isolates and cotton cultivars. Isolates V117I and V176I caused the predicted D- and ND-reaction, respectively, in both Acala SJ-2 and Crema 111 cultivars. Symptoms caused by VCG1 isolates cot200 and cot242 from Israel on 'Acala SJ-2' cotton were as severe as those caused by the reference D isolate V117I. Conversely, severity of disease caused by isolates cot200 and cot242 on cv. Crema 111 was significantly lower ($P<0.05$) and similar to that caused by ND isolate V176I when the isolates were compared by severity of symptoms or AUDPC. However, the isolates from Israel caused plant fresh weight reductions in both cultivars similar to that caused by D isolate V117I from Spain (Table 4).

Pathogenicity of *V. dahliae* isolates on non-cotton plant species

Reactions of five additional host plants to isolates of three VCG-associated cotton pathotypes (D, DL, and ND) are summarized in Tables 2 and 3 and Figs. 4 and 5. Okra was the most susceptible host to all isolates and pathotypes, being severely affected by chlorosis, necrosis and defoliation. Four weeks after inoculation, all isolates caused close to 100% mortality of okra plants regardless of VCG or pathotype. Nevertheless, statistically significant differences were evident between VCGs: as a group, isolates of VCG1 were the most, and those of VCG2B the least virulent to okra based on AUDPC (Fig. 5), although AUDPC caused by individual isolates from different VCGs did not always differ significantly. On eggplant, isolates of VCG4B caused the most severe symptoms, rapid disease progress, and a high (67%) level of plant mortality; VCG1 and VCG2B isolates caused milder symptoms and did not differ significantly according to AUDPC. However, the disease progress curve of VCG1 isolates reached a plateau 3 weeks after inoculation whereas that of VCG2B isolates continued to rise, resulting in a mortality level higher than that caused by VCG1 isolates (Fig. 4). All isolates were pathogenic to watermelon, inducing moderate to severe symptoms, although disease caused by VCG2B isolates progressed at a slower rate than that of the two other VCGs. Safflower and sunflower were mildly affected by isolates of all three VCGs, with slightly more severe symptoms caused by VCG1 and VCG4B compared with those caused by VCG2B isolates; VCG1 caused higher plant mortality than the other two VCGs. None of the isolates tested was pathogenic to tomato.

RAPD and specific PCR assays

RAPD assays of VCG2B and VCG4B *V. dahliae* isolates from Israel consistently produced the DNA bands diagnostic for the ND pathotype, i.e., 0.9 kb, OPH04; 2.0 kb, OPH19; and 0.65 kb, KS. Similarly, assays of VCG1 isolates from Israel yielded the RAPD markers diagnostic for the D pathotype, i.e., 1.65 kb, OPH19;, 1.0 kb, OPH 20; and 0.75 kb, KS (Pérez-Artés et al. 2000). An exception was that primer OPH20 failed to amplify the D-specific 1.0 kb band from VCG1 isolates cot200, cot213, and cot242 (Fig. 6a). Because of this inconsistency, RAPD results for all isolates were further examined by specific PCR assays using D- and ND-specific primer pairs (Pérez-Artés et al. 2000). Results of these specific PCR assays confirmed that all VCG2B and VCG4B

Table 2 Pathogenicity of *V. dahliae* isolates from cotton on cotton and additional hosts based on AUDPC

