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Differentiation of cotton-defoliating and nondefoliating pathotypes of *Verticillium dahliae* by RAPD and specific PCR analyses

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Abstract

Severe Verticillium wilt of cotton in southern Spain is associated with the spread of a highly virulent, defoliating (*D*) pathotype of *Verticillium dahliae*. Eleven of the *D* and 15 of a mildly virulent, nondefoliating (*ND*) pathotype were analyzed by random amplified polymorphic DNA (RAPD) using the polymerase chain reaction (PCR). Six of 21 primers tested generated pathotype-associated RAPD bands. Another 21 *V. dahliae* isolates were compared in blind trials both by RAPD-PCR using the six selected primers and pathogenicity tests on cotton cultivars. There was a 100% correlation between pathotype characterization by each method. Unweighted paired group method with arithmetic averages cluster analysis was used to divide the 47 *V. dahliae* isolates into two clusters that correlated with the *D* or *ND* pathotypes. There was more diversity among *ND* isolates than among *D* isolates, these latter isolates being almost identical. *ND*- and *D*-associated RAPD bands of 2.0 and 1.0 kb, respectively, were cloned, sequenced, and used to design specific primers for the *D* and *ND* pathotypes. These pathotype-associated RAPD bands were present only in the genome of the pathotype from which they were amplified, as shown by Southern hybridization. The specific primers amplified only one DNA band of the expected size, and in the correct pathotype, when used for PCR with high annealing temperature. These specific primers successfully characterized *V. dahliae* cotton isolates from China and California as to *D* or *ND* pathotypes, thus demonstrating the validity and wide applicability of the results.

Introduction

About 98,000 ha of upland cotton (*Gossypium hirsutum* L.) are grown in the Guadalquivir Valley of Andalucía, southern Spain, accounting for ca. 95% of the national area for cotton production (Anonymous, 1998). In this area, Verticillium wilt caused by *Verticillium dahliae* Kleb. is widespread and is now considered to be the most important disease of the crop (Bejarano-Alcázar et al., 1996; 1997). Verticillium wilt is also recognized as one of the major production constraints of irrigated cotton in the USA (Cotton et al., 1969) and in most cotton-growing areas of the world (Bell, 1992).

Isolates of *V. dahliae* show variation in morphology and virulence (Bell, 1994). Schnathorst and Mathre (1966) first showed that different levels of virulence (i.e., amount of disease caused in a host genotype) occur among *V. dahliae* isolates from cotton in California, and classified them as defoliating (*D*) or

nondefoliating (*ND*) pathotypes according to their ability to completely defoliate cotton or to cause only mild wilt and no defoliation, respectively. While the *ND* pathotype of *V. dahliae* is widespread, the *D* pathotype has been reported only from a few places in the Americas (Mathre et al., 1966; Schnathorst, 1969), China (Zhengjun et al., 1998) and Spain (Bejarano-Alcázar et al., 1996).

Differences in virulence between *D* and *ND* pathotypes of *V. dahliae* bear significance in terms of disease severity and management. Thus, the increased importance of Verticillium wilt of cotton in southern Spain was associated with the spread of the *D* pathotype in the lower Guadalquivir Valley in the early 1980s (Bejarano-Alcázar et al., 1996). At equal inoculum densities, epidemics caused by the *D* pathotype develop earlier and more rapidly, and produce significantly greater yield loss as compared to those caused by the *ND* pathotype (Bejarano-Alcázar et al., 1995; 1997). Management of the disease is best achieved by tolerant cotton cultivars, the effectiveness of which is overcome by the highly virulent *D* pathotype (Bell, 1994; Schnathorst and Mathre, 1966). Therefore, the identification of pathotypes in populations of *V. dahliae* infecting cotton is of importance for disease resistance breeding, as well as for the efficient use of available cultivars with tolerance to the disease.

Cotton isolates of *V. dahliae* can be characterized as to pathotype by means of morphological and physiological traits as well as by virulence tests on cotton cultivars (Bejarano-Alcázar et al., 1996; Schnathorst and Mathre, 1966). However, typing isolates by these procedures can be cumbersome, time-consuming, and results are not informative on the genetic diversity within, or relationships among, populations of the pathogen. Consequently, improved methods are needed for the rapid and informative characterization of *D* and *ND V. dahliae* isolates. In recent years, different molecular genetic techniques have been applied for the characterization of *V. dahliae*. Studies using the polymerase chain reaction (PCR) (Nazar et al., 1991) and restriction fragment length polymorphisms (RFLPs) (Carder and Barbara, 1991) showed the utility of these techniques for differentiating *V. albo-atrum* Reinke & Berthold from *V. dahliae*, as well as in detecting a high degree of genetic diversity within each species. Cluster analysis of RFLP data from isolates of *V. dahliae* from different host species was partially correlated with host specificity (Carder and Barbara, 1994; Okoli et al., 1993; 1994). More recently, random amplified polymorphic DNA (RAPD) analysis (Williams et al., 1990) was used for the characterization of *V. dahliae* isolates from several crop species, and to differentiate isolates of the pathogen into groups that correlate with their host range (Koike et al., 1996; Messner et al., 1996). RAPD analysis of cotton isolates of *V. dahliae* in Australia enabled the genetic similarity of isolates to be estimated, but no correlation was found between banding patterns and virulence characteristics (Ramsay et al., 1996). Therefore, more research is needed for the molecular characterization of cotton *D* and *ND* pathotypes of *V. dahliae*.

