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Detection of the defoliating pathotype of *Verticillium dahliae* in infected olive plants by nested PCR

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Abstract

Spread of Verticillium wilt into newly established olive orchards in Andalucía, southern Spain, has caused concern in the olive industry in the region. This spread may result from use of *Verticillium dahliae*-infected planting material, which can extend distribution of the highly virulent, defoliating (D) pathotype of *V. dahliae* to new areas. In this study, a molecular diagnostic method for the early *in planta* detection of D *V. dahliae* was developed, aimed especially at nursery-produced olive plants. For this purpose, new primers for nested PCR were designed by sequencing a 992-bp RAPD marker of the D pathotype. The use of the specific primers and different nested-PCR protocols allowed the detection of *V. dahliae* pathotype D DNA in infected root and stem tissues of young olive plants. Detection of the pathogen was effective from the very earliest moments following inoculation of olive plants with a *V. dahliae* pathotype D conidia suspension as well as in inoculated, though symptomless, plants.

Introduction

The use of pathogen-free planting material is a key control measure for the efficient management of diseases caused by systemic pathogens in asexually propagated plants. For woody hosts, the diagnosis of vascular-infecting fungi such as *Verticillium dahliae* Kleb. by means of isolation of the pathogen from plant tissue is time consuming, destructive, and suffers from inconsistency (Blanco-López et al., 1984). Therefore, new diagnostic methods are desirable for the early, rapid and reliable detection of *V. dahliae* in woody host tissues.

Verticillium wilt, caused by *V. dahliae*, is one of the important diseases of olive (*Olea europaea* L.) throughout its range of cultivation (Jiménez-Díaz et al., 1998). Reported incidence of Verticillium wilt in olive orchards ranged from 2% to 3% of 14 million trees in Greece (Thanassoulopoulos et al., 1979) and from 0.85% to 4.5% of 6.5 million trees in Syria (Al-Ahmad and Mosli, 1993). In southern Spain, 38.5% of 122 adult olive orchards were affected by Verticillium wilt in 1980–1983 with an incidence ranging from 10% to 90% (Blanco-López et al., 1984), and 39.3% of 112 newly established olive orchards surveyed in 1994 and 1996 were affected by the disease (Sánchez-Hernández et al., 1998). In the last few years, Verticillium wilt has been detected in other olive-growing areas throughout Spain (R.M. Jiménez-Díaz, unpublished). Spread of the disease may be a regrettable consequence of new orchards being established in soil infested by the pathogen and/or the use of *V. dahliae*-infected planting material (Rodríguez-Jurado et al., 1993; Thanassoulopoulos, 1993).

Severity of Verticillium wilt in olive depends upon virulence of the pathogen isolates. Artificial inoculation studies indicated that isolates of *V. dahliae* infecting olive can be classified as defoliating (D) or

non-defoliating (ND) pathotypes according to their ability to defoliate the plant (López-Escudero, 1999; Rodríguez-Jurado et al., 1993). Differential virulence is also exhibited in cotton, with isolates from cotton and olive showing cross virulence (Rodríguez-Jurado et al., 1993; Schnathorst and Mathre, 1966; Schnathorst and Sibbett, 1971). While infections by the D pathotype can be lethal to the plant, olive plants infected with the ND pathotype can recover (Jiménez-Díaz et al., 1998; Rodríguez-Jurado, 1993). Therefore, the proper characterization of pathotypes in *V. dahliae* infecting olive is of importance for control of the disease. Although the first report of the D pathotype in Spain was in an area of intensive cotton cultivation (Bejarano-Alcázar et al., 1996), the D pathotype has now spread to nearby olive-growing areas, causing severe Verticillium wilt in newly established olive orchards (López-Escudero, 1999; R.M. Jiménez-Díaz, unpublished).

The early, rapid and reliable detection of plants infected by the D pathotype would be of importance for the management of Verticillium wilt. Detection would help to avoid spread of the D pathotype to new areas, especially if they are free of *V. dahliae*, and for improved use of available control measures. This is of particular relevance now, since the crop is expanding into Australia and South American countries, which import large amounts of rooted, nursery-produced olive plants (J. Samsó, Agromillora Catalana S.A., Barcelona, Spain; E.C. Tjamos, Agricultural University of Athens, Greece; personal communication). In recent years, several molecular techniques have been used for the characterization of *V. dahliae* isolates differing in host range or virulence (Heale, 2000). PCR primers based upon ITS rDNA sequences have been previously designed for detection of *V. dahliae* (Morton et al., 1995; Nazar et al., 1991), and the PCR procedure has been used for the detection and quantification of Verticillium wilt pathogens in herbaceous host plants (Heinz et al., 1998; Moukhamedov et al., 1994; Robb et al., 1994). However, the differentiation of the D and ND pathotypes of *V. dahliae* was not addressed in those studies. Work in our laboratory has shown that *V. dahliae*-specific primers derived from a random genomic clone (Carder et al., 1994) do not differentiate between the D and ND pathotypes (Mercado-Blanco et al., unpublished). Furthermore, such studies had not been carried out for the specific detection of *V. dahliae* pathotypes in woody hosts. In a recent study using 26 D isolates and 41 ND isolates from cotton and olive of diverse geographical origin, our laboratory succeeded in the design of PCR primers that differentiate between the D and ND pathotypes from Spain and other countries using DNA from fungal cultures (Pérez-Artés et al., 2000). From this finding a procedure for the specific *in planta* detection of the *V. dahliae* pathotype ND in infected olive was developed (Mercado-Blanco et al., 2001).

The objective of this research was to develop a new, specific method for the early detection of the D pathotype of *V. dahliae* in nursery-produced olive plants that would be of use in programs for the certification of *V. dahliae*-free planting material. The approach consisted of developing: (i) new specific primers for nested PCR; and (ii) a nested-PCR protocol for the *in planta* detection of the *V. dahliae* pathotype D in olive tissues.

Materials and methods

Fungal isolates and culture conditions

Reagents used in this work were from Sigma Chemical Co, St. Louis, MO, USA; Merck, Darmstadt, Germany; or Panreac, Barcelona, Spain, unless otherwise indicated. Potato-dextrose agar (PDA) and bacto agar were from Difco Laboratories, Detroit, MI, USA. Media were made with deionized water and autoclaved at 121 °C for 20 min.

Verticillium dahliae isolates V4I, V143I, V144I, V152I, and C2F48, representatives of the ND pathotype, and V117I, V138I, and 102D-3, representatives of the D pathotype, were used in this study. These isolates are deposited in the culture collection of Departamento de Protección de Cultivos, Instituto de Agricultura Sostenible, CSIC, Córdoba, Spain. They had been characterized as pathotype both by pathogenicity tests (Bejarano-Alcázar et al., 1996), and RAPD and specific-PCR analyses (Pérez-Artés et al., 2000; Jiménez-Díaz et al., unpublished). Isolates were stored by covering cultures on plum-extract agar with liquid paraffin (Bejarano-Alcázar et al., 1996) at 4 °C in the dark. Active cultures of isolates were obtained by placing small agar plugs from stock cultures on chlortetracycline (30 mg/l)-amended water agar and further subculturing on PDA. Cultures on PDA were grown for 7 days at 24 °C in the dark.

