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Comparative study of genetic diversity and pathogenicity among populations of *Verticillium dahliae* from cotton in Spain and Israel

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Abstract

Genetic diversity and phenotypic diversity in *Verticillium dahliae* populations on cotton were studied among 62 isolates from Spain and 49 isolates from Israel, using vegetative compatibility grouping (VCG), virulence and molecular assays. In Spain, defoliating *V. dahliae* isolates (D pathotype) belong to VCG1, and non-defoliating isolates (ND) belong to VCG2A (often associated with tomato) and VCG4B (often associated with potato). The D pathotype was not identified in Israel. The ND pathotype in Israel is comprised of VCG2B and VCG4B. Isolates in VCG2B and VCG4B ranged in virulence from weakly virulent to highly virulent. The highly virulent isolates induced either partial defoliation or no defoliation. Virulence characteristics varied with inoculation method and cotton cultivar. Highly virulent isolates from Israel were as virulent as D isolates from Spain under conditions conducive to severe disease. The D pathotype is pathologically and genetically homogeneous, whereas the ND pathotype is heterogeneous with respect to virulence, VCG, and molecular markers based on single-primer RAPD and on PCR primer pairs.

Introduction

Verticillium wilt, caused by *Verticillium dahliae* Kleb., is one of the most damaging diseases of upland cotton (*Gossypium hirsutum* L.) in Spain (Bejarano-Alcázar et al., 1996) and in most cotton-growing countries (Bell, 1992). Isolates of *V. dahliae* from cotton vary in their morphology, virulence and genetics (Bell, 1994). Schnathorst and Mathre (1966) first showed a major difference in virulence (i.e., amount of disease caused in a host genotype) among cotton isolates of *V. dahliae*, based on an isolate's ability to completely defoliate cotton (defoliating pathotype, D), or to only cause wilt without defoliation (non-defoliating pathotype, ND). While the ND pathotype is widespread, the D pathotype has been reported only from a few locations in the Americas (Mathre et al., 1966; Schnathorst, 1969), China (Zhengjun et al., 1998), central Asia (Daayf et al., 1995) and Spain (Bejarano-Alcázar et al., 1996). 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). Therefore,

the proper characterization of pathotypes in *V. dahliae* infecting cotton is of importance for disease-resistance breeding as well as for the efficient use of available tolerant cultivars.

The D and ND *V. dahliae* pathotypes belong to separate vegetative compatibility groups (VCGs) (Bell, 1994; Daayf et al., 1995; Joaquim and Rowe, 1990; Puhalla and Hummel, 1983; Zhengjun et al., 1998). All the D strains (from America and China) tested to date belong to VCG1 subgroup A (Bell, 1994). The relationship between VCG and virulence to cotton of the D and ND strains from Spain is not known. In Israel, severe Verticillium wilt with partial defoliation has been observed in certain cotton fields, whereas rather mild wilting has been observed in others. VCG analysis of a large collection of *V. dahliae* isolates from cotton and 12 additional host-plant species placed these isolates in VCG2A, VCG2B and VCG4B (Korolev et al., 2000b). Isolates from cotton belonged to VCG2B and VCG4B, the two major VCGs found and were described as cotton non-defoliating. However, considerable variation in virulence to cotton was evident among those isolates. Two pathotypes were described, based on the disease reactions induced in root-dip inoculated 'Acala SJ-2' cotton plants: isolates of the ND pathotype induced mild-to-moderate symptoms, while isolates of the defoliating-like (DL) pathotype induced severe foliar symptoms, stunting and often death, with occasional partial defoliation. Of the 32 cotton isolates tested, all of the 19 VCG2B isolates and three of the 13 VCG4B isolates were of the DL pathotype, whereas the remaining 10 VCG4B isolates were of the ND pathotype. These results suggested that vegetative compatibility grouping in *V. dahliae* from cotton might be associated with differential virulence traits (Korolev et al., 2000b), and prompted an examination of the highly virulent DL strains from Israel for possible emergence of a new D pathotype among the non-VCG1 local populations. However, in the absence of the D pathotype in Israel, it was not possible to compare the DL pathotype with an authentic D pathotype *in vivo*. In recent years, randomly amplified polymorphic DNA (RAPD) analysis has been used for the characterization of *V. dahliae* isolates from several crop species (Heale, 2000). RAPD analysis allowed the differentiation of the D and ND pathotypes of *V. dahliae* from Spain and the development of specific PCR primers that differentiate between the D and ND pathotypes from cotton in Spain and other countries (Pérez-Artés et al., 2000a). No similar information is available on the *V. dahliae* pathotypes from cotton in Israel.

The objectives of this study were (i) to estimate the spectrum of VCG diversity among isolates from Spain and to compare it with that of isolates from Israel; (ii) to compare the virulence of *V. dahliae* isolates from Israel to that of the D and ND pathotypes from Spain, and to re-examine the distinction between the ND and DL pathotypes from Israel, using three cotton cultivars and two inoculation methods; (iii) to assess the validity of the molecular markers designed to differentiate the D from the ND pathotypes, using new isolates from Israel; and (iv) to reveal any association that exists between pathogenicity, VCG, molecular markers and geographical distribution in the studied *V. dahliae* collections. Preliminary reports of this study have already been published (Korolev et al., 2000a; Pérez-Artés et al., 2000b).

Materials and methods

Fungal isolates and culture conditions

A collection of 111 *V. dahliae* isolates from cotton in Spain (62 isolates) and Israel (49 isolates) was used (Table 1). Isolates from Israel were obtained from 1992 to 1998 from 13 sites (six in the north and seven in the south of the country) (Korolev et al., 2000b). Isolates from Spain were obtained from 1984 to 1994 from cotton stems collected in fields scattered along the Guadalquivir Valley in southern Spain. The isolates from Spain had been previously characterized as D ($n = 28$ isolates) or ND ($n = 34$ isolates) by pathogenicity testing, using the stem-injection inoculation method (Bejarano-Alcázar et al., 1996). Using the root-dip inoculation method, 37 of the isolates from Israel had been previously characterized as non-defoliating, with further subdivision into ND and DL based on the level of virulence. The VCG of the isolates from Israel had been determined (Korolev et al., 2000b). Monoconidial cultures were stored on plum-extract agar covered with sterile liquid paraffin at 5 °C (isolates from Spain), or on Czapek-Dox agar (CDA) at 5–7 °C (isolates from Israel). Active cultures were obtained by placing small mycelial plugs from stock cultures on CDA or on aureomycin-amended water agar, and further subculturing on potato dextrose agar (PDA). For inoculum preparation, cultures were grown on PDA for 7–10 days at 25 °C in the dark.

