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Variation of pathotypes and races and their correlations with clonal lineages in *Verticillium dahliae*

R. M. Jiménez-Díaz[ab]*, C. Olivares-García[a], J. L. Trapero-Casas[b], M. M. Jiménez-Gasco[c], J. A. Navas-Cortés[b], B. B. Landa[b] and M. G. Milgroom[d]

[a]*Departamento de Agronomía, College of Agriculture and Forestry (ETSIAM), Universidad de Córdoba, Campus de Excelencia Internacional Agroalimentario ceiA3, Edificio C-4 Celestino Mutis, Campus Rabanales, Ctra. de Madrid, km 396, 14071 Córdoba,* [b]*Consejo Superior de Investigaciones Científicas (CSIC), Instituto de Agricultura Sostenible (IAS), Campus de Excelencia Internacional Agroalimentario, Avenida Menéndez Pidal s/n, 14004 Córdoba, Spain;* [c]*Department of Plant Pathology and Environmental Microbiology, The Pennsylvania State University, University Park, PA 16802, and* [d]*Plant Pathology and Plant–Microbe Biology Section, School of Integrative Plant Science, Cornell University, Ithaca, NY 14853, USA*

Understanding pathogenic variation in plant pathogen populations is key for the development and use of host resistance for managing verticillium wilt diseases. A highly virulent defoliating (D) pathotype in *Verticillium dahliae* has previously been shown to occur only in one clonal lineage (lineage 1A). By contrast, no clear association has yet been shown for race 1 with clonal lineages. Race 1 carries the effector gene *Ave1* and is avirulent on hosts that carry resistance gene *Ve1* or its homologues. The hypothesis tested was that race 1 arose once in a single clonal lineage, which might be expected if *V. dahliae* acquired *Ave1* by horizontal gene transfer from plants, as hypothesized previously. In a diverse sample of 195 *V. dahliae* isolates from nine clonal lineages, all race 1 isolates were present only in lineage 2A. Conversely, all lineage 2A isolates displayed the race 1 phenotype. Moreover, 900-bp nucleotide sequences from *Ave1* were identical among 27 lineage 2A isolates and identical to sequences from other *V. dahliae* race 1 isolates in GenBank. The finding of race 1 in a single clonal lineage, with identical *Ave1* sequences, is consistent with the hypothesis that race 1 arose once in *V. dahliae*. Molecular markers and virulence assays also confirmed the well-established finding that the D pathotype is found only in lineage 1A. Pathogenicity assays indicated that cotton and olive isolates of the D pathotype (lineage 1A) were highly virulent on cotton and olive, but had low virulence on tomato.

Keywords: cotton, *Gossypium hirsutum*, *Olea europaea*, olive, VCG, verticillium wilt

Introduction

Verticillium wilts caused by *Verticillium dahliae* are amongst the most devastating and challenging diseases to manage in agricultural production worldwide (European Food Safety Authority Panel on Plant Health, 2014). These diseases can reach high incidences and cause yield losses of 50% or more in high-value crops such as artichoke, cotton, lettuce, olive, potato and strawberry (Friebertshauser & DeVay, 1982; Cirulli *et al.*, 2010; Johnson & Dung, 2010; Atallah *et al.*, 2011; Jiménez-Díaz *et al.*, 2012). *Verticillium dahliae* is a vascular-colonizing, soil- and seedborne, mitosporic ascomycete that is found worldwide (du Toit *et al.*, 2005; Atallah *et al.*, 2011; Inderbitzin *et al.*, 2011; Göre *et al.*, 2014; www.mycobank.org), and has one of the broadest host ranges of any fungal plant pathogen (European Food Safety Authority Panel on Plant Health, 2014). *Verticillium dahliae*'s wide host range and its ability to survive long-term in soil as melanized microsclerotia make verticillium wilts difficult to manage (Wilhelm, 1955; European Food Safety Authority Panel on Plant Health, 2014).

Host resistance is the single most practical and cost-efficient method for managing verticillium wilt, but its efficiency is compromised by two types of pathogenic variation in *V. dahliae*. First, a highly virulent defoliating (D) pathotype overcomes disease resistance in cotton and olive that is still effective against a nondefoliating (ND) pathotype. The D pathotype was first described for isolates causing severe defoliation in California in the early 1960s in a cotton variety that had previously been verticillium-wilt tolerant (Schnathorst & Mathre, 1966) and the pathotype now occurs worldwide (reviewed in Jiménez-Díaz *et al.*, 2012; Milgroom *et al.*, 2016). Isolates of the D pathotype cause rapid and severe defoliation only on cotton, okra and olive, but they are also pathogenic on artichoke, celery, eggplant, flax, safflower, sunflower and watermelon, with a range of virulence (defined as the relative capacity of a pathogen to cause disease, as indicated by the amount of damage it causes a host) (Schnathorst & Mathre, 1966; Jiménez-Díaz *et al.*, 2006; Korolev *et al.*, 2008). By contrast, isolates that

*E-mail: ag1jidir@uco.es

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cause leaf necrosis and wilting, but not defoliation, on cotton, okra and olive are referred to as having a ND pathotype (Bell, 1994; Korolev *et al.*, 2008; Jiménez-Díaz *et al.*, 2012).

The second type of pathogenic variation in *V. dahliae* is the presence of two pathogenic races. Race 1 is defined by the presence of the effector gene *Ave1*, which confers avirulence to cultivars of tomato or lettuce that carry the resistance gene *Ve1* or its homologue *Vr1*, respectively (Kawchuk *et al.*, 2001; Hayes *et al.*, 2011; de Jonge *et al.*, 2012). These resistance genes are conserved across many plant species, including other members of the Solanaceae (Song *et al.*, 2016). *Ve1* and its homologues encode pattern-recognition receptors that recognize products encoded by *Ave1*, leading to a defence response against infection by race 1 (de Jonge *et al.*, 2012). On the other hand, *Ave1* is a virulence factor and confers greater virulence to race 1 isolates in tomato and *Arabidopsis* plants lacking *Ve1* (de Jonge *et al.*, 2012). Conversely, race 2 isolates are defined by the lack of *Ave1* and therefore are potentially virulent on plants carrying *Ve1* because they evade recognition (de Jonge *et al.*, 2012; Short *et al.*, 2014). Race 2 occurs worldwide in sympatric populations with race 1, and it causes disease on a range of crops for which the use of *Ve1* has not been reported (Dobinson *et al.*, 1996; Maruthachalam *et al.*, 2010; Ligoxigakis & Markakis, 2012). Race 2 isolates pathogenic on tomato are genetically diverse, and were hypothesized to have multiple origins (Dobinson *et al.*, 1998). Such diversity is not surprising, however. Analyses of nucleotide sequences strongly suggest that *V. dahliae* acquired *Ave1* from plants by horizontal gene transfer (HGT) (de Jonge *et al.*, 2012). Under this scenario, because HGT from plants to fungi is a rare event, the hypothesis emerges that race 1 arose only once and is genetically much less diverse than race 2.

To fully understand pathogenic variation in *V. dahliae* it must be viewed in the context of the highly clonal structure of *V. dahliae* populations (Jiménez-Díaz *et al.*, 2012; Jiménez-Gasco *et al.*, 2014; Milgroom *et al.*, 2014). Clonality in *V. dahliae* was first described by vegetative compatibility groups (VCGs), which comprise isolates that can form stable heterokaryons through anastomosis (Katan, 2000). Because of strong linkage disequilibrium in clonal populations, VCGs in *V. dahliae* are highly correlated with molecular markers (Jiménez-Díaz *et al.*, 2012; Jiménez-Gasco *et al.*, 2014), and with clonal lineages. Nine distinct clonal lineages were recently identified by analysis of more than 26 000 single nucleotide polymorphisms (SNPs), and were shown to have arisen originally by recombination (Milgroom *et al.*, 2014). *Verticillium dahliae*'s current clonal population structure is probably a consequence of selection for adaptation to agricultural crops (Douhan & Johnson, 2001; Jiménez-Díaz *et al.*, 2006; Korolev *et al.*, 2008) and the clonal expansion of fit genotypes on agricultural crops (Milgroom *et al.*, 2016). Therefore, if race 1 arose once, as hypothesized above, then race 1 isolates would be expected to be found in a single clonal lineage, just as the D pathotype is found in a single clonal lineage (Jiménez-Díaz *et al.*, 2012; Milgroom *et al.*, 2014, 2016).

Although the correlation of clonal lineages and pathogenic variation is very clear for the D pathotype, limited information is currently available on the relationships of races 1 and 2 with clonal lineages. The information that does exist is sometimes contradictory, and does not support any general conclusions (Dobinson *et al.*, 1998; Maruthachalam *et al.*, 2010; Ligoxigakis & Markakis, 2012; Jabnoun-Khiareddine *et al.*, 2013; Short *et al.*, 2014; Hu *et al.*, 2015). Because knowledge of relationships among race, pathotype and clonal lineage is likely to be helpful for breeding and deployment of cultivars with resistance to verticillium wilt, the aim of this study was to clarify these relationships in *V. dahliae*. The specific objectives were to test the hypotheses that (i) race 1 arose once in *V. dahliae* and therefore is found in a single clonal lineage, and (ii) conversely, race 2 is diverse because it is the ancestral state from which race 1 evolved, most probably by HGT. Two additional objectives were (iii) to determine if there is any correlation between pathotype and race, and (iv) to assess the virulence and pathotype of race 2 isolates in different lineages to cotton and tomato cultivars and clones of wild olive that are resistant to the D pathotype.

Materials and methods

Verticillium dahliae isolates and culture conditions

In this study, 195 isolates of *V. dahliae* from nine countries, eight crops and five woody hosts were used (Tables 1 & S1). A large number of isolates were from cotton and olive because of the agricultural and phytopathological significance of the D and ND pathotypes on these crops (Bell, 1994; Jiménez-Díaz *et al.*, 2012). Isolates were selected from the *V. dahliae* collections in the Department of Crop Protection, Institute for Sustainable Agriculture, CSIC, Córdoba, Spain and the Department of Plant Pathology and Environmental Microbiology, The Pennsylvania State University, PA, USA.

Isolates were stored on plum-lactose-yeast extract agar (PLYA; Talboys, 1960) covered with sterile liquid paraffin, at 4 °C in the dark. Isolates were obtained on *Verticillium*-semi-selective sodium polypectate agar (which contains streptomycin sulphate, chloramphenicol and chlortetracycline) to ensure they were free from bacterial contamination, and were further subcultured on fresh potato dextrose agar (PDA) (250 g unpeeled potato, 20 g agar, 20 g glucose, 1 L distilled water). Cultures were derived from single conidia, and identified by the formation of verticillate conidiophores and microsclerotia on PDA. They were further confirmed as *V. dahliae sensu stricto* by PCR assay according to Inderbitzin *et al.* (2013).