Plant inoculation

Plants were inoculated by dipping their thoroughly washed, trimmed roots in a suspension of 10^5 or

10^7 conidia/ml of D *V. dahliae* isolate V138I for 1 h. These conidia were obtained by flooding 7-day-old cultures on PDA and filtering the suspension through sterile cheesecloth. Thirty-five 'Picual' plants were inoculated with each inoculum concentration and 18 plants, similarly treated except for the absence of inoculum, served as uninoculated controls. These inoculated and uninoculated plants were sampled in a time course for PCR detection of *V. dahliae* DNA. In addition, 18 plants were inoculated with each inoculum concentration and kept throughout the experiment as a reference for symptom development. Eighteen 'Picual' plants were inoculated as above with 10^7 conidia/ml of ND *V. dahliae* isolate V4I, as additional controls in further PCR experiments to check for specificity of the primers used for the *in planta* detection of the D pathotype. The experiment was arranged as a completely randomized design. After inoculation, the plants were transplanted into sterile soil (sand : loam, 2 : 1 v/v) in pots and were incubated at 22/24 °C light/dark and a 14-h photoperiod of fluorescent light of 262 $\mu E\,m^{-2}\,s^{-1}$. Plants were fertilized with 20-5-32 + microelements hydro–sol (Haifa Chemicals, LTD, Haifa, Israel) every week. The disease reaction was assessed by severity of symptoms on a 0–4 scale according to percentage of affected leaves and twigs (0 = no symptoms, 1 = 1–33%, 2 = 34–66%, 3 = 67–100%, and 4 = dead plants) at weekly intervals from 24 to 52 days after inoculation (Rodríguez-Jurado, 1993). Data were subjected to analysis of variance using Statistix (NH Analytical Software, Roseville, MN). Treatment means were compared using Fisher's protected least significant difference test (LSD) at $P = 0.05$.

Plants from the above experiment were used for PCR detection of *V. dahliae* DNA. PCR experiments were conducted as described below using DNA samples from roots and stems of olive plants (three plants per time interval) collected at time 0 (just after dipping roots in water or in the conidia suspensions), and at 2, 4, 7, 18, 24, and 52 days after inoculation. Plants inoculated with 10^5 conidia/ml were not sampled 4 days after inoculation. Uninoculated plants were also sampled at 0, 7, and 52 days after root dip in sterile water.

Fungal and plant DNA extraction

DNA from *V. dahliae* isolates V4I and V138I was extracted from lyophilized mycelia according to the method of Raeder and Broda (1985). Fungal mycelia were obtained from cultures in potato-dextrose broth as previously described (Pérez-Artés et al., 2000), lyophilized and ground to a fine powder using an autoclaved pestle and mortar. Fifty milligrams of powdered mycelia were used for DNA extraction.

Total genomic DNA was extracted from roots and stems of pathotype infected and non infected 3- to 6-month-old olive plants of cv Picual. These plants were root-dip inoculated with *V. dahliae* isolate V117I (Pérez-Artés et al., 2000) in resistance screening tests performed by the authors or by López-Escudero (1999). All inoculated plants showed symptoms of Verticillium wilt by 4 weeks after inoculation and *V. dahliae* was isolated from them. Total genomic DNA was extracted using the commercially available DNeasy Plant Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For DNA extraction, the roots of a plant were cut off from the stem, the bark was removed from stems with a clean scalpel, and these roots and stems were thoroughly washed and surface-disinfested in NaClO (0.5% available chlorine) for 1.5 min (stems) or 2 min (roots). The disinfested roots and stems were freeze-dried, cut into 8–10 mm long pieces and ground to a fine powder for 0.5–1 min in stainless steel vessels with balls of a mixer mill (Retsch Mod. MM-2, Eurocomercial, Sevilla, Spain) (Rodríguez-Jurado, 1993). Powdered tissue samples were kept at −20 °C. To avoid cross contamination among samples, vessels and balls were thoroughly washed, disinfested in two steps using 1% v/v Armil® (benzalkonium chloride 10% w/v) (Squibb Industria Farmaceútica, Barcelona, Spain) and 95% ethanol, flamed, and chilled before each use. A sample of 20 mg of the fine powder was used for DNA extraction. DNA purity and concentration were determined spectrophotometrically and by agarose gel electrophoresis according to standard procedures (Sambrook et al., 1989).

DNA sequencing and Southern hybridization

A D-associated 1.0 kb RAPD band identified in previous work (Pérez-Artés et al., 2000) was sequenced completely as follows: plasmid pD1 (Pérez-Artés et al., 2000) was purified by the Qiagen Plasmid Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA sequencing was performed on both strands in overlapping fashion. The universal pUC/M13 forward (−21) and reverse primers as well as specific oligonucleotides synthesized by Genset Oligos were used. The DNA sequence of 992 bp (Figure 1) was determined using an Applied Biosystems Model 373

