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Detection of the defoliating and nondefoliating pathotypes of *Verticillium dahliae* in artificial and natural soils by nested PCR

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Abstract

In Spain, Verticillium wilt, caused by *Verticillium dahliae*, is the most important disease of cotton and olive. Isolates of *V. dahliae* infecting these crops can be classified into highly virulent, defoliating (D), and mildly virulent, nondefoliating (ND), pathotypes. Infested soil is the primary source of inoculum for Verticillium wilt epidemics in cotton and olive, and severity of disease relates to the prevailing *V. dahliae* pathotype. In this work we have adapted the use of previously developed primer pairs specific for D and ND *V. dahliae* for the detection of these pathotypes by nested PCR in artificial and natural soils. Success in the detection procedure depends upon efficiency in extracting PCR-quality DNA from soil samples. We developed an efficient DNA extraction method from microsclerotia infesting the soil that includes the use of acid washed sand during the grinding process and skimmed milk to avoid co-purification of *Taq*-polymerase inhibitors with DNA. The specific nested-PCR procedure effectively detected 10 or more microsclerotia per gram of soil. The detection procedure has proven efficient when used with a naturally infested soil, thus demonstrating usefullness of the diagnostic method for rapid and accurate assessment of soil contamination by *V. dahliae* pathotypes.

Introduction

The soilborne fungus *Verticillium dahliae* causes severe wilt in many herbaceous (e.g., artichoke, eggplant, cotton, potato) and woody crops (e.g., apricots, avocado, olive, peaches), flowers (e.g., chrysanthemum, rose) and woody ornamentals (e.g., ash, catalpa, maple) worldwide (Pegg and Brady, 2002). In Spain, Verticillium wilt is the most important disease of cotton (*Gossypium hirsutum*) and olive (*Olea europaea*) causing severe yield losses and plant death (Bejarano-Alcázar et al., 1997; Jiménez-Díaz et al., 1998). Severity of attacks by *Verticillium* wilt depends upon virulence (defined as the amount of disease caused in a host genotype) of the pathogen isolates. Isolates of *V. dahliae* infecting cotton and olive show cross-virulence and can be classified into defoliating (D) and non defoliating (ND) pathotypes based on their ability to completely defoliate the plant (D) or to only cause wilt and partial or no defoliation (ND) (Bejarano-Alcázar et al., 1996; Rodríguez Jurado, 1993; Rodríguez Jurado et al., 1993; Schnathorst and Sibbett, 1971). In cotton, Verticillium wilt epidemics caused by the D pathotype develop earlier, more rapidly, and result in a greater reduction of cotton seed yield compared with losses caused by the ND pathotype (Bejarano-Alcázar et al., 1995, 1997). Similarly, olive plants infected by the ND pathotype can show complete remission from symptoms (Jiménez-Díaz et al., 1998; Rodríguez-Jurado et al., 1993). Conversely, infections by the D pathotype can be lethal to olive and cotton cultivars and overcome valuable tolerance to

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ND *V. dahliae* in certain cotton and olive genotypes (Bell, 1994; López-Escudero, 1999; Schnathorst and Mathre, 1996). Therefore, the proper characterization of *V. dahliae* pathotypes would help in the decision-taking process for disease management such as choice of host cultivar and avoidance of soil infested with D *V. dahliae* (Tjamos, 1993).

Plating methods (dry and wet) have traditionally been used for the detection and quantification of *V. dahliae* in soil. However, existing methods are slow, inconsistent, soil dependent, and do not inform about pathotype (Goud and Termorshuizen, 2003; Harris et al., 1993). During the last few years, significant progress has been made in the development of improved methods for the detection and quantification of plant pathogenic fungi, based mainly on the detection of DNA sequences by the polymerase chain reaction (PCR) (Martin et al., 2000; Miller, 1996). These PCR-based techniques allow for the amplification of species-specific sequences and have the advantage of being highly specific, sensitive, and rapid, with the potential to be automated (Taylor et al., 2001). Those methods were successfully used for the detection (Mahuku et al., 1999; Volossiouk et al., 1995) and quantification (Krishnamurthy et al., 2001; Mahuku and Platt, 2002) of *V. dahliae* in soil, but differentiating between D and ND *V. dahliae* was not addressed in those studies.

Recently, we developed a PCR-based method for specific *in planta* detection of D and ND *V. dahliae* pathotypes in infected cotton and olive plants which consists of a nested-PCR procedure using external and internal primer pairs specific for D and ND *V. dahliae* (Mercado-Blanco et al., 2001, 2002; Pérez-Artés et al., 2000). In this study we report the development and application of these PCR-based techniques for detection of D and ND *V. dahliae* isolates in different artificial and natural soils.