VCG	Pathotype	Isolate	AUDPC[a], %						
			Cotton	Eggplant	Okra	Safflower	Sunflower	Tomato	Watermelon
1	D	cot200	66.3a	15.6b	78.4a	15.1b	13.1b	5.7a	48.8a
1	D	cot242	64.4a	20.1ab	89.2a	28.2a	21.9a	5.7a	48.4a
1	D	cot213	63.0a	14.9b	79.6a	22.9a	23.0a	10.1a	39.8a
1	D	cot356	50.7a	9.0b	75.7a	27.9a	11.9b	7.6a	33.6ab
1	D	cot308	44.3ab	10.5b	84.2a	22.1a	19.6a	6.2a	47.9a
Mean			57.7A	14.0B	81.4A	23.2A	17.9A	7.1A	43.7A
2B	DL	cot63	47.3ab	24.8a	64.9b	12.8ab	17.1ab	6.3a	30.1ab
2B	DL	cot112	45.5ab	12.7b	43.8b	13.8b	12.1b	9.3a	20.2b
2B	DL	cot299	44.8ab	15.8b	68.1ab	12.6b	8.0b	10.0a	22.4b
2B	DL	cot117	34.8b	23.6a	56.1b	13.8b	12.8b	8.0a	23.3b
2B	DL	cot149	26.7b	13.1b	87.9a	12.2b	8.5b	10.2a	22.5b
Mean			39.8B	18.0B	64.2C	13.0B	11.7B	8.8A	23.7B
4B	ND	cot24	22.8bc	44.7a	67.9b	20.8b	11.3b	12.6a	35.4ab
4B	ND	cot129	21.8bc	29.3a	73.6a	18.2ab	16.4ab	5.5a	58.6a
4B	ND	cot93	14.9c	41.2a	64.2b	23.8a	20.6a	7.4a	22.7b
4B	ND	cot85	14.3c	31.0a	68.1b	24.3b	10.9b	7.3a	53.6a
4B	ND	cot175	9.3c	25.4a	82.4a	17.5b	14.4b	11.2a	34.8ab
Mean			16.6C	34.3A	71.2B	20.9AB	14.7AB	8.8A	41.0A

VCG vegetative compatibility group, *D* cotton defoliating, *DL* cotton defoliating-like, and *ND* cotton non-defoliating pathotype

[a] AUDPC, expressed as percentage of the maximum possible area for a 28 day period. In each column, values followed by the same letter do not differ significantly according to contrast *t* tests (P=0.05). Lower-case letters refer to differences between isolates, and upper-case letters refer to differences between VCGs.

isolates tested were of the ND pathotype (not shown), as well as that all VCG1 isolates except for cot 200, cot213, and cot242 were of the D pathotype (Fig. 6b). The latter three VCG1 isolates yielded no amplification products with either D- or ND-specific primers. To further investigate such a lack of amplification, template DNA of isolates cot 200, cot213, and cot242 were used for an additional specific PCR assay using primers DB19/DB22/espdef01 as described by Mercado-Blanco et al. (2003). The use of the three-primer set together in a single reaction mixture yielded either a single 523 bp band characteristic of ND isolates, or both a 539 bp band together with a 334 bp amplicon characteristic of D *V. dahliae* isolates from cotton and olive. *Verticillium dahliae* VCG1 isolates cot200, cot213, and cot242 yielded both the 539 and 334 bp markers characteristic of the D pathotype (Fig. 6c).

Discussion

Genetic and phenotypic characterization of *V. dahliae* populations prevailing in cotton-growing areas is of much importance for the effective management of Verticillium wilt. In previous studies, examination of 200 *V. dahliae* isolates from cotton in different areas in Israel for vegetative compatibility, virulence and pathotype-specific molecular markers indicated that VCG1 did not occur in this population, which was comprised of VCG2B and VCG4B (Korolev et al. 2000, 2001). In the present study, we report that VCG1 was found to prevail at a new, extensive focus of Verticillium wilt in the Hula Valley of northeastern Israel, where cotton crops were severely defoliated. In that Valley, VCG1 was the only VCG found in three sites, while coexisting with VCG2B at the Shamir site. Also, this study further confirms that VCG2B and VCG4B prevail in northern and southern Israel, respectively.

VCG1 isolates were divided into two subgroups, A and B, based on the vigour of complementation between *nit* mutants, of which only VCG1A isolates cause severe defoliation of cotton (Bell 1994). In this work, all VCG1 isolates from Israel caused complete defoliation of inoculated cotton plants comparable to that caused by reference VCG1/D strains from Spain,

Table 3 Pathogenicity of *V. dahliae* isolates from cotton on cotton and additional hosts based on mortality

