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

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An increasing incidence and distribution of verticillium wilt has occurred in the last few years in newly established olive orchards in southern Spain. This spread of the disease may result from use of *Verticillium dahliae*-infected planting material. The early *in planta* detection of the pathogen would aid the implementation of certification schemes for pathogen-free planting material. In this work, a nested polymerase chain reaction (PCR) method was developed for the *in planta* detection of the nondefoliating (ND) *V. dahliae* pathotype, aimed especially at nursery-produced olive plants. For this purpose, specific primers were designed from the sequence of a 1958-bp random amplified polymorphic DNA (RAPD) marker of ND *V. dahliae*, and a procedure for the extraction of PCR-quality total genomic DNA from infected root and stem tissues of young olive plants was tested and further optimized. Nested PCR assays detected ND *V. dahliae* in 4- to 14-month-old artificially infected plants of three olive cultivars. The ND-specific PCR product was not amplified from total genomic DNA extracted from olive plants infected with the defoliating *V. dahliae* pathotype. Detection of the ND pathotype was effective from the very earliest moments following artificial inoculation of olive plants with a *V. dahliae* conidial suspension. Also, detection was achieved in inoculated, though symptomless, olive plants as well as in plants that were symptomatic but became symptomless by 217 days after inoculation.

Keywords: *in planta* detection, *Olea europaea*, olive, pathotypes, PCR, *Verticillium dahliae*

Introduction

Verticillium wilt, caused by *Verticillium dahliae*, affects olive (*Olea europaea*) throughout its range of cultivation (Jiménez-Díaz *et al*., 1998) and causes severe yield losses and tree mortality (Thanassoulopoulos *et al*., 1979; Al-Ahmad & Mosli, 1993; Tsror *et al*., 2000). In southern Spain, 38·5% of 122 adult olive orchards inspected in 1980–83 were affected by verticillium wilt with an incidence ranging from 10 to 90% (Blanco-López *et al*., 1984), and 39·3% of 112 newly established orchards surveyed in 1994 and 1996 were affected by the disease (Sánchez-Hernández *et al*., 1998). In the last few years, verticillium wilt has also been detected in other olive-growing areas in Spain (R. M. Jiménez-Díaz, unpublished data). This 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 attacks by *V. dahliae* depends upon virulence (here defined as the amount of disease caused in a host genotype) of the pathogen isolates. Isolates of *V. dahliae* infecting olive can be classified as defoliating (D) and nondefoliating (ND) pathotypes according to their ability to defoliate the plant (Rodríguez-Jurado, 1993; Rodríguez-Jurado *et al*., 1993). This differential virulence is also exhibited in cotton (*Gossypium hirsutum*), with isolates from cotton and olive showing cross-virulence (Schnathorst & Mathre, 1966; Schnathorst & Sibbett, 1971; Rodríguez-Jurado *et al*., 1993). While infections by the D pathotype can be lethal to the plant, olive plants infected by the ND pathotype can show complete remission from symptoms (Rodríguez-Jurado, 1993; Jiménez-Díaz *et al*., 1998), thus facilitating spread of the pathogen in nonsymptomatic planting material. Although the differential virulence of *V. dahliae* pathotypes to olive has been documented in artificial inoculation studies (Schnathorst & Sibbett, 1971; Rodríguez-Jurado, 1993; López-Escudero, 1999), until now verticillium wilt attacks in olive orchards in

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Spain have been caused by the ND pathotype (Jiménez-Díaz *et al*., 1998). Recently, natural infections by the D pathotype in olive orchards were found in Spain (López-Escudero, 1999; R.M. Jiménez-Díaz *et al*., unpublished data), but are not yet known to occur in other olive-growing countries of the Mediterranean basin (Jiménez-Díaz *et al*., 1998). Therefore, although the D pathotype is more virulent, the ND pathotype is still a serious threat in olive orchards.

Management of verticillium wilt in olive is mainly by means of an integrated disease management strategy (Tjamos, 1993). A key control measure in this strategy is the use of pathogen-free plant material for propagation and for the establishment of new olive orchards, especially when planting is done in areas free of *V. dahliae*. This is of particular relevance now, since the crop is expanding in Australia and South American countries, which import large amounts of rooted, nursery-produced olive plants from Spain (J. Samsó, Agromillora Catalana SA, Barcelona, Spain; and E. C. Tjamos, Agricultural University of Athens, Athens, Greece, personal communication,). Characterization of olive plants as pathogen-free by means of isolation suffers from the inconsistency of recovering *V. dahliae* from affected woody tissues (Blanco-López *et al*., 1984). Furthermore, isolation of the pathogen from plant tissues is time-consuming and does not provide information about the pathotype infecting the plant. Therefore, new diagnostic methods are desirable for the early, rapid, specific and reliable detection of *V. dahliae* in plant material. In recent years, several different types of molecular techniques have been used for the characterization of *V. dahliae* isolates differing in host range or virulence (Heale, 2000). The polymerase chain reaction (PCR) procedure has been used for the detection and quantification of verticillium wilt pathogens in herbaceous host plants (Moukhamedov *et al*., 1994; Heinz *et al*., 1998). However, the differentiation of D and ND pathotypes of *V. dahliae* was not tested in those studies, nor have such studies 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, PCR primers were designed that differentiate between the D and ND pathotypes from Spain and other countries using DNA from fungal mycelia (Pérez-Artés *et al*., 2000). This discovery raised the possibility of using these specific primers for the *in planta* detection of the two *V. dahliae* pathotypes in infected olive.

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

Materials and methods

Chemicals and media

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

Fungal isolates and culture conditions

Cotton *V. dahliae* isolates V4I and V138I, representatives of the ND and D pathotypes, respectively, and olive ND *V. dahliae* isolate V143I were used in this study. These isolates have been characterized in previous studies (Rodríguez-Jurado *et al*., 1993; Bejarano-Alcázar *et al*., 1996; Pérez-Artés *et al*., 2000) and are deposited in the culture collection of the Departamento de Protección de Cultivos, Instituto de Agricultura Sostenible, Córdoba, Spain. 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 on chlorotetracycline-amended (30 mg L^{-1}) water agar (CWA) and further subculturing on PDA. Cultures on PDA were grown for 7 days at 24°C in the dark.