RAPD analysis is useful in identifying variation and pathotypes in a wide variety of phytopathogenic fungi (e.g., Assigbetse et al., 1994; Balardin et al., 1997; Kelly et al., 1994). It can be applied without prior genetic information about the organism, is rapid, inexpensive, requires minimal amounts of DNA, and does not require radioactivity. Sequence characterized amplified regions (SCARS) have been used for the specific amplification of the target DNA (Kelly et al., 1998; McDermott et al., 1994). The aim of this study was to test the hypothesis that isolates of *D* and *ND* pathotypes of *V. dahliae* could be differentiated by means of RAPD analysis, and to design specific PCR primers based on sequences of pathotype-associated RAPD bands that would provide a rapid method for the specific diagnosis of the above pathotypes.

Materials and methods

Fungal isolates and culture conditions

In the first part of this study, a total of 47 monosporic isolates of *V. dahliae* was used, including 35 from cotton and 12 from olive trees (*Olea europaea* L.) mainly from southern Spain (Table 1). Twenty-six of the 47 isolates had been previously characterized as *D* ($n = 11$) or *ND* ($n = 15$) pathotypes by means of biological pathotyping (Bejarano-Alcázar et al., 1995; 1996). The remaining 21 *V. dahliae* isolates were typed by RAPD analysis and pathogenicity tests in blind trials in this study. In addition, the following soilborne fungi were used for RAPD studies: *V. albo-atrum* Reinke and Berthold, *V. nigrescens* Pethybr., *V. tricorpus* Isaac, *Fusarium culmorum* (W.G. Sm.) Sacc., *F. graminearum* Schwäbe, *F. moniliforme* Sheldon, *F. solani* (Mart.) Sacc., *F. oxysporum* Schlechtend.: Fr. f.sp. *ciceris* (Padwick) Matuo & Sato,

Table 1. Collection information and pathotype of isolates of *V. dahliae* used in this study

Isolate(s)[a]	Host origin	Geographical location[b]	Pathotype by	
			Pathogenicity test	RAPD analysis
Group A				
V4I, V200I, V204I, V209I–V211I	Cotton	Central valley	ND	ND
V176I, V179I, V183I, V185I, V188I, V192I, V197I	Cotton	Lower valley	ND	ND
V213I, V217I	Cotton	Upper valley	ND	ND
V117I, V177I, V180I, V184I, V186I, V187I, V190I, V219I–V22	Cotton	Lower valley	D	D
Group B				
V173I, V174I	Cotton	Southern Spain	ND	ND
V142I	Cotton	Upper valley	ND	ND
V147I–V149I	Olive tree	Central Spain	ND	ND
V152I	Olive tree	Central valley	ND	ND
V143I–V145I	Olive tree	Upper valley	ND	ND
V135I	Olive tree	Central valley	D	D
V136I, V150I, V151I	Olive tree	Lower valley	D	D
V153I	Olive tree	Upper valley	D	D
V138I	Cotton	Upper valley	D	D
V168I–V172I	Cotton	Southern Spain	D	D

[a]Isolates in group A were characterized to pathotype by host inoculation before RAPD analysis. Isolates in group B were characterized to pathotype by host inoculation and RAPD analysis in 'blind trials'.

[b]Lower, Central and Upper areas in the Guadalquivir Valley of Andalucia, southern Spain (Bejarano-Alcázar et al., 1996). For some isolates, the specific area of location within the valley was not determined. Location of these isolates is indicated as southern Spain.

F. oxysporum f.sp. *melonis* W.C. Snyder & H.N. Hans., *F. oxysporum* f.sp. *niveum* (E.F. Smith) W.C. Snyder & H.N. Hans. and *F. oxysporum* f.sp. *phaseoli* Kendr. & W.C. Snyder. These represent fungi within a range of phytopathological and ecological habits and were used to check for cross-reaction. All fungal isolates are from the culture collection of the Departamento de Protección de Cultivos, Instituto de Agricultura Sostenible, Córdoba, Spain.

In the second part of this study, 20 additional *V. dahliae* isolates from diverse geographical origins were used, to further test the validity of molecular markers developed for the characterization of *D* and *ND* pathotypes. These included eight olive isolates from Italy (I1 through I8), three cotton isolates from China (Vd8, JY and HB3) and nine cotton isolates from the USA (T9-1, T9-2, SS4-1, SS4-2 and V318I through V322I). *V. dahliae* isolates from Italy were kindly provided by Professor A. Zazzerini (Instituto di Patologia Vegetale, Universitá degli Studi di Perugia, Italy); isolates Vd8, JY, HB3, T9-1 and SS4-1 by Professor J. Katan (Department of Plant Pathology and Microbiology, The Hebrew University of Jerusalem, Israel); and isolates T9-2 and SS4-2 by Dr. E. Paplomatas (Department of Plant Pathology, Benaki Phytopathological Institute, Greece) and Professor J. De Vay (Department of Plant Pathology, University of California, Davis, California), respectively. Isolates V318I through V322I were collected by the senior author from cotton in California. Isolates T9-1 and T9-2, and isolates SS4-1 and SS4-2, are representatives of the *D* and *ND* pathotypes from California (Puhalla and Hummel, 1983; Schnathorst and Mathre, 1966), respectively; while isolates Vd8 and HB3 have been assigned to VCG 1 in China and belong to the *D* pathotype (Zhengjun et al., 1998).