Table 1. Isolates of *Verticillium dahliae* from cotton, listed by vegetative compatibility group (VCG), geographic origin, and pathotype

VCG[a]	Isolate	Geographic origin		Pathotype by	
		Country	Site	Pathogenicity test	RAPD pattern[c]
VCG1	V138I	Spain	El Carpio	D	D
	V117I	Spain	Lebrija	D	D**
	V184I, V186I, V187I, V219I–V221I	Spain	Lebrija	D	D
	V181I, V182I, V189I, V229I, V231I	Spain	Lebrija	D	nt[d]
	V177I	Spain	Los Palacios	D	D
	V175I, V222I, V223I, V226I	Spain	Los Palacios	D	nt
	V180I, V190I	Spain	S. Leandro	D	D
	V218I, V224I	Spain	S. Leandro	D	nt
	V191I	Spain	Trajano	D	D
	V168I–V172I	Spain	Unknown	D	D
VCG2A	V217I	Spain	Andújar	ND	ND
	V249I	Spain	Andújar	ND	nt
	V194I	Spain	Brenes	ND	nt
	V209I	Spain	Córdoba	ND	ND
	V207I	Spain	Córdoba	ND	nt
	V142I, V210I, V211I	Spain	El Carpio	ND	ND
	V212I, V253I	Spain	El Carpio	ND	nt
	V183I, V185I	Spain	Lebrija	ND	ND
	V230I	Spain	Lebrija	ND	nt
	V176I, V179I	Spain	Los Palacios	ND	ND
	V197I, V200I	Spain	Palma del Rio	ND	ND
	V196I, V198I, V201I, V237I	Spain	Palma del Rio	ND	nt
	V204I	Spain	Posadas	ND	ND
	V206I	Spain	Posadas	ND	nt
	V213I	Spain	Villanueva	ND	ND
	V214I	Spain	Villanueva	ND	nt
	V173I, V174I	Spain	Unknown	ND	ND
	V149I	Spain	Unknown	ND	nt
VCG2B	cot56, cot59	Israel	Dalia	ND	ND
	cot78	Israel	Dalia	ND*	ND
	cot79	Israel	Dalia	ND (DL)**	ND
	cot3	Israel	Ein Shemer	ND	ND
	cot17	Israel	Ein Shemer	ND (DL)**	ND
	cot117	Israel	Ein Shemer	PD (DL)**	nt
	cot8, cot21, cot69, cot81, cot83	Israel	Galed	ND	ND
	cot14, cot70, cot72	Israel	Galed	ND*	ND
	cot2, cot20	Israel	Gan Shmuel	ND	ND
	cot18	Israel	Kfar Hahoresh	ND	ND
	cot112	Israel	Kfar Hahoresh	ND (DL)**	ND
	cot15, cot62, cot108	Israel	Ramat David	ND	ND
	cot60, cot128	Israel	Ramat David	ND (DL)**	ND
	cot67, cot102, cot104	Israel	Ramat David	ND*	ND
	cot125, cot138, cot140	Israel	Unknown	ND	ND
VCG4B	V192I	Spain	Brenes	ND	ND
	V193I, V239I	Spain	Brenes	ND	nt
	V188I	Spain	Lebrija	ND	ND
	V227I	Spain	Los Palacios	ND	ND*
	cot24	Israel	Be'ery	ND (ND)**	ND
	cot26, cot28, cot122, scot6	Israel	Be'ery	ND	ND
	cot40, cot120, cot127	Israel	Be'ery	PD*	ND

Table 1. Continued

VCG[a]	Isolate	Geographic origin		Pathotype by	
		Country	Site	Pathogenicity test	RAPD pattern[c]
	cot13, cot84	Israel	Bet Kamma	ND	ND
	cot87	Israel	Bet Kamma	ND*	ND
	cot129, cot133	Israel	Ein Hashlosha	ND	ND
	cot1	Israel	Kfar Azza	ND	ND
	cot92	Israel	Kfar Azza	ND (ND)**	ND
	cot75	Israel	Nir Am	ND*	ND
	cot12	Israel	Re'im	ND (DL)**	ND
	cot22, cot23	Israel	Zorrah	PD (DL)**	ND
HSI[e]	V4I	Spain	Córdoba	ND	ND**

[a]VCG assignment of isolates from Israel as determined by Korolev et al. (2000b); VCG assignment of isolates from Spain was determined in this study.
[b]Pathogenicity of isolates from Spain as determined by Bejarano-Alcázar et al. (1996). Pathogenicity of isolates from Israel was determined by Korolev et al. (2000b), except for those marked with an asterisk (∗), which were tested only in this work; ∗∗ = isolates tested both by Korolev et al. (2000b) and in this work. D = Defoliating; ND = Non-defoliating; PD = Partially defoliating; (DL) and (ND) = Designated defoliating-like and non-defoliating, respectively, in Korolev et al. (2000b).
[c]RAPD characterization of isolates from Spain was determined by Pérez-Artés et al. (2000a), except for that marked with an asterisk (∗), which was tested only in this work; ∗∗ = isolates tested both by Pérez-Artés et al. (2000a) and in this work. Isolates classified as ND or PD by pathogenicity test had the same RAPD pattern, designated ND.
[d]Not tested.
[e]Heterokaryon self-incompatible.