DNA extraction

DNA used in the study was from *V. dahliae* isolates, which served as controls in PCR reactions, as well as from *V. dahliae*-infected and -noninfected olive plants and endophytic, nonpathogenic fungi isolated from these plants. 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 according to Raeder & Broda (1985).

Total genomic DNA was extracted from roots and stems of ND *V. dahliae*-infected and -noninfected 4- to 14-month-old olive plants of cvs Hendeño, Oblonga and Picual. These plants were root-dip inoculated with the ND olive isolate V143I (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 and *V. dahliae* was isolated from them. Total genomic DNA was extracted using the commercially available DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). 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 stems and roots 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- to 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, Seville, 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 100 g L^{-1}) (Squibb Industria Farmaceútica, Barcelona, Spain) and 95% ethanol, then flamed and chilled before use. A sample of 20 mg of the fine powder was used for DNA extraction according to the manufacturer's instructions. Additional validation of the methodology developed was performed by using the DNeasy method for extraction and purification of DNA from 'Coker 310' cotton plants which were both infected with ND *V. dahliae* isolate V4I and noninfected.

Sequencing of the nondefoliating-associated random amplified polymorphic DNA marker and design of new specific primers

A ND-associated 2·0-kb random amplified polymorphic DNA (RAPD) band identified in previous work (Pérez-Artés *et al*., 2000) was sequenced completely as follows: plasmid pND2 (Pérez-Artés *et al*., 2000) was purified by the Qiagen Plasmid Minikit (Qiagen) according to the manufacturer's instructions. DNA sequencing was performed on both strands in overlapping fashion. The universal pUC/M13 forward (-1) and reverse primers as well as specific oligonucleotides synthesized by Genset Oligos were used. The DNA sequence of 1958 bp was determined using an Applied Biosystems Model 373 A automated DNA sequencer. The complete sequence was deposited in the EMBL/GenBank/DDBJ nucleotide sequence databases under accession number AJ302675. Sequence analysis was done using the GeneMark Predictions (Borodovsky & McIninch, 1993) and GeneID (Guigo *et al*., 1992) servers programs. A homology search was performed with the BLASTX 2.1.1 program (Altschul *et al*., 1997) of the NCBI network service. From the resulting sequence, external (NDf/NDr) and internal (INTNDf/INTNDr; INTND2f/INTND2r) primer pairs were developed as shown in Table 1.

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

The 2·0-kb ND-associated RAPD marker cloned in plasmid pND2 was released by digestion with *Pst*I and *Eco*RI endonucleases. The band was resolved by electrophoresis in a 1% agarose gel and eluted using the QIAEX II Gel Extraction kit (Qiagen). DNA was labelled using DIG-11-dUTP (digoxigenin-3-O-methyl-carbonyl-amino-caproyl-5-(3-aminoallyl)-uridine-5′-triphosphate) (Boehringer-Mannheim Biochemicals, Barcelona, Spain) according to the manufacturer's instructions. For Southern blots using PCR products, 25 μL of amplification mixture was resolved on a 1% agarose gel and transferred to Zeta-Probe® Blotting Membranes (Bio-Rad Lab. SA, Madrid, Spain) according to standard procedures (Sambrook *et al*., 1989). Hybridization was performed with the nonradioactive detection kit from Boehringer-Mannheim Biochemicals at 68°C, and the chemiluminescence method was used to detect hybridization bands according to the instructions in the kit.

Table 1 Nucleotide sequence (5′-3′) of primers developed in this study from the sequence of a 1958-bp random amplified polymorphic DNA (RAPD) band associated with the nondefoliating pathotype of *Verticillium dahliae*

Primer[a]	Sequence	Nucleotide position
NDf (+)	ATCAGGGGATACTGGTACGAGA	277–298
NDr (–)	GAGTATTGCCGATAAGAACATG	1686–1665
INTNDf (+)	CCACCGCCAAGCGACAAGAC	377–396
INTNDr (–)	TAAAACTCCTTGGGGCCAGC	1539–1520
INTND2f (+)	CTCTTCGTACATGGCCATAGATGTGC	570–595
INTND2r (–)	CAATGACAATGTCCTGGGTGTGCCA	1393–1369

[a]Matching (+) or complementary (–) sequence.

In planta PCR detection of ND *V. dahliae*

Random amplified polymorphic DNA assays using primer OPH-19 (Pérez-Artés *et al*., 2000) were carried out as a preliminary test of the purity of the DNA extracted from plant tissue and to compare the RAPD profile of saprophytic fungi isolated from olive plants with that of ND *V. dahliae* V4I. Amplifications were carried out as described by Pérez-Artés *et al*. (2000), except that the time for denaturation was reduced to 4 min and 10 ng of fungal DNA (controls) or 1–3 μL (10–30 ng) of total genomic DNA extracted from *V. dahliae*-infected or -noninfected plants was used.