Isolates of *Verticillium* spp. were stored axenically by covering cultures on plum extract agar with liquid paraffin (Bejarano-Alcázar et al., 1996), and those of *Fusarium* spp. in sterile soil in test tubes. All isolates were kept at 4 °C in the dark. Active cultures of isolates were obtained by placing small agar plugs or soil aliquots from stock cultures on aureomycin-amended water agar and further subculture on potato–dextrose

agar (PDA). Cultures on PDA were grown for 7–10 days at 25 °C in the dark, then transferred as single-spore cultures to PDA.

DNA extraction

For DNA extraction, 1 ml of conidial suspension (5×10^6 conidia/ml) was transferred to flasks containing 100 ml of Czapeck–Dox broth supplemented with yeast extract, malt extract, mycological peptone and caseine hydrolysate (2 g/l each) (Typas et al., 1992). Cultures were incubated for 4–9 days at 25 °C on an orbital shaker at 125 rpm, in the dark. After incubation mycelia were recovered by filtration, lyophilized and ground with an autoclaved pestle and mortar. DNA was extracted from 50 mg of lyophilized ground mycelium using a scaled-down method of Raeder and Broda (1985), and quantified by measuring optical density at 260 nm. Aliquots of samples were also analyzed on a 0.7% agarose gel in Tris–acetate EDTA buffer (40 mM Tris–acetate and 1 mM EDTA) as described by Sambrook et al. (1989).

Pathogenicity tests

Cotton cultivar Acala SJ-2 and line PI 70–110 were used for pathotyping 21 *V. dahliae* isolates by stem-puncture inoculation as described previously (Bejarano-Alcázar et al., 1996). *V. dahliae* isolates V4I and V117I , previously characterized as *ND* and *D* isolates, respectively, were used as reference isolates (Bejarano-Alcázar et al.,1996). Seeds were disinfested in 1% NaOCl for 2.5 min, germinated and sown in 15-cm diameter clay pots (one plant per pot) filled with a sterilized potting mixture (sand/clay loam/peat; 1 : 1 : 1, vol/vol/vol; autoclaved twice at 121 °C for 90 min). Plants were grown in a growth chamber adjusted to a 14-h photoperiod of fluorescent light of 216–270 $\mu E\, m^{-2}\, s^{-1}$. Temperature and relative humidity, respectively, were 21–27 °C and 50–90% during the light period, and 18–22 °C and 60–100% during the dark period. Plants were watered as required and fertilized every 2 weeks with a water- soluble fertilizer (20–10–20, N–P–K). Plants 6-weeks-old were inoculated with a suspension of $2.5–3 \times 10^6$ conidia/ml in sterile distilled water. Control plants were treated similarly with sterile distilled water (Bejarano-Alcázar et al., 1996). Disease symptoms were observed daily and symptom severity was assessed on a 0–4 scale according to the percentage of foliage affected by chlorotic, necrotic, and wilt symptoms and/or defoliation, in an acropetal progression (0 = no symptoms; 1 = 1–33% foliage affected; 2 = 34–66% foliage affected; 3 = 67–100% foliage affected; 4 = dead plant) at 2, 3, 4 and 5 weeks after inoculation. The experiment had a randomized complete block design with 10 replicated plants per isolate/cultivar combination. Analysis of variance and mean comparisons were performed with data on final disease severity. The experiment was repeated once.

RAPD analysis

RAPD reactions (Williams et al., 1990) were carried out with 21 primers, of which 20 were 10-mer oligonucleotide primers corresponding to the OPH primer set (Operon Technology, Alameda, CA). The additional primer was KS (Kelly et al., 1994) derived from the polycloning site of the phagemid pBluescript (Stratagene, Cambridge, UK). The RAPD-PCR amplification reactions (25 µl) consisted of 0.5 µM of primer, 200 µM of each dNTP, 2.5 µl of 10 × reaction buffer (50 µM KCl, 10 mM Tris–HCl pH 9.0 (25 °C), 1% v/v triton X-100), 1.5 U of *Taq* DNA polymerase (Promega, Madison, WI), 2.5 mM $MgCl_2$ and 100 ng of fungal DNA. Reactions were performed in a 9600 thermocycler (Perkin-Elmer, Norwalk, CT). Reaction conditions were: denaturation at 94 °C for 5 min followed by 30 cycles of 1 min of annealing at 37 °C (for 10-mer primers) or 42 °C (for the KS primer), extension for 3 min at 72 °C, and denaturation for 1 min at 94 °C. The final cycle consisted of 1 min of annealing followed by 6 min at 72 °C to produce fully double-stranded fragments. Temperature between annealing and extension steps increased at 0.6 °C/s. Amplification products were separated on 1.5% agarose gels at $1.5\ V\ cm^{-1}$ and visualized under UV light after they were stained with ethidium bromide. The DNA markers used for electrophoresis were from Boehringer-Mannheim (Barcelona, Spain). All reactions were repeated at least three times and always included negative controls (no template DNA).