VCG	Pathotype	Isolate	Mortality[a], %						
			Cotton	Eggplant	Okra	Safflower	Sunflower	Tomato	Watermelon
1	D	cot242	100.0a	6.7c	100.0a	16.7ab	3.3a	0.0	56.7a
1	D	cot308	100.0a	0.0c	100.0a	16.7ab	3.3a	0.0	57.5a
1	D	cot356	100.0a	0.0c	100.0a	30.0a	0.0a	0.0	55.6a
1	D	cot200	93.3a	0.0c	96.0a	6.7b	3.3a	0.0	65.0a
1	D	cot213	93.3a	0.0c	100.0a	13.3ab	6.7a	0.0	55.0a
Mean			97.1A	2.2C	99.2A	16.7A	3.1A	0.0	58.3A
2B	DL	cot117	86.7ab	13.3bc	100.0a	3.3b	0.0a	0.0	51.7a
2B	DL	cot299	80.0ab	3.3c	100.0a	0.0b	0.0a	0.0	50.0a
2B	DL	cot63	66.7ab	26.7b	99.3a	0.0b	0.0a	0.0	60.0a
2B	DL	cot149	60.0b	35.0b	83.3b	0.0b	0.0a	0.0	50.0a
2B	DL	cot112	46.7abc	10.0c	90.0ab	0.0b	0.0a	0.0	43.3a
Mean			68.0B	15.7B	96.4A	0.7B	0.0B	0.0	52.4A
4B	ND	cot24	20.0c	60.0a	96.7a	0.0b	0.0a	0.0	50.0a
4B	ND	cot93	20.0c	50.0a	100.0a	10.0b	0.0a	0.0	33.3a
4B	ND	cot129	13.3c	88.3a	100.0a	0.0b	0.0a	0.0	66.7a
4B	ND	cot175	6.7c	88.3a	90.0ab	10.0b	0.0a	0.0	47.5a
4B	ND	cot85	6.7c	80.0a	100.0a	0.0b	0.0a	0.0	66.7a
Mean			12.9C	67.1A	97.2A	5.0B	0.0B	0.0	51.3A

VCG vegetative compatibility group, *D* cotton defoliating, *DL* cotton defoliating-like, *ND* cotton non-defoliating pathotype

[a] Percentage of dead plants, 28 days after inoculation. In each column, values followed by the same letter do not differ significantly according to contrast *t* tests (P=0.05). Lower-case letters refer to differences between isolates, and upper-case letters refer to differences between VCGs.

and should accordingly be regarded as belonging to VCG1A. Moreover, our results further confirm the present status of the VCG1/D-pathotype as a subspecific population within *V. dahliae* which was based on studies of D strains from America, Asia, and Europe (Bell 1994; Daayf et al. 1995; Korolev et al. 2001; Zhengjun et al. 1998). VCG1/D-pathotype is considered indigenous to southern USA and northern Mexico, and now has spread to other geographic areas in America, Asia, China, Greece and Spain (Bell 1994; Bejarano-Alcázar et al. 1996; Daayf et al. 1995; Elena and Paplomatas 2001; Gore 2007; Korolev et al. 2001; Mathre et al. 1966; Zhengjun et al. 1998). Whether VCG1/D-pathotype in Israel is endemic or introduced is unclear. Worldwide spread of the D pathotype may have occurred with contaminated cotton seed (Bell 1992), but defoliating VCG1 isolates of certainly endemic origin were recovered from cotton and cotton field soil in Iran and Tadjikistan (Daayf et al. 1995; Hamdolla-Zadeh 1993; Portenko and Akimov 1997). A feature that might relate to the origin of VCG1/D isolates concerns their molecular diversity. Thus, isolates of VCG2B and VCG4B from Israel appeared molecularly homogeneous since all of the tested ones yielded the DNA bands characteristic of the ND pathotype in RAPD and specific-PCR assays (Pérez-Artés et al. 2000). Conversely, Israeli VCG1 isolates appeared molecularly heterogeneous because three of them from the Shamir site failed to amplify the D-associated 0.55 kb marker defined by primer pair D-1/D-2, as well as the RAPD band from which these specific-PCR primers were derived (Pérez-Artés et al. 2000), although they caused defoliation in susceptible 'Acala SJ-2'cotton. However, all VCG1 isolates from Israel amplified the 539 bp band specific for *V. dahliae* and the D-associated 334 bp amplicon when their DNAs were assayed by specific PCR using primer set DB19/DB22/espdef01 (Mercado-Blanco et al. 2003). Similar diversity in PCR patterns among VCG1/D isolates from cotton was recently reported by Collado-Romero et al. (2006), whereby most of the isolates yielded the so-called A pattern (amplification of 462 bp and 334 bp D-associated markers) but some isolates from Greece and Turkey produced a B pattern characterized by amplification of the 334 bp marker

