European Journal of Plant Pathology (2005) 112:43–52
DOI 10.1007/s10658-004-8232-0

Development of specific PCR primers for identification and detection of *Phytophthora capsici* Leon.

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Accepted 13 December 2004

Key words: capsicum, diagnosis, ITS, pepper, soil, zoospores

Abstract

A PCR-based method was developed for the identification and detection of *Phytophthora capsici* in pepper plants. Three PCR primers (CAPFW, CAPRV1 and CAPRV2) specific for *P. capsici* were designed based on the sequence of its internal transcribed spacer regions. CAPFW/CAPRV1 amplify a 452 bp product from *P. capsici* DNA whereas CAPFW/CAPRV2 a 595 bp fragment; neither set amplifies DNA from pepper or several fungi pathogenic to pepper. In conventional (single-round) PCR, the limit of detection was 5 pg DNA for both primer sets, whereas in nested PCR the detection limit for both was of 0.5 fg. However, when the dilution series of target DNA were spiked with plant DNA, amplification declined two-fold in both conventional and nested PCR. The CAPFW/CAPRV2 set in conventional PCR was used to detect *P. capsici* DNA in inoculated plants. Detection occurred as soon as 8 h post-inoculation in stem samples from infected but still symptomless plants. The method was also tested to detect fungal DNA in infected soils.

Introduction

Phytophthora capsici is an important soilborne pathogen of pepper. It belongs to the genus *Phytophthora*, order *Pythiales*, phylum *Oomycota*, and several reports have grouped it together with *P. citricola* and *P. citrophthora* in a distinct group of papillate species (Oudemans and Coffey, 1991; Lee and Taylor, 1992; Cooke and Duncan, 1997). It is a heterothallic species with papillate sporangia and amphigynous antheridia. *Phytophthora capsici* is of worldwide distribution and causes multiple diseases not only in pepper but also in tomatoes, cucurbits and other plant species by infecting roots (Phytophthora root rot), leaves (Phytophthora blight) and fruits (Phytophthora fruit rot) (Leonian, 1922; Bowers and Mitchell, 1990; Ristaino, 1991; Erwin and Ribeiro, 1996). Among these, Phytophthora blight and Phytophthora root rot are the most economically important soilborne diseases of pepper throughout the world (Kim et al., 1989, Bosland and Lindsey, 1991). In Spain, Phytophthora root rot is the most potentially destructive disease of pepper cultures, being Phytophthora blight and Phytophthora fruit rot nonhabitual (Nuez et al., 1996; Pomar et al., 2001). In Phytophthora root rot the pathogen penetrates into the plant through the collar causing first necrosis in that area. At the same time the plant becomes withered while the fungus advances towards both the stem and the root causing an obstruction in the vascular system. The infected tissues become dry, sunken, parchment-like, and turn dark in colour. Finally, the death of the plant occurs at the last stage of infection. These symptoms are characteristic of *P. capsici;* however recently in Galicia (NW Spain) the presence of *Phytophthora nicotianae* isolates which are able to infect pepper plants causing similar symptoms to those caused by *P. capsici* has been reported (Andrés et al., 2003).

There are chemical treatments, especially fungicides, which are used to combat *P. capsici*, but these can result in soil pollution. Therefore, the design of an early method to detect the pathogen would be useful not only for preventing the disease but also to avoid more environmental contamination. So far, detection methods have included visual examination based on the taxonomic key of Stamps et al. (1990) and isolation in selective media, but these traditional methods are time-consuming, labour-intensive and very often they require extensive knowledge of fungal taxonomy. Serological techniques have also been developed in different *Phytophthora* species (Jones and Shew, 1988; McDonald et al., 1990; Grote and Gabler, 1999), but the lack of specificity of some antibodies and the necessity of obtaining monoclonal antibodies complicate the technique (Bonants et al., 1997). Molecular methods and in particular the polymerase chain reaction (PCR) have been successful in identifying and detecting different fungal plant pathogens (Lacourt and Duncan, 1997; Schena et al., 2002 a, b; Ippolito et al., 2002). The advantages of PCR are its high specificity, sensitivity and rapidity with regard to traditional techniques. PCR has been widely used for detection of fungal plant pathogens based on the internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA) (Willits and Sherwood, 1999; Wallenhammar and Arwidsson, 2001; Grote et al., 2002). The 18S, 5.8S, and 28S nuclear rDNA genes are highly conserved for different fungal species and have been used for phylogenetic studies, whereas the ITS regions evolve more quickly and may vary among species within a genus (White et al., 1990; Cooke and Duncan, 1997; Cooke et al., 2000), allowing the development of PCR primers that uniquely amplify one species.

The aim of this work was to develop a rapid and reliable method for *P. capsici* detection in plants and soils using PCR technology.