Analysis of similarity using RAPD bands

Six primers were selected for their capacity to produce polymorphic DNA bands. RAPDs generated by single-primer PCR were used to compare relatedness

of isolates. Bands were scored for presence or absence with '1' or '0', repectively. A binary matrix combined all the data records for all 47 isolates for all six primers. The numerical taxonomic software package NTSYS 1.80 (Rohlf, 1988) was used to order the isolates by an unweighted paired group method with arithmetic averages (UPGMA) based on Jaccard's similarity coefficient (Sneath and Sokal, 1973), which disregards negative matches between pairs of isolates and provides a more accurate picture of relatedness.

Cloning and sequencing of pathotype-associated RAPD markers

Two pairs of specific PCR primers were designed from pathotype-associated bands generated by RAPD-PCR using primers OPH-19 (2.0 kb, *ND*) and OPH-20 (1.0 kb, *D*). The specific DNA fragments were amplified for cloning by using *Ultma*™ (Perkin-Elmer) instead of *Taq* DNA polymerase (Promega), to reduce the rate of error and generate blunt-end fragments. Amplification conditions were the same as described previously for RAPD studies. The amplification products were resolved on 1.5% agarose gels in 1 × TAE, and the pathotype-associated bands were recovered using Gene-Clean (Bio 101, Vista, CA) or Magic™ clean-up columns (Promega) according to the manufacturer's instructions. The fragments were then ligated into *Sma* I-digested plasmid pUC118 (Promega), and used to transform competent *E. coli* DH5α cells. Colonies harboring recombinant plasmids were detected by blue/white colony screening on ampicillin/IPTG/X-gal Luria-Bertani plates according to the manufacturer's instructions. Plasmids from white colonies were subsequently screened for insert size. Plasmid DNA was isolated from 3 ml of overnight cultures of recombinant clones using Magic™ Mini Preps (Promega), according to the manufacturer's instructions. Clones containing an insert of the correct size were further analyzed by labelling and hybridization back to the original RAPD Southern blots. These clones were also probed against genomic *Eco*RI-digested DNA from *D* and *ND* isolates of *V. dahliae*.

The inserts from one plasmid harboring the *ND*-associated 2.0 kb band, named pND2, and from another containing the *D*-associated 1.0 kb band, named pD1, were sequenced using the dye-terminator primer cycle-sequencing kit and the Applied Biosystems apparatus (model 373A). Both strands were sequenced using the universal pUC/M13 primers forward (−21) and reverse. From the resulting sequences, the following specific PCR primers were designed using the PRIMER routine of GCG (Devereux et al., 1984): D-1 (5′-CATGTTGCTCTGTTGACTGG-3′); D-2 (5′-GACACGGTATCTTTGCTGAA-3′); ND-1 (5′-CAGGGGATACTGGTACGAGACG-3′); and ND-2 (5′-ATGAGTATTGCCGATAAGAACA-3′).

Labelling of probe DNA, Southern blotting and hybridization of transferred DNA

The *D*- and *ND*-associated RAPD DNA bands were labelled using DIG-11-dUTP (digoxigenin-3-O-methylcarbonyl-amino-caproyl-5-(3-aminoallyl)-uridine-5′-triphosphate, Boehringer-Mannheim) according to the manufacturer's instructions. For Southern blots using RAPD products, 15 µl of a 25 µl amplification mixture were resolved on 1.5% agarose gels and transferred onto Hybond N membranes (Amersham International, Uppsala, Sweden) by capillarity. When genomic DNA was used, 3–5 µg of total DNA from 11 *D* and 15 *ND V. dahliae* isolates (Table 1 – Group A) were digested with *Eco*RI, resolved on 0.7% agarose gels and transferred onto Hybond N membranes as above.

Specific PCR reactions

The specific PCR oligonucleotide primers D-1/D-2 and ND-1/ND-2 were synthesized by Operon (Operon Technologies, Alameda, CA). PCR reactions were performed in a 9600 Perkin-Elmer thermocycler using the same amplification conditions as those described for RAPD studies, except for the following modifications: the annealing temperature was increased to 56 °C; 1.25 mM of $MgCl_2$; and 0.75 U of *Taq* DNA polymerase were used, and the time for extension was reduced to 1 min.

Characterization of V. dahliae *isolates from diverse geographical origins*

The 20 additional *V. dahliae* isolates from China, Italy and the USA were characterized to pathotype both by means of RAPD and specific PCR analyses using primers OPH-19, OPH-20 and KS, and primers D-1/D-2 and ND-1/ND-2, respectively. Amplification conditions were as described above.