Determination of vegetative compatibility groups and clonal lineages

Most of the 195 isolates were characterized for VCG and clonal lineage in previous studies (Table S1). For 18 isolates that had not been assayed previously (Table S1), VCGs were determined by complementation of nitrate-nonutilizing (*nit*) mutants with *nit* mutants of the international reference strains of *V. dahliae*

Table 1 Summary of *Verticillium dahliae* isolates used in this study by country and host of origin, lineage and genotype

Country (*n* by country)	Host (*n* by country and host)	VCG/lineage (*n* by country, host and lineage)	PCR genotype[a]	Race 2 sequence haplotype[b]
Australia (13)	Cotton (13)	1A (7[c])	A	V
		2A (4[c])	C	NA
		4B (2[c])	C	I
China (2)	Cotton (2)	1A (2)	A	V
Greece (7)	Cotton (7)	1A (6)	A	V
		$2B^{824}$ (1)	C	II
Israel (22)	Cotton (17)	1A (12)	A	V
		$2B^{824}$ (4)	C	II
		4B (1)	C	I
	Aubergine (2)	2A (2)	C	NA
	Tomato (3)	2A (1)	C	NA
		$2B^{824}$ (1)	C	II
		4B (1)	C	I
Italy (1)	Olive (1)	2A (1[c])	C	NA
Poland (1)	Pepper (1)	$2B^{824}$ (1[c])	C	II
Spain (113)	Cotton (31)	1A (28)	A	V
		2A (2)	C	NA
		4B (1)	C	I
	Olive (63)	1A (41)	A	V
		2A (11)	C	NA
		$2B^{R1}$ (2)	C	III
		4B (9)	C	I
	Artichoke (15)	2A (2)	C	NA
		$2B^{334}$ (10)	B	III
		$2B^{824}$ (3)	C	II
	Tomato (5)	2A (3[c])	C	NA
		$2B^{824}$ (2[c])	C	II
Turkey (10)	Cotton (1)	1A (1)	B	V
	Olive (9)	1A (9)	B	V
USA (26)	Cotton (4)	1A (3)	A	V
		1A (1)	B	V
	Woody plants (5)	1B (5)	B	V
	Tomato (2)	2A (2)	C	NA
	Potato (9)	4A (7)	E	II
		4B (2)	C	I
	Soil (4)	4A (2)	E	II
		4B (2)	C	I
	Pepper (2)	6 (2)	C	—[d]

[a]PCR assays were carried out as described by Mercado-Blanco *et al.* (2003) and Collado-Romero *et al.* (2006). The four PCR markers patterns were: A = 334 bp (+), 462 bp (+), 824 bp (−); B = 334 bp (+), 462 bp (−), 824 bp (−); C = 334 bp (−), 462 bp (−), 824 bp (+); and E = 334 bp (+), 462 bp (−), 824 bp (+). PCR genotypes were determined for the new isolates in this study, along with a subset of other isolates; all others were determined in previous studies as indicated by references in Table S1.

[b]Sequence haplotype for the 256-bp amplicon from race-2-specific primers VdR2F/VdR2R as defined in Table 2. NA, not applicable because this sequence is not present in race 1 isolates.

[c]Isolates for which new VCG and PCR data were developed in this study include: from Australia, seven isolates in lineage 1A (PP61, PP280, C25, 75 BRF (Back) W5 East, 74 BRF East, Lachlan NAss Split 74 BRF, W4 74 BRF), four in 2A (5M, PP36, PP242, PP245), and two in 4B (A36, PP94); one from olive in Italy (V384); one from pepper in Poland (VdPp); one 2A isolate (V11) from tomato in Spain; and two $2B^{824}$ isolates (Ver12 and Ver13) from tomato in Spain.

[d]No sequence was obtained for two lineage 6 isolates because no amplicon was produced by either race 1-specific or race 2-specific PCR.

from the Ohio Agricultural Research and Development Center (OARDC, Ohio State University, Wooster, OH, USA) for VCG1, VCG3 and VCG4A and *nit* testers from Israel for VCG1A, VCG2A, VCG2B and VCG4B (Korolev *et al.*, 2000, 2008) as described previously (Jiménez-Díaz *et al.*, 2006). All reference testers were kindly provided by T. Katan (ARO, The Volcani Center, Bet Dagan, Israel). No complementation tests were done in this work to type isolates to VCG1B and VCG6. Israeli *nit* testers were previously demonstrated to correlate with the international OARDC reference strains of *V. dahliae* VCGs (Katan, 2000; Korolev *et al.*, 2000). Cultures were incubated at 24 ± 1 °C in the dark for up to 4 weeks. Unknown isolates were assigned to a VCG when a dense, aerial growth (Fig. S1) or black microsclerotia formed where mycelia from the unknowns and the VCG tester strain had met.

Clonal lineages were previously defined for 141 of the 195 isolates by analysis of 26 748 SNPs derived from genotype by sequencing (GBS) (Milgroom *et al.*, 2014; Table S1). Because molecular markers that define clonal lineages consistently correspond to VCGs in almost all cases (Jiménez-Díaz *et al.*, 2012; Jiménez-Gasco *et al.*, 2014; Milgroom *et al.*, 2014), lineages were inferred from VCGs. For VCG2B, lineages were further assigned to $2B^{334}$ or $2B^{824}$ based on PCR markers for pathotype, as described below.

Extraction of fungal DNA

Isolates were grown on PDA overlain with a sterile cellophane disk for 7 days at 24 °C in the dark. Mycelia scraped off the cellophane were lyophilized and 50 mg of lyophilized mycelia were ground to a fine powder in 2 mL tubes with glass beads in a Fast Prep Instrument (BIO 101). DNA was extracted from ground mycelia using the i-Genomic Plant DNA Extraction Mini kit (Intron Biotechnology, Inc.), according to manufacturer's instructions. DNA purity and concentration were determined using a ND-1000 spectrophotometer (NanoDrop Technologies) and by 1% agarose gel electrophoresis.

PCR assays for D and ND pathotypes

Pathotype was previously shown to be correlated with two *V. dahliae*-specific PCR markers. The first marker (primers DB19/DB22/espdef01) produces amplicons 539 or 523 bp in length in D and ND isolates, respectively (Mercado-Blanco *et al.*, 2003). These primers also amplify a 334-bp amplicon in D isolates and also in ND isolates from VCG1B and VCG2B (Jiménez-Díaz *et al.*, 2012), which were assigned to lineages 1B and $2B^{334}$, respectively. The second marker (primer pairs INTD2f/INTD2r and INTND2f/INTND2r) was used jointly in duplex PCRs to produce 462- or 824-bp amplicons in D and ND pathotypes, respectively (Jiménez-Díaz *et al.*, 2006, 2012). Amplification of the 824-bp amplicon in VCG2B isolates was used to assign them to lineage $2B^{824}$. These markers have been used previously to genotype isolates of *V. dahliae*, and were used on an additional 38 isolates in this study in order to compare results with previous genotyping of 157 isolates (Table S1) and to assign isolates in VCG2B to either lineage $2B^{334}$ or $2B^{824}$.

PCR assays for races 1 and 2

All 195 *V. dahliae* isolates were characterized to race 1 or race 2 by three PCR assays: two markers that were designed to detect race 1 by amplifying parts of effector gene *Ave1*, using

primer pairs Tr1/Tr2 (Usami *et al.*, 2007) and VdAve1F/VdAve1R (de Jonge *et al.*, 2012) that produce 900- and 680-bp amplicons, respectively, in race 1 isolates; and primer pair VdR2F/VdR2R that produces a 256-bp amplicon in race 2 isolates, and not in those of race 1 (Short *et al.*, 2014).

Amplification reactions using primer pair Tr1/Tr2 were performed as described by Usami *et al.* (2007). Amplification reactions using primer pairs VdAve1F/VdAve1R and VdR2F/VdR2R were performed as described by Hu *et al.* (2015). Reactions were performed in a PTC-100 programmable thermal controller (MJ Research, Inc.). All PCR products were separated on 1% agarose gels, stained with 5% ethidium bromide and visualized under UV light. The Gene-ruler DNA ladder mix (Fermentas) was used as a DNA size marker for electrophoresis. Reactions were repeated twice and always included negative controls (no DNA) and two positive controls: DNAs of race 1 isolate V11 and race 2 isolate W-83.

Sequencing of PCR products for race 1 and race 2

Sequences were obtained of the 900-bp amplicons from all race 1 isolates, and the 256-bp amplicons from a sample of 112 race 2 isolates. Amplicons were excised from the gel and purified using the Geneclean turbo gel extraction kit (Q-BIOgen) according to manufacturer's instructions, quantified as described above for genomic DNA, and used for direct DNA sequencing using both forward and reverse primers at the STABVIDA sequencing facilities (Caparica, Portugal). All sequences obtained were edited and aligned using BIONUMERICS v. 6.1 software (Applied Maths) and compared to those available in GenBank.

Pathogenicity assays for pathotype and race

A sample of 15 race 2 isolates from five different clonal lineages was tested for virulence on cotton (*Gossypium hirsutum*), tomato (*Solanum lycopersicum*), cultivated olive (*Olea europaea* subsp. *europaea* var. *europaea*) and wild olive (*O. europaea* subsp. *europaea* var. *sylvestris*) in four experiments (I to IV) in a growth chamber. In addition, race 1 isolate V11 (lineage 2A) and race 2 isolates Ver12 and Ver13 (lineage $2B^{824}$) from tomato in Spain were used as controls. Details of each experiment are described below.

Inocula consisted of conidial suspensions or infested cornmeal-sand mixture (CMS) (Jiménez-Fernández *et al.*, 2016). Conidia were harvested from 7-day-old cultures in potato-dextrose broth (Difco Laboratories Inc.) incubated at 24 ± 1 °C in the dark on an orbital shaker (Adolf Kühner AG) at 125 rpm. Conidia in the liquid cultures were filtered through sterile cheesecloth and the inoculum concentration was adjusted to 10^7 conidia mL^{-1} with sterile deionized water. CMS inocula were produced in Erlenmeyer flasks containing autoclaved CMS infested with PDA discs from 7-day-old cultures and incubated at 24 ± 1 °C in the dark for 4 weeks. The infested CMS was thoroughly mixed with a pasteurized soil mixture (sand: clay loam, 2:1, v/v; pH 8.3; 27% water holding capacity) at a rate of 10%, v/v. The inoculum density in the mixture, determined by a dilution-plating assay on semiselective NP-10 medium (Jiménez-Díaz *et al.*, 2012), ranged from 1.5 to 5.3 × 10^7 colony-forming units (cfu) per g soil, depending on the isolate. Plants were inoculated either by transplanting to the infested soil mixture, or root dip or stem injection with a conidial suspension, depending on the experiment, as described below. After inoculation, plants were incubated in a growth chamber for variable periods of time, depending on the experiment, at 22 ± 2 °C, 60–80% relative humidity and a 14 h photoperiod of fluorescent light of 360 μE m^{-2} s^{-1}. Plants were watered every 1–2 days, as needed, and fertilized weekly with 50 mL Hoagland's nutrient solution.

Experiments were not aimed at comparing among plant species for disease reaction to different *V. dahliae* isolates. Therefore, for each experiment, each host plant was arranged in a completely randomized design with *V. dahliae* isolates as single factors. There were eight cotton (Experiment I), eight tomato (Experiment II), six tomato (Experiment III) or six olive and 10 tomato (Experiment IV) completely randomized replicated plants (one plant per pot) for each plant genotype × isolate combination. Experiments I and II were conducted twice.