Materials and methods

Fungal and plant material

One of the Spanish *P. capsici* isolates was collected in northwest Spain (Galicia) from infected pepper plants during a survey conducted in 1998 (Pomar et al., 2001), while the other was obtained from Centro de Investigaciones Agrarias Mabegondo (CIAM, Spain). Other *P. capsici* isolates were obtained from CABI Bioscience (United Kingdom) (Table 1). The rest of the fungi (*Phytophthora* spp. isolates and other fungal species) were either obtained from collection at, the Scottish Crop Research Institute (SCRI) or purchased from Colección Española de Cultivos Tipo (CECT, Spain) (Table 2).

Pepper (*Capsicum annuum*) plants of cultivar Yolo Wonder were grown in a growth chamber at 25 °C and a photoperiod of 16 h light and 8 h darkness. When plants were one month old they were placed into independent pots for further inoculation. Infected plants were returned to the chamber under the same growth conditions.

DNA extraction

To extract DNA, isolates were grown in still culture in pea broth (Cooke et al., 2000). After one week of incubation at 24 °C, mycelia were collected and freeze-dried for extended storage at −20 °C. Total genomic DNA was extracted according to Raeder and Broda (1985) with some modifications. Briefly, freeze-dried mycelia were ground in a mortar with SDS extraction buffer (200 mM Tris HCl, 250 mM NaCl, 25 mM EDTA and 5% SDS); extraction with phenol/chloroform/isoamyl alcohol (25:24:1, v/v) was then carried out. The precipitation was done with cold isopropanol,

Table 1. Phytophthora capsici isolates used in this work

Isolate name	Country of origin	Source	Host
UDC196Pc	Spain	UDC (Spain)	*Capsicum annuum*
RO-4	Spain	CIAM (Spain)	*Capsicum annuum*
IMI 352321	India	CABI (U.K.)	*Piper nigrum*
IMI 379384	Pakistan	CABI (U.K.)	*Capsicum annuum*
IMI 149561	Mexico	CABI (U.K.)	*Capsicum annuum*
IMI 230564	Nigeria	CABI (U.K.)	*Capsicum annuum*
IMI 223314	Iran	CABI (U.K.)	*Soil*

the pellet was washed with 70% ethanol and finally it was resuspended in sterile double-distilled water. The amount of DNA was measured spectrophotometrically at 260 nm.

In order to extract DNA from both naturally and artificially infected plants, samples were ground with liquid nitrogen using a mortar and pestle. The powder was transferred to a tube and mixed with extraction buffer (0.1 M Tris-HCl, pH 7, 1.4 M NaCl, 0.2% β-mercaptoethanol, 1% PVP, 2% CTAB). Extraction with phenol/chloroform/isoamyl alcohol (25:24:1, v/v) was carried out. DNA was precipitated with isopropanol, washed with cold ethanol (70%) and resuspended in sterile distilled water (SDW).

DNA was extracted from soil according to Volossiouk et al. (1995) with few modifications. Briefly, 0.25 g of soil sample were ground with a mortar and pestle until a fine powder remained. The powdered soil was suspended in 0.5 ml of skimmed milk powder solution (0.1 g of milk powder in 25 ml of H_2O) by vigorous vortexing. The soil and debris were removed by centrifugation and the supernatant was mixed with SDS extraction buffer (200 mM Tris HCl, 250 mM NaCl, 25 mM EDTA and 5% SDS). An equal volume of phenol was then added and mixed by vortexing. Then, chloroform: isoamyl alcohol (24:1, v/v) was added and the precipitation was carried out with isopropanol. Finally, the DNA was washed with ethanol (70%) and the pellet was resuspended in SDW.

Primer design and PCR amplification

Three specific primers for *Phytophthora capsici* (CAP primers) were used: one forward primer (CAPFW; 5′TTTAGTTGGGGGTCTTGTACC3′), and two reverse primers (↑CAPRV1; 5′CCTCCACAACCAGCAACA3′ and CAPRV2; 5′TACGGTTCACCAGCCCATCA3′) were designed by comparison of the internal transcribed spacer regions of different *Phytophthora* species, looking for a region of high dissimilarity (for details about species and GenBank accession numbers, see Cooke et al., 2000). First, to ensure DNA quality, all extracts were amplified by PCR using universal primers ITS 4 (White et al. 1990) and ITS 6 (Cooke et al., 2000). PCR reactions were performed in a total volume of 10 µl containing 100 ng of genomic DNA, 1X PCR reaction buffer (Roche Diagnostics SL), 50 µg of bovine serum albumin (Sigma), 100 µM of each dNTP (Bioline), 0.2 µM of each primer (Roche Diagnostics SL) and 0.4 U of Taq DNA polymerase (Roche Diagnostics SL). The PCR reaction was incubated in a programmable thermal cycler (Primus 25 PCR-System) starting with 2 min dena-