Materials and methods

Fungal isolates and inoculum production

Monoconidial *V. dahliae* isolates V4I and V138I that are representatives of the ND and D pathotypes, respectively, were used in this study. These isolates originated from diseased cotton plants in the Guadalquivir Valley of southern Spain and were characterized both by biological and molecular pathotyping in previous studies (Bejarano-Alcázar et al., 1996; Pérez-Artés et al., 2000; Mercado-Blanco et al., 2001, 2002). The isolates are deposited in the culture collection of Departamento de Protección de Cultivos, Instituto de Agricultura Sostenible, CSIC, Córdoba, Spain. Plum-extract agar cultures of isolates covered with liquid paraffin were stored at 4 °C in the dark (Bejarano Alcázar et al., 1995). Active cultures were obtained by placing small agar plugs from stock cultures on chlorotetracycline-amended (30 mg L^{-1}) water agar and further subculturing on potato-dextrose agar (PDA) at 24 °C in the dark for 7 days. Conidia suspensions of isolates were obtained from cultures in potato-dextrose broth incubated at 125 rpm on an orbital shaker (Adoff Kuhner AG, Birsfelden, Switzerland) at 24 °C in the dark for 7 days. Conidia in the liquid cultures were filtered through three layers of sterile cheesecloth and conidia concentration was adjusted as needed using a haemocytometer.

Soil samples for experiments were artificially infested with microsclerotia of *V. dahliae* V4I and V138I produced on fresh harvested, autoclaved (121 °C, 20 min) potato stems. For microsclerotia production, 10 to 15-cm-long stem pieces were dipped in a 10^7 conidia ml^{-1} suspension in sterile distilled water for 20 min. Inoculated stem pieces were dried between sterile filter paper sheets in a flow of filtered air, and incubated in a moist chamber at 24 °C in the dark for 30 days. Stem pieces heavily colonized by *V. dahliae* were dried as above and stored in paper bags at 22 ± 1 °C until use. To obtain free microsclerotia, the dried stem pieces were cut into 0.5 cm fragments and ground using an electric mixer containing sterile distilled water. Then, the powder was sifted through 125, 105 and 40 μm sieves in a tandem and microsclerotia retained on the 40 μm sieve were collected in sterile distilled water. The concentration of microsclerotia in the suspension was adjusted as needed using a haemocytometer. To assess viability of microsclerotia, a diluted suspension of microsclerotia was plated onto ethanol-streptomycin agar (ESA) (ethanol, 5.6 g L^{-1}; streptomycin sulphate, 0.1 g L^{-1}; agar, 5 g L^{-1}). Cultures were incubated at 24 °C in the dark for 11 days, and the number of colonies developed were counted assuming that a colony developed from a single microsclerotium.

Soil samples

Several soil types, differing in texture and composition, were sampled and used in this study. These were: an artificial soil mixture consisting of sand and silt in

1:1 proportion; and three (A,B,C) natural soils. Soils A and B were representative of agricultural soils in Córdoba province (southern Spain) where cotton and olive were grown regularly. Soil A was a Vertisol (51.4% clay, 26.4% silt, and 22.2% sand), pH 8.1, 2.0% organic matter, and 40 m.e.q. per 100 g soil cation exchange capacity. Soil B was a sandy soil (15.9% clay, 14.9% silt, and 69.2% sand), pH 8.3, 1.5% organic matter, and cation exchange capacity of 12 m.e.q. per 100 g soil. Soil C was sampled from a hilly forest near Córdoba, and was used as a representative of a non-agricultural soil considered as being unlikely infested by *V. dahliae*. This soil was a Inceptisol (27% clay, 9% silt, and 64% sand), pH 6.1, 2.2% organic matter, and cation exchange capacity of 14 m.e.q. per 100 g soil.

Four 200 g soil samples were randomly collected from the selected fields using a cylindrical auger ($2.5 \cdot 20$ cm) to a depth of 20 cm, after removing 3–5 cm of surface soil. Soil samples from each plot were bulked, dried at room temperature, sieved through a 2-mm pore-size screen, and then thoroughly mixed. All soil samples were sterilised by autoclaving for 1 h at 121 °C on three consecutive days, leaving the samples at room temperature between each autoclaving round, and then artificially infested with *V. dahliae*.