Severity of symptoms in individual plants was assessed on a 0–4 rating scale according to the percentage of foliage affected in acropetal progression: 0 = no symptoms; 1 = 1–33%; 2 = 34–66%; 3 = 67–100%; and 4 = dead plant (Korolev *et al.*, 2008; Jiménez-Fernández *et al.*, 2016). Assessments were made at 3-day intervals throughout the duration of the experiments. For those hosts that developed foliar symptoms (i.e. cotton in Experiment I and olive in Experiment IV), disease ratings were plotted over time to obtain disease severity progress curves and the area under the curves standardized by duration of disease development in days (SAUDPC) was calculated using the trapezoidal integration method (Campbell & Madden, 1990). Also, plant height and fresh weight (Experiments I and II), cotton plant height (Experiment I) and tomato fresh weight (Experiment III) were determined from the cotyledonary node and above at the end of experiments.

Upon termination of the experiments, infection by *V. dahliae* was determined in each plant, regardless of visible symptoms, by isolating the fungus on water agar amended with chlortetracycline (20 g L^{-1} agar, 30 mg L^{-1} chlortetracycline). For each plant, four (cotton and tomato) or eight (olive and wild olive clones) stem pieces representative of the plant height were thoroughly washed under running tap water for 30 min and the bark of olive pieces aseptically removed. Stem pieces were surface disinfested in 0.5% NaOCl for 1.5 min, and one 5- to 10-mm-long segment from each piece was plated onto the medium. Cultures were incubated at 24 ± 1 °C in the dark for 7 days. Colonies of *V. dahliae* were identified by microscopic observation of verticillate conidiophores and formation of microsclerotia (Inderbitzin *et al.*, 2011).

Experiment I: Virulence assays on cotton to determine pathotype

The objective of this experiment was to use virulence to confirm the pathotypes of isolates determined previously by PCR markers (see above). Twelve race 2 isolates from diverse lineages were inoculated onto cotton cv. Acala SJ-2 to distinguish between D and ND pathotypes. This host is highly susceptible and shows severe defoliation when inoculated with isolates of the D pathotype, but shows leaf necrosis and wilting when inoculated with isolates of the ND pathotype (Jiménez-Díaz *et al.*, 2012).

Seeds of Acala SJ-2 were supplied by IFAPA Research Center (Córdoba, Spain). Seeds were surface-disinfested (1% NaOCl for 2 min), dried under a stream of filtered air and germinated in a sterile moist chamber at 25 °C for 30 h. Germinated seeds, selected for uniformity (length of radicle = 1–2 cm), were sown in 11 × 11 × 12 cm pots filled with the pasteurized soil mixture, and grown for 6 weeks in a growth chamber (2-true-leaf stage). Plants were then inoculated by injecting 5 μL conidial inoculum (10^7 conidia mL^{-1}) with a hypodermic syringe at the

bases of the first and second stem internodes, on opposite sides (Korolev *et al.*, 2008). Control plants were mock-inoculated by injection of sterile distilled water. Inoculated and control plants were incubated for 7 weeks in a growth chamber with conditions described above.

Experiment II: Pathogenicity assays on tomato to determine race

Tomato cvs. San Pedro and Rio Grande were inoculated with the same isolates as in Experiment I to confirm races 1 and 2 inferred from PCR markers. San Pedro is susceptible to races 1 and 2 (A. de Cal, INIA, Madrid, Spain, personal communication) whereas Rio Grande is resistant to race 1 (Jabnoun-Khiareddine *et al.*, 2013). Race 1 isolate V11 from tomato was used as a control. Seeds of San Pedro and Rio Grande were obtained from Ramiro Arnedo S.A. (Calahorra, La Rioja, Spain) and Nunhems Spain S.A., Bayer CropScience Vegetable Seeds (Cartagena, Spain), respectively. Seeds were sown in vermiculite in 50-well trays and seedlings were grown in a growth chamber for 4 weeks (2-true-leaf stage). Seedlings were then uprooted and rinsed free of soil under tap water. Roots were lightly trimmed, dipped in a suspension of 10^7 conidia mL^{-1} for 20 min and transplanted to 11 × 11 × 12 cm pots filled with the pasteurized soil mixture. Control plants were mock-inoculated by dipping lightly trimmed roots in sterile distilled water. Inoculated and control plants were incubated for 7 weeks in a growth chamber as described above.

Experiment III: Pathogenicity and virulence of additional V. dahliae *isolates on tomato*

This experiment was performed to test race 2 isolates from tomato in Spain (Ver12 and Ver13, lineage $2B^{824}$) and a potato isolate from the USA (W-83, lineage 4A), as well as race 1 isolate V11 from tomato, for pathogenicity on tomato cvs San Pedro, Boa, Marmande and Vemone. The susceptibilities of Boa, Marmande and Vemone to *V. dahliae* were previously unknown; seeds of these cultivars were obtained from Vilmorin Ibérica (Alicante, Spain). Tomato seedlings were grown, root-dip inoculated, and incubated as in Experiment II.

Experiment IV: Verticillium wilt resistance in olive

The objective of this experiment was to determine if wild olive clones (Ac-4, Ac-13, Ac-18 and Outvert) recently shown to be highly resistant to two isolates with the D pathotype, one each from cotton and olive in Spain (Jiménez-Fernández *et al.*, 2016; Palomares-Rius *et al.*, 2016), would also be resistant to additional D pathotype isolates in lineage 1A (V669 and V1365), and also to an isolate from olive in Spain with the ND pathotype (V1236) from lineage $2B^{R1}$. Picual olive was used as a susceptible control, and isolate V781 was used as a control for the D pathotype (Palomares-Rius *et al.*, 2016). Tomato cvs Bonny Best and Rio Grande were used to confirm races inferred for these isolates by PCR markers, as in Experiment II. Bonny Best is susceptible to races 1 and 2, whereas Rio Grande is resistant to race 1 (Okie & Gardner, 1982; Jabnoun-Khiareddine *et al.*, 2013). Seeds of tomato cvs Bonny Best and Rio Grande were obtained from Nunhems Spain S.A., Bayer CropScience Vegetable Seeds (Cartagena, Spain). Tomato seedlings were grown as in Experiment II.

Own-rooted olive plants of Picual, Ac-13 and Ac-18 were propagated commercially by Plantas Continental, S.A. (Posadas, Córdoba, Spain) by rooting leafy stem cuttings in a pasteurized potting mixture under mist conditions in plastic tunnels. Plants of Ac-4 were provided by F. Pliego-Alfaro (Dpto. de Biología Vegetal, Universidad de Málaga, 29071, Málaga, Spain); they were axenically micropropagated from axillary buds, with further rooting in an autoclaved potting mixture. Plants of Outvert were provided by Vitroplant Italia S.R.L. Società Agricola (Cesena, Italy); they were axenically micropropagated from axillary buds and rooted as above.

One-year-old plants, 60 cm in height, of Ac-13, Ac-18 and Picual, and 6-month-old, 40-cm-tall plants of Ac-4, were uprooted from the potting substrate, shaken to retain the rhizosphere soil, and transplanted into 13 × 13 × 13 cm pots filled with the infested soil mixture. Tomato plants were root-dip inoculated as for Experiment II. Plants of Picual were also inoculated by root-dipping as for tomato. Negative control plants were mock-inoculated with sterile distilled water (root-dip inoculation), or grown in non-infested CMS mixed with the pasteurized soil mixture at the same rate as infested CMS (transplanting inoculation). Inoculated and control plants were incubated for 12 (olive and wild olive) or 7 weeks (tomato) in a growth chamber adjusted to the conditions described above.

Data analysis

Disease incidence and stem colonization data were analysed with the GENMOD procedure using the binomial distribution and the logit as link function in SAS v. 9.4 (SAS Institute Inc.). A likelihood ratio test was used to determine whether or not the characteristics of *V. dahliae* isolates influenced ($P < 0.05$) the incidence of disease and degree of stem colonization by the pathogen. The statistical significance ($P < 0.05$) of the likelihood ratio was determined by a chi-square test and contrast statements were used to determine significant differences ($P < 0.05$) among treatments (Agresti, 2007). The overall effect of *V. dahliae* isolates on disease severity, assessed on the 0–4 rating scale described above, was determined by the analysis of variance type statistic of ranked data using the MIXED procedure in SAS to generate relative effects; the LD_CI macro was used to generate 95% confidence intervals (Shah & Madden, 2004). Data for SAUDPC, plant height and fresh weight were subjected to standard analysis of variance (ANOVA) with the GLM procedure in SAS to determine whether or not the characteristics of *V. dahliae* isolates significantly influenced ($P < 0.05$) disease reaction and plant growth. Data fulfilled the assumptions for ANOVA according to appropriate statistics. Similarity among repetitions in experiments I and II, tested by preliminary ANOVA using experimental repetitions as blocks, made it possible to combine data for analyses. Orthogonal single-degree of freedom contrasts were computed to test the effect of selected experimental treatment combinations.

Results

Assignment of isolates to VCGs and clonal lineages

Of the 18 isolates newly typed to VCGs in this study, seven isolates from cotton in Australia were strongly compatible with VCG1A tester strain T9 and were assigned to VCG1A (Tables 1 & S1; Fig. S1a,c). Similarly, four other isolates from cotton in Australia, one isolate from olive in Italy and one isolate from tomato in Spain were assigned to VCG2A (Fig. S1b); two isolates from tomato in Spain and one from pepper in Poland were assigned to VCG2B (Fig. S1e). Finally, two additional cotton isolates from Australia were assigned to VCG4B (Fig. S1d).

The combination of PCR markers for pathotypes (Jiménez-Díaz *et al.*, 2012) resulted in four distinct genotypes among the 195 isolates. These genotypes are denoted A, B, C and E, based on the presence or absence of a 334-bp amplicon, and the alternate amplicons of sizes 462 or 824 bp (Fig. S2; see footnotes in Tables 1 and S1 for definitions of genotypes). PCR genotypes were independent of hosts but were highly correlated with VCGs and clonal lineages (Tables 1 & S1). Most isolates in lineage 1A had genotype A. However, as found previously, a few isolates in lineage 1A and all isolates in lineages 1B and $2B^{334}$ had genotype B, characterized by the presence of the 334-bp amplicon and absence of the 462-bp amplicon (Jiménez-Díaz *et al.*, 2012). Isolates from potato and potato soil from the USA in lineage 4A had genotype E, characterized by the presence of the 334 and 824-bp amplicons. All isolates in lineages 2A, $2B^{824}$ and 4B had genotype C, characterized by the presence of the 824-bp amplicon. Using a combination of VCG assignment and diagnostic PCR markers, 38 isolates were assigned to clonal lineages. Isolates Ver12, Ver13 and VdPp in VCG2B were assigned to lineage $2B^{824}$. A full listing of VCGs, PCR results and clonal lineages for all 195 isolates is provided in Table S1.