Table 2. Fungal species used to test the specificity of *Phytophthora capsici*-specific primers

Species	Isolate name	Source
Botrytis cinerea	B05-10	WWU (Germany)
Fusarium oxysporum f.sp. *lycopersici*	2715	CECT (Spain)
Phytophthora cactorum.	CAC23	SCRI (Scotland)
Phytophthora citrophthora	IMI 332632	CABI (U.K.)
Phytophthora citricola	CIT 2	SCRI (Scotland)
Phytophthora clandestina	CLA 2	SCRI (Scotland)
Phytophthora cryptogea	CRY 1	SCRI (Scotland)
Phytophthora gonapodyides	P245	SCRI (Scotland)
Phytophthora fragariae var. *rubi*	FVR	SCRI (Scotland)
Phytophthora infestans	97.39.7.2	SCRI (Scotland)
Phytophthora nicotianae	NIC 1	SCRI (Scotland)
Phytophthora palmivora	P488	SCRI (Scotland)
Phytophthora porri	P1720	SCRI (Scotland)
Phytophthora quercina	L50/2	SCRI (Scotland)
Verticillium albo-atrum	2693	CECT (Spain)
Verticillium dahliae	2694	CECT (Spain)
Verticillium nigrescens	2696	CECT (Spain)
Verticillium tricorpus	2695	CECT (Spain)

turation at 95 °C, followed by 30 cycles at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 60 s, with a final extension step of 5 min at 72 °C. PCR products were analysed by electrophoresis in 1% (w/v) agarose gel in 1X Tris-borate-EDTA buffer (Maniatis et al. 1982) and stained with ethidium bromide (5 μg ml^{-1}) for visualisation. Conventional PCR with CAP primers was carried out as described above except for the annealing temperature, which was 56 °C. In some cases, a nested PCR was developed to increase the sensitivity of the method. In this case, either *Phytophthora* specific primers (PHYTO primers) - which included one forward (5′CTTTCCACGTGAACCGTWTC3′) and two reverse primers (5′CAAAATGGATCGACCCCTCG3′ and 5′CCAAATGGATCGACCCTCG3′)- or primer pairs DC6/ITS4 (Cooke et al., 2001) were used in the first round. The PCR conditions for PHYTO primers and DC6/ITS4 were the same as for ITS4/ITS6 primers but with an annealing temperature of 59 °C or 56 °C, respectively. One microlitre of the first round was used as template in the second round of amplification with CAP primers, performed according to the PCR protocol described above.

Primer specificity and sensitivity

Specificity was tested against DNA from a range of *Phytophthora* species, pepper pathogens and different *P. capsici* isolates (Tables 1 and 2). To determine the sensitivity, DNA from *P. capsici* was used to prepare a ten-fold serial dilution from 500 ng μl^{-1} to 0.05 fg μl^{-1}. Then, to test the negative effect of plant DNA on PCR, the ten-fold dilutions of fungal DNA were mixed with 500 ng μl^{-1} of plant DNA extracted from Yolo Wonder pepper plants. Sensitivity was determined with conventional and nested PCR.

Detection of Phytophthora capsici *in plants and soils*

One-month-old pepper plants were inoculated with 10,000 zoospores ml^{-1} of isolate UDC196Pc by adding 5 ml of the inoculum into the soil. Samples of leaves, stems and roots were taken at 8, 24, 48, 72 and 96 h post-inoculation. Additionally, pepper plants showing symptoms of Phytophthora root rot and healthy plants were collected in the field from five farms in northwest Spain. Soil samples were also collected in the same farms where infected plants had been harvested.

Detection of Phytophthora capsici *zoospores*

A suspension of zoospores was obtained according to Larkin et al. (1995). *Phytophthora capsici* isolate UDC196PC was grown in V8 agar at 24 °C for 7 days to obtain zoospores. V8 agar cultures were then cut into small pieces and incubated with SDW at 24 °C for 72 h. Zoospore release was induced by chilling cultures at 5 °C for 1 h and then incubating at 24 °C for 30–60 min. Zoospore suspensions were filtered through a 10 μm gauze to remove hyphal and sporangial debris. Zoospore concentration was counted using a haemocytometer. An aliquot of two-fold zoospore suspension (from 512 to 1 zoospores) was added directly into the PCR reaction. Nested PCR was also performed to determine the sensitivity of detection.

Results

Primer design, specificity and sensitivity

Multiple sequences of the ITS region of several *Phytophthora* species were aligned to identify a characteristic region for *P. capsici*. Three specific primers, one forward (CAPFW) and two reverse (CAPRV1 and CAPRV2), were designed. The first set (CAPFW/CAPRV1) gave a 452 bp product (data not shown) whereas the second (CAPFW/CAPRV2) one of 595 bp (Figure 1). Both amplification products contained parts of ITS1, ITS2 and the whole 5.8S subunit.