VCG analysis

Nitrate non-utilizing (*nit*) mutants were generated from 62 *V. dahliae* isolates from Spain grown on water-agar-chlorate (WAC) medium (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., 2000b); *nit* mutants derived from reference strain T9, kindly provided by R.C. Rowe (The Ohio State University/OARDC, Wooster, OH), were used to identify VCG1. According to Bell (1994), the cotton-D strain T9 belongs to subgroup VCG1A (see Discussion). Israeli *nit* testers, previously developed from local isolates and correlated with the international OARDC reference strains of *V. dahliae* VCGs (Katan, 2000; Korolev et al., 2000b; Rowe, 1995), were used for identification of VCG2A, VCG2B and VCG4B.

Pathogenicity tests

Three experiments (I, II and III) were carried out in Spain using 23 VCG-characterized isolates from Israel. Eleven of the isolates were chosen to represent a range of virulence within the ND and DL pathotypes (Korolev et al., 2000b), and 12 were new. Virulence of these isolates was compared with that of isolates V117I and V138I (D pathotype) and V179I (ND pathotype) from Spain, which served as pathotype references (Bejarano-Alcázar et al., 1996). Experiment I (Table 2) included 19 isolates from Israel, 10 of which belonged to VCG2B and nine to VCG4B. In particular, DL isolates of both VCG2B and VCG4B were included. Four isolates (designated R in Table 2) constituting duplicate cultures were included as internal controls. Experiment II (Table 3) included 12 isolates from Israel, four of which (cot40, cot70, cot102 and cot120) had been tested in experiment I. Experiment III (Table 4) included three isolates from Israel also tested in experiment II.

Plants were inoculated by the stem-injection method (Bejarano-Alcázar et al., 1996) in experiments I and II, or by the root-dip method in experiment III. For stem-injection inoculation, disinfested (1% NaOCl for 2 min), germinated seeds were sown in 12 cm diam plastic pots (one plant per pot) filled with a non-sterilized potting mixture (sand/clay loam/peat; 2 : 1 : 2, vol/vol/vol). Plants were grown in a greenhouse (experiment I: cvs Coker 310 and Delta Acala 90) at

Table 2. Virulence of *Verticillium dahliae* isolates from Spain and Israel to cotton cvs Coker 310 and Delta Acala 90 inoculated by the stem-injection method (experiment I)[a]

VCG[b]	Isolate	Pathotype[c]	AUDPC[d] (%)	
			Coker 310	Delta Acala 90
1	V117I	D	44.6	47.0
	V138I	D	44.5	53.4
2A	V179I	ND	27.3	39.1
4B	cot24 (ND)[e]	ND	22.6	30.7
	cot127 R	PD	40.2	33.8$^{\circ,\bullet,f}$
	cot92 R (ND)	ND	27.3	37.8
	cot127	PD	39.7	37.9$^{\bullet}$
	cot40	PD	43.9	39.6$^{\bullet}$
	cot92 (ND)	ND	34.8	42.0
	cot120 R	PD	31.4$^{\circ,\bullet}$	42.6
	cot22 (DL)	PD	43.9	43.0
	cot120	PD	33.2	45.7
2B	cot78	ND	9.5*	6.4*
	cot104	ND	5.7*	7.8*
	cot14	ND	3.2*	10.6*
	cot102	ND	9.7*	16.0*
	cot112 R (DL)	ND	37.7	29.2$^{\circ,\bullet}$
	cot67	ND	36.5	32.6
	cot17 (DL)	ND	35.6	33.3$^{\circ,\bullet}$
	cot112 (DL)	ND	35.9	33.8$^{\circ,\bullet}$
	cot60 (DL)	ND	36.9	36.1$^{\bullet}$
	cot70	ND	35.9	37.9

[a]Plants were inoculated by injecting a conidial suspension into the base of the first and second internodes.
[b]Vegetative compatibility group.
[c]D = Defoliating; ND = Non-defoliating; PD = Partially defoliating.
[d]Area under disease progress curve, expressed as percentage of the maximum possible area for the five-week period.
[e]Designated non-defoliating (ND) and defoliating-like (DL) in Korolev et al. (2000b).
[f]Mean AUDPC of isolates from Israel, designated DL in Korolev et al. (2000b) and/or PD in this study, was compared with mean AUDPC of the reference D isolates from Spain using the Dunnett's test ($P = 0.05$): $\circ$ = significantly different from V117I; $\bullet$ = significantly different from V138I. ND isolates from Israel were similarly compared with the reference ND isolate V179I from Spain: $*$ = significantly different.

18–24 °C, or in a growth chamber (experiment II: cvs Acala SJ-2 and Delta Acala 90) at 22–26°C, with a 14 h photoperiod of fluorescent light of 216–270 $\mu Em^{-2}s^{-1}$. Relative humidity in the growth chamber ranged from 50% to 90% during the light period and from 60% to 100% during the dark period. Six-week-old plants were inoculated with 5 µl of a 3×10^6 conidia ml^{-1} suspension in sterile distilled water (SDW). Control plants were treated similarly with SDW (Bejarano-Alcázar et al., 1996). After inoculation, the plants were maintained in the greenhouse or growth chamber as before. For root-dip inoculation (experiment III), disinfested, germinated seeds of cvs Acala SJ-2 and Delta Acala 90 were sown in trays filled with a non-sterilized sandy soil. Plants were grown in a growth chamber as in experiment II. Seedlings at the cotyledon stage were uprooted from the substrate, their roots thoroughly washed in tap water, trimmed, and dipped for 3 min in a conidial suspension (1.5×10^6 conidia ml^{-1}). Non-inoculated control seedlings were dipped in SDW. Seedlings were then transplanted (five per pot) to 12 cm diam plastic pots filled with non-sterilized potting mixture (clay loam/peat; 2 : 1, vol : vol), and maintained in the growth chamber. Plants were watered as needed and fertilized every two weeks with a water-soluble fertilizer (20-10-20, N-P-K). Plants were observed daily for the development of foliar symptoms and defoliation. Disease severity in individual plants was rated on a scale of 0–4 (0 = no symptoms, 4 = dead plant) 2, 3, 4 and 5 weeks after inoculation (Bejarano-Alcázar et al., 1996). Disease ratings were plotted over time to obtain disease progress curves. The area under the disease progress curve (AUDPC) calculated by the trapezoidal integration method (Campbell and Madden, 1990) was expressed as percentage of the maximum possible area for the five-week-period of the experiment. At the termination of experiments II and III, plants were excised above the cotyledon node for fresh weight determination, and mean weight was expressed as percentage of the corresponding weight of control plants.