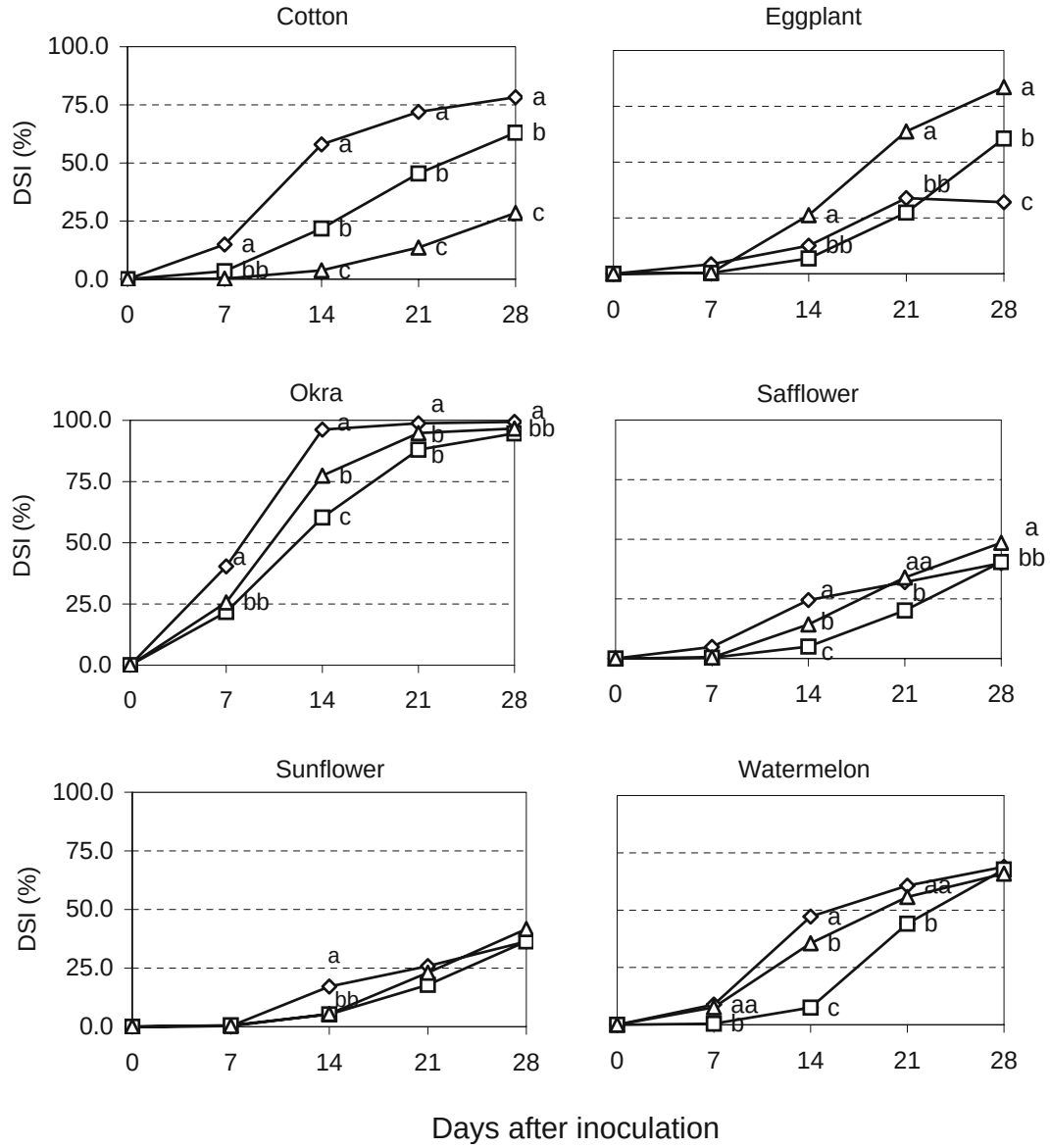

Fig. 4 Disease progress caused by *V. dahliae* isolates of three vegetative compatibility groups (VCG; five isolates of each VCG) in six host plant species. *Diamond* VCG1, *square* VCG2B, *triangle* VCG4B. *DSI* disease severity index. For each test plant, DSI values for the same day after inoculation designated by the same letter are not significantly different according to contrast *t* tests at $P \leq 0.05$