Single-PCR assays using primers ND1/ND2 (Pérez-Artés *et al*., 2000), NDf/NDr, INTNDf/INTNDr and INTND2f/INTND2r were carried out for the specific *in planta* detection of *V. dahliae*. The use of primer pairs ND1/ND2 and NDf/NDr yielded a product of 1410 bp; and that of primer pairs INTNDf/INTNDr and INTND2f/INTND2r yielded a PCR product of 1163 and 824 bp, respectively (Fig. 1, Table 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; 0·25 μM of each primer, 2·5 mM of $MgCl_2$ 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 first round using primer pair NDf/NDr and the conditions indicated above was transferred to a fresh tube containing the mixture for the second amplification reaction, and the annealing temperature was set at 64°C for 1 min. Two different primer pairs were used in the second round of

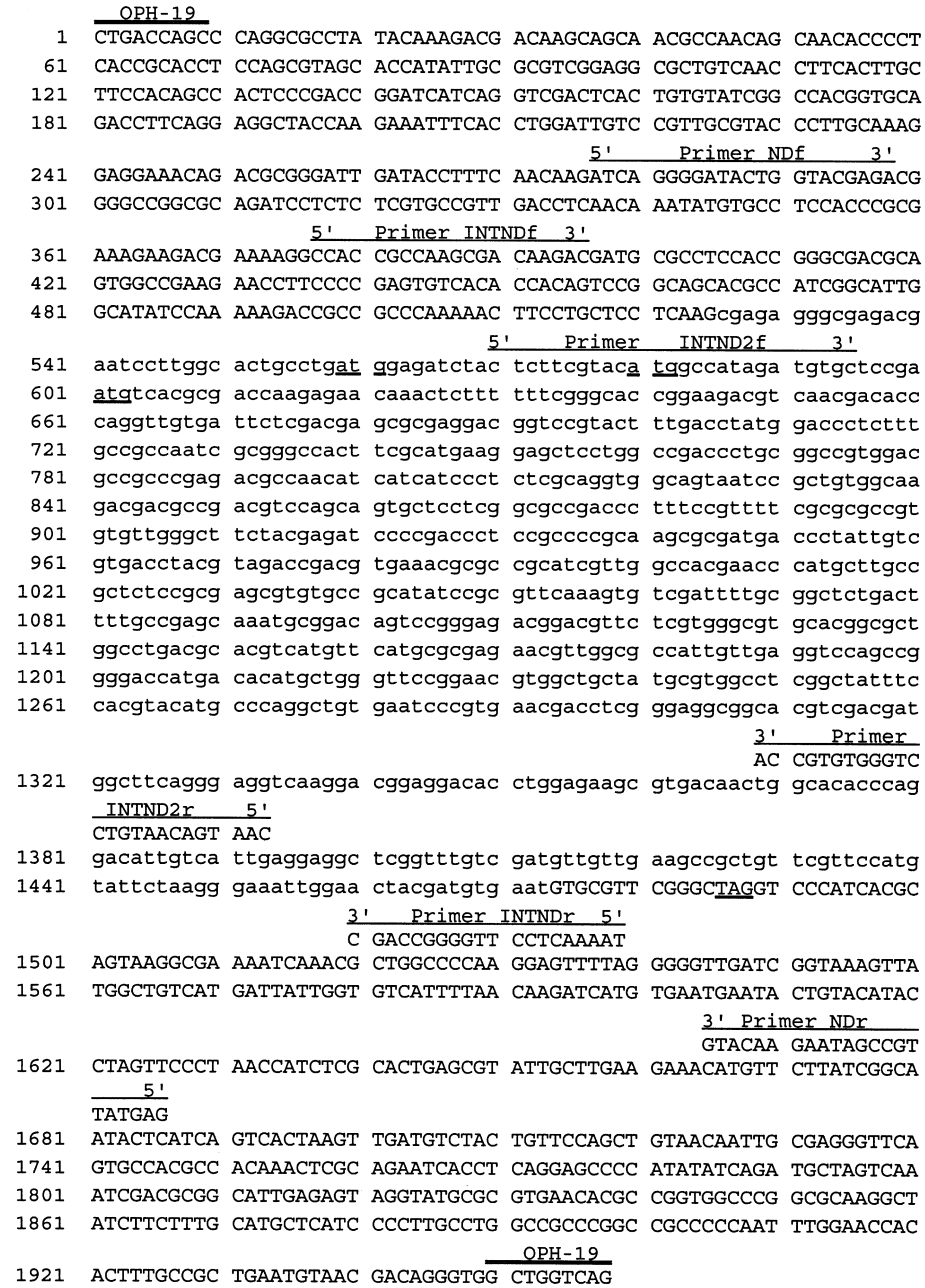

Figure 1 Nucleotide sequence of the 1958-bp ND-specific random amplified polymorphic DNA (RAPD) marker amplified by primer OPH-19. Upper double lines show the sequence of the OPH-19 primer. Upper single lines indicate the position of the specific primers used in this study. A predicted exon (from potential acceptor site to potential donor site) is shown in lower case letters. Three in-frame potential start codons (positions 559, 580 and 601) and a stop codon (position 1486) are indicated by thick underlining.

amplification: (i) INTNDf/INTNDr and (ii) INTND2f/INTND2r. Reactions were performed in a PTC-100™ Programmable Thermal Controller (MJ Research, Inc., Watertown, MA, USA). Amplification products were separated on 1% agarose gels, stained with ethidium bromide and visualized under UV light. The DNA size markers used for electrophoresis were from Boehringer-Mannheim. Reactions were repeated at least three times and always included negative controls (no DNA) and positive controls (DNA from *V. dahliae* isolates V4I and V138I purified from mycelia grown in pure culture).

Time course of *in planta* PCR detection of ND *V. dahliae*

Results of the *in planta* detection of the pathogen were validated by additional studies using 4-month-old plants of cv. Picual artificially inoculated with the ND

V. dahliae isolate V4I (Pérez-Artés *et al*., 2000). This cultivar–pathotype combination was chosen because: (i) V4I induces progressive symptom development in cv. Picual; and (ii) plants infected by this pathotype show symptom recovery when held for an extended time after inoculation (Rodríguez-Jurado, 1993).