Experiments had a factorial treatment design (*V. dahliae* isolates × cotton cultivars) with 12 (experiment I), nine (experiment II), or three (experiment III) replicates (pots) in a randomized complete block design. Percentage data were arcsin-transformed, and transformed data were subjected to analysis of variance. Mean plant fresh weight, and mean disease rating caused by each of several selected isolates from Israel, were compared with those of the D or ND reference isolates from Spain using the Dunnett's test at $P = 0.05$.

RAPD and specific PCR assays

Eighty-nine *V. dahliae* isolates from cotton in Israel (53 isolates) and Spain (36 isolates) were compared. The isolates from Spain had already been characterized as D ($n = 17$ isolates) or ND ($n = 19$ isolates) by means of biological pathotyping (Bejarano-Alcázar et al., 1996)

Table 3. Virulence of *Verticillium dahliae* isolates from Spain and Israel to cotton cvs Acala SJ-2 and Delta Acala 90 inoculated by the stem-injection method (experiment II)[a]

VCG[b]	Isolate	Pathotype[c]	AUDPC[d] (%)			Fresh weight[e] (%)		
			Acala SJ-2	Delta Acala 90	Mean	Acala SJ-2	Delta Acala 90	Mean
1	V117I	D	44.7	47.1	45.9	55.4	65.7	60.5
4B	cot120	PD	24.7	36.1	30.4[*,f]	57.6	55.3	56.4
	cot75	ND	28.6	33.1	30.8	70.8	85.8	78.3
	cot87	ND	28.6	35.8	32.2	76.5	67.5	72.0
	cot40	PD	31.0	34.4	32.7[*]	64.8	76.1	70.5
	cot23 (DL)[g]	PD	32.5	36.1	34.3[*]	59.8	73.5	66.6
	cot12 (DL)	ND	35.1	39.2	37.2[*]	65.3	87.4	76.3
2B	cot102	ND	10.3	4.6	7.4	77.4	84.9	81.1
	cot79 (DL)	ND	10.9	10.1	10.5[*]	67.1	93.2	80.1
	cot128 (DL)	ND	26.7	22.4	24.5[*]	78.2	79.0	78.6
	cot72	ND	32.1	27.8	29.9	65.1	77.7	71.4
	cot70	ND	30.0	30.3	30.1	74.1	64.2	69.2
	cot117 (DL)	PD	29.3	31.1	30.2[*]	40.3	71.9	56.1

[a]Plants were inoculated by injecting a conidial suspension into the base of the first and second internodes.
[b]Vegetative compatibility group.
[c]D = Defoliating; ND = Non-defoliating; PD = Partially defoliating.
[d]Area under disease progress curve, expressed as percentage of the maximum possible area for the five-week-period.
[e]Expressed as percentage of corresponding weight in control plants injected with water.
[f]Mean AUDPC and fresh weight of isolates from Israel, designated DL in Korolev et al. (2000b) and/or PD in this study, were compared with those of the reference D isolate V117I from Spain using the Dunnett's test ($P = 0.05$). Isolates for which means are significantly different are marked with an asterisk (∗).
[g]Designated defoliating-like (DL) in Korolev et al. (2000b).

Table 4. Virulence of *Verticillium dahliae* isolates from Spain and Israel to cotton cvs Acala SJ-2 and Delta Acala 90 inoculated by the root-dip method (experiment III)[a]

VCG[b]	Isolate	Pathotype[c]	AUDPC[d] (%)		Fresh weight[e] (%)		
			Acala SJ-2	Delta Acala 90	Acala SJ-2	Delta Acala 90	Mean
1	V117I	D	70.6	55.8	2.9	2.2	2.5
4B	cot120	PD	17.8[*,f]	36.7[*]	54.6	28.3	41.4[*]
	cot23 (DL)[g]	PD	35.0[*]	35.2[*]	43.3	25.1	34.2[*]
2B	cot79 (DL)	ND	35.5[*]	39.5[*]	42.7	41.1	41.9[*]

[a]Seedlings at the cotyledon stage were uprooted from the substrate, and their roots were washed, trimmed and dipped in a conidial suspension.
[b]Vegetative compatibility group.
[c]D = Defoliating; ND = Non-defoliating; PD = Partially defoliating.
[d]Area under disease progress curve, expressed as percentage of the maximum possible area for the five-week-period.
[e]Expressed as percentage of corresponding weight in control plants dipped in water.
[f]Mean AUDPC and fresh weight of isolates from Israel, designated DL in Korolev et al. (2000b) and/or PD in this study, were compared with those of the reference D isolate V117I from Spain using the Dunnett's test ($P = 0.05$). Isolates for which means are significantly different are marked with an asterisk (∗).
[g]Designated defoliating-like (DL) in Korolev et al. (2000b).

and by RAPD assays (except for ND isolate V227I) (Pérez-Artés et al., 2000a). Similarly, all the isolates from Israel had been characterized for VCG, and some of them also for virulence to cotton (Korolev et al., 2000b). Mycelia of isolates grown in supplemented Czapek-Dox broth were harvested, lyophilized and ground as previously described (Pérez-Artés et al., 2000a). DNA extracted from ground mycelium by the method of Raeder and Broda (1985) was used for RAPD and specific PCR assays as described by Pérez-Artés et al. (2000a). Amplification was carried out in two different thermocyclers: Perkin-Elmer 9600 (Perkin-Elmer, Norwalk, CT); PTC 100 (MJ Research Inc., Watertown, MA), using primers OPH 04, OPH 19 and OPH 20 (Operon Technology, Alameda, CA), and primer KS (Pérez-Artés et al., 2000a). These primers have been shown to reliably amplify D- and ND-specific DNA fragments from *V. dahliae* DNA when used in RAPD assays (Pérez-Artés et al., 2000a). All reactions were repeated at least three times and always included negative controls (no template DNA). Oligonucleotide primer pairs D-1/D-2 and ND-1/ND-2 were used for specific PCR as indicated by Pérez-Artés et al. (2000a). Primers D-1/D-2 and ND-1/ND-2 amplify single bands of 0.55 or 1.5 kb from DNAs of D and ND strains, respectively.