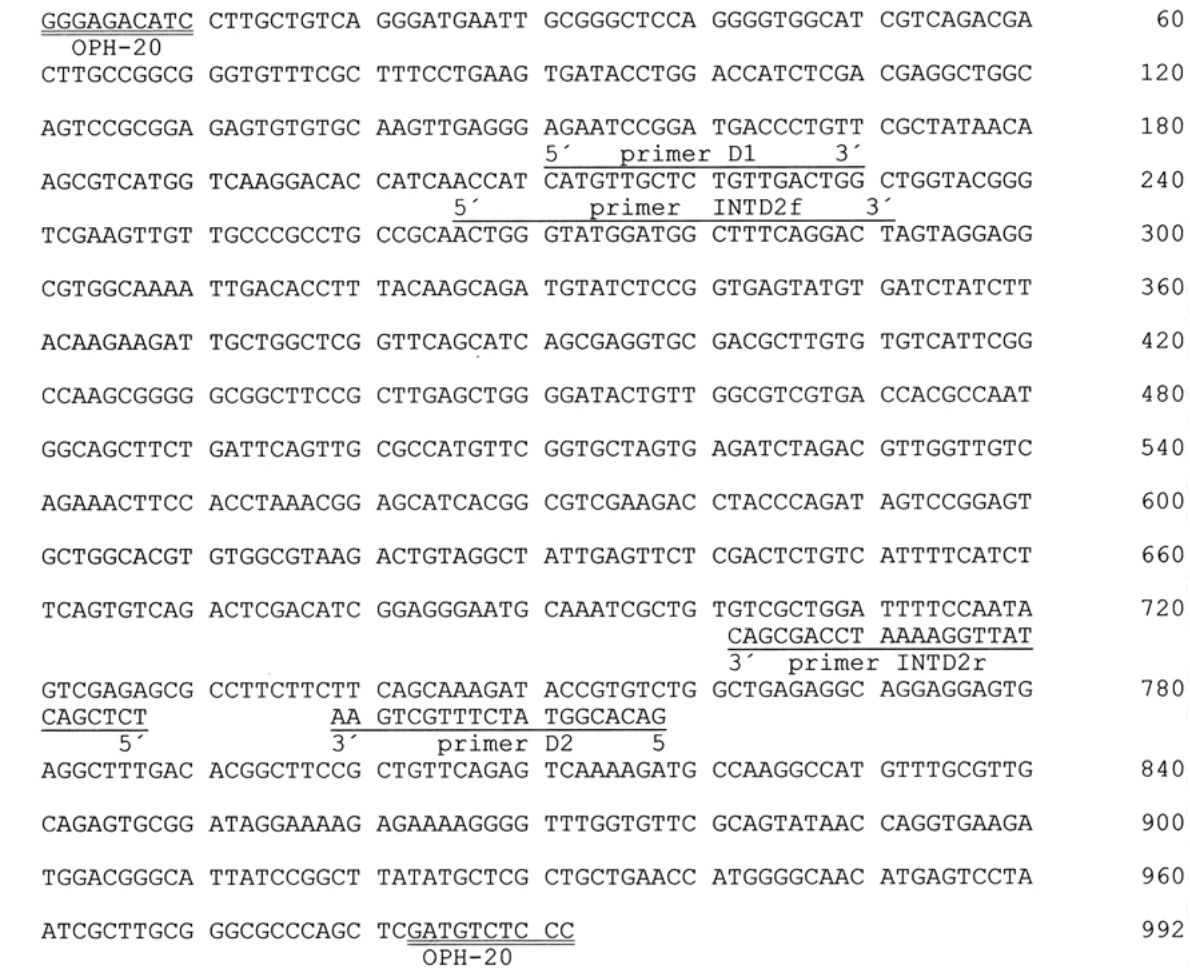

Figure 1. Nucleotide sequence of the 992-bp *V. dahliae* defoliating-associated RAPD marker amplified by primer OPH-20 (double underlined). Single lines indicate the position of the specific primers used in this study.

automated DNA sequencer. The complete sequence has been deposited in the EMBL nucleotide sequence database under accession number AJ302674. A similarity search was performed with the BLASTN and BLASTX 2.1.1 programs (Altschul et al., 1997) of the NCBI network service. From the resulting sequence, the following primers internal to primers D1/D2 (Pérez-Artés et al., 2000) were developed: INTD2f (5′-ACTGGGTATGGATGGCTTTCAGGACT-3′) and INTD2r, (5′-TCTCGACTATTGGAAAATCCAGCG-AC-3′).

The 992 bp D-associated RAPD marker cloned in plasmid pD1 was released by digestion with *Eco*RI and *Pst*I endonucleases. The band was resolved by electrophoresis in a 1% agarose gel and eluted using the Qiaex II Gel Extraction kit (Qiagen, Hilden, Germany). DNA was labelled using DIG-11dUTP (digoxigenin-3-O-methylcarbonyl-aminocaproyl-5-(3-aminoallyl)-uridine-5′ triphosphate) (Roche Diagnostic S.L., Barcelona, Spain) according to the manufacturer's instructions. This labelled DNA was used as a probe in Southern hybridization experiments of PCR products. For Southern blots of PCR products, DNA from amplification mixtures (25 µl) was resolved on 1% agarose gels and transferred to Biohylon-Z+ membranes (Quantum Biotechnologies Inc., Montreal, Canada) according to standard procedures (Sambrook et al., 1989). Hybridization experiments were also carried out using the 548-bp PCR product produced with the D1/D2 primer pair (see below). This DNA

fragment was purified from the amplification mixture using the QIAquick kit (Qiagen, Hilden, Germany), labelled as above, and used as a probe against Southern blots of undigested and complete *Eco*RI-digested genomic DNAs of ND isolates C2F48, V4I, V143I, and V152I, and D isolates 102D-3, V117I, and V138I (Pérez-Artés et al., 2000; Jiménez-Díaz et al., unpublished). Hybridization was performed with the non-radioactive detection kit from Roche Molecular Bochemicals (Roche Diagnostic S.L., Barcelona, Spain) overnight at 68 °C, and the chemiluminescence method was used to detect hybridizing bands according to instructions in the kit.

PCR experiments

RAPD analyses using primer OPH-20 (Operon Technology, Alameda, CA) were carried out as a preliminary test on the purity of the DNA extracted from plant tissue. Amplifications were carried out as described by Pérez-Artés et al. (2000), except for the following modifications: the time for denaturation was reduced to 4 min; 20 μM KCl was used in the reaction buffer; and 10 ng of fungal DNA (controls) or 1–3 μl (10–30 ng) of total genomic DNA extracted from *V. dahliae* infected or non-infected plants were used. For specific *in planta*-PCR detection of D *V. dahliae*, primer pairs D1/D2 (D1, 5′-CATGTTGCTCTGTTGACTGG-3′; D2, 5′-GACACGGTATCTTTGCTGAA-3′) (Pérez-Artés et al., 2000) or INTD2f/INTD2r were used in single PCR assays. Amplification with primer pairs D1/D2 and INTD2f/INTD2r yielded PCR products of 548 bp and 462 bp, respectively (Figure 1). Amplification conditions were as described by Pérez-Artés et al. (2000), except for the following modifications: the time for denaturation was reduced to 4 min; the reaction buffer contained 20 μM KCl; 0.25 μM of each primer, 2.5 mM $MgCl_2$ and 1–3 μl (10–30 ng) of DNA extracted from plant material (stems or roots) were used; and the annealing temperature was increased to 58 °C. For nested PCR, 1 μl of the products synthesized using primer pair D1/D2 and the conditions indicated above was transferred to a fresh tube containing the mixture for the second amplification reaction, and amplification was done as described above, with the annealing temperature set at 64 °C for 1 min. The INTD2f/INTD2r pair of nested primers was used in the second round of amplification. Reactions were performed in a PTC-100™ Programmable Thermal Controller (MJ Research, Inc., Watertown, Mass.), were repeated at least three times, and always included negative (no DNA) and positive (10 ng of *V. dahliae* V138I and V4I DNA purified from mycelia grown in pure culture) controls.

Results

DNA sequencing

Pérez-Artés et al. (2000) identified and partially sequenced a 1.0 kb D-pathotype-associated RAPD band amplified using primer OPH-20. In this study, the sequencing of the 992 bp (53.93% G + C content) sequence of this RAPD marker was completed (Figure 1). No relevant features were found in the analysis of the sequence. A BLAST search of the EMBL/GenBank/DDBJ databases showed no significant similarity at either nucleotide or amino acid sequence level. The complete sequence of this RAPD marker allowed specific primers to be selected for the nested-PCR experiments. Positions of the primers used are indicated in Figure 1.