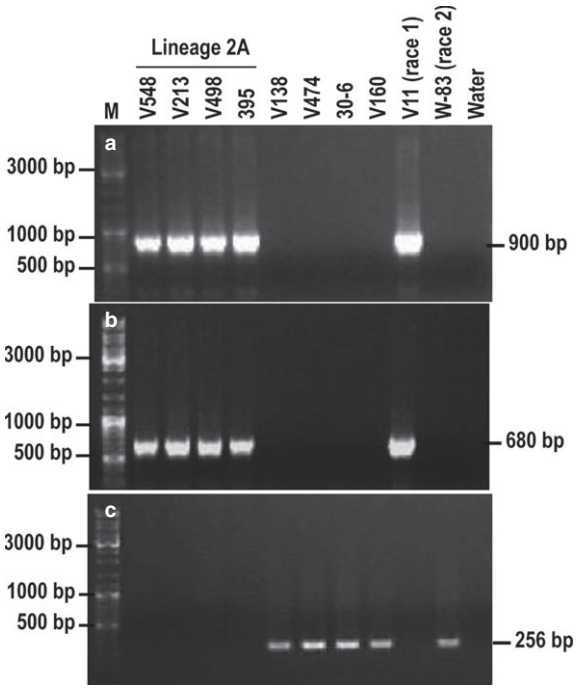

Figure 1 Gel electrophoresis of race-specific PCR markers for race 1 and race 2 in isolates of *Verticillium dahliae*. (a) Race 1-specific 900-bp amplicons generated with primers VdAve1F and VdAve1R. (b) Race 1-specific 680-bp amplicons generated with primers Tr1 and Tr2. (c) Race 2-specific 256-bp amplicons generated with primers VdR2F and VdR2R. Lineage 2A isolates (V213, isolated from cotton in Spain; V498, aubergine, Israel; V548, artichoke, Spain; and 395, tomato, USA) are in race 1. Isolates V138 (lineage 1A/D pathotype, cotton, Spain), V474 (lineage $2B^{824}$, artichoke, Spain), 30-6 (lineage 4A, potato, USA) and V160 (lineage 4B, cotton, Israel) are in race 2. Isolates V11 (lineage 2A, tomato, Spain) and W-38 (lineage 4A, potato, USA) were used as positive controls for races 1 and 2, respectively. Sterile distilled water was used as a negative control. Lane M, Gene-ruler DNA ladder mix (Fermentas) was used as a DNA size marker.

Correlation of pathotype and race to clonal lineages

All but two isolates amplified either the race 1-specific or race 2-specific amplicons. Among the remaining 193 isolates, all 27 isolates in lineage 2A were identified as race 1 by PCR by both pairs of race 1-specific primers (Fig. 1a,b; Table 1). Thus, race 1 was found in a single clonal lineage, lineage 2A, sampled from five different hosts in diverse geographic areas (Table 1). The remaining 166 isolates were identified by PCR as race 2 (Figs 1c & 2; Table 1). Race 2 isolates were in lineages 1A, 1B, $2B^{334}$, $2B^{824}$, $2B^{R1}$, 4A and 4B (Table 1). Note, however, that only isolates in lineage 1A had the D pathotype (see below) (Jiménez-Díaz *et al.*, 2012); race 1, found only in clonal lineage 2A, and race 2, in all other lineages, had no association with the D pathotype. PCR using template DNA from two lineage 6 isolates (V560 and V561) failed to amplify any products, despite repeated attempts. However, PCR using the same template DNA successfully amplified *V. dahliae*-specific markers (Fig. S2a; Jiménez-Díaz *et al.*, 2012; Inderbitzin *et al.*, 2013) and subsequent sequencing of these amplicons further confirmed their identity as *V. dahliae* (results not shown).

Sequence analysis of race 1 and race 2 markers

Sequences of the 900-bp amplicon from *Ave1*, which is specific to race 1, were identical for all 27 race 1 isolates, and identical to sequences of other race 1 isolates available in GenBank (JR2, CP009078; CBS381.66, JQ625339; St14.01, JQ625341; and TomR1, AB095266). By contrast, the 256-bp amplicon from the race 2-specific marker was polymorphic and sequence variation correlated with clonal lineages. Sequencing of this amplicon from 112 isolates resulted in four haplotypes (designated I to III, and V), which differed at five nucleotide sites when compared among themselves and with homologous sequences from *V. dahliae* available in GenBank (Table 2). Three of the four haplotypes were identical to one of those in GenBank; the fourth (haplotype V) was unique to this study. The sequence of GenBank accession KC935944 was not present among isolates in the study; this haplotype was designated here as IV (Table 2).

Haplotypes of the 256-bp amplicon of race 2 were clearly correlated with clonal lineages. Haplotype I was found in lineage 4B; haplotype II in lineages 4A and $2B^{824}$; haplotype III in lineages $2B^{334}$ and $2B^{R1}$; and haplotype V in lineages 1A and 1B (Tables 1 & 3). Clonal lineages with the same haplotype for this sequence are also genetically closely related to SNP genotypes (Milgroom *et al.*, 2014).

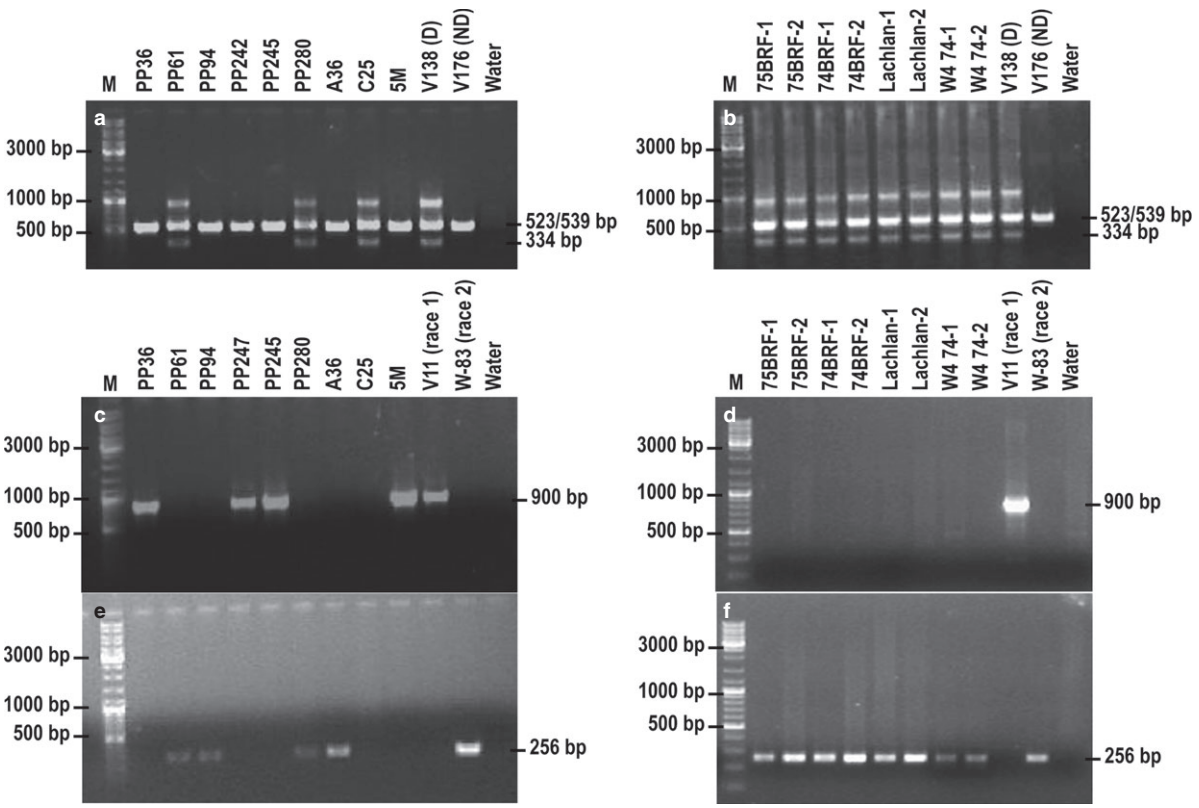

Figure 2 Gel electrophoresis of PCR amplifications to identify defoliating (D) and nondefoliating (ND) pathotypes, and races 1 and 2 in isolates of *Verticillium dahliae*. (a,b) PCR amplification of *V. dahliae*- (523/539 bp) and D-specific (334 bp) amplicons (Mercado-Blanco *et al.*, 2003). Isolates V138 and V176 from cotton, Spain, are in lineages 1A and 2A and were used as positive controls for the D and ND pathotypes, respectively. (c,d) Race 1-specific 900-bp amplicons generated with primers VdAve1F and VdAve1R. (e,f) Race 2-specific 256-bp amplicons generated with the race 2-specific primers VdR2F and VdR2R. Isolates V11 from tomato, Spain (lineage 2A) and W-38 from potato, USA (lineage 4A) were used as positive controls for races 1 and 2, respectively. Isolates C25 PP61, PP280, 74 BRF, 75 BRF, Lachlan and W4 74 from cotton in Australia are in lineage 1A and of D pathotype. Isolates 5M, PP36, PP242, and PP245 from cotton in Australia are in lineage 2A and race 1. Isolates A36 and PP94 from cotton in Australia are in lineage 4B and of ND pathotype. Numbers 1 and 2 appended to isolate names indicate replications of PCR assays of these isolates. Sterile distilled water was used as a negative control. Lane M, Gene-ruler DNA ladder mix (Fermentas) used as a DNA size marker.

Virulence and pathotypes of *V. dahliae* isolates from different lineages

No symptoms developed in noninoculated or mock-inoculated plants.

Experiment I: Pathogenicity on cotton to determine pathotype

Virulence on cotton cv. Acala SJ-2 varied significantly by clonal lineage, although isolates from all lineages reached 100% disease incidence (Table 4). Isolates in lineage 1A induced stunting and epinasty, followed by severe leaf chlorosis, necrosis and defoliation (Fig. 3). This disease syndrome was characteristic of the D pathotype (Schnathorst & Mathre, 1966; Bell, 1994). Symptoms started to develop 7–9 days after inoculation. All lineage 1A isolates, except for isolate T9, caused complete defoliation and death (mean symptom severity, 4.0, on a 0–4 rating scale) by 7 weeks after inoculation. Isolate T9 caused defoliation of all but the uppermost leaves and had a mean symptom severity of 3.2 (Fig. 3; Table 4). By contrast, isolates in all other lineages, including those of race 1 in lineage 2A, induced leaf chlorosis and necrosis but no defoliation; the only exception was for isolate V1242, which caused defoliation of the lowermost leaves (Fig. 3). This disease syndrome was characteristic of the ND pathotype (Schnathorst & Mathre, 1966; Bell, 1994). ND isolates had mean severities ranging from 2.1 to 3.2 (Table 4). Based on SAUDPC, race 1/lineage 2A isolate V11 was significantly ($P < 0.05$) less virulent than isolates of D pathotype (lineage 1A), but more virulent than other ND pathotype isolates. Two exceptions were isolates T9 (D pathotype) and V1242 (lineage 4B/ND pathotype), which were not significantly different from isolate V11 ($P \geq 0.05$; Table 4; Fig. 3). Collectively, isolates in lineage 1A were significantly ($P < 0.05$) more virulent on Acala SJ-2 cotton than those of ND pathotype, regardless of their clonal lineage (Table 4). When ND isolates in lineage 4B were considered as a group, they were significantly ($P < 0.05$) more virulent that those in lineages 4A, $2B^{334}$ and $2B^{R1}$, in that order, as indicated by mean symptom severity and SAUPC values (Table 4).