only. Interestingly, two tested VCG1 cotton isolates from Shamir differed from the VCG1 representative D-pathotype isolate from Spain also in pathogenicity: Both Shamir and Spanish isolates showed the D pathotype on cv. Acala SJ-2 but the isolate from Spain was more aggressive than the Shamir isolates on cv. Crema111.

Isolates of VCG1/D-pathotype, VCG2B/DL-pathotype, and VCG4B/ND-pathotype from cotton in Israel differed in biological properties (virulence to crops within host range, and response to high temperature and carbendazim) that may have a bearing on their adaptation to environments and crops. Overall, virulence of *V. dahliae* isolates on 'Acala SJ-2' cotton correlated with their VCG: isolates of VCG1/D-pathotype and VCG4B/ND-pathotype were the most and least virulent, respectively, and those of VCG2B were intermediate in

Table 4 Virulence of *V. dahliae* isolates to cotton cvs Acala SJ-2 and Crema 111

Isolate	VCG	Pathotype		DSI[a] (%)		AUDPC[b] (%)		Fresh weight[c] (g)	
		Acala SJ-2	Crema 111	Acala SJ-2	Crema 111	Acala SJ-2	Crema 111	Acala SJ-2	Crema 111
Control	–	–	–	0.0	0.0	0.0	0.0	29.8 a	32.5 a
V176I	2A	ND	ND	1.8[d] b	1.7 b	25.2 a	18.9 b	14.0 b	21.5 b
V117I	1	D	D	3.1 a	2.8 a	29.7 a	31.3 a	7.7 c	13.4 c
Cot 242	1	D	ND	3.0 a	1.9 b	25.4 a	18.2 b	8.0 c	17.7 c
Cot 200	1	D	ND	2.9 a	2.0 b	28.5 a	18.3 b	8.2 c	16.0 c

Plants were inoculated by injecting a conidial suspension into the base of the first and second internodes.

VCG vegetative compatibility group, *D* cotton defoliating, *ND* cotton non-defoliating pathotype

[a] Disease severity index, assessed on a 0 to 4 scale based upon the percentage of foliage with Verticillium wilt symptoms (0 = no symptoms; 4 = plant dead) 4 weeks after inoculation.

[b] AUDPC, expressed as percentage of the maximum possible area for a 28 day period.

[c] Fresh weight was determined in plants excised above the cotyledon node.

[d] Values in each column followed by the same letters are not significantly different ($P \leq 0.05$) according to Fisher's protected least significant difference.

virulence and caused partial defoliation (i.e., DL, or PD, pathotype) which is in agreement with results from previous studies (Korolev et al. 2000, 2001). That kind of correlation has been found for *V. dahliae* VCGs from other crops such as artichoke and potato in Spain and California, respectively (Jiménez-Díaz et al. 2006; Strausbaugh et al. 1992).