Detection of V. dahliae *pathotype D in plant tissues by single PCR*

The D1/D2 specific primers were used repeatedly in PCR assays aimed at the detection of the D pathotype (isolate V117I) in both symptomatic and symptomless plants in this study. To determine the amount of *V. dahliae* DNA that could be detected in a total genomic DNA sample of plant tissue, a constant amount of 12.5 ng of DNA extracted from the stem of a non-infected olive plant was mixed in a serial dilution (1 : 5) with *V. dahliae* DNA extracted from pure fungal mycelia. Results from repeated experiments indicated that as little as 8 pg of *V. dahliae* DNA was detected in a sample (12.5 ng) of total genomic DNA (Figure 2B). In contrast, up to 2 pg of *V. dahliae* DNA were still detectable without mixing fungal DNA with total genomic DNA from olive (Figure 2A). In the above PCR assays, the PCR product of 548 bp specific for the D pathotype was detected as a faint band in 40% of the DNA samples (10–30 ng) extracted from roots, but not in those from stems, of isolate V117I-infected olive plants. Further PCR assays were carried out using the INTD2f/INTD2r primer pair with samples of the same total genomic DNAs as above, to test improvement of the level of detection. In these PCR assays, the

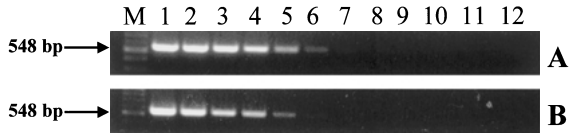

Figure 2. Detection of *V. dahliae* isolate V138I DNA in a serial dilution (1 : 5) when present alone (A) or mixed with a constant amount (12.5 ng) of total genomic DNA extracted from non-infected olive stem tissue (B). Amplifications were carried out using primers D1/D2, yielding a PCR product of 548 bp. PCR reaction mixtures (25 µl) contained 25 ng (lane 1), 5 ng (lane 2), 1 ng (lane 3), 200 pg (lane 4), 40 pg (lane 5), 8 pg (lane 6), 1.6 pg (lane 7), 0.32 (lane 8), 0.064 (lane 9), and 0.0128 pg (lane 10) of V138 DNA purified from mycelia. Negative controls with no DNA (lane 11) or with 12.5 ng of total genomic olive DNA (lane 12) were also included. M: molecular weight marker.

predicted 462-bp band was amplified (sometimes only as a faint band) in a large majority of the root tissue samples, but in less than 10% of the stem tissue samples of pathotype D *V. dahliae*-infected plants assayed.

Time course of in-planta-*PCR detection of pathotype D* V. dahliae *by nested PCR*

A nested-PCR procedure using the D1/D2 and INTD2f/INTD2r primer pairs was designed to improve amplification consistency of the D-pathotype-specific PCR marker. The aim was to detect the D pathotype *in planta* during a time interval spanning the initial stages of infection following inoculation to development of symptoms in infected plants.

No symptoms developed in uninoculated control plants. The first symptoms in inoculated plants were visible 24 days after inoculation, at which time disease incidence was 67% and 100% for plants inoculated with 10^5 and 10^7 conidia/ml, respectively. By 52 days after inoculation, all inoculated plants had foliar symptoms but disease severity was significantly ($P < 0.05$) higher in plants inoculated with 10^7 conidia/ml than in plants inoculated with 10^5 conidia/ml (Table 1). Conversely, 86% of plants inoculated with 10^7 conidia/ml ND isolate V4I were affected 52 days after inoculation with 1.4 mean disease severity. These plants served as a reference for comparison of severity of disease reaction between the D and ND isolates used in the study, as well as an additional control for specificity of the D PCR marker.

Nested-PCR analyses using total genomic DNA from olive plants sampled in a time course after inoculation revealed that pathotype D was detected very soon after inoculation. Thus, the expected product of 462 bp was amplified from roots of plants sampled at time 0 and day 2 after inoculation with 10^7 and 10^5 conidia/ml of pathotype D isolate V138I, respectively. From this sampling time on, the 462-bp product was amplified consistently when total genomic DNA from roots was used as template. This allowed detection of pathogen DNA in roots of all plants sampled 7 days after inoculation with 10^5 conidia/ml of isolate V138I (Figure 3). Furthermore, the single-PCR product of 548 bp specific for the D pathotype was amplified in the first PCR round at that time as well as in roots sampled 18 and 24 days after inoculation. Interestingly, the 548-bp PCR product was not amplified in plant roots sampled 52 days after inoculation. Conversely, detection of pathogen DNA in stem tissues using the two-separate-rounds procedure was achieved only in plants sampled 18 days after inoculation or later (Figure 3). From this sampling time on, the 462-bp product was amplified consistently and the 548-bp band was never detected. Nested-PCR analyses using total genomic DNA from plants inoculated with 10^7 conidia/ml yielded the same pattern of results as above, except for stems of two plants sampled at time 0 of inoculation from which the 462-bp product was amplified (data not shown). There were no PCR

Table 1. Disease reaction of olive cv Picual inoculated with the defoliating *V. dahliae* isolate V138I by the root-dip method[a]

Inoculum concentration (conidia/ml)	Time after inoculation (days)	Disease	
		Incidence (%)	Severity (0–4)[b]
10^5	18	0	0
	24	66.7	0.5a
	52	100	1.6b
10^7	18	0	0
	24	100	0.7a
	52	100	3.3b*

[a]Four-month-old plants were uprooted from the substrate, and their roots were thoroughly washed, trimmed, and dipped in a conidial suspension for 1 h. Plants were incubated in a growth chamber at 22/24 °C light/dark and a 14-h photoperiod of fluorescent light of 262 µE m^{-2} s^{-1}; disease reaction was assessed at weekly intervals after inoculation.

[b]Mean symptom severity assessed on a 0–4 scale according to percentage of affected leaves and twigs (0 = no symptoms, 1 = 1–33%, 2 = 34–66%, 3 = 67–100%, and 4 = dead plant). Means followed by the same letter for each inoculum concentration are not significantly different according to Fisher's protected LSD ($P = 0.05$). The mean followed by an asterisk (∗) is significantly larger than the mean for the corresponding time of assessment at the lower inoculum concentration.

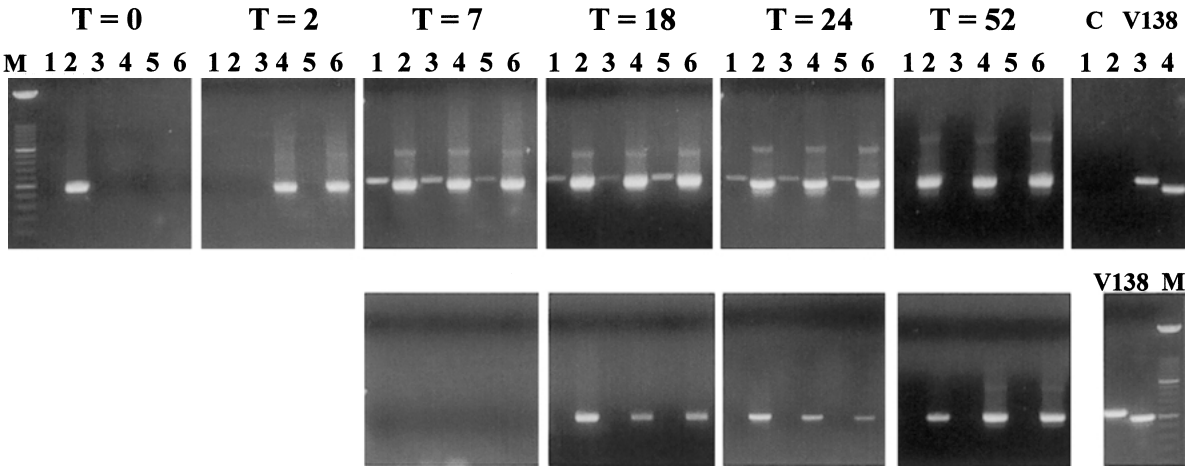

Figure 3. Detection of *V. dahliae* defoliating isolate V138I in infected 'Picual' olive in a time course of infection by nested PCR in two consecutive, independent reactions. A composite is shown with results from roots (upper panel) and stems (lower panel) of three plants sampled at the times after inoculation indicated (T, days); M, molecular weight marker; C, control reaction with no DNA; V138, control reaction with V138I DNA (10 ng) purified from mycelia. Odd-numbered lanes show products of the first amplification (24 μl) using primers D1/D2 (PCR product of 548 bp); even-numbered lanes show products of the second amplification (25 μl) using 1 μl of the first amplification product and primers INTD2f/INTD2r (PCR product of 462 bp).