To test the specificity of CAPprimers, purified DNA from 12 *Phytophthora* species and six isolates from other fungal species, some pathogenic to pepper (Table 2), was amplified using CAPFW, CAPRV1 and CAPRV2. First, to ensure good quality DNA, extracts were amplified using primers ITS4 and ITS6. These primers yield a fragment of 900 bp for *Phytophthora* species (Cooke and Duncan, 1997) and a fragment ranging from 400 to 600 bp for the other pepper pathogens (data not shown). However, no PCR products were obtained for either the CAPFW/CAPRV2 set (Figure 1) or the CAPFW/CAPRV1 set (data not

shown) when they were used with fungal species other than *P. capsici*. On the contrary, when the primers were tested against different *P. capsici* isolates (Table 1) amplification products of predicted size (595 bp and 452 bp) were obtained for both primer sets, CAPFW/CAPRV2 (Figure 2) and CAPFW/CAPRV1 (data not shown).

Sensitivity of primer pairs was tested using serial dilutions of total DNA extracted from *P. capsici*. In conventional PCR, the lowest amount of amplified DNA was 5 pg for both primer pairs, CAPFW/CAPRV2 (Figure 3A) and CAPFW/CAPRV1 (data not shown). In nested PCR, after the first round amplification with PHYTO primers, the detection limit was 0.5 fg for CAPFW/CAPRV2 (Figure 3B) and the same for CAPFW/CAPRV1 (data not shown). The inclusion of plant DNA in the PCR reaction decreased the sensitivity limit two-fold in both conventional (Figure 4A) and nested PCR (Figure 4B).

Detection of Phytophthora capsici *in plants and soils*

To test whether the PCR assay could detect the fungal DNA in plants, susceptible cultivar Yolo Wonder plants were inoculated with a suspension of zoospores and samples of leaves, stems and roots were taken in a time course period. Only the primer set CAPFW/CAPRV2 was used to detect fungal DNA in the plants. Using conventional PCR with this primer pair, fungal DNA was detected at 8 h post-inoculation in infected stems but still pre-symptomatic. After 24 h the fungus was also detected in roots but in no case was it detected in leaves using single PCR (Figure 5A). Only by nested PCR, with PHYTO primer set in the first round, was fungal DNA detected in leaves at 8 h after inoculation (Figure 5B). No amplification products were obtained after conventional and

Figure 1. Specificity of *Phytophthora capsici*-specific primers CAPFW/CAPRV2. M, 100 bp marker; lane 1, *Phytophthora capsici* UDC196Pc (positive control); lane 2, *P. nicotianae*; lane 3, *P. cactorum*; lane 4, *P. quercina*; lane 5, *P. palmivora*; lane 6, *P. gonapodyides*; lane 7, *P. cryptogea*; lane 8, *P. clandestina*; lane 9, *P. citricola*; lane 10, *P. fragariae* var. *rubi*; lane 11, *P. infestans*; lane 12, *P. porri*; lane 13, *P. citrophthora*; lane 14 V*erticillium dahliae*; lane 15, *Verticillium albo-atrum;* lane 16, *Verticillium nigrescens;* lane 17, *Verticillium tricorpus*; lane 18, *Botrytis cinerea*; lane 19, *Fusarium oxysporum* and lane 20, no DNA template.

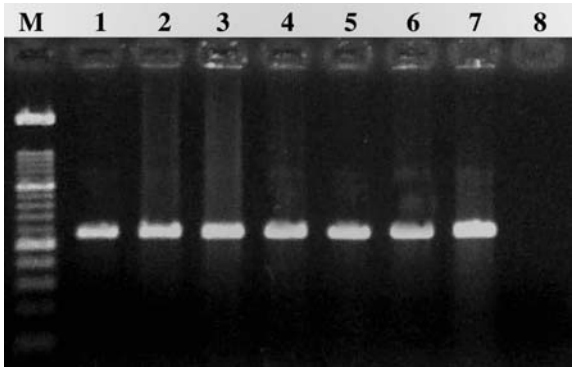

Figure 2. Amplification of different *Phytophthora capsici* isolates using CAPFW/CAPRV2. M, 100 bp marker; lane 1, UDC196Pc; lane 2, RO-4; lane 3, isolate from India; lane 4, from Pakistan; lane 5, from Mexico; lane 6, from Nigeria; lane 7, from Iran and lane 8, no DNA template.