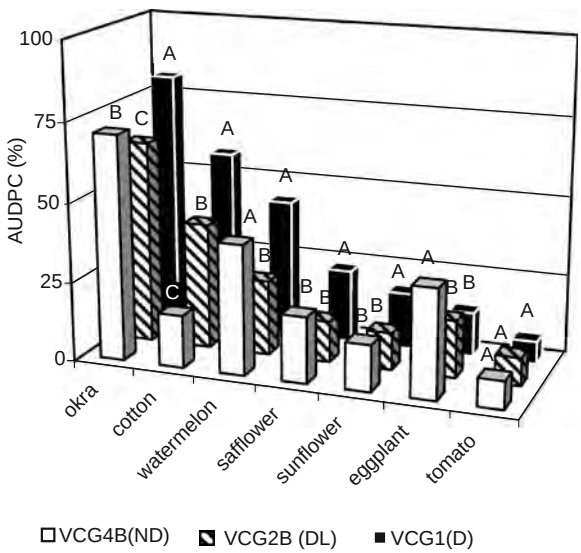

Fig. 5 Comparative pathogenicity of three VCG-associated cotton pathotypes of *V. dahliae* to seven host plant species. *AUDPC* area under disease progress curve, expressed as percentage of the maximum possible area for a 28 day period. *VCG* vegetative compatibility group. *D* cotton defoliating, *DL* cotton defoliating-like, and *ND* cotton non-defoliating pathotype. For each host plant, *columns* designated by the *same letter* do not differ significantly according to contrast *t* tests ($P \leq 0.05$)

All tested *V. dahliae* isolates were pathogenic on cotton, eggplant, okra, safflower, sunflower, and watermelon, but tomato showed a consistent resistance reaction to them. However, the pattern of virulence (i.e., amount of disease caused in a host genotype) on susceptible plants varied with the VCG of isolates. Isolates of VCG1/D-pathotype were highly virulent and defoliated cotton and okra, but were moderately virulent to safflower, sunflower, and watermelon, and mildly virulent to eggplant and caused no defoliation to any of them. Collectively, VCG1 isolates were more virulent to cotton and okra, and less virulent to eggplant, than isolates of any other VCG. Schnathorst and Mathre (1966) reported that the cotton-defoliating T-1 strain of *V. dahliae* from California (later defined as VCG1 by Joaquim and Rowe 1990) was not pathogenic to watermelon. Similarly, Baht and Subbarao (1999) found that a VCG1 cotton isolate from California was not pathogenic to artichoke whereas Jiménez-Díaz et al. (2006) found that isolates of VCG1/D-pathotype from artichoke and cotton in Valencia, Spain, were moderately virulent to artichoke. Differences in the nature of isolates and host cultivars, as well as methods of inoculation used may account for those discrepancies. Schnathorst and Mathre (1966) also found that the T-1 (VCG1) and SS-4 (VCG2A; Joaquim and Rowe 1990) strains from cotton were mildly and highly virulent to tomato, respectively, whereas in this study tomato cv. Rehovot13 was not affected by isolates of VCG1/D-pathotype, VCG2B/DL-pathotype, and

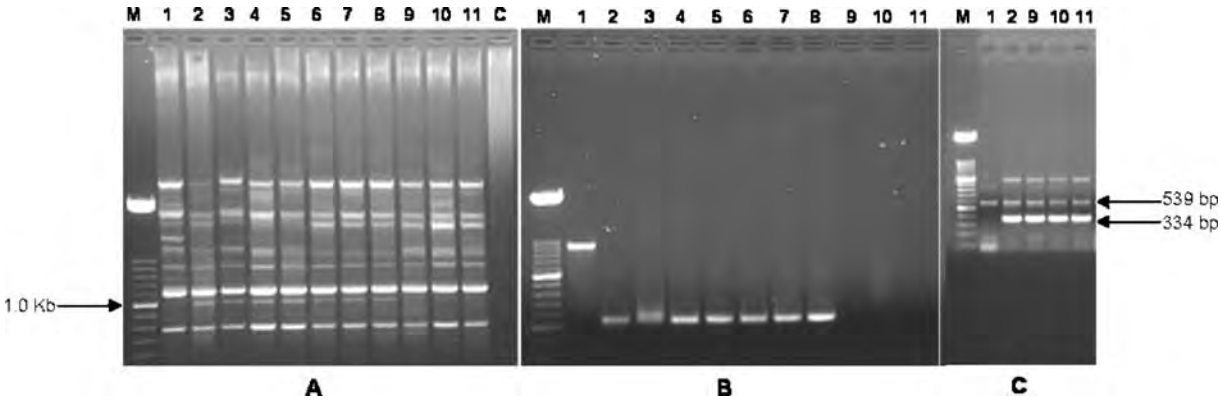

Fig. 6 Amplifications obtained with **a** random primer OPH20, **b** D-specific primers, and **c** DB19/DB22/espdef 01 primer set, using DNA from Israeli VCG1 isolates. DNA products were separated on a 1.5% agarose gel and stained with ethidium bromide. *Numbers on the left and right sides* are the sizes of the respective marker bands that identify Defoliating *V. dahliae* isolates. *Lane M* 0.1 kb DNA ladder XIV (Boehringer–Mannheim). *Lanes 1* and *2* Reference Spanish V176I (ND pathotype) and V138I (D pathotype) isolates. *Lanes 3–11* Israeli VCG1 isolates cot308, cot350, cot351, cot 352, cot354, cot356, cot200, cot213, and cot242. *Lane C* Negative (no template DNA) control