products amplified from non-inoculated control plants. Control nested-PCR reactions using DNA extracted from pure mycelium of isolate V138I yielded the predicted DNA bands, that is, both the 548-bp single-PCR product in the first amplification and the 462-bp nested-PCR product. Similar control reactions using V4I DNA did not yield the 548-bp product; unexpectedly, however, these reactions also yielded the 462-bp PCR product after nested PCR (Figures 4, 6, and 7).

DNA–DNA hybridization using the 992-bp D-RAPD marker as a probe demonstrated homology between the nested-PCR product from the V4I isolate DNA and the pathotype D-associated band (Figure 4). Nested PCR using total genomic DNA from roots of plants inoculated with 10^7 conidia/ml of the D isolate showed the diagnostic D marker band (Figure 4B). A faint band of size similar to that of the D marker was amplified in nested PCR using total genomic DNA from roots of some of the plants inoculated with 10^7 conidia/ml of isolate V4I. This nested-PCR product also yielded a weak signal after hybridization with the RAPD-marker probe (Figure 4A). Control nested-PCR reactions using DNA extracted from pure mycelia of isolates V4I and V138I both yielded a positive signal after hybridization. However, the hybridization signals both for mycelial DNA and total genomic DNA from V4I-infected roots showed less intensity as compared to hybridization signals using samples containing DNA from isolate V138I. Similarly, Southern hybridization of *Eco*RI-digested genomic DNA from different ND (V4I, V143I, V152I) isolates revealed a hybridization signal when probed with the 548-bp PCR fragment amplified from V138I DNA with primers D1/D2. These hybridization signals were also of lesser intensity as compared to hybridization signals using DNA from D isolates 102-D3, V117I, and V138I (Figure 5). To further determine homology between the 462-bp DNA fragment amplified from D and ND isolates, sequencing of this fragment was carried out using ND isolates V4I, V143I, V144I, and C2F48, and D isolate V138I. DNA from these isolates were submitted to nested PCR using primer pairs D1/D2 and INTD2f/INTD2r. No PCR products were visible after the first PCR for any of the isolates except for D isolate V138I. The 462-bp band was amplified from all tested isolates after the second round of amplification. The 462-bp band from each of the tested D and ND isolates was purified by the QIAquick Purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and sequenced using the INTD2f primer. Sequence comparison revealed that all sequences were identical except for a shift G → C in position 716 of the sequence in DNA of ND isolates (Figure 1).

A nested, single-reaction-mixture PCR protocol using the D1/D2 and INTD2f/INTD2r primer pairs was

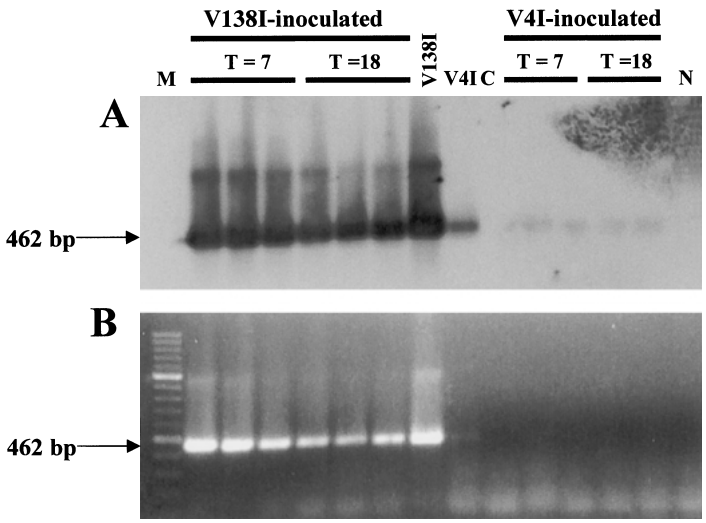

Figure 4. Southern analysis (A) using the agarose gel resolved products of a single-tube, nested-PCR amplification (25 µl) (B) for the specific detection of the *V. dahliae* defoliating-associated marker in infected 'Picual' olive using primers D1/D2 and INTD2f/INTD2r. Roots of three plants inoculated with 10^7 conidia/ml of *V. dahliae* defoliating isolate V138I or two plants inoculated with *V. dahliae* non-defoliating isolate V4I were sampled at 7 and 18 days after inoculation and the total genomic DNA was extracted. Control reactions were carried out using DNA (10 ng) extracted from pure mycelia of isolates V4I and V138I, no DNA (lane C), and the total genomic DNA (10 ng) extracted from an uninoculated control plant sampled 52 days after inoculation (lane N). Exposure time was 20 min.

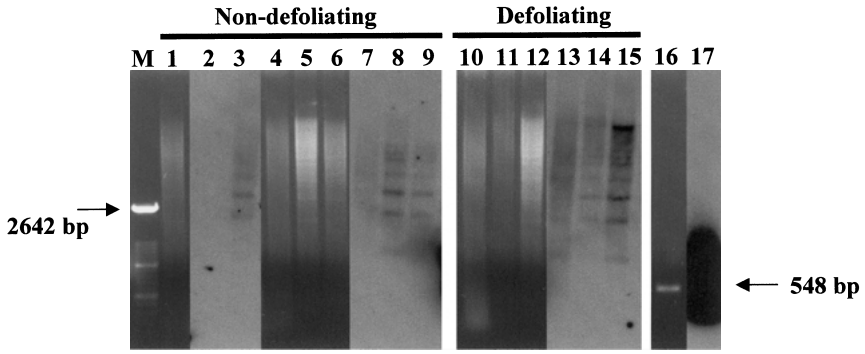

Figure 5. Southern analysis of complete *Eco*RI-digested genomic DNA of defoliating (V117I, V138I, 102-D3) and non-defoliating (V4I, V143I, V152I, C2F48) isolates of *V. dahliae* resolved on agarose gels. M and lane 2: Molecular weight marker; lanes 1 and 3: V4I; lanes 4 and 7: V143I; lanes 5 and 8: V152I; lanes 6 and 9: C2F48; lanes 10 and 13: V117I; lanes 11 and 14: V138I; lanes 12 and 15: 102D-3; lanes 16 and 17: 548-bp DNA fragment used as a probe. The 548-bp fragment was amplified using DNA from isolate V138I and primers D1/D2. Exposure time was 20 min.