RAPD patterns generated by single-primer PCR were used to determine relatedness among isolates. For each isolate and primer, a data record was constructed on the basis of 1 being assigned to an isolate if a band of a given size was present and 0 if it was absent. A binary matrix combined all data records for all 89 isolates used in the study. Data were analyzed using the NTSYS-Spc2.0 software package (Exeter Software, Setauket, NY) and the unweighted paired group method with arithmetic averages (UPGMA) with Jaccard's similarity coefficient (Sneath and Sokal, 1973).

Results

VCGs of V. dahliae *isolates from Spain*

Based on their positive complementation reactions with specific testers, 62 *V. dahliae* isolates from Spain were assigned to VCGs. All 28 isolates previously identified as pathotype D belonged to VCG1, whereas isolates of the ND pathotype belonged to VCG2A (28 isolates) and VCG4B (five isolates); one ND isolate (V4I) was self-incompatible. All VCG1 isolates originated from the lower area of the Guadalquivir Valley (Bejarano-Alcázar et al., 1996) except for one isolate from the upper Valley. VCG2A isolates originated from the upper, central and lower areas of the Valley in similar proportions. VCG4B isolates were recovered from three locations in the central and lower Valley (Table 1).

Pathogenicity of isolates from Israel

Disease reactions in experiments I through III varied with *V. dahliae* isolate, cotton cultivar and inoculation method. Inoculation by the stem-injection method (experiments I and II) resulted in a range of disease reactions. None of the isolates from Israel caused a D-reaction (i.e., complete defoliation) in any of the cotton cvs Coker 310, Acala SJ-2 or Delta Acala 90 which are highly susceptible, moderately susceptible and tolerant to Verticillium wilt, respectively. Rather, these isolates were classified into four main virulence categories (Figure 1): (i) weakly virulent isolates that induce slight foliar symptoms in a few lower leaves; (ii) moderately virulent isolates that induce mild foliar symptoms in the lower leaves, with little or no stunting or defoliation; (iii) highly virulent isolates that induce severe foliar symptoms, often stunting and death of plants but little or no defoliation; and (iv) highly virulent isolates that induce severe foliar symptoms with occasional stunting and death of plants, and partial defoliation affecting mainly lower to mid-leaves. In Tables 2–4, isolates included in categories (i)–(iii) are designated ND (non-defoliating), whereas isolates in category (iv) are designated PD (partially defoliating).

There was a statistically significant isolate × cultivar interaction in disease severity as determined by the AUDPC for cvs Coker 310 and Delta Acala 90 in experiment I (Table 2). No interaction was evident for either AUDPC or plant fresh weight for cvs Acala SJ-2 or Delta Acala 90 in experiment II (Table 3). Disease reactions in cv Delta Acala 90 (common to experiments I and II), caused by the same *V. dahliae* isolates, were slightly more severe in experiment I than in experiment II (Tables 2 and 3). However, the relative ranking of those five isolates with respect to virulence on cv Delta Acala 90 was consistent between experiments. In addition, disease reactions induced by duplicate isolates (designated R in Table 2) in experiment I were similar to those of the coded isolates. These

Figure 1. Disease reactions in cotton cv Acala SJ-2, 5 weeks after stem-injection inoculation with 3×10^6 conidia ml^{-1} of isolates of *Verticillium dahliae* representating the main virulence categories. Left to right: defoliating (V117I); highly virulent, partially defoliating (category iv: cot127R); highly virulent, non-defoliating (category iii: cot17); moderately virulent, non-defoliating (category ii: cot24); non-inoculated control.

results indicate consistency within, as well as between, experiments.

Eleven of the Israeli isolates examined in this study had been previously pathotyped as highly virulent DL (nine isolates) or less virulent ND (two isolates) based solely on AUDPC (Korolev et al., 2000b). In the present study, defoliation was used as an additional major criterion for pathotyping (Figure 1). Seven out of nine isolates previously designated DL (Tables 2 and 3) were highly virulent. Of these seven isolates, three (cot22, cot23 and cot117) showed the PD pathotype (category iv), while the remaining four (cot12, cot17, cot60 and cot112) were highly virulent ND (category iii). Two additional DL isolates (cot79 and cot128) were weakly virulent (category i) and moderately virulent (category ii) ND, respectively. The two previously ND-designated isolates (cot24 and cot92) were highly virulent ND (category iii). Of the 12 newly tested isolates from Israel, seven VCG2B isolates were either weakly (cot14, cot78, cot102 and cot104), moderately (cot72), or highly (cot67 and cot70) virulent ND; and five VCG4B isolates were highly virulent PD (cot40, cot120 and cot127) or ND (cot75 and cot87) (Tables 2 and 3).

In experiment I, all the DL isolates from Israel characterized as either ND or PD in this study, as well as three newly characterized PD isolates (cot40, cot120 and cot127), were as virulent ($P = 0.05$) to cv Coker 310 as were the reference D isolates from Spain (Table 2). However, this pattern of reactions changed slightly with cv Delta Acala 90. In this latter cultivar, disease reactions induced by most of the isolates (i.e., cot 17, cot 40, cot 60, cot 112 and cot 127) were less severe than that induced by the reference D isolates from Spain. Virulence distinction between DL and PD isolates on one hand, and the reference D isolate on the other, was more obvious in experiment II: irrespective of cultivar, all DL and PD isolates were significantly less virulent ($P < 0.05$) than the D isolate as determined by AUDPC (Table 3). Nevertheless, these differences did not correlate with the levels of fresh-weight reduction in infected plants. Representative DL isolates of both VCG4B and VCG2B, and a newly characterized PD isolate (cot120), were compared with a representative D isolate by root-dip inoculation of cvs Acala SJ-2 and Delta Acala 90 in experiment III (Table 4). Symptoms developed faster and more severely, and defoliation induced by PD and D isolates was more pronounced compared with disease reactions in stem-injection inoculations. There was a statistically significant isolate × cultivar interaction based on AUDPC, but not on plant fresh-weight reduction. All tested isolates, either of the previous DL or the present PD pathotypes, were significantly less virulent ($P < 0.05$) than the D reference isolate on cvs Acala SJ-2 and Delta Acala 90 by both the AUDPC and the fresh-weight reduction criteria (Table 4). Finally, four new VCG2B isolates (cot14, cot78, cot102 and cot104) were significantly ($P < 0.05$) less virulent than the reference ND isolate from Spain (Table 2).