VCG4B/ND-pathotype. That VCG1 isolates were mildly virulent to eggplant is worth noting since Verticillium wilt is one of the most destructive diseases on eggplant (Cirulli et al. 1990), and without considering the VCG of isolates this plant species has been used earlier as the most susceptible test plant to *V. dahliae* (Korolev and Katan 1999; Bejarano-Alcázar et al. 1999). As shown in the present study, susceptibility of eggplant to *V. dahliae* varies significantly with the VCG of isolates so that the most severe disease reaction was caused by isolates of VCG4B/ND-pathotype.

Isolates of VCG2B were second in virulence to 'Acala SJ-2' cotton and caused partial defoliation of plants (i.e., DL or PD pathotype), but were the least virulent to okra, sunflower, safflower and watermelon, and as virulent as VCG1 to eggplant. VCG2B is a rather heterogeneous group of *V. dahliae* isolates in physiological and morphological traits, as well as in host range, and it appears that native populations of this group can evolve high virulence to specific hosts in some regions, as for example cotton and artichoke (Jiménez-Díaz et al. 2006; Katan 2000; Korolev et al. 2000, 2001). Compared with VCG2B/DL-pathotype, isolates of VCG4B/ND-pathotype were collectively less virulent to cotton but more virulent to eggplant, okra, and watermelon, and as virulent to safflower and sunflower. This lesser virulence to cotton compared with VCG2B isolates agrees with that found previously by Korolev et al. (2000, 2001). VCG4B, as VCG4A, occurs closely linked with potato cultivation (Joaquim and Rowe 1991; Strausbaugh 1993; Korolev et al. 2000), although only VCG4B has been found in Europe so far (Elena and Paplomatas 1998; Gore 2007; Katan 2000; Joaquim and Rowe 1991). In Israel and Spain, cotton VCG4B isolates were found in the main potato-growing regions (Korolev et al. 2000; 2001), so that once established by potato cultivation, VCG4B populations infect cotton if it is grown as part of crop rotation systems. In a previous study, most VCG4B isolates from Israel were of the ND pathotype while three cotton VCG4B isolates from a separate site near Jerusalem were described as VCG4B/DL pathotype (Korolev et al. 2000). In the present study all VCG4B isolates tested were of the ND pathotype.

In summary, the present study indicates that *V. dahliae* VCG1/D-pathotype had been established in north-eastern Israel in the late 1990s which potentially posed a severe threat to susceptible crops in that region because of the wide host range and high virulence of this strain. Besides overcoming valuable cotton tolerance to ND isolates, D isolates can develop devastating epidemics with initial inoculum six to ten times lower than that needed by ND isolates (Bejarano-Alcázar et al. 1995; Schnathorst and Mathre 1966). Compared with VCG2B isolates, VCG1 isolates form rapidly growing colonies that sporulate more profusely, and are more tolerant to high temperatures and some fungicides. That seems to provide VCG1 higher fitness for survival and may facilitate the substitution of VCG2B by VCG1

populations in the presence of susceptible hosts. In fact, such a situation has already occurred in southern Spain, where the D pathotype, first reported in cotton crops at a restricted area of the Guadalquivir Valley, has now spread to the northern part of the valley and poses a severe risk to both cotton and olive crops in that region (Bejarano-Alcázar et al. 1996; Mercado-Blanco et al. 2003). The detection of isolates highly virulent to cotton in the north-eastern part of Israel stimulated the authorities and the cotton-growers association to take measures to avoid the spread of the pathotype to other areas. Cotton was not planted in the foci of severe defoliating wilt during the last decade, and the fields were used for cereals or fallow. The greater emphasis was given to sanitation. At present, there are no data about new foci of the defoliating wilt in that part of Israel.

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