The study was carried out in a sequence that started with assays using soil samples amended with purified DNA, then continued with soil samples infested with microsclerotia. By doing so, we aimed to gradually increase complexity in the substrate to be used in the DNA extraction procedure. As a first step, samples of the sterilised, artificial soil mixture were mixed with DNA extracted from mycelia of ND *V. dahliae* V4I within a range of concentration of 0.25 to 40 ng DNA mg^{-1} soil. The sterilised, artificial soil mixture without added DNA, and DNA extracted from mycelia of *V. dahliae* V4I, were used as negative and positive controls, respectively. To determine whether soil composition might influence quality of the extracted DNA and, therefore, the efficiency of the nested-PCR procedure, we prepared samples of soil infested using a natural soil (soil C). Samples of soil C were autoclaved as above and mixed with DNA of *V. dahliae* isolate V4I at a rate of 20 ng DNA mg^{-1} soil. This rate was selected as adequate for the assay because of results from the previous assays.

In a second step, efficiency of the diagnostic procedure was determined using natural soils infested with *V. dahliae* microsclerotia. To infest the soil samples, aliquots of 1 g each of the autoclaved soil samples were thoroughly mixed with the appropriate volume of microsclerotia suspension to generate a range of inoculum densities of 10, 10^2, 10^3, 10^4, and 10^5 microsclerotia of the ND pathotype per gram of soil, and 5, 50, $5 \cdot 10^2$, $5 \cdot 10^3$ and $5 \cdot 10^4$ microsclerotia of the D pathotype per gram of soil. The artificially infested soil samples were dried at room temperature for 10 to 15 days, and then lyophilized. Both autoclaved soil samples without addition of the fungus, and soil samples infested in the same way as before but then sterilised by autoclaving (121 °C, 1 h), were used as negative controls. The infested lyophilized soil samples and negative controls were stored in sterile closed glass tubes at 4 °C until used. DNA extracted from mycelia of ND *V. dahliae* V4I, and D *V. dahliae* V138I were used as positive controls.

Finally, soil sampled from a cotton field affected by Verticillium wilt was used as representative soil naturally infested with *V. dahliae*. Four soil samples (200 g each) were taken close to affected plants, thoroughly mixed, dried at room temperature, and sieved before lyophilizing. A subsample of this naturally-infested soil was sterilised by autoclaving (121 °C, 1 h), on three consecutive days, and used as negative control.DNA extracted from mycelia of ND *V. dahliae* V4I, and D *V. dahliae* V138I were used as positive controls.

Extraction of DNA from soil samples

DNA from infested soil samples was extracted according to the procedure developed by García-Pedrajas et al. (1999) with minor modifications. Briefly, each of 1 g samples of the infested, lyophilized soils was amended with 30% acid-washed autoclaved sand and ground to a fine powder using an autoclaved (121 °C, 30 min) pestle and mortar. Samples of 75 mg of the fine powder were transferred to 1.5 ml microcentrifuge tubes, thoroughly mixed by vortexing with 200 μl of 3.2% (w/v) commercial skimmed milk (Nestlé, 99% fat free) and the mixtures were centrifuged at 12,000 *g* for 1 h at 4 °C. After centrifugation, supernatants were transferred to a fresh tube and thoroughly mixed with 6 μl of 10% SDS and an equal volume of phenol:chloroform:isoamylalcohol (25:24:1) by vortexing. This mixture was centrifuged at 12,000 *g* for 30 min at room temperature, and the upper phase was collected and used directly for amplification.

PCR assays

The extracted DNA was used as template for nested-PCR assays using primer pairs NDf/NDr and