RAPD assays

RAPD patterns generated by primers OPH 04, OPH 19, OPH 20 and KS from 53 *V. dahliae* isolates from Israel consistently showed the diagnostic DNA bands specific for the ND pathotype in Spain, i.e., OPH 04 (0.9 kb), OPH 19 (2.0 kb) and KS (0.65 kb) (Figure 2). In contrast, RAPD amplification with primers OPH 19,

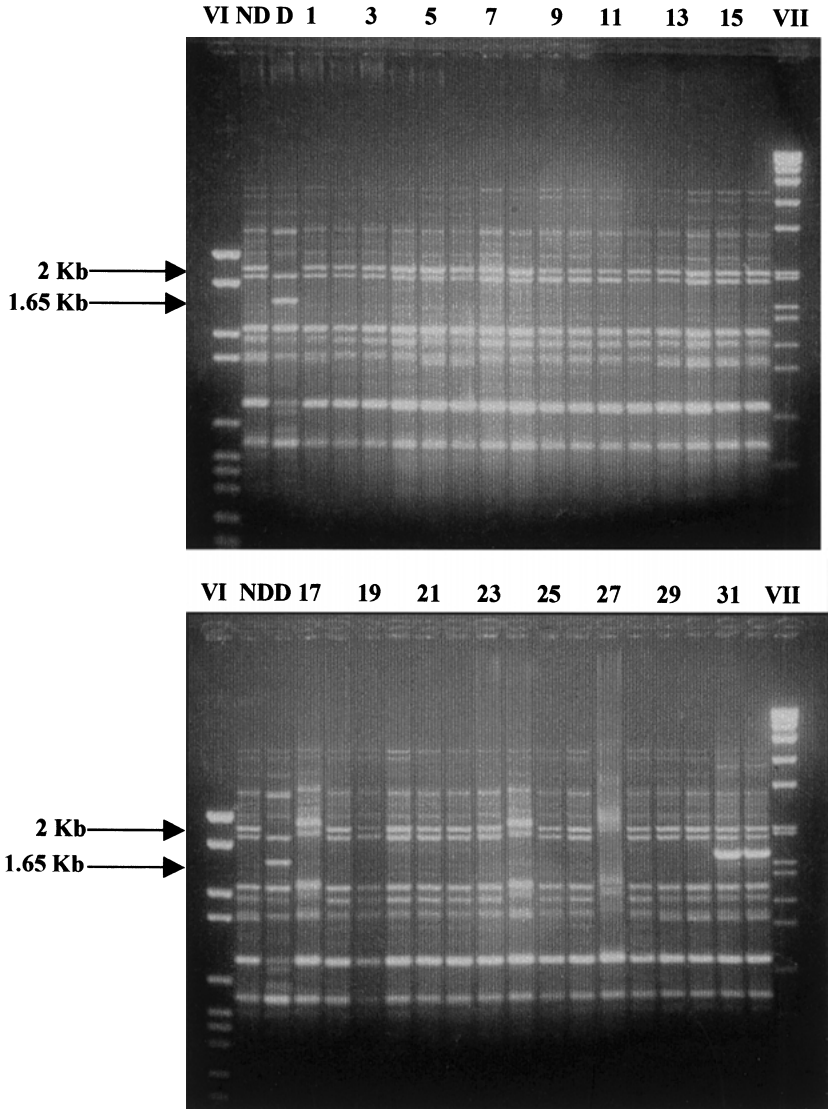

Figure 2. RAPD patterns generated by primer OPH 19 using total DNA from *Verticillium dahliae* isolates from Israel previously characterized as non-defoliating (ND) by pathogenicity test. Assignment of isolates to vegetative compatibility groups (2B and 4B) is indicated in brackets. HSI = heterokaryon self-incompatible. Numbers on the side indicate size (kb) of the DNA marker bands for ND (2.0 kb) and defoliating (D) (1.65 kb) pathotypes. Lanes VI and VII: DNA size markers VI and VII (Boehringer–Mannheim). Lane ND: ND reference isolate V4I (HSI) from Spain. Lane D: D reference isolate V117I (VCG1) from Spain. Lanes 1–32: *V. dahliae* isolates from Israel: 1, cot1 (4B); 2, cot2 (2B); 3, cot3 (2B); 4, cot8 (2B); 5, cot12 (4B); 6, cot13 (4B); 7, cot14 (2B); 8, cot15 (2B); 9, cot17 (2B); 10, cot18 (2B); 11, cot20 (2B); 12, cot21 (2B); 13, cot22 (4B); 14, cot23 (4B); 15, cot26 (4B); 16, cot28 (4B); 17, cot40 (4B); 18, cot56 (2B); 19, cot59 (2B); 20, cot67 (2B); 21, cot69 (2B); 22, cot70 (2B); 23, cot72 (2B); 24, cot75 (4B); 25, cot78 (2B); 26, cot79 (2B); 27, cot81 (2B); 28, cot83 (2B); 29, cot84 (4B); 30, cot87 (4B); 31, cot92 (4B); 32, cot120 (4B).