designed to shorten the time and reduce the possibility of contamination in the detection procedure. This protocol was optimized as follows: the concentration of primers was established at 25 nM for D1/D2 and 250 nM for INTD2f/INTD2r; the initial step of denaturation at 94 °C for 4 min was followed by 15 cycles of 1 min denaturation at 94 °C and 1 min of annealing at 58 °C using primers D1/D2, and 30 additional cycles using primers INTD2f/INTD2r that were run at the same conditions as above except for the annealing temperature of 64 °C. A final extension step of 6 min at 72 °C was run to complete the reaction. Other components in the reaction mixture were the same as indicated before. Use of this protocol with total genomic DNA from olive plants sampled in a time course after inoculation confirmed that DNA of pathotype D was detected soon after inoculation (Figure 6). However, results were less consistent compared with those from

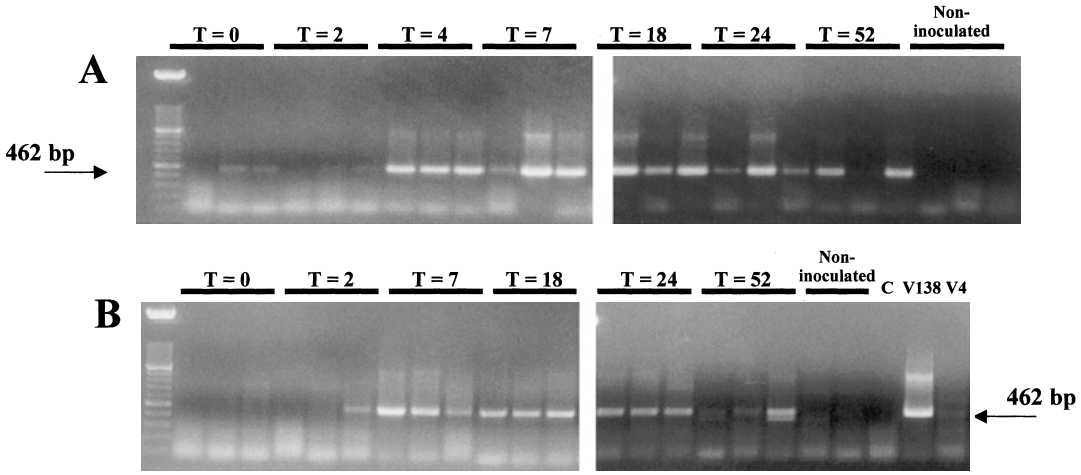

Figure 6. Nested-PCR detection of *V. dahliae* defoliating isolate V138I in roots of 'Picual' olive in a time course of infection. The single-tube, nested-PCR procedure described in the text was used. Plants were inoculated by dipping their washed, trimmed roots in a suspension of 10^7 conidia/ml (A) or 10^5 conidia/ml (B) of *V. dahliae* for 1 h. Three plants per sampling time (T, days after inoculation) were used for DNA extraction. Control reactions included the use of: no DNA (C), 10 ng of DNA extracted from pure mycelia of *V. dahliae* isolate V138I (V138), and 10 ng of DNA extracted from pure mycelia of *V. dahliae* nondefoliating isolate V4I (V4). Each lane was loaded with 25 μl of the amplification reaction mixture.

the two separate rounds of nested-PCR amplification using the same DNA samples (Figure 3). A 462-bp band was also amplified with the single-reaction-mixture protocol when DNA of ND isolate V4I was used as a control, which was fainter compared to that amplified in the two separate rounds of amplifications.

Discussion

The early, rapid and reliable detection of infections by the D *V. dahliae* pathotype in planting material should help the management of Verticillium wilt in olives (Tjamos, 1993). In this study a nested-PCR procedure was developed for the detection of pathotype D *V. dahliae* in symptomatic and nonsymptomatic nursery-propagated olive plants. This required optimizing a procedure for extracting from infected roots and stems pathogen DNA suitable for PCR assays, and designing new primers for amplification of the extracted DNA. This procedure may help enforce the zero-tolerance policy for pathogen-free certification that is prevalent in European countries. Verticillium wilt pathogens have been detected by PCR-based methods in herbaceous host plants like tomato (Heinz et al., 1998) and potato (Moukhamedov et al., 1994; Robb et al., 1994). On the other hand, the use of *V. dahliae*-specific primers for *in-planta*-PCR detection did not differentiate *V. dahliae* pathotypes (Mercado-Blanco et al., unpublished). To the best of our knowledge this is the first report of PCR-based detection of pathotype D *V. dahliae* in a woody host.

DNA extracted from plants can pose difficulty as template for PCR since accompanying inhibitory substances may hinder performance of the amplification reactions (De Boer et al., 1995). This is relevant for woody plants such as olive, whose tissues contain large amounts of phenolic compounds (Akillioğlu and Tanrisever, 1997; Tsukamoto et al., 1984) that might be co-extracted with total genomic DNA. Several procedures for the extraction of PCR-quality DNA from woody plant tissues have been published which have proved suitable in the detection of phytopathogenic fungi infecting woody hosts (Bahnweg et al., 1998; Rollo et al., 1990). Some of these methods use the cationic detergent CTAB. A CTAB-based procedure was tested; several modifications were necessary, such as diluting the DNA samples and/or adding the skim-milk-based buffer BLOTTO (De Boer et al., 1995) into the PCR for consistent success in DNA amplification (data not shown). In contrast, the commercially available DNeasy method produced a satisfactory yield of PCR-quality total genomic DNA from olive roots and stems. The DNA obtained needed neither dilution nor BLOTTO amendment for PCR amplification. The roots and stems of young plants were hard to break down

for the extraction procedure. Grinding freeze-dried olive root and stem tissues in a mixer mill was the best option for obtaining a fine-powered material suitable for efficient DNA extraction. With this procedure, total genomic DNA was routinely extracted with yields of 3–10 μg per 20 mg of lyophilized tissue sample. Occasionally, DNA yield from stem samples was lower than that from root samples of the same plant. The DNA yield from stem was not increased when more tissue was harvested, probably because an excess of starting plant material likely affected some of the steps in the extraction procedure (as anticipated by the manufacturer's instructions).

Detection of pathotype D in total genomic DNA from infected plants by single PCR using D1/D2 or INTD2f/INTD2r primer pairs gave inconsistent results. This could be a consequence of the concentration of fungal DNA being significantly lowered relative to the plant DNA extracted from the tissues. A nested-PCR strategy was necessary to consistently demonstrate the presence of pathotype D in an infected olive plant. This strategy proved successful in plants infected with each of two different D isolates. Since the primers for nested PCR were developed from a D-associated RAPD band common to some 26 D isolates of diverse geographical origin (Pérez-Artés et al., 2000), the usefulness of the procedure should not be restricted to specific isolates. Furthermore, this detection method has already been validated using samples of symptomatic 1- to 2-year-old twigs from naturally infected olive trees of different cultivars at several locations in southern Spain (Mercado-Blanco et al., unpublished).