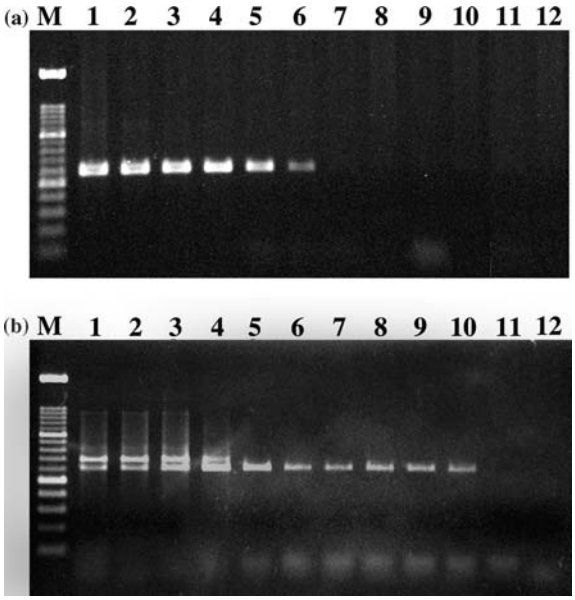

Figure 3. Sensitivity of single PCR (A) or nested PCR (B) for detection of *Phytophthora capsici*. M, 100 bp marker; lane 1, undiluted (500 ng ml^{-1}); lane 2, 10^1 diluted; lane 3, 10^2; lane 4, 10^3 ; lane 5, 10^4; lane 6, 10^5; lane 7, 10^6; lane 8, 10^7; lane 9, 10^8 ; lane 10, 10^9; lane 11, 10^{10} and lane 12, no DNA template.

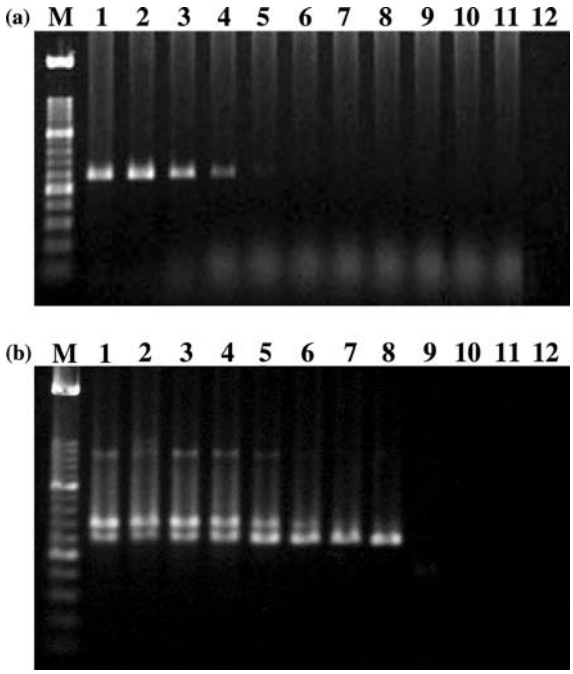

Figure 4. Sensitivity of single PCR (A) or nested PCR (B) for detection using *Phytophthora capsici* DNA plus plant DNA. M, 100 bp marker; lane 1, undiluted (500 ng ml^{-1}); lane 2, 10^1 diluted; lane 3, 10^2; lane 4, 10^3; lane 5, 10^4; lane 6, 10^5; lane 7, 10^6; lane 8, 10^7; lane 9, 10^8, lane 10, 10^9; lane 11, 10^{10} and lane 12, no DNA template.

nested PCR for samples that were healthy and for negative controls.

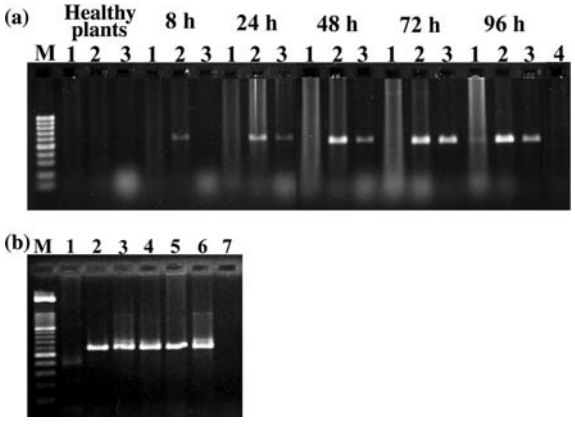

Figure 5. A, single PCR detection of *Phytophthora capsici* in artificially-infected plants using CAPFW/CAPRV2. M, 100 bp marker; lane 1, leaves; lane 2, stems and lane 3, roots; lane 4, no template DNA. B, nested PCR detection of *P. capsici* in artificially-infected leaves. M, 100 bp marker; lane 1, healthy leaves; lane 2, 8 h after inoculation; lane 3, 24 h; lane 4, 48 h; lane 5, 72 h; lane 6, 96 h and lane 7, no DNA template.