OPH 20 and KS never produced the DNA fragments diagnostic for the D pathotype, i.e., OPH 19 (1.65 kb), OPH 20 (1.0 kb) and KS (0.75 kb) (Pérez-Artés et al., 2000a). The RAPD results were further examined by specific PCR amplifications using the D- and ND-specific primer pairs developed by Pérez-Artés et al. (2000a) and template DNA from selected *V. dahliae* isolates from Israel (cot22, cot40, cot92, cot92R, cot127 and cot127R). These isolates were selected because the biological pathotyping (highly virulent, including partial defoliation of cotton plants induced by four of them; Table 2) appeared inconsistent with the molecular characterization (ND by RAPD assay). Consequently, these isolates were recovered from infected 'Delta Acala 90' and 'Coker 310' cotton plants, and their DNAs were extracted and amplified using the D- and ND-specific PCR primer pairs. Genomic DNAs from D and ND isolates served as references. In all cases, only the ND-specific band was amplified. These results confirmed the RAPD characterization, and identified these isolates as ND despite the observed variation in virulence and disease symptoms.

RAPD amplification of DNAs from the 36 *V. dahliae* isolates from Spain and 53 isolates from Israel using the four aforementioned single primers produced an average of 64 bands per isolate. UPGMA analysis of these RAPD banding patterns separated the isolates into two major clusters (Figure 3). All the D isolates from Spain (VCG1) were uniform and grouped together in one cluster, while a second cluster included the ND isolates from Spain and Israel (including pathotype PD). There was some variation among isolates in the ND cluster. Both clusters had about 25% dissimilarity.

Discussion

Phenotypic and genetic characterization of prevailing *V. dahliae* populations is of importance for the effective management of Verticillium wilt in cotton-growing areas. In this study, examination of representative isolates from Israel for vegetative compatibility (Korolev et al., 2000b), virulence and pathotype-specific molecular markers demonstrated that the D pathotype does not occur in this population. Conversely, VCG analysis of a representative collection of isolates from Spain, previously pathotyped by virulence (Bejarano-Alcázar et al., 1996) and molecular markers (Pérez-Artés et al., 2000a), placed all D isolates in VCG1 and all ND isolates in VCG2A or VCG4B. These results further confirm the present status of the VCG1/D-pathotype as a distinct subspecific population within *V. dahliae* which was based on studies of D strains from America and Asia (Bell, 1994; Daayf et al., 1995; Zhengjun et al., 1998). In previous PCR-based genetic analyses, pathotype D from Spain was found to be closely related to American and Asian D strains, and clearly distinct from the ND pathotype from Spain (Pérez-Artés et al., 2000a). In the present study, isolates from Israel ranging in virulence from highly virulent ND and PD to very mild ND were all characterized as ND by RAPD patterns and pathotype-specific PCR markers. These results corroborate the distinctness of the D and ND (including PD) pathotypes, and confirm the validity of the D- and ND-specific PCR primer pairs as diagnostic tools for making this distinction.

The exclusive association of the D pathotype with VCG1, or with its subgroup A, needs further consideration. Bell (1994) divided VCG1 into subgroups A and B based on the vigour of complementation between *nit* mutants. Only isolates of subgroup VCG1A caused severe defoliation of cotton. In this and in similar studies (Chen, 1994; Daayf et al., 1995; Zhengjun et al., 1998), pathotype-D isolate T9 has been used as the primary reference strain of VCG1. Since T9 belongs to subgroup VCG1A (Bell, 1994), all isolates strongly compatible with it (or with other testers strongly compatible with it) should accordingly be assigned to VCG1A. Nevertheless, complementation tests with testers of VCG1B are required for final confirmation.

Most of the ND isolates from Spain were assigned to VCG2A. Because VCG2A is often associated with tomato (Bell, 1994; Korolev et al., 2000b; Puhalla and Hummel, 1983), our results are the first report of VCG2A as a major component of the ND *V. dahliae* population in cotton fields. The origin of VCG2A in cotton fields in Spain in not known. Conversely, VCG4B was less common among the ND isolates collected in southern Spain but it is one of the two major VCGs on cotton in Israel (Korolev et al., 2000b). VCG4B is widespread in North American potato fields (Strausbaugh, 1993; Strausbaugh et al., 1992) and it can be effectively transmitted by infected seed potatoes (Omer et al., 1997). Interestingly, the few VCG4B isolates found in our study are from sites where potato is grown (Bejarano-Alcázar et al., 1996), which might relate to the origin of VCG4B in the sampled cotton fields.

A similar situation exists for Verticillium wilt of cotton in Spain and in Israel as indicated by the presence in both countries of highly virulent forms, as