Plants were root-dip inoculated for 1 h in a suspension of 10^5 or 10^7 conidia mL^{-1} of isolate V4I obtained from 7-day-old cultures on PDA (Rodríguez-Jurado, 1993). Thirty-five 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*. In addition, 18 plants were inoculated with each inoculum concentration and kept throughout the experiment as a reference for symptom development. Eighteen Picual plants inoculated as above with 10^7 conidia mL^{-1} of the D *V. dahliae* isolate V138I served for comparison of disease severity between the ND and D isolates used in the study, as well as internal controls for PCR assays. Seven uninoculated Picual plants served as controls. 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 watered with a hydro-sol fertilizer 20-5-32 + microelements (Haifa Chemicals, LTD, Haifa, Israel) every week. Disease reaction was assessed by severity of symptoms on a 0–4 scale according to the percentage of affected leaves and twigs (0, no symptoms; 1, 1–33%; 2, 34–66%; 3, 67–100%; 4, dead plant) at weekly intervals from 18 to 52 days, as well as at days 157 and 217 after inoculation (Rodríguez-Jurado *et al*., 1993). Data were subjected to analysis of variance using Statistix (NH Analytical Software, Roseville, MN, USA). Treatment means were compared using Fisher's protected least significance difference test (LSD) at $P = 0{\cdot}05$.

Plants from the above experiment were used for PCR detection of ND *V. dahliae*. PCR was conducted as described above using DNA samples from roots and stems of olive plants (three plants per time interval) collected at time 0 (immediately after dipping roots in water or in conidial suspensions) and 2, 7, 18, 24, 52, 157 and 217 days after inoculation with 10^5 conidia mL^{-1} of isolate V4I. Plants inoculated with 10^7 conidia mL^{-1} of isolate V4I were also sampled 4 days after inoculation. Two control plants were sampled at time 0 and 7 and 217 days after inoculation. Vascular colonization by *V. dahliae* was also determined in each of the olive plants sampled 157 and 217 days after inoculation by isolation of the fungus on CWA. For each plant, six 5-mm-long surface-disinfested stem pieces were plated onto the medium and incubated at 24°C in the dark for at least 9 days (Rodríguez-Jurado *et al*., 1993).

Results

Sequencing of the 1958-bp RAPD band associated with the ND pathotype of *V. dahliae* and design of new specific primers

In previous work (Pérez-Artés *et al*., 2000), a 2·0-kb ND pathotype-associated DNA band, which is PCR-amplified using primer OPH-19, was identified and partially sequenced. In this study, the sequence of 1958 bp (56·95% G + C content) of this RAPD marker (Fig. 1) was extended and completed. A possible open reading frame spanning positions 601–1488 and a possible exon from positions 526 (predicted acceptor site) to 1473 (predicted donor site) were identified in the sequence. No significant homology at either nucleotide or amino acid sequence level was found in the homology search. From the complete sequence of this 1958-bp marker the new specific primers NDf/NDr, INTNDf/INTNDr and INTND2f/INTND2r were developed for the single and nested PCR experiments carried out in this work. Positions of the primers used are indicated in Fig. 1 and Table 1.

Detection of ND *V. dahliae* in plant tissues by use of specific primers and nested PCR

The DNeasy extraction procedure yielded about 3–10 μg of PCR-quality genomic plant DNA routinely. Samples of olive stems and roots were freeze-dried and subsequently ground to a fine powder to obtain a suitable plant material for extraction of total DNA. A tissue sample of 20 mg of this tissue powder was quite adequate for DNA extraction. All of the DNA samples extracted by the DNeasy procedure were suitable for RAPD (data not shown) and specific PCR amplifications (see below) using both diluted or undiluted DNA. Similar results were obtained with cotton plants. This method was therefore used for plant DNA extraction in subsequent experiments.

Both the ND1/ND2 primer pair and the newly designed NDf/NDr specific primers were used repeatedly in PCR assays aimed at the detection of ND *V. dahliae* in both symptomatic and nonsymptomatic 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, 12·5 ng of DNA extracted from roots of a noninfected olive plant were mixed in a serial dilution (1:5) with *V. dahliae* DNA extracted from pure fungal mycelia. Two independent series of amplifications were performed using 1 μL samples of these mixtures. Results indicated that *V. dahliae* DNA was detectable in a sample (12·5 ng) of total genomic DNA when present in amounts less than 8 pg (data not shown). Results in the PCR assays above were not consistent. Further single PCR assays were carried out using either of the internal primer pairs and samples of the same total genomic DNA as above to test improvement of the level of detection. In these PCR

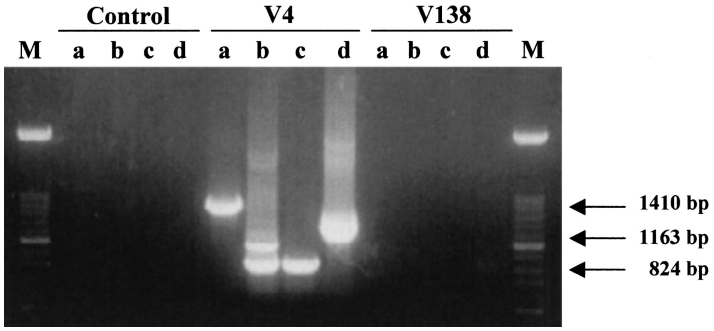

Figure 2 Polymerase chain reaction (PCR) products obtained with the different primer pairs used in this study. Lanes a, amplification reactions with primer pair NDf/NDr, yielding a PCR band of 1410 bp; lanes b, nested PCR reactions with primer pair INTND2f/INTND2r, yielding a PCR band of 824 bp. In these reactions (as well as those in lanes d), 1 μL of product from a first PCR round (primers NDf/NDr) was used as a template. An additional upper band is usually obtained under these PCR conditions, probably as a result of an excess of DNA template in the nested reaction. Lanes c, first amplification reaction with primer pair INTND2f/INTND2r (no additional band is detected); lanes d, nested PCR reactions with primer pair INTNDf/INTNDr, yielding a PCR band of 1163 bp. The PCR reactions were carried out using DNA extracted from pure mycelia of *V. dahliae* V4I (nondefoliating) and V138I (defoliating) isolates as templates, and no DNA as a negative control PCR reaction. M, molecular weight marker.

assays the predicted products were amplified, sometimes only as a faint band, but results were not yet satisfactory (data not shown). Detection of ND *V. dahliae* could be difficult because of the low concentration of fungal DNA in the total genomic DNA extracted from plant tissue samples. Therefore, a nested PCR strategy was tested. Preliminary assays were carried out using *V. dahliae* DNA extracted from pure fungal mycelia to establish the appropriate conditions for amplification with the new internal primers (Fig. 2). Thus, the annealing temperature was established at 64°C while the remaining conditions were kept identical for the two rounds of PCR.