To study the efficiency of the method in detecting *P. capsici* in naturally-infected plants, healthy and symptomatic plants were collected from five different farms in NW Spain. Four different plants from each farm were sampled, three of them symptomatic and one with no symptoms. Using conventional PCR with primer set CAPFW/CAPRV2 no amplification product was obtained in any of the samples tested (data not shown). In nested PCR with PHYTO primer set in the first round and CAPFW/CAPRV2 in the second, the predicted fragment of 595 bp was observed in plants from three out of five farms (Figure 6). In these three farms the fungus was detected in the three infected plants, except for Farm 1, where *P. capsici* was only detected in two out of three symptomatic plants. In no case was the fungus detected in asymptomatic plants. Since there were a few symptomatic plants where the fungus was not detected, another assay was carried out to check the presence of any other *Phytophthora* species causing similar symptoms to those produced by *P. capsici*. In this case, a nested PCR was carried out using the set DC6/ITS4 in the first round and PHYTO primers in the second. An amplification product of predicted size (700 bp) was only obtained in those samples where *P. capsici* had been found previously. No other *Phytophthora* species were detected in plants coming from five different farms (Figure 7).

Regarding the detection in soil, three sub-samples from each sample coming from five different farms were assayed to test the presence of *P. capsici*. Four soils out of five tested positive for *P. capsici*. In each one the fungus was detected in the

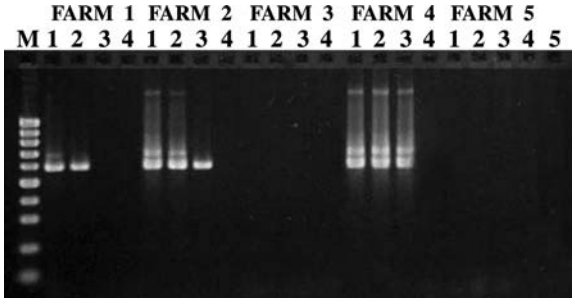

Figure 6. Nested PCR detection of *Phytophthora capsici* in field plants. M, 100 bp marker; lanes 1, 2 and 3 symptomatic plants; lane 4, symptomless plant and lane 5, no DNA template.

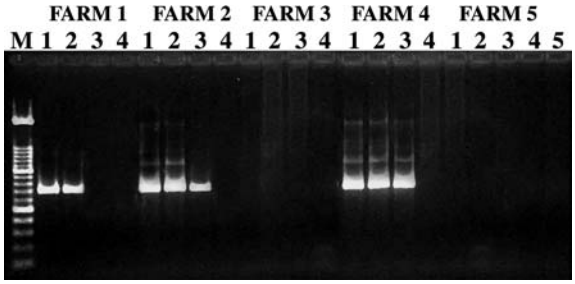

Figure 7. Nested PCR detection of *Phytophthora* spp. in field plants. M, 100 bp marker; lanes 1, 2 and 3 symptomatic plants; lane 4, symptomless plant and lane 5, no DNA template.

three sub-samples analysed (Figure 8). It must be noted that *P. capsici* was detected in the soil from Farm 5 but it was not present in any of the plants sampled in that farm (Figure 6).

Detection of Phytophthora capsici *zoospores*

A suspension of zoospores was obtained from *P. capsici* cultures and one aliquot of a dilution series from 512 to 1 zoospores was included in the PCR tube. CAPFW and CAPRV2 in single PCR were able to detect 16 zoospores, whereas a nested PCR allowed the detection of as few as two zoospores (Figure 9).

Discussion

Phytophthora capsici is one of the most important pathogenic fungi to pepper. An accurate and timely diagnosis of the presence of the pathogen is therefore necessary to prevent huge losses and restrict the spread of the disease to uninfected areas. The main goal of this work was to develop a sensitive and effective diagnostic method used to identify and detect *P. capsici* in plant tissue and soils. PCR has emerged as a powerful tool for the diagnosis of plant diseases because it is more sensitive, robust, rapid and less labour-intensive than traditional diagnostic methods. For designing the species-specific primers to *P. capsici*, rDNA sequences were selected for several reasons. Firstly, they have evolved quickly, showing variation among related taxa and even among species of the same genus (Cooke and Duncan, 1997). Secondly, rDNA is found in many copies in the genome (Lee and Taylor, 1990); in fact, genes coding for rDNA

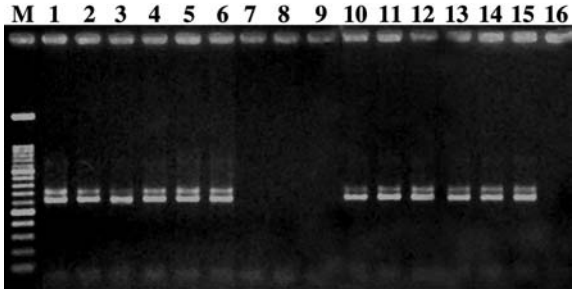

Figure 8. Nested PCR detection of *Phytophthora capsici* in soil using CAPFW/CAPRV2. M, 100 bp marker; lanes 1, 2 and 3 sub-samples from Farm 1; lanes 4, 5 and 6, sub-samples from Farm 2; lanes 7, 8 and 9, sub-samples from Farm 3; lanes 10, 11 and 12, sub-samples from Farm 4; lanes 13, 14 and 15, samples from Farm 5 and lane 16, no DNA template.