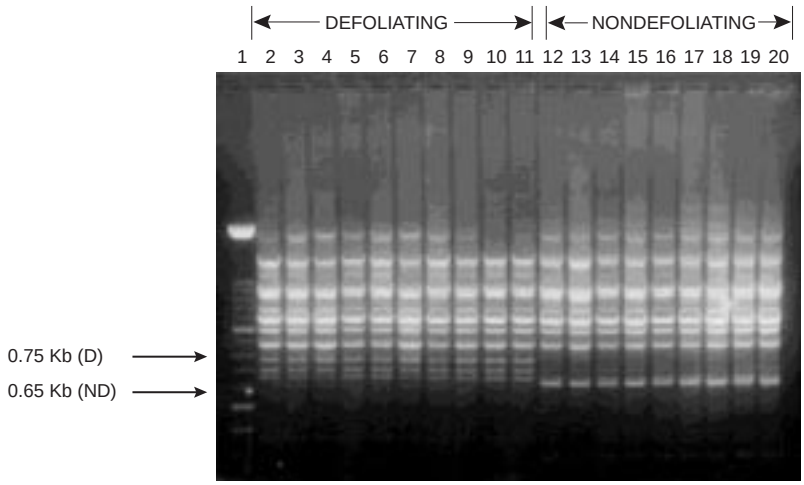

Figure 1. RAPDs generated by primer KS using total DNA from *V. dahliae* isolates previously characterized to pathotype by pathogenicity test. Numbers on the side are sizes in kb pairs of the DNA bands associated with the *D* and *ND* pathotypes. Lane 1: DNA marker XIV (Boehringer-Mannheim). Lanes 2–11: *V. dahliae* cotton-*D* isolates: 2, V117I; 3, V177I; 4, V180I; 5, V184I; 6, V187I; 7, V190I; 8, V191I; 9, V219I; 10, V220I; 11, V221I. Lanes 12–20: *V. dahliae ND* isolates: 12, V4I; 13, V176I; 14, V179I; 15, V183I; 16, V185I; 17, V188I; 18, V192I; 19, V197I; 20, V200I.

Results

RAPD analysis

Eighteen of the 20 Operon primers and primer KS generated banding patterns from all 26 *V. dahliae* isolates (including 3–18 DNA bands) which were reproducible in repeated amplifications. From these patterns, five DNA bands were associated with the *D* pathotype and five others with the *ND* pathotype, which produced distinct RAPD banding patterns of use for a quick characterization of *V. dahliae* isolates. Several DNA fragments were amplified by primers OPH-09, OPH-19, OPH-20, and KS only from DNA of *D* isolates of *V. dahliae* (Figure 1). Similarly, a few RAPD fragments associated with the *ND* pathotype were obtained using primers OPH-04, OPH-19, and KS (Figure 1). Primer OPH-04 amplified a band of 1.0 kb that was present in all *D* isolates tested as well as in a few *ND* isolates, including *V. dahliae* isolate V4I which was used as the *ND* type isolate. Similarly, the primers OPH-09 and OPH-13 produced, respectively, RAPD bands of 0.68 and 0.6 kb from most, but not all, of the *ND* isolates used in the study.

Primers OPH-04, OPH-09, OPH-13, OPH-19, OPH-20, and KS were selected to determine the reliability of the RAPD methodology for the characterization of isolates of *V. dahliae* as *D* or *ND* pathotypes. For that purpose, 21 new isolates of *V. dahliae* of unknown pathotype from cotton (9) and olive tree (12) collected in different parts of Spain (Table 1 – Group B) were used for simultaneous RAPD analysis and biological pathotyping in 'blind trials'. Severity of disease reaction caused by *D* isolates averaged 3.6 (range 2.6–4.0) in line PI 70–110 and 2.7 (range 2.0–3.8) in cv Acala SJ-2. Conversely, disease severity caused by *ND* isolates averaged 2.1 (range 1.6–2.8) in line PI 70–110 and 1.6 (range 1.1–2.3) in cv Acala SJ-2. Results of both assays indicated a 100% matching in the pathotype characterization of each isolate tested (Table 1). Furthermore, when DNA from selected *D* and *ND V. dahliae* isolates and of isolates of other *Verticillium* and *Fusarium* species was employed for RAPD analysis using primers OPH-09 and KS and the same amplification conditions as above, the characterization of the *D* and *ND V. dahliae* isolates was clearly distinctive from all other fungi tested.

RAPD analyses of DNA from all 47 *V. dahliae* isolates using the six primers listed in Table 2 produced a total of 88 bands. UPGMA analysis of these RAPD banding patterns separated the isolates into two distinct clusters (Figure 2). Isolates belonging to the *D* pathotype grouped together in one cluster, while a second cluster included the *ND* isolates. There was very little variation among isolates within either cluster. Both clusters had a 23% dissimilarity.

Table 2. *V. dahliae* pathotype-associated DNA fragments identified in RAPD-PCR analysis using arbitrary primers from Operon and the sequencing KS primer

Code	Primer sequence 5′–3′	MW (kb) of the amplified DNA band	
		ND[a]	D
OPH-04	GGAAGTCGCC	0.9	1.0
		1.0[b]	
OPH-09	TGTAGCTGGG	0.68[b]	1.7
			0.6
OPH-13	GACGCCACAC	0.6[b]	
OPH-19	CTGACCAGCC	2.0	1.65
OPH-20	GGGAGACATC		1.0
KS	CGAGGTCGACGGTATCG	0.65	0.75

[a]ND: Cotton-*ND* pathotype, D: cotton-*D* pathotype of *V. dahliae*; [b]DNA band which was amplified from many, but not all, *ND V. dahliae* used in this study.