Table 2 Sequence haplotypes among a sample of 112 isolates from diverse lineages and defoliating (D) and nondefoliating (ND) pathotypes amplified with primers VdR2F/VdR2R for the *Verticillium dahliae* race 2-specific PCR marker, plus one (haplotype IV) from GenBank

Sequence haplotype	Nucleotides[a]					GenBank accession number[b]
	1345363	1345395	1345486–1345487	1345506	1345535	
I	C	G	A	C	G	KC935942; KX034393
II	T	G	–	C	A	CP010983; KX034394; KX034395
III	C	G	–	C	A	KC935943; KX034396; KX034397
IV	C	A	–	C	A	KC935944
V	C	G	–	G	A	KX034398; KX034399

[a]Nucleotide position is relative to sequence CP010983 *V. dahliae* VdLs.17 chromosome 4 from GenBank. All other nucleotides in the 256-bp sequence are identical for all isolates sequenced to date.
[b]GenBank accession numbers KX034393 to KX034399 are for sequences for representative isolates in this study. Accession numbers KC935942 to KC935944 and CP010983 are for sequences of isolates in GenBank used for comparisons. No information was available about lineage and pathotype for these latter isolates.

Table 3 Summary of relationships among lineage, pathotype, race, PCR patterns and race 2-specific sequence haplotype for 195 isolates of *Verticillium dahliae*

Lineage	*n* (by lineage)	Pathotype	Race	PCR genotype	Race 2 sequence haplotype
1A	110	D	2	A	V
1B	5	ND	2	B	V
2A	27	ND	1	C	NA
$2B^{334}$	10	ND	2	B	III
$2B^{824}$	12	ND	2	C	II
$2B^{R1}$	2	ND	2	C	III
4A	9	ND	2	E	II
4B	18	ND	2	C	I
6	2	ND	–	C	–

NA, not applicable because this sequence is not present in race 1 isolates.

There were no significant differences between lineages $2B^{334}$ and $2B^{R1}$.

Reduction in plant height in Acala SJ-2 caused by infection with *V. dahliae* was correlated with virulence. Thus, all isolates in lineage 1A (D pathotype), including T9, and ND isolate PP94 (lineage 4B) significantly ($P < 0.05$) reduced the final height of plants by 30.2–50.9% compared with plants inoculated with race 1 isolate V11 or noninoculated controls. Collectively, isolates in lineage 1A reduced plant height the most ($P < 0.05$) followed by those in lineages 4B, 2B ($2B^{334}$, $2B^{R1}$) and 4A, in order of decreasing growth reduction (Table 4).

Experiment II: Pathogenicity on tomato to determine race

Using the same isolates as in Experiment I, only race 1 isolate V11 (lineage 2A) induced leaf chlorosis and necrosis on tomato (100% disease incidence, 0.94 mean symptom severity), and that occurred only in San Pedro plants (Table 5). Race 2 isolates induced almost no visible symptoms; incipient chlorosis and necrosis were observed on some of the lowermost leaves on Rio Grande (12.5–62.0% incidence) or San Pedro (12.5–25.0% incidence); mean disease severity was <0.2 on both cultivars (Table 5). Race 1 isolate V11 colonized 9.4% and 40.6% of the stems of Rio Grande and San Pedro, respectively. Except for isolate V549 (lineage $2B^{334}$), stem colonization for all other race 2 isolates was unrelated ($P \geq 0.05$) to clonal lineage, ranging from 3.1% to 31.2% in Rio Grande and from 3.1% to 15.6% in San Pedro (Table 5).

Development of foliar symptoms in tomato was correlated with reduction in plant growth, but differed considerably between cultivars and *V. dahliae* isolates. None of the isolates significantly ($P \geq 0.05$) reduced plant height compared with the noninoculated control plants. By contrast, significant reductions ($P < 0.05$) in fresh weight compared with the control were caused by isolate V1242 (lineage 4B) in Rio Grande, and isolates PP61 (lineage1A) and V11 (race 1, lineage 2A) in San Pedro, (Table 5). Although the overall effect of race 1 isolate V11 on plant growth of Rio Grande did not differ significantly ($P \geq 0.05$) from that caused by most other (race 2) isolates, this isolate significantly ($P < 0.05$) reduced the fresh weight of San Pedro more than most race 2 isolates, irrespective of clonal lineage (Table 5). Race 2 isolates reduced plant fresh weight ($P < 0.05$) according to clonal lineage, with slight differences between tomato cultivars. In Rio Grande, isolates in lineage 4B caused the greatest reduction in fresh weight ($P < 0.05$), and this reduction was significantly ($P < 0.05$) more than isolates from lineages 1A and 4A, but did not differ from that caused by isolates from lineage 2B ($P \geq 0.05$). Isolates in lineage 1A did not differ from the other lineages collectively in Rio Grande (Table 5), but they reduced fresh weight of San Pedro the most ($P < 0.05$), followed by isolates in lineages 4B and 2B in decreasing order. The isolate in lineage 4A caused the smallest ($P < 0.05$) reduction in fresh weight of all lineages on both tomato cultivars (Table 5).

Experiment III: Virulence of additional V. dahliae *isolates on tomato*

Race 1 isolate V11 induced leaf chlorosis and necrosis in San Pedro and Vemone. Symptoms started to develop 12 days after inoculation and reached 100% incidence,

Table 4 Virulence of race 2 *Verticillium dahliae* isolates of the defoliating (D) and nondefoliating (ND) pathotypes, in different clonal lineages, to cotton cv. Acala SJ-2 (Experiment I)[a]

Lineage	Isolate	Race[b]	Pathotype	Disease incidence (%)	Disease severity (0–4)[c]	SAUDPC[d]	Stem colonization (%)[e]	Plant height (cm)[f]
–	Control	–	–	0	0	0	0	28.3 ± 1.87
1A	PP61	2	D	100	4*	3.3 ± 0.04*	100*	14.4 ± 0.61*+
	T9	2	D	100	3.2 ± 0.21*	2.3 ± 0.10	64.3 ± 17.98	18.7 ± 1.78*+
	V138	2	D	100	4*	3.4 ± 0.10*	100*	14.6 ± 0.43*+
	V465	2	D	100	4*	3.6 ± 0.07*	100*	14.4 ± 0.46*+
	V641	2	D	100	4*	3.1 ± 0.06*	100*	13.9 ± 0.59*+
	V1365	2	D	100	4*	3.3 ± 0.10*	100*	15.9 ± 0.72*+
	V1900	2	D	100	4*	3.4 ± 0.11*	100*	14.5 ± 0.60*+
$2B^{334}$	V549	2	ND	100	2.6 ± 0.21	1.3 ± 0.07*	18.8 ± 13.15	25.4 ± 0.99
$2B^{R1}$	V1374	2	ND	100	2.1 ± 0.35	2.0 ± 0.21*	25.0 ± 9.45	23.3 ± 2.02
4A	W-83	2	ND	100	2.1 ± 0.17*	1.9 ± 0.10*	21.4 ± 10.10	25.8 ± 1.64
4B	PP94	2	ND	100	3.2 ± 0.15*	1.7 ± 0.09*	100*	19.7 ± 2.18+
	V1242	2	ND	100	2.6 ± 0.14	2.6 ± 0.13	64.3 ± 17.98	23.9 ± 1.30
2A	V11	1	ND	100	2.9 ± 0.10	2.6 ± 0.07	50.0 ± 15.43	24.7 ± 1.58
				P	*P*	*P*	*P*	*P*
Source of variation								
Isolates				nt	0.0006	<0.0001	0.0133	<0.0001
Contrasts[g]								
Lineage 1A versus all others				nt	nt	<0.0001	nt	0.0104
Lineage 1A versus $2B^{334}$ and $2B^{R1}$				nt	nt	0.0004	nt	<0.0001
Lineage 1A versus 4A				nt	nt	<0.0001	nt	<0.0001
Lineage 1A versus 4B				nt	nt	<0.0001	nt	0.0002
Lineage $2B^{334}$ versus $2B^{R1}$				nt	0.4607	0.0491	0.6685	0.7992
Lineage $2B^{334}$ and $2B^{R1}$ versus 4A				nt	0.0326	<0.0001	0.9828	0.8109
Lineage $2B^{334}$ and $2B^{R1}$ versus 4B				nt	0.0406	<0.0001	<0.0001	<0.0001
Lineage 4A versus 4B				nt	<0.0001	<0.0001	<0.0001	<0.0001

[a]Data are means (±standard error) of 16 replicated plants per treatment combination. See text for experiment details.

[b]Determined by PCR assays using primer pairs VdAve1F/VdAve1R (de Jonge *et al.*, 2012; race 1) and Tr1/Tr2 (Usami *et al.*, 2007; race 1) and VdR2F/VdR2R (Short *et al.*, 2014; race 2).

[c]Mean disease severity assessed 7 weeks after inoculation according to the percentage of foliage affected in acropetal progression (0 = no symptoms, 1 = 1–33%, 2 = 34–66%, 3 = 67–100%, and 4 = dead plant). Mean for an isolate followed by an asterisk (*) is significantly different from that of race 1 isolate V11 at $P < 0.05$.

[d]Area under the disease severity progress curve over time calculated by the trapezoidal integration method (Campbell & Madden, 1990) standardized by duration of disease development. Mean for an isolate followed by an asterisk (*) is significantly different from that of race 1 isolate V11 at $P < 0.05$.

[e]Determined by isolation of *V. dahliae* from four stem pieces of each inoculated plant and incubating at 24 ± 1 °C for 7 days. Mean for an isolate followed by an asterisk (*) is significantly different from that of race 1 isolate V11 at $P < 0.05$.

[f]Determined from the cotyledonary node 7 weeks after inoculation. Mean plant height for an isolate followed by an asterisk (*) and/or a plus sign (+) is significantly different from that corresponding to race 1 isolate V11 (*) or the noninoculated control (+) at $P < 0.05$.