Thereafter, nested PCR assays were carried out using total genomic DNA extracted from olive tissues. A first round of amplification was carried out using total genomic DNA from roots and stems of olive plants (cvs Hendeño, Oblonga and Picual) infected with ND *V. dahliae* isolate V143I and cotton plants (cv. Coker 310) infected with ND *V. dahliae* isolate V4I. Approximately 30 ng of total genomic DNA were used as the template in the first round of PCR with the NDf/NDr primer pair. Then, 1 μL of the amplification products was submitted to a second round of amplification (20 or 30 cycles) using the INTNDf/INTNDr primer pair (Fig. 3). No amplification of the predicted 1410-bp band was detectable after the first PCR except for the control reaction (lane 15, Fig. 3a). However, nested PCR revealed the predicted 1163-bp nested PCR band in DNA samples extracted from root tissues of V143I-inoculated olive and V4I-inoculated cotton plants. No amplification products were obtained using DNA extracted from uninoculated controls. The size of bands was in accordance with that of bands obtained when DNA from *V. dahliae* V4I mycelia was used for amplification. Furthermore, Southern blots of the amplification products of the total genomic DNA from inoculated plants revealed hybridization signals even using the product of the first PCR. This indicated that amplification took place for all samples examined in the first round of PCR, although no visible detection on agarose gels was possible (Fig. 3b).

Time course of *in planta* PCR detection of ND *V. dahliae*: testing the detection method in young symptomatic and nonsymptomatic plants

Disease reactions in olive cv. Picual plants inoculated with *V. dahliae* isolates ND V4I and D V138I agreed with previous results (Rodríguez-Jurado *et al*., 1993). No symptoms developed in uninoculated control plants. The first symptoms in inoculated plants were visible 24 days after inoculation, at which time disease incidences were 28·6 and 57·1% for plants inoculated with 10^5 and 10^7 conidia mL^{-1} of isolate V4I, respectively. Disease incidence and severity increased with time after inoculation and were higher in plants inoculated with 10^7 conidia mL^{-1} than in those inoculated with 10^5 conidia mL^{-1} (Table 2). All plants inoculated with 10^7 conidia mL^{-1} of the D isolate V138I were affected 52 days after inoculation with a mean disease severity score of 3·3.

Approximately 50% of plants inoculated with *V. dahliae* isolate V4I recovered from symptoms by the end of the experiment, 217 days after inoculation. At this time, both nonsymptomatic and symptomatic plants were sampled for isolation of the fungus from the stem, as was done with similar plants 157 days after inoculation. *Verticillium dahliae* was isolated both from symptomatic and symptomless plants. However, plants inoculated with 10^7 conidia mL^{-1} yielded a higher percentage of *V. dahliae* isolation from stem pieces than plants inoculated with 10^5 conidia mL^{-1}. Thus, 0% (10^5 conidia mL^{-1}) and 41·7% (10^7 conidia mL^{-1}) of stem pieces sampled 157 days after inoculation yielded *V. dahliae*, whereas 11·1 and 50%, respectively, of

Table 2 Disease reaction of olive cv. Picual inoculated with the nondefoliating *Verticillium dahliae* isolate V4I by the root-dip method[a]

Inoculum concentration (conidia mL^{-1})	Time after inoculation (days)	Disease	
		Incidence (%)	Severity (0–4)[b]
10^5	18	0	0
	24	28·6	0·5 a
	52	71·4	0·8 a
10^7	18	0	0
	24	57·1	0·5 a
	52	85·7	1·4 b*

[a]Plants 4 months old were uprooted from the substrate and their roots thoroughly washed, trimmed and dipped in a conidial suspension for 1 h. Plants were incubated in a growth chamber adjusted to 22/24°C light/dark and a 14-h photoperiod of fluorescent light of 262 $\mu E\ m^{-2}\ s^{-10}$. Plants were assessed for disease reaction 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%; 4, plant dead). Means followed by the same letter for each inoculum concentration are not significantly different according to Fisher's protected LSD ($P = 0{\cdot}05$). The mean followed by an asterisk is significantly larger than the mean for the corresponding assessment time at the lower inoculum concentration.

similar stem pieces yielded *V. dahliae* when sampled 217 days after inoculation.

Nested PCR assays using either the INTNDf/INTNDr or INTND2f/INTND2r primer pairs revealed that ND *V. dahliae* was detectable very soon after inoculation. The predicted 1163-bp product was amplified using total genomic DNA from olive tissues sampled at time 0 and day 2 after inoculation and primer pair INTNDf/INTNDr. Similarly, the use of the INTND2f/INTND2r primer pair with this DNA above yielded an 824-bp band, which was the predicted product when this primer pair was used for amplification. The two primer pairs were used in independent reactions with the same DNA sample as a supporting proof of each amplification experiment (Fig. 4). DNA from olive roots yielded the predicted nested-amplification products consistently, but that from stems of the same plants showed these products only sporadically. When plant tissues were analysed on a time course, two out of three plants inoculated with 10^7 conidia mL^{-1} and sampled at time 0 gave positive results (Fig. 4a). Conversely, none of the plants inoculated with 10^5 conidia mL^{-1} sampled at the same time yielded any amplification product (data not shown). However, these differences associated with the two inoculum concentrations used were of no significance with plants sampled 2 days after inoculation. All three plants inoculated with 10^7 conidia mL^{-1} sampled at this latter time yielded the predicted