Cloning, sequencing, and characterization of pathotype-associated RAPD markers

The sequences of 500 nucleotides from each end of the pathotype-associated RAPD bands showed the presence of the initial RAPD primers (either OPH-19 or OPH-20) at both ends of the insert DNA. Primers forward and reverse for the *D* pathotype of *V. dahliae* were located at 210 and 235 nucleotides from each end of the specific RAPD band, respectively. Therefore, these primers amplified a fragment of 0.55 kb in PCR reactions using DNA from *D* isolates of the pathogen. Primers forward and reverse for the *ND* pathotype were located at 281 and 271 nucleotides from each end of the specific band, respectively. Consequently, these primers amplified a fragment of 1.5 kb in PCR reactions using DNA from *ND* isolates of *V. dahliae*.

Southern blotting of the specific RAPD gels with labelled insert DNA from pND2 showed hybridization with the 2.0 kb band only; no other signal was observed in lanes corresponding to *ND* isolates, and no signal was observed in lanes corresponding to *D* isolates (data not shown). The same behavior was observed with the insert from pD1, which hybridized with the 1.0 kb band in lanes corresponding to *D* isolates only (Figure 3). When the pND2 insert was probed against *Eco*RI-digested genomic DNA, a single band was obtained and only in lanes corresponding to the 15 *ND* isolates. Similarly, when the insert from pD1 was used as a probe, a single band was observed in tracks of the 11 *D* isolates

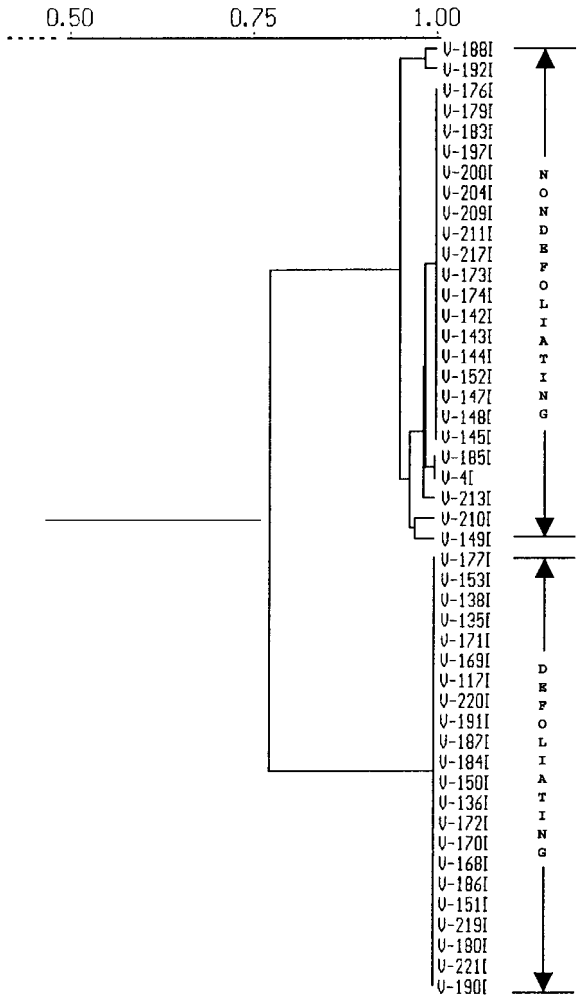

Figure 2. Dendrogram derived from cluster analysis (UPGMA) of RAPD data of *D* and *ND V. dahliae* isolates listed in Table 1, using the software package NTSYS. Top scale is percentage similarity based on the Jaccard similarity coefficient.

only (data not shown). This shows that in both cases the DNA fragment specific for a pathotype of *V. dahliae* has no homology with DNA of the other pathotype.

PCR of fungal DNA using specific primers

Fungal DNA of a range of *D* and *ND V. dahliae* isolates was used for PCR using the specific primers. Reaction mixtures contained a pair of each of the two specific primers simultaneously, and genomic DNA from either *D* or *ND* isolates. There was a complete correlation between the expected pathotype-specific DNA