[g]Linear single-degree of freedom contrasts were computed to test the effect of selected experimental treatment combinations at $P < 0.05$. Underlined values indicate that estimated value for the first term of the contrast is lower than that of the second term. nt, not tested.

with mean severity of 1.63 in San Pedro and 1.25 in Vemone 5 weeks later (Table 6). On San Pedro, race 2 isolates were either not pathogenic (isolate Ver12) or caused incipient chlorosis and necrosis on some of the lowermost leaves in 25% (isolate Ver13) or 50% (W-83) of the plants. None of the isolates tested in this experiment caused foliar symptoms in tomato cv. Marmande (Table 6). Similarly, isolates Ver12 and Ver13 were not pathogenic on Vemone or Boa, but isolate W-83 was weakly virulent, causing incipient leaf chlorosis in 66.7% of the plants of these cultivars. All isolates, except Ver12 or Ver13, infected and systemically colonized stem tissues of tomato. *Verticillium dahliae* could not be recovered from Boa plants inoculated with Ver12 or Ver13 (Table 6). There were no significant differences ($P \geq 0.05$) in stem colonization among cultivars, and no isolate × cultivar interaction was found. Overall, stem colonization differed among isolates ($P < 0.05$). Across all cultivars, stem colonization was highest for race 1 isolate V11, and was less for race 2 isolates W-83, Ver12 and Ver13, in decreasing order. Infection by *V. dahliae* reduced fresh weight of plants to a variable degree depending on the isolates. Across tomato cultivars, race 1 isolate V11 reduced plant growth by 20.0%, and race 2 isolates W-86, Ver12 and Ver13 reduced growth by 14.2%, 11.8% and 4.1%, respectively (Table 6).

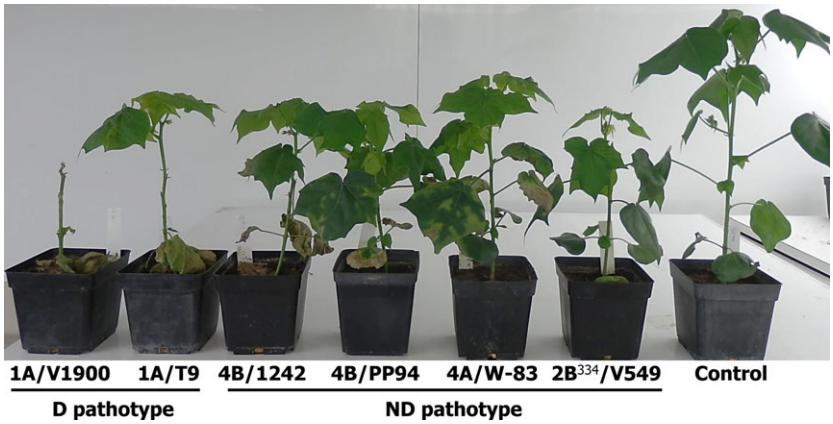

Figure 3 Disease reactions in cotton cv. Acala SJ-2 showing the defoliating (D) and nondefoliating (ND) pathotypes of *Verticillium dahliae* 3 weeks post-inoculation. Plants were stem-injection inoculated with 10^7 conidia mL^{-1} of *V. dahliae* isolates from clonal lineages 1A, $2B^{334}$, 4A and 4B. Isolates V1900 and T9 in lineage 1A caused severe defoliation typical of the D pathotype. Isolate V1242 in lineage 4B caused shedding of a few of the lowermost leaves. Isolates PP94, W-83 and V549 in lineages 4B, 4A and $2B^{334}$ varied in the severity of foliar symptoms but caused no defoliation, which is characteristic of the ND pathotype. Noninoculated controls were stem-injected with sterile distilled water.

Experiment IV: Verticillium wilt resistance in olive

Disease reaction of Picual olive to transplanting to infested soil or root-dip inoculation varied with *V. dahliae* isolates. No symptoms developed in plants of wild olive clones and Picual olive inoculated with the ND isolate V1236 (lineage $2B^{R1}$) by either method of inoculation. Nevertheless, *V. dahliae* was isolated from 8.3% of symptomless Picual plants. Symptoms induced by D isolates V669, V781 and V1365 (lineage 1A) started to develop 30–42 days after transplanting to infested soil or root-dip inoculation, respectively. Symptoms consisted of early dropping of symptomless, green leaves from individual twigs that eventually resulted in complete defoliation, necrosis and death of the plant; these symptoms are typical for the defoliating syndrome caused by D *V. dahliae* (Jiménez-Díaz *et al.*, 2012). Isolate V1365 was more virulent than isolates V781 and V669 according to differences in the disease incidence and severity or SAUDPC, regardless of inoculation method (Table 7). However, these differences were statistically significant ($P < 0.05$) only for plants grown in infested soil. Final disease incidence and severity induced by isolate V1365 were higher in plants inoculated by transplanting to infested soil (83.3% and 2.1%, respectively) than in plants that were root-dip inoculated (50.0% and 1.5%, respectively). Disease incidence and severity induced by isolates V669 and V781 varied from 17% to 50% and 0.3–1.4, respectively, depending on isolate and method of inoculation. Stem colonization was significantly higher ($P < 0.05$) for isolate V1365, which was recovered from 75% to 50% of plants inoculated by transplanting to infested soil and root dip, respectively. Inoculation with isolates V669 and V781 gave rise to a range of 13.8–28.1% stem colonization across isolates and method of inoculation.

No symptoms developed in wild olive clones Ac-4, Ac-13 and Ac-18 inoculated by transplanting to soil infested with lineage 1A isolates V669, V781 and V1365, or with ND isolate V1236 in lineage $2B^{R1}$. Similarly, no symptoms developed on Outvert wild olives inoculated with isolates V669 or V1236 and no *V. dahliae* was isolated from the stems of these plants. By contrast, isolates V781 and V1365 caused incipient mild chlorosis on some of the lowermost leaves of 17% of Outvert plants, and *V. dahliae* was isolated from 66.7% of plants inoculated with either isolate, with 12.5% stem colonization.

Root-dip inoculation of tomato cultivars with D isolates (lineage 1A) gave rise to symptomless reactions in Bonny Best (isolates V669 and V1365), and Rio Grande (isolates V669 and V781; Table 7). In contrast, isolates V781 and V1365 induced incipient chlorosis and necrosis in a few of the lowermost leaves on 10% plants of Bonny Best and Rio Grande. Also, a similar reaction was induced by race 2, ND isolate V1236 in Bonny Best and Rio Grande. However, there was a significant difference in stem colonization ($P < 0.05$) among isolates and tomato cultivars. Stem colonization by D isolates was significantly higher in Bonny Best than in Rio Grande. For both tomato cultivars, stem colonization was the lowest for isolate V669 (2.5% and 10.0% in Rio Grande and Bonny Best, respectively) but it increased to a range from 22.5% to 60.0% for isolates V781 and V1365. The ND isolate V1236 (lineage $2B^{R1}$) gave rise to 47.5% stem colonization in Bonny Best but reached the highest colonization ($P < 0.05$) in Rio Grande (80.0%; Table 7).

Discussion

Use of race 1-specific and race 2-specific PCR markers (Usami *et al.*, 2007; de Jonge *et al.*, 2012; Short *et al.*, 2014) and 195 diverse *V. dahliae* isolates (in nine clonal lineages, from six crop species and five woody plant species, from nine countries, with both D and ND pathotypes) showed that all race 1 isolates were present only

Table 5 Virulence of race 2 *Verticillium dahliae* isolates of the cotton-defoliating (D) and -nondefoliating (ND) pathotypes, in different clonal lineages, to tomato cvs Rio Grande and San Pedro (Experiment II)[a]

				Rio Grande					San Pedro				
Lineage	Isolate	Race[b]	Pathotype on cotton	Disease incidence (%)	Disease severity (0–4)[c]	Stem colonization (%)[d]	Plant height (cm)[e]	Fresh weight (g)[f]	Disease incidence (%)	Disease severity (0–4)[c]	Stem colonization (%)[d]	Plant height (cm)[e]	Fresh weight (g)[f]
–	Control	–	–	0.0	0.00	0.0	30.2 ± 1.5	34.9 ± 1.9	0.0	0.00	0.0	28.9 ± 1.0	39.8 ± 0.8
2A	V11	1	ND	0.0	0.00	9.4 ± 6.6	29.1 ± 1.7	32.5 ± 1.3	100	0.94 ± 0.11	40.6 ± 9.4	23.5 ± 1.7	30.3 ± 1.7+
1A	PP61	2	D	25.0 ± 16.4	0.13 ± 0.08	15.6 ± 8.1	28.6 ± 1.8	33.9 ± 0.9	12.5 ± 12.50	0.06 ± 0.06	3.1 ± 3.1*	27.3 ± 1.0	36.5 ± 1.1*
	T9	2	D	0.0	0.00	15.6 ± 8.1	28.5 ± 1.5	33.7 ± 0.9	0.0	0.00	12.5 ± 6.7*	29.7 ± 1.0*	39.0 ± 1.1*
	V138	2	D	37.5 ± 18.3	0.19 ± 0.09	21.9 ± 7.4	27.5 ± 1.2	31.8 ± 1.0	12.5 ± 12.50	0.13 ± 0.13	15.6 ± 6.6*	29.4 ± 0.7*	37.7 ± 0.8*
	V465	2	D	62.5 ± 18.3	0.31 ± 0.09	31.3 ± 10.3	27.4 ± 0.5	34.0 ± 0.9	0.0	0.00	6.3 ± 4.1*	27.9 ± 1.3	37.8 ± 1.7*
	V641	2	D	0.0	0.00	3.1 ± 3.1	27.6 ± 1.0	37.4 ± 1.1	12.5 ± 12.50	0.06 ± 0.06	6.3 ± 6.3*	31.8 ± 1.1*	47.9 ± 1.6*
	V1365	2	D	0.0	0.00	9.4 ± 6.6	30.4 ± 1.4	36.0 ± 1.5	12.5 ± 12.50	0.06 ± 0.06	9.4 ± 4.6*	28.4 ± 1.4	40.4 ± 0.8*
	V1900	2	D	25.0 ± 16.4	0.13 ± 0.08	18.8 ± 7.8	28.6 ± 1.5	32.0 ± 0.5	0.0	0.00	15.6 ± 8.1*	29.4 ± 2.0*	40.6 ± 2.0*
$2B^{334}$	V549	2	ND	0.0	0.00	0.0	27.6 ± 1.5	35.9 ± 1.0	0.0	0.00	0.0	27.5 ± 1.4	44.5 ± 1.9*
$2B^{R1}$	V1374	2	ND	0.0	0.00	15.6 ± 6.6	30.3 ± 1.2	38.2 ± 0.9*	25.0 ± 16.37	0.13 ± 0.08	15.6 ± 8.1*	27.9 ± 0.8	40.7 ± 0.9*
4A	W-83	2	ND	12.5 ± 12.5	0.06 ± 0.06	25.0 ± 6.7	29.3 ± 1.4	39.6 ± 1.3*	0.0	0.00	15.6 ± 6.6*	30.0 ± 1.6*	44.2 ± 1.1*
4B	PP94	2	ND	12.5 ± 12.5	0.06 ± 0.06	15.6 ± 6.6	28.3 ± 1.7	35.1 ± 1.6	12.5 ± 12.50	0.06 ± 0.06	6.3 ± 4.1*	30.8 ± 1.7*	46.4 ± 1.7*
	V1242	2	ND	37.5 ± 18.3	0.19 ± 0.09	25.0 ± 8.2	25.9 ± 1.6	28.8 ± 0.8+	12.5 ± 12.50	0.06 ± 0.06	9.4 ± 4.6*	26.0 ± 1.8	37.3 ± 1.9*
				P	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*
Source of variation													
Isolates				nt	nt	0.1240	0.6680	<0.0001	nt	nt	0.0087	0.0121	<0.0001
Contrasts[g]													
Lineage 1A versus all others				nt	nt	nt	nt	0.2050	nt	nt	0.5054	0.3799	<0.0001
Lineage 1A versus $2B^{334}$ and $2B^{R1}$				nt	nt	nt	nt	0.2696	nt	nt	0.2504	0.1409	0.0001
Lineage 1A versus 4A				nt	nt	nt	nt	0.0356	nt	nt	0.2504	0.4169	0.1364
Lineage 1A versus 4B				nt	nt	nt	nt	0.0100	nt	nt	0.7929	0.6770	0.0003
Lineage $2B^{334}$ versus $2B^{R1}$				nt	nt	nt	nt	0.0525	nt	nt	nt	0.0487	<0.0001
Lineage $2B^{334}$ and $2B^{R1}$ versus 4A				nt	nt	nt	nt	0.0115	nt	nt	1.0000	0.7952	0.1927
Lineage $2B^{334}$ and $2B^{R1}$ versus 4B				nt	nt	nt	nt	0.2438	nt	nt	0.2409	0.1304	0.8575
Lineage 4A versus 4B				nt	nt	nt	nt	0.0005	nt	nt	0.2409	0.3270	0.2469

[a]Data are means (±standard error) of 16 replicated plants per treatment combination. See text for experimental details.