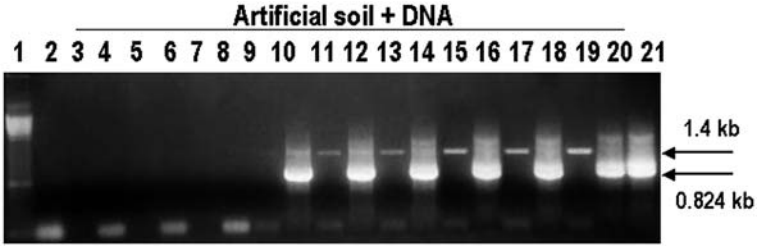

Figure 1. Detection of DNA of *V. dahliae* isolate V4I [nondefoliating (ND) pathotype] in artificial soil by PCR using ND specific nested primers. Samples of soil were infested with a range of concentrations of DNA: lanes 3/4, 0.25 ng of DNA mg^{-1} soil; lanes 5/6, 0.5 ng DNA mg^{-1} soil; lanes 7/8, 1 ng DNA mg^{-1} soil; lanes 9/10, 2 ng DNA mg^{-1} soil; lanes 11/12, 5 ng DNA mg^{-1} soil; lanes 13/14, 10 ng DNA mg^{-1} soil; lanes 15/16, 20 ng DNA mg^{-1} soil; lanes 17/18, 30 ng DNA mg^{-1} soil; lanes 19/20, 40 ng DNA mg^{-1} soil. First and second lane at each concentration of DNA correspond to the first and second reaction of the nested PCR , respectively. Lane 1: molecular weight marker; lane 2: negative control (product of the second reaction of PCR using the extract from soil without DNA); lane 21: positive control (product of the second reaction of PCR using DNA extracted from mycelia of the *V. dahliae* isolate V4I)

Df/Dr (Pérez-Artés et al., 2000; Mercado Blanco et al., 2001) for the first round of amplification, and primers INTND2f/INTND2r and INTD2f/INTD2r (Mercado Blanco et al., 2001, 2002) for the second round. Amplifications with primer pairs NDf/NDr and INTND2f/INTND2r yielded PCR products of 1.4 kb and 0.824 kb (Mercado Blanco et al., 2002) from DNA of ND *V. dahliae*, respectively. Amplification with primer pairs Df/Dr and INTD2f/INTD2r yielded PCR products of 0.548 kb (Pérez-Artés et al., 2000) and 0.462 kb (Mercado Blanco et al., 2001) from DNA of D *V. dahliae*, respectively. For nested-PCR assays, the reaction mixture (25 μl) for the first reaction consisted of: 0.25 μM of each primer (NDf/NDr or Df/Dr), 200 μM of each dNTP, 2.5 μl of 10× reaction buffer [50 μM KCl, 10 mM Tris-HCl pH 9.0 (25 °C), 1% v/v Triton X-100] 0.75 U of Taq DNA polymerase (Promega, Madison, WI), 2.5 mM $MgCl_2$ and 1 μl of soil extract. Amplification conditions were: denaturation at 94 °C for 5 min followed by 30 cycles of 1 min annealing at 58 °C, 3 min extension at 72 °C, and 1 min denaturation at 94 °C. The final cycle consisted of 1 min annealing followed by 6 min at 72 °C to produce fully double stranded DNA fragments. For the second reaction, 1 μL of the PCR product from the first reaction was added to a fresh tube containing a new reaction mixture and the internal primer pairs INTND2f/INTND2r or INTD2f/INTD2r. Amplification conditions were same as for the first round of amplification, except for the annealing temperature that was raised to 64 °C. PCR reactions were performed in a Perkin-Elmer 9600 or 2400 thermocycler (Perkin-Elmer, Norwalk, CT). All reactions were repeated twice and always included negative (no DNA) and positive (template DNA purified from pure mycelia of *V. dahliae* V4I and V138I) controls.

Results

Experiments were conducted to establish the best conditions for detection of D and ND *V. dahliae* in soil as well as for assessing efficiency and sensibility of the procedures. In a first step, samples of soil amended with *V. dahliae* V4I DNA were subjected to the DNA extraction procedure described in Materials and methods, and used as template for PCR assays. Nested-PCR assays using these extracts as template yielded the expected diagnostic DNA band in those samples containing 2 ng of *V. dahliae* V4I DNA mg^{-1} soil or more (Figure 1). The diagnostic DNA bands were visible both after the first (1.4 kb) and the second (0.824 kb) rounds of amplification (Figure 1). Intensity of the DNA band obtained after de first round of amplification increased with DNA concentration in soil (Figure 1). Conversely, when this experiment was repeated using a natural soil, soil C, amended with 20 ng DNA mg^{-1} soil, the diagnostic DNA band was visible only after the second round of nested PCR (Figure 2).