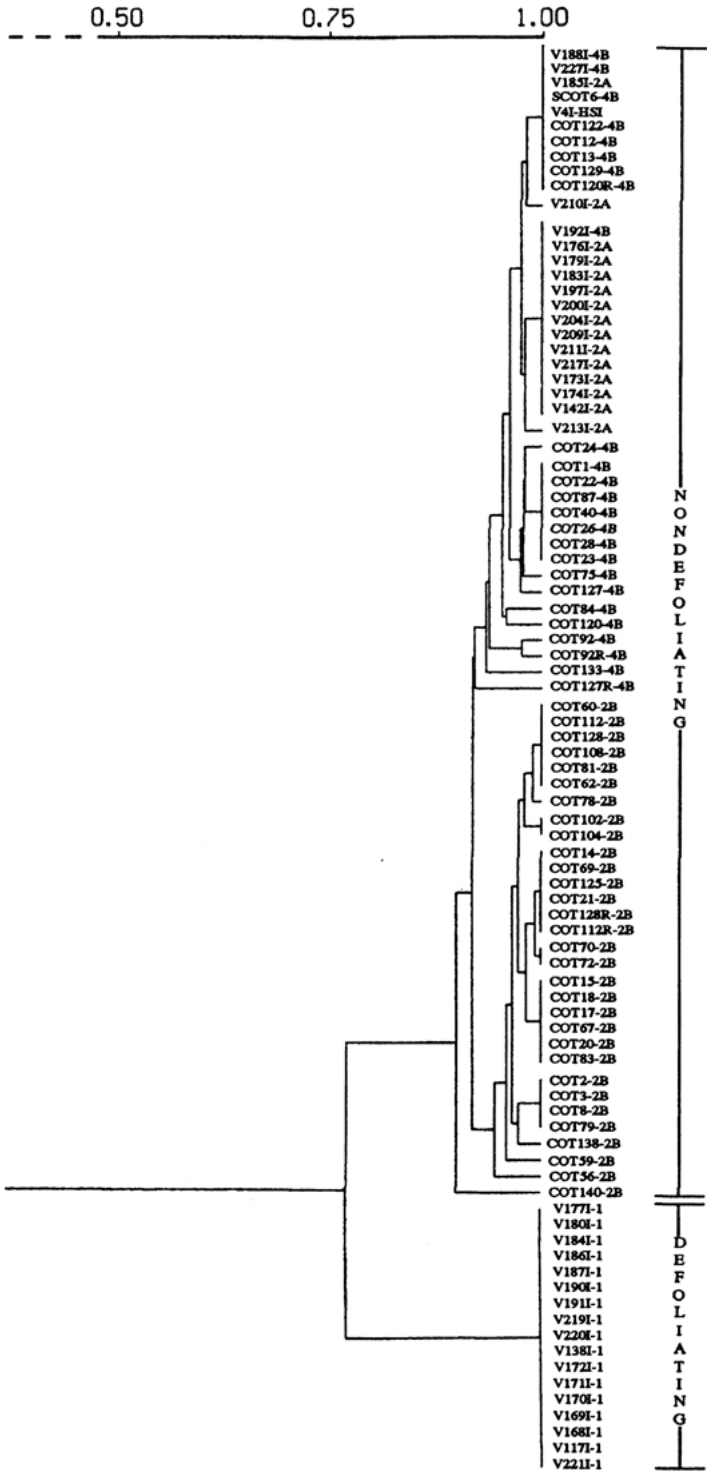

Figure 3. Dendrogram derived from cluster analysis (UPGMA) of RAPD data of *Verticillium dahliae* isolates from Israel and Spain belonging to the indicated vegetative compatibility groups (VCG1, 2A, 2B and 4B), using software package NTSYS Spc2.0. Top scale is percentage similarity based on the Jaccard similarity coefficient.

well as moderate or mild forms of *V. dahliae*. However, the pathogen populations in the two countries differ in their specific characteristics. The highly virulent form of *V. dahliae* in Spain consists of the VCG1/D-pathotype, whereas in Israel a VCG2B/ND-pathotype is associated with severe disease in certain cotton fields. Mild-to-moderate disease in other fields in Israel is associated with VCG4B, whereas the mild pathogen population in Spain is dominated by VCG2A, with VCG4B as a minor component. VCG2B and VCG4B in Israel are regionally separated, and their local histories are different (Korolev et al., 2000b). In Spain, VCG2A occurs throughout the Guadalquivir Valley in southern Spain, the main cotton-growing area in the country, whereas VCG1 occurs in the lower valley, where cotton is grown most intensively (Bejarano-Alcázar et al., 1996).

The D and ND pathotypes of *V. dahliae* in Spain are clearly distinct in their virulence characteristics. Conversely, the ND (including PD) isolates from Israel exhibit a broad range of virulence levels, with partial overlap between VCGs in artificial inoculation. In addition to the AUDPC criterion, partial defoliation was a distinctive reaction used to distinguish between highly virulent PD or ND strains, and to demonstrate that partial defoliation can be induced by isolates that differ in the severity of symptoms they cause in cotton cultivars. Results in this study demonstrated that disease reaction could vary with inoculation method (root-dip versus stem-injection) and cotton cultivar. Thus, three isolates out of nine previously designated DL based on an AUDPC (Korolev et al., 2000b) were categorized as PD, the remaining six isolates being weakly, moderately or highly virulent ND. Similarly, 12 new VCG2B and VCG4B isolates from Israel were categorized as ND, and PD or ND, respectively. Four of the VCG2B isolates were weakly virulent, further illustrating the high variability previously noted in this VCG (Korolev et al., 2000b). However, based on repeated large-scale virulence tests and continuing field observations in Israel, VCG2B is considered overall more virulent to cotton than VCG4B. In artificial inoculation tests, partial overlap in disease ratings between these VCGs can be contributed by individuals (weakly virulent VCG2B and highly virulent VCG4B isolates) that deviate from the means of their groups (Katan, 2000; Korolev et al., 2000b). The broad range of virulence levels found among cotton *V. dahliae* isolates from Israel agrees with previous reports from California and Spain, where isolates with a virulence level intermediate between that of D and ND isolates cause severe Verticillium wilt irrespective of whether they induce partial defoliation (Ashworth, 1983; Schnathorst, 1973) or not (Bejarano-Alcázar et al., 1996; Mathre et al., 1966; Schnathorst, 1973; Schnathorst and Mathre, 1966).

Highly virulent *V. dahliae* isolates from Israel, either ND or PD, were as virulent as D isolates from Spain when compared under conditions most conducive to severe disease (i.e., susceptible cv Coker 310 and the temperature range of experiment I). However, these isolates were less virulent than D isolates on the less susceptible cvs Acala SJ-2 and Delta Acala 90 and the less favorable temperatures used in experiments II and III (Barrow, 1970; Bell and Presley, 1969). This indicates the potential of Verticillium wilt for devastating cotton in soils in Israel where highly virulent strains are prevalent, as demonstrated in Spain for the D pathotype (Bejarano-Alcázar et al., 1995; 1997). Therefore, there is a need to characterize the *V. dahliae* pathotypes prevailing in any given cotton-growing area for efficient disease control. Distinguishing between complete or partial defoliation in field situations of severe disease can be difficult or even impossible. This difficulty may be compounded by the presence of a mixed population of D, PD and/or ND strains in a given cotton field. This study shows that VCG tests and PCR assays employing the pathotype-specific primer pairs, alone or in combination, provide reliable diagnostic tools for distinguishing between the D and ND (including PD) pathotypes. Further work is needed to develop similar diagnostic tools for distinguishing between highly virulent isolates within the ND pathotype.

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