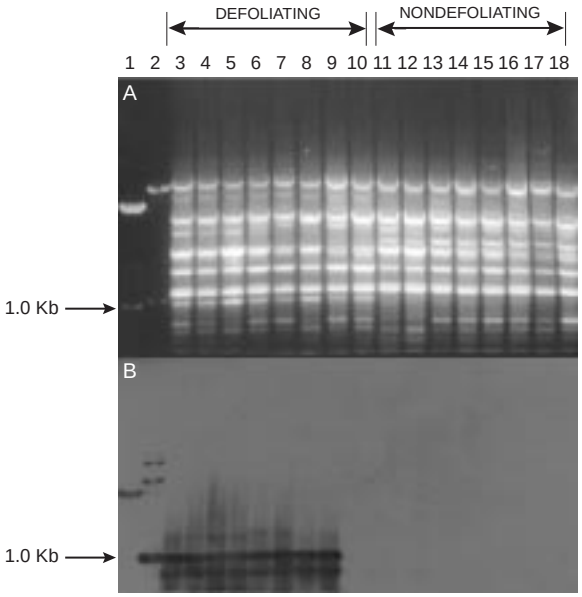

Figure 3. (A) RAPDs generated by primer OPH-20 using total DNA from *V. dahliae* isolates previously characterized to pathotype by pathogenicity test. Numbers on the side are sizes in kb pairs of the pathotype-associated DNA bands. Lane 1, DNA marker XIV (Boehringer-Mannheim); Lane 2, Plasmid pUC 118 (Promega) digested with *Eco*RI and *Pst*1 to free the 1 kb fragment. Lanes 3–10: *V. dahliae D* isolates: 3, V117I; 4, V177I; 5, V180I; 6, V184I; 7, V187I; 8, V190I; 9, V191I; 10, V219I; Lanes 11–18: *V. dahliae ND* isolates: 11, V4I; 12, V176I; 13, V179I; 14, V183I; 15, V185I; 16, V188I; 17, V192I; 18, V197I. (B) Southern blot hybridization and chemiluminiscence detection results using the same gel as in A. The 1 kb *D*-associated RAPD marker was used as nonradioactive probe.

band amplified and the pathotype identity of the isolate of *V. dahliae*. When DNA of *ND* isolates was used, only the 1.5 kb band was obtained. Similarly, when DNA of *D* isolates was used, only the 0.55 kb fragment was amplified (Figure 4).

Characterization of V. dahliae *isolates from diverse geographical origins*

Of the 20 additional *V. dahliae* isolates characterized both by RAPD and specific PCR analyses, isolates T9-1, T9-2, Vd8 and HB3 were characterized as *D* isolates, and isolates SS4-1 and SS4-2 were characterized as *ND* isolates. This molecular characterization was fully coincident with the pathotype assignment of those isolates made by biological or vegetative compatibility tests (Puhalla and Hummel, 1983; Schnathorst and Mathre, 1966; Zhengjun et al., 1998). The remaining 14 *V. dahliae* isolates were characterized as *ND*. RAPD profiles obtained for these 14 *ND* isolates were identical to that of the *ND* V4I type isolate from Spain. Similarly, the four *D* isolates from China and California shared identical RAPD profiles with the *D* V117 I type isolate from Spain.

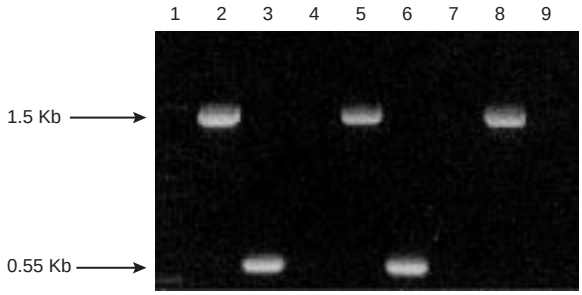

Figure 4. Specific PCR of total DNA from *D* and *ND V. dahliae* isolates using the *D*- and *ND*-specific primers jointly in the reaction mixture. Numbers on the side are sizes in kb pairs of the *ND* (1.5 kb) and *D* (0.55 kb) specific bands. Lane 1: 100 bp DNA ladder (Promega). Lanes 2, 5 and 8: *ND V. dahliae* isolates V4I, V176I, and V173I, respectively. Lanes 3 and 6: *D V. dahliae* isolates V117I and V180I, respectively. Lanes 4 and 7 correspond to amplifications using genomic DNA from *F. oxysporum* f.sp. *ciceris* and *F. oxysporum* f.sp. *niveum*, respectively. Lane 9: negative control (no template DNA).

Discussion

Isolates of *V. dahliae* from cotton are classified as *D* and *ND* pathotypes, which differ much in virulence (Bejarano-Alcázar, 1995; 1996; Bell, 1994; Schnathorst and Mathre, 1966). The quick and proper identification of *V. dahliae* isolates as *D* or *ND* is important for a better understanding of the epidemiology of Verticillium wilt and for improving efficiency in the management of the disease in cotton and olive (Bejarano-Alcázar, 1995; 1997; Bell, 1994; Rodríguez-Jurado et al., 1993; Schnathorst and Sibbet, 1971). The use of molecular genetic diagnostic techniques, particularly those based on the PCR, is one of the best choices for the rapid identification and epidemiological surveillance of plant pathogens (Henson and French, 1993). The goal of this study was to determine whether or not the *D* and *ND* pathotypes of *V. dahliae* could be differentiated by RAPD analysis and, if so, to design specific PCR primers from DNA sequences of pathotype-associated RAPD bands. Results in the study

support that hypothesis. Banding patterns obtained by using arbitrary single primers in a PCR-based reaction differentiated *V. dahliae* isolates as *D* or *ND* (Table 2, Figure 1). Furthermore, results from RAPD analysis placed *V. dahliae* isolates into one of two groups (Figure 2). These groupings correlated well with the *D* or *ND* nature of isolates, as previously determined by biological assays (Bejarano-Alcázar et al., 1996). The reliability of results in the study was further tested and demonstrated both by the characterization of previously untested isolates as *D* or *ND* in the 'blind trial' (Table 1), and the correct pathotype characterization of *V. dahliae* isolates from different geographical origins.