The developed nested-PCR strategy made it possible to detect the fungus in roots and stems of nursery-produced olive plants sampled very soon (time 0, day 2) after inoculation with conidia of D *V. dahliae*. This early detection of the pathogen may relate to a rapid translocation of *V. dahliae* conidia along xylem vessels in the plant, as reported for other tree species (Banfield, 1941; Emechebe et al., 1975). The early detection of the pathogen after inoculation may also have been related to the high inoculum concentration used. The high inoculum concentration was needed for consistent infection in pathogenicity experiments (Rodríguez-Jurado, 1993). The nested-PCR strategy also made possible the consistent detection of the pathogen in roots of symptomless plants sampled up to 3 weeks after inoculation. This satisfied the major aim of this study of early detection of pathotype D with latent infections in nursery-propagated olive plants. Detection of the pathogen in olive stems was less consistent and occurred later compared to that in the roots. However, the consistency of stem detection increased during the time course of disease development.

That the nested-PCR procedure yielded a faint, D-associated PCR product from the total genomic DNA of some ND pathotype-infected plants was unexpected. In fact, this 462-bp band was not detected either in a single PCR using the D1/D2 primers and DNA from a number of pathotype ND *V. dahliae* isolates (Pérez-Artés et al., 2000) or after one round of amplification in the *in planta*-detection experiments of *V. dahliae* V4I-infected plants (this study). Also, Southern blotting of the pathotype D-specific RAPD gels containing products produced with primer OPH-20, using the 992-bp D-associated RAPD band as a probe, showed no signal in lanes corresponding to a number of ND isolates including isolate V4I (Pérez-Artés et al., 2000). However, Southern blotting of *Eco*RI-digested genomic DNA from several ND isolates (including isolate V4I) yielded hybridization bands when probed with the 548-bp PCR-amplifed fragment of D isolate V138I. These hybridization bands were of less intensity and copy number compared with those in the hybridized, *Eco*RI-digested DNA of D isolates. Low intensity hibridization spots located in the main mass of genomic DNA were also found in Southern blots of undigested DNA of ND isolates (data not shown). This indicates that no homology occurs in extrachromosomal DNA (i.e., plasmids, viruses, or mitochondrial genomes). Therefore, the nested-PCR procedure developed in this study allowed the detection of a previously unnoticed sequence in the DNA of some ND isolates, including V4I. Since this 462-bp DNA fragment is present both in D and ND pathotypes, although less abundant in ND isolates, DNA sequence heterology in ND isolates could explain why a single PCR assay was not enough to amplify this sequence from DNA of ND isolates to the same extent than from DNA of D isolates. Furthermore, since the 462-bp fragments from D and ND isolates were nearly identical, as showed by comparison of DNA sequences, the possible divergency in the RAPD sequence must be located outside this DNA region which may affect the primer binding site of the D1 and/or D2 primers. However, this point remains to be elucidated, since no amplification was obtained from DNA of ND isolates when the D1/D2 primer pair was used. The few cases in this study of the 462-bp band being amplified in V4I-infected plants might reduce the specificity of the nested-PCR procedure in the detection of the

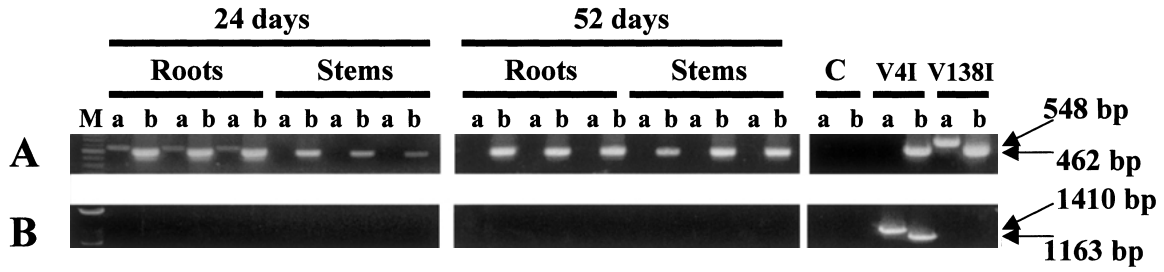

Figure 7. Nested-PCR analysis using samples of total DNA from roots and stems of three V138I-infected olive plants sampled at each of 24 and 52 days after inoculation. Two sets of nested PCR were carried out to demonstrate the sole presence of a defoliating isolate in the DNA samples. A: Nested PCR using primer pairs D1/D2 and INTD2f/INTD2r; B: Nested PCR using primer pairs NDf/NDr and INTNDf/INTNDr (Mercado-Blanco et al., 2001). M: Molecular weight marker; a: lanes showing the result after one round of amplification; b: lanes showing the result after the second round of amplification; C: control reaction (no DNA): V4I DNA (10 ng); V138I DNA (10 ng).

D pathotype. However, this effect can be counteracted by including an additional negative control in the assay; that is, a simultaneous nested-PCR assay for the specific detection of the ND pathotype using a NDf/NDr primer pair in the first round of amplification and either INTNDf/INTNDr or INTND2f/INTND2r primer pairs in the second round (Mercado-Blanco et al., 2001). In this second round of amplification, the INTNDf/INTNDr and INTND2f/INTND2r primer pairs amplify fragments of 1163 and 824 bp, respectively, only from DNA of the ND pathotype (Mercado-Blanco et al., 2001; EMBL accession number AJ302675). An example of this approach is shown in Figure 7. Total genomic DNA extracted from roots and stems of V138I-inoculated (10^5 conidia/ml) olive plants used in this study were submitted to nested PCR using NDf/NDr and INTNDf/INTNDr primer pairs. None of the plants tested yielded the ND-specific nested-PCR product of 1163 bp, indicating the presence of a D isolate (V138I) in the infected plants. In any case, the detection of a pathotype in an unknown olive sample would require that the two nested-PCR assays using primer pairs D1/D2 and INTD2f/INTD2r, or NDf/NDr and INTNDf/INTNDr or INTND2f/INTND2r, are run in parallel with the same total DNA extracted from the tissues.

While carrying out the nested-PCR assays in a time course of infection, consistent differences in the fluorescense intensity of the specific PCR products were found, depending on the time of sampling of infected plants. Intensity of the PCR product was maximum with samples from days 7–24 in the experiment and decreased 52 days after inoculation, at which time a high disease incidence and severity had already developed in inoculated plants. Interestingly, the most intense PCR bands occurred in plants sampled before or at the time of symptom development regardless of the inoculum concentration used. The results of the nested-PCR assays performed in this study are not informative about the total amount of pathogen DNA in the sampled tissue. However, it is tempting to speculate that the smaller amount of PCR products found for plants sampled at the time the disease was fully developed could indicate a decrease of the fungal biomass in the plant. Fluctuations of *V. dahliae* biomass in infected olive tissues were reported by Rodríguez-Jurado (1993) while assessing colonization of the plant by the pathogen by dilution plating of tissues ground by the same procedure used in this study. Similar findings were also reported in alfalfa (*Medicago sativa* L.) (Hu et al., 1993), and tomato (*Lycopersicon esculentum* Mill.) (Heinz et al., 1998) infected by *Verticillium albo-atrum* Reinke et Berth., by means of a PCR-based quantitative approach. These findings suggest that once symptoms develop the pathogen biomass may decrease, although a certain amount of the fungus remains in the plant and gives rise to cyclical colonization by the pathogen (Heinz et al., 1998). It might be hypothesized that fluctuations in the plant colonization by *V. dahliae* can also take place in a woody host such as olive, which warrants further studies.