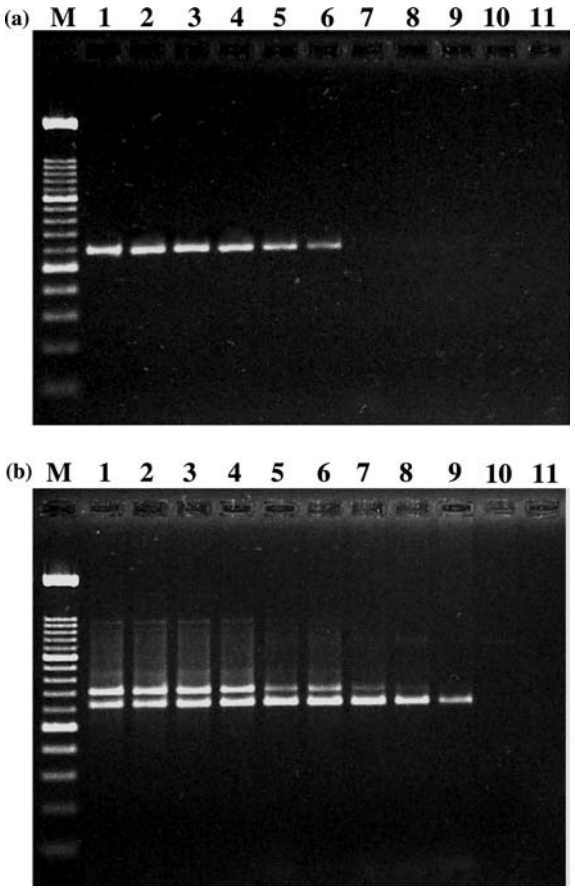

Figure 9. Detection of zoospores using single PCR (A) or nested PCR (B) with CAPFW/CAPRV2. M, 100 bp marker; lane 1, 512 zoospores; lane 2, 256; lane 3, 128; lane 4, 64; lane 5, 32; lane 6, 16; lane 7, 8; lane 8, 4; lane 9, 2; lane 10, 1 and lane 11, no DNA template.

in fungi are found on a single chromosome in repeated units arranged tandemly with 60–220 copies represented in the haploid genome (Russell et al., 1984; Martin, 1990). Finally, the availability of the ITS sequence database (Cooke et al., 2000) facilitated the alignment of sequences of a wide range of *Phytophthora* species. Based on these characteristics, three specific primers were designed for *P. capsici*; CAPFW, CAPRV1 and CAPRV2. The predicted fragment obtained with the set CAPFW and CAPRV1 would be of 452 bp, whereas the amplicon obtained with the pair CAPFW and CAPRV2 would be of 595 bp. Both of these products would contain parts of ITS1, ITS2 and the whole 5.8S subunit.

The primers proposed in the present study were tested for specificity and sensitivity. Specificity was verified by the absence of cross-reaction with DNA from different *Phytophthora* species, several fungal species and the pepper plant. The results showed that both primer sets (CAPFW/CAPRV1 and CAPFW/CAPRV2) were species-specific. Moreover, these primers amplified a range of *P. capsici* isolates representing worldwide diversity, showing that they can amplify *P. capsici* DNA across *P. capsici* populations. To the best of our knowledge, previous assays have been performed to design specific primers for *P. capsici* in the ITS region. However, they could differentiate between *P. capsici*, *P. citricola* and *P. citrophthora* only by using a digestion with restriction enzymes (Ersek et al., 1994; Ristaino et al., 1998). It has been shown that there is sequence homology in spacer region I among these three species and they have been grouped into a phylogenetically distinct cluster (Lee and Taylor, 1992; Cooke and Duncan, 1997; Cooke et al., 2000). Therefore, great care must be taken when designing specific primers to ensure that these primers will not amplify other close species. The present study demonstrates using a specificity test that the primers designed here amplify neither for *P. citrophthora* nor *P. citricola*. Moreover, as expected, no amplification is obtained with any other *Phytophthora* species or pepper pathogens.