[b]Determined by PCR assays using primer pairs VdAve1F/VdAve1R (de Jonge *et al.*, 2012; race 1) and Tr1/Tr2 (Usami *et al.*, 2007; race 1) and VdR2F/VdR2R (Short *et al.*, 2014; race 2).

[c]Mean disease severity assessed 7 weeks after inoculation according to the percentage of foliage affected in acropetal progression (0 = no symptoms, 1 = 1–33%, 2 = 34–66%, 3 = 67–100%, and 4 = dead plant). Mean for an isolate followed by an asterisk (*) is significantly different from that of race 1 isolate V11 at $P < 0.05$.

[d]Area under the disease severity progress curve over time calculated by the trapezoidal integration method (Campbell & Madden, 1990) standardized by duration of disease development. Mean for an isolate followed by an asterisk (*) is significantly different from that of race 1 isolate V11 at $P < 0.05$.

[e]Determined by isolation of *V. dahliae* from four stem pieces of each inoculated plant and incubating at 24 ± 1 °C for 7 days. Mean for an isolate followed by an asterisk (*) is significantly different from that of race 1 isolate V11 at $P < 0.05$.

[f]Determined from the cotyledonary node 7 weeks after inoculation. Mean plant height for an isolate followed by an asterisk (*) and/or a plus sign (+) is significantly different from that corresponding to race 1 isolate V11 (*) or the noninoculated control (+) at $P < 0.05$.

[g]Linear single-degree of freedom contrasts were computed to test the effect of selected experimental treatment combinations at $P < 0.05$. Underlined values indicate that estimated value for the first term of the contrast is lower than that of the second term. nt, not tested.

Table 6 Virulence of *Verticillium dahliae* isolates, in different clonal lineages, to tomato cultivars (Experiment III)[a]

Tomato cultivar	Lineage	Isolate	Disease incidence (%)	Disease severity (0–4)[b]	SAUDPC[c]	Stem colonization (%)[d]	Fresh weight (g)
San Pedro	–	Control	–	–	–	–	41.7 ± 4.6
	2A	V11	100	1.63 ± 0.13	1.10 ± 0.05	62.5 ± 8.0	24.0 ± 2.2
	$2B^{824}$	Ver12	0.0	0.00	0.00	41.7 ± 21.0	32.9 ± 3.3
	$2B^{824}$	Ver13	25.0 ± 25.0	0.13 ± 0.13	0.12 ± 0.12	20.8 ± 12.5	39.9 ± 5.8
	4A	W-83	50.0 ± 28.9	0.25 ± 0.14	0.16 ± 0.09	50.0 ± 11.8	39.6 ± 3.5
Vemone	–	Control	–	–	–	–	36.0 ± 0.6
	2A	V11	100	1.25 ± 0.14	0.89 ± 0.09	66.7 ± 6.8	27.8 ± 2.9
	$2B^{824}$	Ver12	0.0	0.00	0.00	16.7 ± 16.7	41.9 ± 4.1
	$2B^{824}$	Ver13	0.0	0.00	0.00	8.3 ± 8.3	37.3 ± 10.3
	4A	W-83	0.0	0.00	0.00	16.7 ± 9.6	37.3 ± 3.0
Boa	–	Control	–	–	–	–	43.9 ± 0.1
	2A	V11	0.0	0.00	0.00	50.0 ± 13.6	38.4 ± 5.2
	$2B^{824}$	Ver12	0.0	0.00	0.00	0.0	28.9 ± 2.7
	$2B^{824}$	Ver13	0.0	0.00	0.00	0.0	40.8 ± 6.0
	4A	W-83	66.7 ± 33.3	0.67 ± 0.33	0.15 ± 0.07	38.9 ± 5.6	39.7 ± 2.3
Marmande	–	Control	–	–	–	–	37.5 ± 0.0
	2A	V11	0.0	0.00	0.00	62.5 ± 4.2	36.8 ± 2.0
	$2B^{824}$	Ver12	0.0	0.00	0.00	25.0 ± 25.0	30.0 ± 3.5
	$2B^{824}$	Ver13	0.0	0.00	0.00	8.3 ± 8.3	26.0 ± 2.3
	4A	W-83	0.0	0.00	0.00	50.0 ± 20.4	36.5 ± 2.9

[a]Data are means (±standard error) of six replicated plants per treatment combination. See text for experimental details.
[b]Mean disease severity assessed 7 weeks after inoculation according to the percentage of foliage affected in acropetal progression (0 = no symptoms, 1 = 1–33%, 2 = 34–66%, 3 = 67–100%, and 4 = dead plant).
[c]Area under the disease severity progress curve over time calculated by the trapezoidal integration method (Campbell & Madden, 1990) standardized by duration of disease development.
[d]Determined by isolation of *V. dahliae* from four stem pieces of each inoculated plant and incubating at 24 ± 1 °C for 7 days.

Table 7 Virulence of race 2 *Verticillium dahliae* isolates, in different clonal lineages, to olive cultivar Picual and tomato cultivars Bonny Best and Rio Grande (Experiment IV)[a]

Host	Cultivar	Lineage	Isolate	Disease incidence (%)	Disease severity (0–4)[b]	SAUDPC[c]	Stem colonization (%)[d]
Olive	Picual[e]	1A	V669	16.7 ± 16.7	0.67 ± 0.67	0.57 ± 0.57	22.2 ± 16.5
		1A	V781	33.3 ± 21.1	0.75 ± 0.66	0.26 ± 0.22	22.9 ± 16.6
		1A	V1365	83.3 ± 16.7	2.08 ± 0.72	1.02 ± 0.41	75.0 ± 15.5
	Picual[f]	1A	V669	50.0 ± 28.9	0.33 ± 0.24	0.15 ± 0.13	13.8 ± 10.1
		1A	V781	50.0 ± 28.9	1.38 ± 0.94	0.62 ± 0.47	28.1 ± 24.1
		1A	V1365	50.0 ± 28.9	1.50 ± 0.96	0.71 ± 0.56	50.0 ± 28.9
Tomato	Bonny Best[f]	1A	V669	0.0	0.00	0.00	10.0 ± 5.5
		1A	V781	10.0 ± 10.0	0.05 ± 0.05	0.04 ± 0.04	45.0 ± 10.4
		1A	V1365	0.0	0.00	0.00	60.0 ± 5.5
		$2B^{R1}$	V1236	10.0 ± 10.0	0.05 ± 0.05	0.03 ± 0.03	47.5 ± 5.8
	Rio Grande[f]	1A	V669	0.0	0.00	0.00	2.5 ± 2.5
		1A	V781	0.0	0.00	0.00	35.0 ± 10.0
		1A	V1365	10.0 ± 10.0	0.05 ± 0.05	0.03 ± 0.03	22.5 ± 8.7
		$2B^{R1}$	V1236	10.0 ± 10.0	0.05 ± 0.05	0.03 ± 0.03	80.0 ± 5.0

[a]Data are means (±standard error) of six (olive) or 10 (tomato) replicated plants per treatment combination. See text for experimental details.
[b]Mean disease severity assessed 7 weeks after inoculation according to the percentage of foliage affected in acropetal progression (0 = no symptoms, 1 = 1–33%, 2 = 34–66%, 3 = 67–100%, and 4 = dead plant).
[c]Area under the disease severity progress curve over time calculated by the trapezoidal integration method (Campbell & Madden, 1990) standardized by duration of disease development.
[d]Determined by isolation of *V. dahliae* from four stem pieces of each inoculated plant and incubating at 24 ± 1 °C for 7 days.
[e]Plants were inoculated by transplanting to a soil mixture infested with 1.5 to 5.3 × 10^7 colony-forming units per g soil depending on the isolate.
[f]Plants were inoculated by root dip in a suspension of 10^7 conidia mL^{-1} for 20 min.

in lineage 2A; and conversely, all lineage 2A isolates were in race 1. Lineage 2A isolates were sampled from five crop species, in four countries, and were ND in pathotype. Moreover, the 900-bp nucleotide sequences of the race 1-specific *Ave1* gene were identical among all 27 lineage 2A isolates, and identical to homologous

sequences in GenBank. The finding of race 1 in a single clonal lineage, with identical *Ave1* sequences among isolates, is consistent with the hypothesis that race 1 arose once in *V. dahliae*. Acquisition of effector gene *Ave1* is likely to have occurred by HGT (de Jonge *et al.*, 2012).

Molecular markers and virulence assays also confirmed the well-established fact that the D pathotype is found only in lineage 1A, and all isolates in lineage 1A have the D pathotype (Korolev *et al.*, 2008; Milgroom *et al.*, 2014). Results from the virulence assays showed that only isolates in lineage 1A, regardless of their host source (cotton or olive) and geographic origin, caused severe defoliation of cotton cultivar Acala SJ-2 (Fig. 3), whereas isolates from all other lineages tested were less virulent and caused the ND syndrome. Lineage 1A isolate T9 was somewhat less virulent compared with other D isolates. However, this isolate has been maintained in culture since the 1960s as a single-spore isolate from isolate T-1, which was the first D pathotype isolate reported, from cotton in California in 1964 (Schnathorst & Mathre, 1966; Puhalla & Mayfield, 1974). Isolate T9 may have lower virulence because it has been in culture for so long. Lineage 1A (D pathotype) isolates also varied in virulence to olive cultivar Picual, although they all caused severe defoliation characteristic of the D syndrome, as described earlier (Jiménez-Díaz *et al.*, 2012). Previous work showed that ND isolates in lineages 2A, $2B^{824}$ and 4B were significantly less virulent on Picual olive compared with lineage 1A isolates (Tsror & Levin, 2003; Jiménez-Díaz *et al.*, 2012). Race 1 isolates were not assayed on olive in this study. By contrast, none of the lineage 1A (D pathotype) isolates were pathogenic to wild olive clones, which had previously been tested with only two other D pathotype isolates (Jiménez-Fernández *et al.*, 2016; Palomares-Rius *et al.*, 2016).