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References

Akillioğlu M and Tanriserver A (1997) Descripción de los compuestos fenólicos en el olivo y determinación de la composición fenólica en dos diferentes órganos y cultivares. Olivae 68: 28–31

Al-Ahmad MA and Mosli MN (1993) Verticillium wilt of olive in Syria. Bulletin OEPP/EPPO Bulletin 23: 521–529

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W and Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research 25: 3389–3402

Bahnweg G, Schulze S, Möller EM, Rosenbrock H, Langebartels C and Sanderman H (1998) DNA isolation from recalcitrant materials such as tree roots, bark, and forest soil for the detection of fungal pathogens by polymerase chain reaction. Analytical Biochemistry 262: 79–82

Banfield WM (1941) Distribution in the sap stream of spores of three fungi that induce vascular wilt diseases of elm. Journal of Agricultural Research 62: 637–681

Bejarano-Alcázar J, Blanco-López MA, Melero-Vara JM and Jiménez-Díaz RM (1996) Etiology, importance, and distribution of Verticillium wilt of cotton in southern Spain. Plant Disease 80: 1233–1238

Blanco-López MA, Jiménez-Díaz RM and Caballero JM (1984) Symptomatology, incidence and distribution of Verticillium wilt of olive trees in Andalucia. Phytopathologia Mediterranea 23: 1–8

Carder JH, Morton A, Tabrett AM and Barbara DJ (1994) Detection and differentiation by PCR of subspecific groups within two *Verticillium* species causing vascular wilts in herbaceous hosts. In: Schots A, Dewey FM and Oliver R (eds) Modern Assays for Plant Pathogenic Fungi: Identification, Detection and Quantification (pp 91–97) CAB International, Oxford

De Boer SH, Ward LJ, Li X and Chittaranjan S (1995) Attenuation of PCR inhibition in the presence of plant compounds by addition of BLOTTO. Nucleic Acids Research 23: 2567–2568

Emechebe AM, Leaky CLA and Banage WB (1975) Verticillium wilt of cacao in Uganda: incidence and progress of infection in relation to time. East African Agricultural and Forestry Journal 41: 184–186

Heale JB (2000) Diversification and speciation in *Verticillium* – an overview. In: Tjamos EC, Rowe RC, Heale JB and Fravel DR (eds) Advances in *Verticillium* Research and Disease Management (pp 1–14) APS Press, St Paul, MN

Heinz R, Lee SW, Saparno A, Nazar RN and Robb J (1998) Cyclical systemic colonization in *Verticillium*-infected tomato. Physiological and Molecular Plant Pathology 52: 385–396

Hu X, Nazar RN and Robb J (1993) Quantification of *Verticillium* biomass in wilt disease development. Physiological and Molecular Plant Pathology 42: 23–36

Jiménez-Díaz RM, Tjamos EC and Cirulli M (1998) Verticillium wilt of major tree hosts: olive. In: Hiemstra JA and Harris DC (eds) A Compendium of Verticillium Wilt in Trees Species (pp 13–16) Ponsen & Looijen, Wageningen

López-Escudero J (1999) Evaluación de la resistencia de olivo a las variantes patogénicas de *Verticillium dahliae* y eficacia de la solarización en el control de la Verticilosis. Ph.D. Thesis, University of Córdoba, Spain

Mercado-Blanco J, Rodríguez-Jurado D, Pérez-Artés E and Jiménez-Díaz RM (2001) Detection of the nondefoliating pathotype of *Verticillium dahliae* in infected olive plants by nested PCR. Plant Pathology 50: 609–619

Morton A, Carder JH and Barbara DJ (1995) Sequences of the internal transcriber spacers of the ribosomal RNA genes and relationships between isolates of *Verticillium albo-atrum* and *V. dahliae*. Plant Pathology 44: 183–190

Moukhamedov R, Hu X, Nazar RN and Robb J (1994) Use of polymerase chain reaction amplified ribosomal intergenic sequences for the diagnosis of *Verticillium tricorpus*. Phytopathology 84: 256–259

Nazar RN, Hu X, Schmidt J, Culham D and Robb J (1991) Potential use of PCR-mediated ribosomal intergenic sequences in the detection and differentiation of Verticillium wilt pathogens. Physiological and Molecular Plant Pathology 39: 1–11

Pérez-Artés E, García-Pedrajas MD, Bejarano-Alcázar J and Jiménez-Díaz RM (2000) Differentiation of cotton-defoliating and -nondefoliating pathotypes *Verticillium dahliae* by RAPD and specific PCR analyses. European Journal of Plant Pathology 106: 507–517

Raeder U and Broda P (1985) Rapid preparation of DNA from filamentous fungi. Letters of Applied Microbiology 1: 17–20

Robb J, Hu X, Platt H and Nazar R (1994) PCR assay for the detection and quantification of *Verticillium* species in potato. In: Schots A, Dewey FM and Oliver R (eds) Modern Detection Assay for Plant Pathogenic Fungi: Identification, Detection and Quantification (pp 83–90) CAB International, Oxford

Rodríguez-Jurado D (1993) Interacciones huésped-parásito en la Verticilosis del olivo (*Olea europaea* L.) inducida por *Verticillium dahliae* Kleb. Ph.D. Thesis, University of Córdoba, Spain

Rodríguez-Jurado D, Blanco-López MA, Rapoport HF and Jiménez-Díaz RM (1993) Present status of Verticillium wilt of olive in Andalucia (southern Spain). Bulletin OEPP/EPPO Bulletin 23: 513–516

Rollo F, Salvi R and Torchia P (1990) Highly sensitive and fast detection of *Phoma tracheiphila* by polymerase chain reaction. Applied and Microbiology Biotechnology 29: 572–576

Sambrook J, Fristsch EF and Maniatis T (1998) Molecular Cloning: A Laboratory Manual, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Sánchez-Hernández ME, Ruíz-Dávila A, Pérez de Algaba A, Blanco-López MA and Trapero-Casas A (1998) Occurrence and etiology of death of young olives trees in southern Spain. European Journal of Plant Pathology 104: 347–357

Schnathorst WC and Mathre DE (1966) Host range and differentiation of a severe form of *Verticillium albo-atrum* in cotton. Phytopathology 56: 1155–1161

Schnathorst WC and Sibbett GS (1971) The relation of strains of *Verticillium albo-atrum* to severity of Verticillium wilt in *Gossypium hirsutum* and *Olea europaea* in California. Plant Disease Reporter 9: 780–782

Thanassolopoulos CC (1993) Spread of Verticillium wilt by nursery plants in olive growes in the Chalkidiki area (Greece). Bulletin OEPP/EPPO Bulletin 23: 517–520

Thanassoulopoulos CC, Biris DA and Tjamos EC (1979) Survey of Verticillium wilt of olive trees in Greece. Plant Disease Reporter 63: 936–940

Tjamos EC (1993) Prospects and strategies in controlling Verticillium wilt of olive. Bulletin OEPP/EPPO Bulletin 23: 505–512

Tsukamoto H, Hisada S, Nishibe S and Roux DG (1984) Phenolic glycosides from *Olea europaea* subspc. *Africana*. Phytochemistry 23: 2839–2841