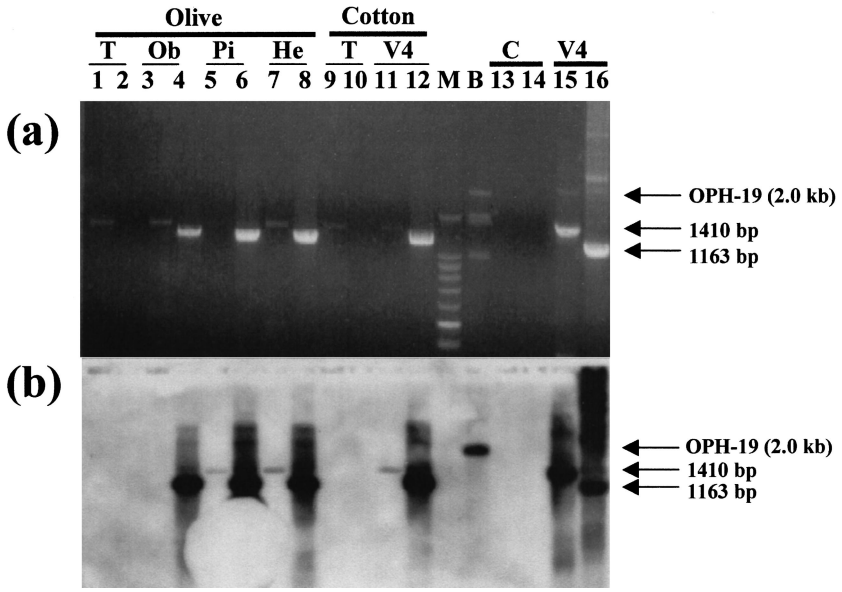

Figure 3 (a) Nested PCR results obtained from DNA samples extracted from roots of three olive cultivars infected with the nondefoliating (ND) *Verticillium dahliae* isolate V143I and cotton cv. Coker 310 infected with the ND *V. dahliae* isolate V4I, or uninoculated. Odd-numbered lanes correspond to the first round of PCR using primer pair NDf/NDr; even-numbered lanes correspond to nested PCR results (after transferring 1 μL of the amplification mixture of the first PCR to fresh tubes), using primer pair INTNDf/INTNDr. M, molecular weight marker; B, mixture of RAPD bands (the uppermost band of this mixture is the 2·0-kb ND pathotype-associated RAPD marker obtained with OPH-19); T, noninoculated plants; Ob, olive cv. Oblonga; Pi, olive cv. Picual; He, olive cv. Hendeño (all of the olive cultivars were infected with the ND *V. dahliae* isolate V143I); C, control reaction with no DNA template (lanes 13 and 14); V4, PCR results using DNA of isolate V4I extracted from pure mycelia (lanes 15 and 16), and total genomic DNA extracted from a cotton plant inoculated with isolate V4I (lanes 11 and 12). (b) Southern blot, hybridization and chemiluminescence detection results using the same gel as in (a). The 2·0-kb ND-specific RAPD marker was used as the nonradioactive probe. The exposure time was 20 min. Numbers on the right show the sizes of the PCR products.

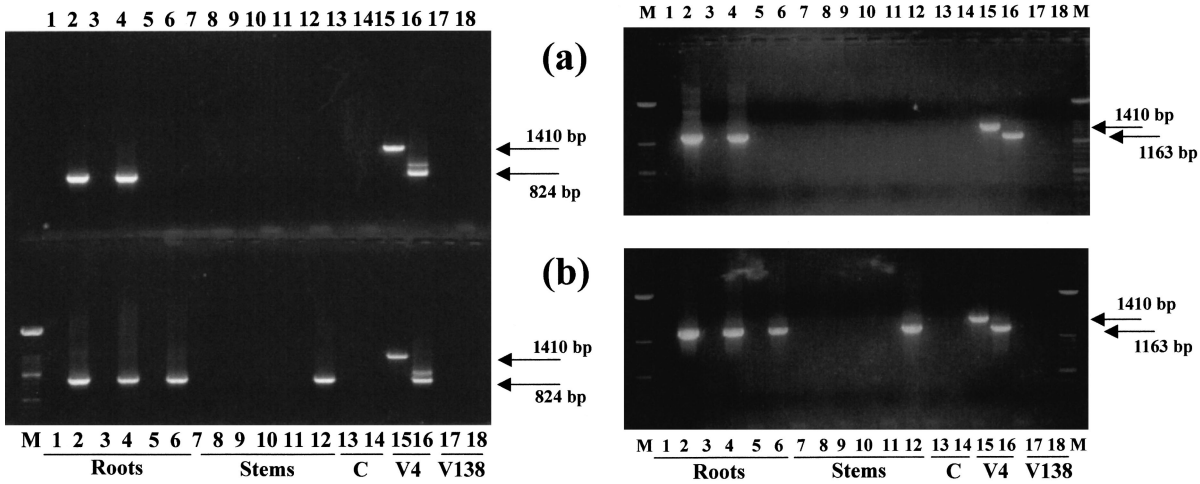

Figure 4 (a) Nested PCR using DNA extracted from three olive cv. Picual plants inoculated with the nondefoliating (ND) *Verticillium dahliae* isolate V4I and sampled at time 0 (i.e. inoculation time). Lanes 1–6 correspond to PCR performed with DNA extracted from roots. Lanes 7–12 correspond to PCR performed with DNA extracted from stems of the same plants (i.e. lanes 1 and 2 correspond to the root DNA of the plant whose stem DNA was analysed in lanes 7 and 8, and so on). Odd-numbered lanes display results of the first round of amplification. Even-numbered lanes show results of the nested PCR. (b) Nested PCR using DNA samples extracted from three olive cv. Picual plants inoculated with the ND *V. dahliae* isolate V4I and sampled 2 days after inoculation. Lane numbering is as described for (a). On the left, nested PCR performed with primer pair INTND2f/INTND2r, yielding a PCR product of 824 pb. On the right, nested PCR performed with primer pair INTNDf/INTNDr, yielding a PCR product of 1163 pb. Lanes 13 and 14, first PCR and nested PCR, respectively, in control reaction (no DNA); lanes 15 and 16, first PCR and nested PCR, respectively, performed with samples of ND *V. dahliae* isolate V4I DNA extracted from pure mycelia; lanes 17 and 18, first PCR and nested PCR, respectively, carried out with samples of defoliating *V. dahliae* isolate V138I DNA extracted from pure mycelia. M, molecular weight marker.

nested PCR product (with one plant yielding positive amplification from both root- and stem-extracted DNA) [Fig. 4(b), lanes 6 (root) and 12 (stem) of the same plant].