In contrast to the simple relationships between race 1 with lineage 2A, and the D pathotype with lineage 1A, several studies in the literature have reported correlations among markers that are confusing and/or contradictory. For example, Hu *et al.* (2015) recently used pathotype-specific and race-specific PCR to characterize *V. dahliae* isolates from cotton in different countries. They concluded that D and ND pathotypes strongly correlate with race 2 and race 1, respectively. Based on results from the present study, an alternative interpretation of this correlation is that the *V. dahliae* population infecting cotton in their study comprised individuals primarily in lineages 1A (D pathotype, race 2) or 2A (ND pathotype, race 1). The correlation would be considerably weaker if samples were more diverse and contained a larger proportion of isolates of different lineages, all of which were characterized in the present study as ND pathotype and race 2. No lineage or VCG data were presented by Hu *et al.* (2015); therefore, it was not possible to assess this alternative interpretation empirically. By contrast, cotton isolates genotyped in the present study were in four lineages: 1A and 2A, as well as $2B^{824}$ and 4B. These latter two lineages, with ND pathotype and race 2, contradict the correlation found by Hu *et al.* (2015); the lack of knowledge of the identity of clonal lineages (or VCGs) in this previous study meant that factors important for understanding pathogenic variation in *V. dahliae* were missing.

Unless the specific genes underlying a phenotype have been identified, as in the case of *Ave1* and race 1, the clonality of *V. dahliae* populations provide the clearest interpretations for any correlations among biological characteristics such as pathotype and genetic markers. For example, markers for the D pathotype identify clonal lineage 1A, whereas those for the ND pathotype identify all other lineages. Although these markers identify clonal lineages, they are functionally unrelated to the phenotype. As such, the designation of the ND pathotype is not evolutionarily informative. The D pathotype arose once (Milgroom *et al.*, 2016), whereas the ND pathotype was found in all other lineages as the ancestral state. The markers to identify *Ave1* are functionally specific for the race 1 phenotype, which was found only in lineage 2A. By contrast, the race 2-specific marker is part of a gene that encodes a hypothetical protein (Short *et al.*, 2014). PCR primers for the race 2-specific marker were designed to amplify sequences from isolates that lack *Ave1*, not because this marker is functionally involved in virulence that determines the race 2 phenotype, but because the primers for it could be designed purposely to exclude amplification from lineage 2A/race 1 individuals. Not surprisingly, sequence variation in this marker correlated with genetic relatedness among these clonal lineages (Jiménez-Díaz *et al.*, 2012; Jiménez-Gasco *et al.*, 2014; Milgroom *et al.*, 2014), as one might expect for any random sample of sequences among lineages.

Results from a small number of isolates in previous studies contradicted the strict relationship found in the present study between race 1 and lineage 2A. For example, one race 2 isolate (Dvd-S85) was found in VCG2A, and one race 1 isolate (Dvd-T3) was found in VCG2B (Dobinson *et al.*, 1998). Genotyping of these two isolates (obtained from the Canadian Collection of Fungal Cultures, Ottawa) with microsatellite markers, however, showed that both isolates had multilocus genotypes nearly identical to each other and to lineage 2A isolates (authors' unpublished results). Similarly, race 1 isolate JR2 was reported to be genetically almost identical (in the same lineage) to race 2 isolate VdLs17, and race 2 isolate DVD-s26 was nearly identical to two race 1 isolates (St14.01 and CBS381.66) (de Jonge *et al.*, 2012, 2013). An assessment of SNPs unique to each clonal lineage (derived from Milgroom *et al.*, 2014) in the genome sequences of these isolates reported by de Jonge *et al.* (2013), showed that race 1 isolate JR2 is in lineage $2B^{824}$ (along with race 2 isolate VdLs17), and race 2 isolate DVD-s26 is in lineage 2A (along with race 1 isolates DVD-s29, St14.01 and CBS381.66; authors' unpublished results). These findings contradict the correlation of race 1 with lineage 2A. Regardless of these few exceptions, results from the present survey of a large sample of

isolates showed a clear correspondence between race 1 and lineage 2A. Discrepancies may have been caused by the loss of *Ave1* by mutation in lineage 2A isolates (making them race 2), or recombination of *Ave1* into individuals of different lineages (making them race 1), which could only be detected by further genotyping (Milgroom *et al.*, 2014). A reanalysis of the original isolates from these other studies for a variety of genetic markers is currently underway to test these hypotheses.

In the present study, all isolates, except two in lineage 6, yielded positive amplification with either race 1-specific primers or race 2-specific primers, but not both. Lineage 6 has only been reported for a few isolates from pepper in California (Bhat *et al.*, 2003) and it was interpreted as a recombinant between lineages $2B^{824}$ and 4A (Milgroom *et al.*, 2014), which in this present work were demonstrated to comprise race 2 isolates. Lineage 6 isolates were previously confirmed as *V. dahliae* by PCR assays using *V. dahliae*-specific primers (Jiménez-Díaz *et al.*, 2012; Inderbitzin *et al.*, 2013) with the same template DNA used in the present study; thus, the DNA was of high enough quality for PCR and should have amplified with the race-specific primers. It is possible that the recombination event that gave rise to lineage 6 could have disrupted the genomic region amplified by the race 2-specific marker. However, no problems were encountered in the amplification of race 2-specific markers in isolates in lineages $2B^{R1}$ and $2B^{334}$, which also arose by recombination (Jiménez-Díaz *et al.*, 2006; Milgroom *et al.*, 2014). Therefore, the absence of amplification from the two isolates of lineage 6 is presently unexplained.

The results of pathogenicity assays on tomato cultivar San Pedro, which lacks *Ve1*, the race 1 isolate V11 (with *Ave1*) caused significantly more severe foliar symptoms and reduced plant fresh weight more than race 2 isolates, which lack *Ave1*, regardless of their clonal lineage. This was in agreement with findings by de Jonge *et al.* (2012, 2013) who showed that *Ave1* acted as a virulence factor in *V. dahliae* inoculated on tomato plants lacking the *Ve1* resistance gene. Race 1 tomato isolate V11 was also more virulent than race 2 isolates on cotton cultivar Acala SJ-2. Race 2 isolates from diverse clonal lineages had low virulence on susceptible tomato cultivars San Pedro and Rio Grande, causing only incipient foliar symptoms, if any, no reduction in plant height, and only small reductions in fresh weight. In particular, isolates of the D pathotype (lineage 1A) are highly virulent on cotton and olive but were only able to cause small reductions in fresh weight of tomato plants, whether or not they carried the *Ve1* gene. These results agree with work by several authors who reported that D isolates from cotton were weakly virulent (Schnathorst & Mathre, 1966) or not pathogenic (Bhat & Subbarao, 1999; Korolev *et al.*, 2008) on tomato cultivars lacking *Ve1*. These results are also consistent with the concept that *V. dahliae* is host adapted, not host specialized; that is, individuals may be pathogenic on multiple hosts but are usually more virulent on some hosts, typically those from which they were isolated (Koike *et al.*, 1994; Bhat & Subbarao, 1999; Douhan & Johnson, 2001; Jiménez-Díaz *et al.*, 2006). Caution is needed in interpreting the underlying cause of differences in virulence. Although race 1 and race 2 may differ in virulence because of the presence or absence of *Ave1*, these races are also present in different clonal lineages that have many additional genetic differences that may affect host adaptation.

In summary, results of this research reinforce the concept of a strong clonal structure of *V. dahliae* populations despite sampling from diverse hosts and geographic regions. Pathogenic variation in this fungus, in particular, the defoliating (D) pathotype and race 1, can be explained best by the emergence of these phenotypes in specific clonal lineages. The D pathotype is restricted to lineage 1A, which arose in North America and subsequently spread widely to other continents (Milgroom *et al.*, 2016). Race 1 was found exclusively in clonal lineage 2A in the present study, consistent with the hypothesis that it arose once, most likely by horizontal gene transfer (de Jonge *et al.*, 2012). Further study will be needed to explore the exceptions to this pattern previously reported for a few isolates (Dobinson *et al.*, 1998; de Jonge *et al.*, 2013). Nonetheless, explaining pathogenic variation in terms of clonal lineages brings a biological interpretation to the study of this fungus instead of a series of correlative studies with results that vary depending on the specific population sampled.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Figure S1. Assays for heterokaryon formation to identify vegetative compatibility groups (VCG) in *Verticillium dahliae*. (a) Complementation of isolate W4 74 BR (left) and lack of complementation of race 1 isolate PP242 (right) with VCG1A tester isolate T9 (centre). (b) Complementation of race 1 isolates PP245 (left) and 5M (right) with VCG2A tester Ep8M (centre). (c) Complementation of isolate PP280 (left) and lack of complementation of isolate PP242 (right) with VCG1A tester isolate T9 (centre). (d) Lack of complementation of isolate PP36 (left) and complementation of isolate PP94 (right) with VCG4B tester Pt15 (centre). (e) Lack of complementation of VCG4B tester Pt15 (left) and complementation of VCG2B tester Cot11 (right) with isolate VdPp (centre).

Figure S2. Gel electrophoresis of PCR amplifications using primers specific for *Verticillium dahliae sensu stricto* (Inderbitzin *et al.*, 2013) and defoliating (D) and nondefoliating (ND) pathotypes (Jiménez-Díaz *et al.*, 2006, 2012). (a) PCR amplification of *V. dahliae* (490 bp) amplicon generated with species-specific primers Df and Dr (Inderbitzin *et al.*, 2013). (b) PCR amplification of *V. dahliae* (523/539 bp) and D-specific (334 bp) amplicons (Jiménez-Díaz *et al.*, 2012). (c) PCR amplification of D- (462 bp) and ND-specific (824 bp) amplicons jointly (Jiménez-Díaz *et al.*, 2006). Isolates Vd, V11, V598 from tomato (Spain) are in lineage 2A. Isolates Ver12 and Ver13, also from tomato (Spain), are in lineage $2B^{824}$. Isolates V560 and V561 from pepper (USA) are in lineage 6. Isolates V138 and V176 from cotton (Spain) are in lineages 1A and 2A, respectively, and were used as positive controls for the D and ND pathotypes, respectively. Numbers 1 and 2 appended to isolate names indicate replications of PCR assays of these isolates. PCR for isolates Ver12 in panel C failed; no amplicon can be detected visually. Sterile distilled water was used as a negative control. Lane M, Gene-ruler DNA ladder mix (Fermentas) used as a DNA size marker.

Table S1. *Verticillium dahliae* isolates used in this study listed by lineage, host source, geographic origin, reference code, pathotype as determined by pathogenicity test, pattern of specific polymerase chain reaction (PCR) genotypes, race as determined by PCR marker, and sequence haplotypes for a race-2-specific sequence.