Regarding sensitivity, using a 10-fold dilution series of pure DNA a visible amplification product was detected up to 5 pg of *P. capsici* DNA with both primer sets in single PCR. To increase the sensitivity of detection, nested PCR was carried out with a first round amplification using PHYTO primers. These primers will only amplify *Phytophthora* species giving a fragment of 700 bp. CAP primers will anneal inside that fragment amplified by PHYTO primers. This technique allowed an increase in the sensitivity of the method up to 0.5 fg of template DNA; this will be especially important when the target concentration is low or PCR inhibitory substances are present. These results of sensitivity are similar to those obtained by other authors with different *Phytophthora* species (Judelson and Tooley, 2000; Ippolito et al., 2002; Grote et al., 2002; Kong et al., 2003). Moreover, the sensitivity of the method was tested by mixing the pure fungal DNA with plant DNA. This mixture could possibly reflect an infection from a range of 1:1 (a very heavy infection) to a range of 1:1000000 (reflecting an early stage of infection). In this case, as shown in the results, the detection decreased two-fold in both single and nested PCR. These low detection levels show that the method could be used safely with low concentrations of fungal DNA. Therefore, in the second part of this work whether the PCR assay could detect fungal DNA in plant tissue and soil was tested. The PCR assay was used effectively to detect *P. capsici* in artificially-infected plants using conventional PCR with primer set CAPFW/CAPRV2. In this case, the method was able to detect the pathogen as soon as 8 h post-inoculation in stems of asymptomatic plants. However, nested PCR was necessary to detect the fungal DNA in leaves.

The capacity of the method to amplify DNA from infected but symptomless plants suggests that it could be a useful method to detect the fungus in field plants, even before they develop typical symptoms. However, when the assay was tested with naturally-infected plants, nested PCR was necessary to detect the fungus and it was only detected in symptomatic plants. This was probably due to the low degree of infection in naturally infected plants in comparison with those artificially infected and the presence in field plants of other pathogens which could interfere with the detection. Moreover, in some cases, even with the use of nested PCR, detection of the pathogen in symptomatic plants was not possible. In these plants there could be some other pathogen, such as *Verticillium dahliae*, infecting the plant, or environmental stress such as flooding, causing the

symptoms. Another assay was carried out with the aim of detecting any other *Phytophthora* species causing the same symptoms as *P. capsici*. A recent study (Andrés et al., 2003) has reported the presence in NW Spain of *Phytophthora nicotianae* isolates with the capacity to infect pepper plants. In this assay a nested PCR was performed using the primer combination DC6/ITS4 in the first round and PHYTO primers in the second round. The universal primers DC6 and ITS4 amplify DNA of all members of the order Peronosporales, such as *Phytophthora*, *Pythium* and downy mildews (Cooke et al., 2001). In this case, the predicted fragment of 700 bp was obtained in the same samples where *P. capsici* had been detected previously. Therefore, in those symptomatic plants where *P. capsici* was not detected, it is not probable that *P. nicotianae* is responsible for the symptoms, although it could be present together with *P. capsici* in those plants where *P. capsici* was detected.

For the detection of *P. capsici* in soil, CAP primers were tested. In soils, the success of PCR-based detection will depend on obtaining high yields of target DNA from samples (Cullen et al., 1999) and avoiding the presence of soil inhibitors. Field soil samples from five different farms were studied for the presence of soilborne inoculum of *P. capsici*. A total of three sub-samples was tested from each sample and the fungus was detected in 12 out of 15 by nested PCR. It was detected in the same fields where it had been detected in plants, apart from Farm 5, where the fungus was detected in soil samples but not in plant tissue. Several reasons could explain this finding. For instance, it is possible that resistant plants were growing in Farm 5 or that *P. capsici* forms present in this farm were non-pathogenic; even some environmental condition could impede infection in this case.

The primers developed were also assayed for their ability to detect zoospores. Zoospores are an important target for detection because they spread the disease in water and infect the host after encystment. They are probably the most common propagule found in water, where numbers can reach as high as 400 per litre in recirculated irrigation water (McDonald et al., 1994). The current assay detected as few as two zoospores *in vitro* using nested PCR by adding aliquots of spore dilutions directly into the PCR tubes. No attempt was made to detect oospores since only A1 mating type has been reported in Spain, having no possibility of sexual reproduction and therefore, of the production of oospores. So low detection levels of zoospores will be useful to detect the pathogen in irrigation water, one of the main sources of inoculum and an efficient means of spreading pathogens (Kong et al., 2003).

Although there are many factors influencing the occurrence of Phytophthora root rot and Phytophthora blight, the method described above used to detect *P. capsici* in plant tissue and soil could form an important part of disease risk assessment. It will allow the corroboration of the presence of the fungus in plants and the monitoring of the pathogen in field soils and irrigation water. Knowing the presence of the fungus at the earliest stages of infection is of vital importance to be able to take action in the prevention of the disease.

Acknowledgements

This work was supported by grants from MEC (project AGF99-0301) and XUGA (project PGDIT99AGR10301). C.S. held a fellowship from MEC. Agar was kindly supplied by Industrias Roko S.A. Work at SCRI was supported by SEERAD and cultures held under licence No. PH/25/2004

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