Although no symptoms developed before day 24 after inoculation, nested PCR assays detected ND *V. dahliae* consistently in root (but not in stem) tissues of plants sampled 4, 7 and 18 days after inoculation. Only in a few cases was it possible to detect *V. dahliae* in samples of stem tissues (data not shown), particularly 2 and 4 days after inoculation. DNA extracted from stems sampled 7 and 18 days after inoculation sometimes failed to yield PCR products. Plants sampled 52 days after inoculation yielded 100% positive detection of ND *V. dahliae* in roots and stems when plants were inoculated with 10^7 conidia mL^{-1}, and 100 and 33% positive detection from root and stem, respectively, when 10^5 conidia mL^{-1} were used for inoculation (Fig. 5a). When inoculated plants were sampled 157 and 217 days after inoculation, ND *V. dahliae* was not detected in stems (Fig. 5b), but positive detection was achieved in every sampled plant using DNA from roots. None of the uninoculated control plants yielded PCR bands from tissues sampled 0, 7 and 217 days after inoculation (Fig. 5c). Furthermore, when DNA from D *V. dahliae* V138I-inoculated olive plants was used as a template, the specific nested PCR product for the ND pathotype was not amplified (data not shown). To further confirm the specificity of *V. dahliae* detection, RAPD (primer OPH-19) and PCR assays were performed using DNA from some 17 isolates of saprophytic fungi that were isolated along with *V. dahliae* from the inoculated olive plants used in this study. These fungi included species of *Aspergillus*, *Fusarium*, *Penicillium* and other unidentified genera. RAPD profiles produced from such DNA were very different from that obtained for ND *V. dahliae* (data not shown). Also, no specific PCR products (from either one round of PCR or nested PCR) were obtained in reactions using DNA of each of the 17 fungal isolates and any of the primer pairs developed in this study (data not shown).

Discussion

The use of pathogen-free planting material is a key control measure for management of verticillium wilt in olives. In this study, a nested PCR procedure was developed for the consistent and early detection of ND *V. dahliae* in root tissue of symptomatic and nonsymptomatic nursery-propagated olive plants. This required optimizing a procedure for extracting the pathogen DNA suitable for PCR assay and designing new specific primers for amplification of the extracted DNA. These specific primers did not amplify the ND-specific product when DNA from D *V. dahliae*-infected plants was used as a template. Although *Verticillium* spp. can be detected in herbaceous hosts by PCR-based methods (Moukhamedov *et al*., 1994; Heinz *et al*., 1998), this is the first report of PCR-based detection of *V. dahliae* pathotypes in a woody host.

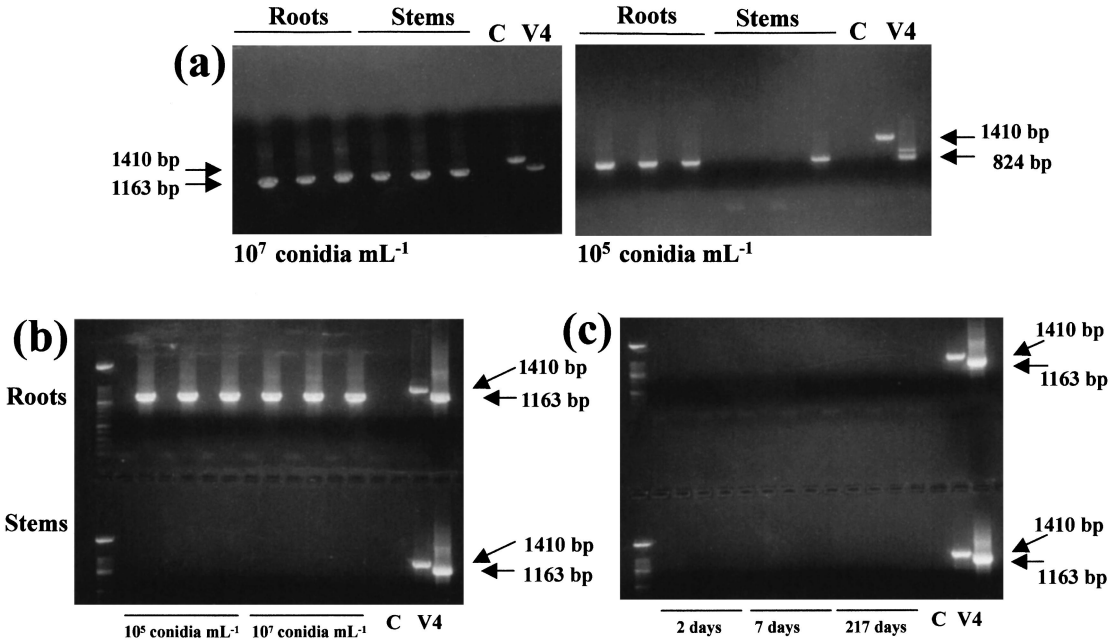

Figure 5 (a) Results of nested PCR using total DNA extracted from three olive cv. Picual plants sampled 52 days after inoculation with conidia of nondefoliating (ND) *Verticillium dahliae* isolate V4I. On the left, the 1163-bp nested PCR product using primer pair INTNDf/INTNDr and DNA extracted from plants inoculated with 10^7 conidia mL^{-1}. On the right, the 824-bp nested PCR product obtained using primer pair INTND2f/INTND2r and DNA extracted from plants inoculated with 10^5 conidia mL^{-1}. Samples are ordered in the gel as explained in Fig. 4. (b) Results of nested PCR using total DNA extracted from six olive cv. Picual plants sampled 217 days after inoculation with ND *V. dahliae* isolate V4I. (c) Absence of nested PCR bands using DNA extracted from noninoculated olive plants (roots, upper gel; stems, lower gel) sampled 2, 7 and 217 days after dipping the root systems in sterile water. Results displayed in (b) and (c) were obtained using the primer pair INTNDf/INTNDr. C, control reaction (no template DNA); V4, positive control reaction with DNA extracted from pure mycelia of ND *V. dahliae* isolate V4I.