The *V. dahliae* detection method was also efficient with natural soils artificially infested with microsclerotia of *V. dahliae* ND and D pathotypes. Effectiveness of the DNA extraction procedure and that of the PCR amplification protocol was not influenced by the different composition of the two natural soils (soils A and B) used in the assays. In both cases, addition of sand was necessary for the efficient DNA extraction from microsclerotia infesting the soil (Figure 3). Similarly, no differences in PCR products (i.e. similar DNA

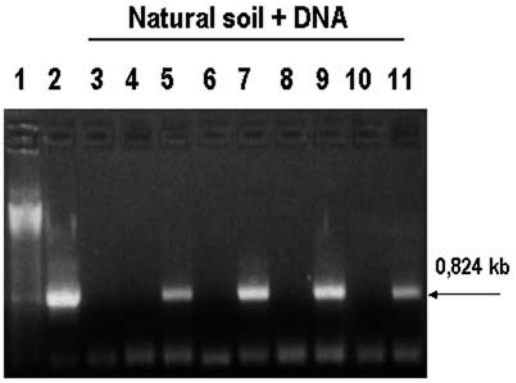

Figure 2. Detection of DNA of *V. dahliae* isolate V4I in a natural soil using ND specific nested primers. Samples of soil were mixed with DNA of *V. dahliae* at a concentration of 20 ng DNA mg^{-1} soil. Lanes 4/5, 6/7, 8/9 and 10/11 correspond to the first and second reaction of the nested PCR amplification from every of four replicate assays. Lane 1: molecular weight marker; lane 2: positive control (product of the second reaction of amplification using DNA extracted from mycelia of isolate V4I); lane 3: negative control (product of the second reaction of amplification using extract from soil without DNA)

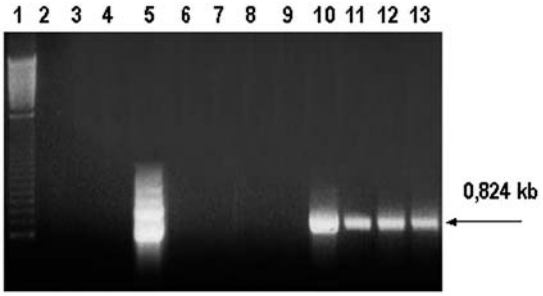

Figure 3. Detection of *V. dahliae* DNA in two natural soils, soil A (lanes 6, 7, 10, 11) and soil B (lanes 8, 9, 12, 13), infested with 10^5 microsclerotia of the ND isolate V4I per gram of soil. Extracts of DNA from each soil were obtained without (lanes 6–9) or with (lanes 10–13) addition of sand. Two concentrations of skimmed milk were used: 3.2% (lanes 6, 8, 10, 12), and 4.8% (lanes 7, 9, 11, 13). Lane 1: molecular weight marker. Lanes 2–4, negative controls: lane 2, extract obtained from a noninfested sterile soil; lanes 3 and 4, extracts obtained from, respectively, samples of A and B soils infested with 10^5 microsclerotia g^{-1} soil and then sterilised. All lines represent the product obtained after the second amplification in the nested-PCR.

band intensities on agarose gels) were found for any of the two natural soil types, regardless the concentration of skimmed milk (3.2% or 4.8% ,w/v) added to the extraction mixture (Figure 3). On the contrary, PCR assays conducted on extracts from soils where skimmed milk was not added did not yield any of the expected diagnostic amplicons (result not shown). Similarly, there was no amplification in PCR assays

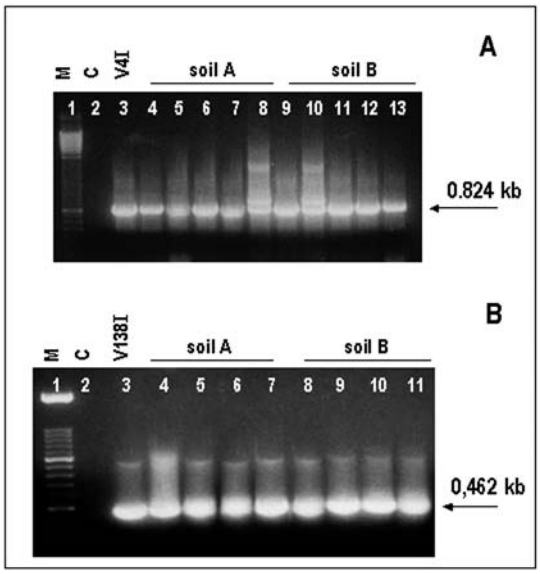

Figure 4. Detection of DNA from *V. dahliae* in natural soils artificially infested with a range of microsclerotia of the ND (Figure 4a) and D (Figure 4b) pathotypes. All lanes show the product obtained after the second reaction of amplification in the nested PCR procedure. In Figure 4a, lanes 4 to 8 correspond to A soil infested with 10, 10^2, 10^3, 10^4, and 10^5 microsclerotia g^{-1} of soil, respectively; and lanes 9 to 13 correspond to B soil infested with the above number of microsclerotia g^{-1} of soil, respectively. In Figure 4b, lanes 4 to 7 correspond to A soil infested with 50, 5×10^2, 5×10^3 and 5×10^4 microsclerotia g^{-1} of soil, respectively; and lanes 8 to 11 correspond to B soil infested with the above number of microsclerotia g^{-1} of soil, respectively. Lane 1: molecular weight marker; lane 2: negative control (extract from noninfested soil); lane 3: positive control [DNA extracted from mycelium of *V. dahliae* isolate V4I (Figure 4a) or V138I (Figure 4b)].