In recent years, several different types of molecular genetic techniques have been used for the characterization of *V. dahliae* isolates differing in host range or virulence: RFLPs (Carder and Barbara, 1994; Okoli et al., 1993; 1994), RAPDs (Koike et al., 1996; Messner et al., 1996; Ramsay et al., 1996), and ITS sequencing (Morton et al., 1995). However, to the best of our knowledge no studies of such a nature had been done before to differentiate the *D* and *ND* pathotypes using these techniques. Success in our study in the molecular characterization of *D* and *ND V. dahliae* isolates from cotton in Spain might relate to the clear-cut differences between the two pathotypes (Bejarano-Alcázar et al., 1996; Bell, 1992; Presley, 1969; Schnathorst and Mathre, 1966). One of the strongest differences concerns vegetative compatibility in *V. dahliae*. Different studies have shown that *D* and *ND* isolates are vegetatively incompatible, with *D* isolates belonging to an unique vegetative compatibility group (VCG 1) (Bell, 1994; Joaquim and Rowe, 1990; Puhalla and Hummel, 1983; Zhengjun et al., 1998). Recent studies have also shown that *D* isolates of *V. dahliae* from cotton in Spain belong to VCG 1 while those of the *ND* pathotype belong to either VCG 2A or VCG 4B (Korolev et al., 1998). RAPD analysis of *V. dahliae* isolates from cotton in Australia revealed genetic differences among isolates with varying degrees of virulence to cotton genotypes (Ramsay et al., 1996). However, this study did not include *D* isolates, as the *D* pathotype has not yet been reported from Australia (Barbara et al., 1998; Bell, 1994), and no clear correlation was found between molecular markers and virulence characteristics. This might be because the isolates in the study did not differ enough in virulence. Overall, the clearest differentiation within *V. dahliae* found in other studies is between groups of isolates with distinct pathogenic capabilities (i.e., brassica isolates from isolates from other hosts) (Koike et al., 1996; Messner et al., 1996).

Results using the *D* and *ND* RAPD markers found in our study were reproducible in repeated amplification experiments. However, because of the random nature of RAPD markers, the reproducibility of the technique might be influenced by several factors including the source and procedure used for DNA isolation, the occurrence of contaminants, the amplification of different DNA sequences of the same size, etc. To overcome that possibility, an additional aim of our study was to design primers for specific PCR based on the DNA sequences of the *D*- and *ND*-associated RAPD bands. This was achieved. Southern hybridizations demonstrated a complete association between the selected RAPD DNA sequences and the *V. dahliae* pathotypes, which was desirable to prevent any nonpathotype-specific amplification when specific primers were designed. Thus, as expected from this complete association of the RAPD DNA sequence with a unique amplified band of the corresponding pathotype, a single, distinctive band is amplified for each pathotype when the specific primers developed are used in reaction mixtures containing DNA from *D* and *ND* isolates. The availability of such primers allows PCR techniques to be applicable under more restrictive conditions for more specific detection of the fungus in the presence of DNA from other microorganisms or from plants.

The molecular markers developed in this study have practical applications in the epidemiology and management of Verticillium wilt in cotton and olive. Thus, they can be very useful for studies to determine the persistence and spread of the *D V. dahliae* pathotype throughout the cropping season and also between cropping seasons and accross the cotton and olive growing areas. Similarly, the molecular markers may be of help in identifying possible origins of inoculum The recent occurrence of the *D* pathotype in Spain led to speculation about its origin in Europe (Bejarano-Alcázar et al., 1996). Because of the distinctness in VCG assignment of *D* and *ND* isolates from Spain, it is unlikely that the *D* pathotype might be a variant of the native *V. dahliae* population. Therefore, the *D* pathotype may have been introduced from outside Spain. In fact, *D* and *ND* isolates from cotton in Spain are very similar in morphological, physiological and pathogenic characteristics to the isolates SS-4 and T-9, which were isolated from cotton in California and are representative of *ND* and *D* pathotypes, respectively (Bejarano-Alcázar et al., 1996; Presley, 1969; Schnathorst, 1973;

Schnathorst and Mathre, 1969). In our study, RAPD profiles of *D* isolates from Spain were identical to those of *D* isolates from California and China. This, and the fact that the group of *D V. dahliae* isolates in our study is so homogeneous and clearly distinctive from all *ND V. dahliae* isolates, supports the hypothesis that the *D* pathotype was introduced into Spain. Also, no differences were found in our study among *D V. dahliae* isolates from cotton and olive trees, suggesting that the inoculum for infection of both crops may originate from the same source. Finally, the development of primers based on sequence characterized amplified regions is of particular interest for implementing certification schemes for the production of *V. dahliae*-free olive planting stock, but also for epidemiological studies on Verticillium wilt of cotton. The design of primers internal to those available, together with a few refinements in the DNA extraction and PCR procedures, might be useful for the specific detection of *D* and *ND* pathotypes *in planta* (e.g. Kelly et al., 1998), and in soil (e.g. García-Pedrajas et al., 1999). Information of this nature would be of much interest in the design and application of potential disease management strategies for Verticillium wilt.

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