Extracting PCR-quality DNA from stems and roots of infected olives can pose difficulty since phenolic compounds contained in large amounts in olive tissues (Tsukamoto *et al*., 1984; Akillioğlu & Tanrisever, 1997) might be coextracted along with DNA and hinder the PCR processes (De Boer *et al*., 1995). Preliminary assays in our study tested a DNA extraction procedure based on the CTAB cationic detergent; several modifications were necessary, such as diluting the DNA samples and/or adding BLOTTO (De Boer *et al*., 1995) in the PCR protocol for consistent success in DNA amplification (data not shown). In contrast, the DNeasy method provided 100% PCR-quality DNA from olive roots and stems without need of dilution or BLOTTO amendment for PCR amplification.

Detection of ND *V. dahliae* in total genomic DNA from infected plants by single PCR using external or internal specific primers either failed or gave inconsistent results. This could be a consequence of the concentration of fungal DNA being significantly lowered relative to the plant DNA sample extracted from the tissues. A nested PCR strategy was necessary to consistently demonstrate the presence of ND *V. dahliae* in an infected olive plant. This strategy proved successful in root and stem samples of three different olive cultivars and in plants infected with each of two different ND *V. dahliae* isolates from cotton and olive. Also, the whole detection procedure developed for infected olive plants was shown to be applicable to ND *V. dahliae*-infected cotton cv. Coker 310. Since the primers for nested PCR were developed from a ND-associated RAPD band common to some 41 ND isolates from cotton and olive of diverse geographical origins (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 in different locations in southern Spain (Mercado-Blanco *et al*., unpublished data).

The nested PCR strategy developed made it possible to detect the pathogen in nursery-produced olive plants very soon after artificial inoculation with conidia of ND *V. dahliae*, as well as in nonsymptomatic plants either recovered from symptoms or sampled too early after infection. The early detection of the pathogen after inoculation must have been related to the high inoculum concentration used. This high inoculum concentration was needed for consistent results in pathogenicity experiments (Rodríguez-Jurado, 1993). However, a lower amount of fungal inoculum in the plant, as would be likely to occur with natural infections, would make it difficult to detect infection this early. ND *V. dahliae* was consistently detected in olive roots from

day 2 to day 217 after inoculation. Detection of the pathogen DNA in stem samples was less consistent over time. It was thought that, in some cases, the negative results for the attempted detection of the pathogen in the stem of inoculated plants were due to the absence of *V. dahliae* in the sampled tissue, as suggested by the disease incidence at sampling time and results from isolations. Reactions were repeated at least three times and always included negative and positive controls. Negative results were consistent in the repeated reactions. Furthermore, negative results were unlikely to be a consequence of the DNeasy-extracted DNA being unsuitable as a template for PCR, since RAPD amplifications of total genomic plant DNA samples were successful.

Nevertheless, in some cases *V. dahliae* was detected in the olive stem as early as 2 days after inoculation. This may relate to a rapid translocation of *V. dahliae* conidia along xylem vessels in the plant, probably with the transpiration stream, as reported for other tree species (Banfield, 1941; Emechebe *et al*., 1975). In any case, the detection procedure was successful in 100% of root and stem samples when plants inoculated with 10^7 conidia mL^{-1} were analysed 52 days after inoculation, i.e. when 85·7% of plants showed symptoms. Nested PCR using either of the two internal primer pairs consistently detected ND *V. dahliae* in olive cv. Picual roots sampled at any time after inoculation, but it failed in the detection of ND *V. dahliae* in stems of plants incubated in a growth chamber for 217 days after inoculation. Plants sampled at this time had recovered during the extended incubation time and were no longer showing the symptoms of infection. Also, in comparable stems where PCR failed to detect *V. dahliae*, isolations could not always detect it either. It is possible that DNA yield from these latter stem samples was poorer than that obtained from earlier samplings. Although samples of total DNA were equalized for the PCR reaction, these samples might have had less *V. dahliae* in the stem. Thus, the fungal biomass in these formerly symptomatic plants could be considerably reduced because of mycelial lysis (Pegg & Dixon, 1969; Vessey & Pegg, 1973). Additionally, stem tissues of these plants may contain higher amounts of lignin-related compounds which might be related to defence mechanisms operating in Picual plants and associated with the moderate susceptibility of this cultivar to ND *V. dahliae* (Rodríguez-Jurado, 1993; López-Escudero, 1999). Results of ND *V. dahliae* detection in Picual stems resembled the cyclical systemic colonization of tomato plants by *Verticillium albo-atrum* that has been reported (Heinz *et al*., 1998). It is possible that a similar phenomenon could take place in *V. dahliae*-infected olive; this remains to be investigated.

In conclusion, this study indicates that nested PCR amplification of ND *V. dahliae* with the specific primers developed may be a useful method for the specific detection of this pathotype in nursery-produced olive plants. This assay may be useful for the early detection of ND *V. dahliae* in root tissue of symptomless olive plants during plant material propagation for certification purposes or epidemiological studies. Furthermore, a similar approach could be developed for the specific detection of D *V. dahliae*. Studies are in progress aimed at this latter objective.

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