of infested soils that had been autoclaved prior the extraction of DNA (Figure 3).

The sensitivity of the detection procedure was challenged using samples of natural soils (A and B), which were artificially infested with an increasing number of microsclerotia of the D and ND *V. dahliae* pathotypes per gram of soil. Extracts from soils infested with microsclerotia of the ND isolate V4I yielded the diagnostic PCR product (0.824 kb) after the second round of amplification, disregarding soil type and inoculum densities (from 10 to 10^5 microsclerotia g^{-1} soil) (Figure 4a). Similarly, DNA extracted from soils (A or B) infested with a range from 5 to 5×10^4 of microsclerotia per gram of soil of the D isolate V138I, also yielded the expected PCR product (0.462 kb) after the second round of amplification, except for soils infested with 5 microsclerotia g^{-1} soil (Figure 4b). The detection procedure was further challenged against a field soil naturally infested

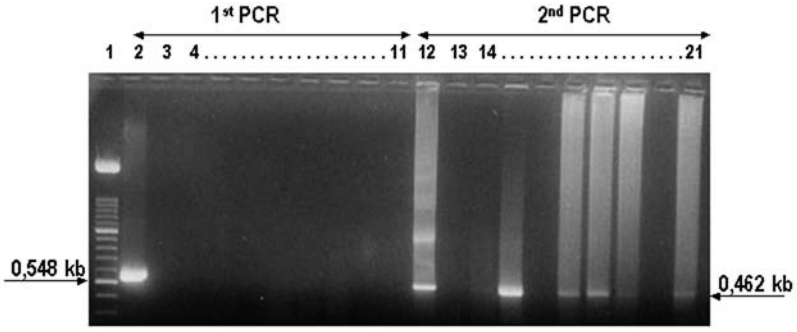

Figure 5. Detection of DNA from microsclerotia of *V. dahliae* in a sample of soil naturally infested with the D pathotype. Lanes 4 to 11 and 14 to 21 correspond, respectively, to the first and second reaction of the nested PCR amplification with eight subsamples of the naturally infested soil. Lane 1: molecular weight marker; lanes 2 and 12: positive control (first and second product of amplification, respectively, using DNA extracted from mycelium of V. dahliae isolate V138I) lanes 3 and 13: negative control (first and second product of amplification, respectively, using the extract from sterilised soil).

with *V. dahliae*. A total of 160 75-mg soil subsamples from 800 g sampled soil were each extracted and assayed. Ten per cent out of 160 assayed samples gave positive detection of *V. dahliae* after the nested-PCR assay. Only the D pathotype was detected. As shown in Figure 5, there was no signal after the first round of amplification with any of the samples, but the specific diagnostic band (0.462 kb) was clearly visible after the second round of the nested PCR.

Discussion

This paper describes the development of a PCR-based assay for the specific detection of D and ND pathotypes of *V. dahliae* in soil. Together with the DNA extraction protocol, the diagnostic method allows for the rapid and accurate assessment of soil contamination by *V. dahliae*.

A simple and inexpensive extraction method has been used in this study, with a small number of efficient lysis and purification steps to allow rapid processing of many samples. The usefullness of the DNA extraction method was confirmed by testing three different types of soil. The extraction procedure was based on the physical disruption of microsclerotia in the soil sample. Regardless the texture (clay or sandy) of the assayed soil, addition of acid-washed sand was necessary for adequate disruption of the fungal structures during the grinding process. Similar results have been shown by other authors working on the extraction of DNA from different fungal structures in soil (García-Pedrajas et al., 1999; Faggian et al., 1999). DNA samples obtained from soils without the addition of sand did not amplify any DNA fragment after nested PCR, thus suggesting that disruption of microsclerotia was not achieved. Extracts from soils that had been infested with microsclerotia of *V. dahliae* and then sterilised by autoclaving never amplified the diagnostic DNA band, thus demonstrating that microsclerotia were efficiently destroyed during the autoclaving process .

One of the main barriers to successful amplification of DNA extracted from soil samples is the presence of humic acids and related compounds produced by the degradation of organic matter (García Pedrajas et al., 1999). These compounds are known to be powerful inhibitors of Taq polymerase (Tsai and Olson, 1992). Addition of skimmed milk prevents the copurification of such inhibitors with DNA (Volossiouk et al., 1995; García-Pedrajas et al., 1999). Our results demonstrate that addition of 3.2% (w/v) of skimmed milk powder to the extraction mixture effectively reduced inhibitors in extracts from different soil types; however, increasing the concentration of skimmed milk in the extraction mixture from 3.2% to 4.8% did not improve the results with any of the soil types studied.

As documented by other authors (Cullen et al., 2002; Faggian et al., 1999; García-Pedrajas et al., 1999; Lees et al., 2002), PCR amplification of DNA extracted directly from soil samples requires a double ('nested') round of amplification to produce clear visible DNA bands. This can probably be due to presence of inhibitors in the soil extract, rather than to a small amount of template DNA being in the reaction mixture. This is supported by comparable results obtained with extracts from artificial and natural soil amended with the same amount of purified DNA. The

lack of amplification after the first round of nested PCR using extracts from the natural soil indicates that components in a natural soil influences efficiency in purification and/or amplification of the target DNA.

PCR assays using extracts from natural soils (clay or sandy types) infested with microsclerotia of D or ND *V. dahliae* pathotypes never amplified the specific diagnostic DNA band after the first round of amplification, regardless the pathogen inoculum density [from 10 to 10^5 microsclerotia g^{-1} soil of isolate V4I (ND), and from 5 to 5×10^4 microsclerotia g^{-1} soil of isolate V138I (ND)] in soil (result not shown). Only after a second round of amplification the specific diagnostic DNA fragment was observed. Both for ND and D pathotypes, the specific nested-primers effectively detected 10 or more microsclerotia per gram of soil. This could be considered a good level of sensibility because the level of *V. dahliae* infestation found in Verticillium wilt-affected cotton and olive crops at southern Spain, as determined by the dry plating method, ranged from 1 to 130 microsclerotia g^{-1} soil (Bejarano et al., 1995, 1996).

The detection procedure developed in this study proved useful with a naturally infested soil. DNA samples were extracted from a total number of 160 soil subsamples, of which 10% amplified the specific diagnostic DNA band for the D pathotype of *V. dahliae*. Previous results carried out in cotton fields suggested a clumping of *V. dahliae* inoculum in soil (Bejarano-Alcázar and Jiménez-Díaz, 1997). This spatial distribution may explain the low percentage of samples that gave a positive detection. Therefore, the PCR-based detection procedure developed must be used in conjunction with adequate soil sampling strategy that take into account the possibility of uneven distribution of the pathogen in soil.

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References

Bejarano-Alcázar J, Melero-Vara J M, Blanco-López M A and Jiménez-Díaz R M 1995 Influence of inoculum density of defoliating and nondefoliating pathotypes of *Verticillium dahliae* on epidemics of Verticillium wilt of cotton (in southern Spain). Phytopathology 85, 1474–1481.

Bejarano-Alcázar J, Blanco-López M A, Melero-Vara J M and Jiménez-Díaz R M 1996 Etiology, importance and distribution of Verticillium wilt of cotton in southern Spain. Plant Dis. 80, 1233–1238.

Bejarano-Alcázar J, Blanco-López M A, Melero-Vara J M and Jiménez-Díaz R M 1997 The influence of Verticillium wilt epidemics on cotton yield in southern Spain. Plant Pathol. 46 168–178.

Bejarano-Alcázar J and Jiménez-Díaz R M 1997 Analysis of the Spatial Pattern of *Verticillium dahliae* in Soil. 7th International Verticillium Symposium, Athens. Book of Abstracts. p. 87.

Bell A A 1994 Mechanisms of disease resistance in *Gossypium* species and variation in *Verticillium dahliae*. *In* Proceedings of the World Cotton Research Conference – 1 Eds. G A Constable and N W Forrester. pp. 225–235. CS/RO, Melbourne, Australia.

Culleen D W, Lees A K, Toth I K and Duncan J M 2002 Detection of *Colletotrichum coccodes* from soil and potato tubers by conventional and quantitative real-time PCR. Plant Pathol. 51, 281–292.

Faggian R, Bulman S R, Lawrie A C and Porter I J 1999 Specific polymerase chain reaction primers for the detection of *Plasmodiophora brassicae* in soil and water. Phytopathology 89, 392–397.

García-Pedrajas M D, Bainbridge B W, Heale J B, Pérez-Artés E and Jiménez-Díaz R M 1999 A simple PCR based method for the detection of the chickpea-wilt pathogen *Fusarium oxysporum* f. sp. *ciceris* in artificial and natural soils. Eur. J. Plant Pathol. 105, 251–259.

Goud J C and Termorshuizen A J 2003 Quality of methods to quantify microsclerotia of *Verticillium dahliae* in soil. Eur. J. Plant Pathol. 109, 523–534.

Harris D C, Yang Y R and Ridout M S 1993 The detection and estimation of *Verticillium dahliae* in naturally infested soil. Plant Pathol. 42, 238–250.

Jiménez-Díaz R M, Tjamos E C and Cirulli M 1998 Verticillium wilt of major tree hosts: olive. *In* A compendium of Verticillium Wilt in Tree Species. Eds. J A Hiemstra and D C Harris. pp. 13–16. Ponsen and Looijen, Wageningen, The Netherlands.

Krishnamurty V, Barbara D and Price A 2001 PCR-based quantification of *Verticillium dahliae* in Soil. 8th International Verticillium Symposium. Book of Abstracts. Córdoba , Spain. pp. 73.

Lees A K, Cullen D W, Sullivan L and Nicolson M J 2002 Development of conventional and quantitative real-time PCR assays for the detection and identification of *Rhizoctonia solani* AG3 in potato and soil. Plant Pathol. 51, 293–302.

López-Escudero F 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 Cordoba, Spain.

Mahuku G S and Platt H W 2002 Quantifying Verticillium dahliae in soils collected from potato fields using a competitive PCR assay. Am. J. Potato Res. 79, 107–117.

Mahuku G S, Platt H W and Maxwell P 1999 Comparison of polymerase chain reaction methods with plating on media to detect and identify *Verticillium* wilt pathogens on potato. Can. J. Plant Pathol. 21, 125–131.

Martín R R, James D and Lévesque C A 2000 Impacts of molecular diagnostic technologies on plant disease management. Annu. Rev. Phytopathol. 38, 207–239.

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

Mercado-Blanco J, Rodríguez-Jurado M D, Pérez-Artés E and Jiménez-Díaz R M 2002 Detection of the defoliating pathotype of *Verticillium dahliae* in infected olive plants by nested PCR. Eur. J. Plant Pathol. 108, 1–13.

Miller S A 1996 Detecting propagules of plant pathogenic fungi. Adv. Bot. Res. 23, 73–102.

Pegg G F and Brady B L 2002 Verticillium Wilts. CAB International, Wallingford, UK.

Pérez-Artés E, García-Pedrajas M D, Bejarano-Alcázar J and Jiménez-Díaz R M 2000 Differentiation of cotton-defoliating and nondefoliating pathotypes of *Verticillium dahliae* by RAPD and specific PCR analyses. Eur. J. Plant Pathol. 106, 507–517.

Rodríguez-Jurado D, Blanco-López M A, Rapoport H F, and Jiménez-Díaz R M 1993 Present status of Verticillium wilt of olive in Andalucia (southern Spain). Bull. OEPP/EPPO 23, 513–516.

Schnathorst W C and Mathre D E 1966 Host range and differentiation of a severe form of *Verticillium albo-atrum* in cotton. Phytopathology 56, 1155–1161.

Schnathorst W C and Sibbett G S 1971 The relationship of strains of *Verticillium albo-atrum* to severity of Verticillium wilt in *Gossypium hirsutum* and *Olea europaea* in California. Plant Dis. Rep. 9, 780–782.

Taylor E, Bates J, Kemion A, Maccaferri M and Thomas J 2001 Modern molecular methods for characterization and diagnosis of seed-borne fungal pathogens. J. Plant Pathol. 83, 75–78.

Tjamos E C 1993 Prospects and strategies in controlling Verticillium wilt in olive. Bull. OEPP/EPPO 23, 505–512.

Tsay Y L and Olson B H 1992 Rapid method for separation of bacterial DNA from humic substances in sediments for polymerase chain reaction. Appl. Env. Microbiol. 58, 2292–2295.

Volossiouk T, Robb E J and Nazar R N 1955 Direct DNA extraction for PCR-mediated assays of soil organisms. Appl. Env. Microbiol. 61, 3972–3976.

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