

AN IMPROVED METHOD FOR THE DETECTION OF *PHYTOPHTHORA CACTORUM* (L.C.) SCHRÖETER IN INFECTED PLANT TISSUES USING SCAR MARKERS

R. Causin, C. Scopel, A. Grendene and L. Montecchio

Dipartimento Territorio e Sistemi Agro-Forestali, Sezione di Patologia Vegetale, Università degli Studi di Padova, AGRIPOLIS - Viale dell'Università 16, 35020 Legnaro (PD), Italy

SUMMARY

Phytophthora cactorum (Lebert *et* Cohn) Schröeter is an important plant pathogen that can cause serious damage in agricultural and ornamental crops as well as in a wide range of forest species. The identification of this pathogen, based on morphological and physiological characters, is time consuming, labour-intensive and requires specialised staff to be correctly performed. Recently, PCR-based methods have partially resolved these problems, but the primers used cross react with *Phytophthora idaei*. To prevent any such reaction the use of a new pair of primers (PC1/PC2) with improved specificity, derived from a specific Random Amplified Polymorphic DNA (RAPD) generated fragment, is proposed. The PC1/PC2 primers, used in a simple PCR protocol, gave a single amplification product of approximately 450 bp; a good degree of specificity, with absence of cross reactions with *Phytophthora pseudotsugae* and *P. idaei*; sensitivity down to 6 pg of *P. cactorum* DNA extracted from pure mycelium; no reactions with the DNA of the host plants tested (downy oak, pear and walnut trees, potato, strawberry, tomato and pea plants). The detection of *P. cactorum* in infected tissues of pear and walnut trees, potato, strawberry, tomato and pea plants was also confirmed. The specificity, sensitivity and robustness of the PC1/PC2 primers together with the possibility of their use in a rapid, simple and reliable diagnostic method are discussed.

Key words: *Phytophthora cactorum*, PCR, RAPD, specific primers, SCAR.

INTRODUCTION

Phytophthora cactorum (Lebert *et* Cohn) Schröeter is an oomycete known as an important plant pathogen. The *Oomycetes* were formerly considered as members

of the fungal kingdom, from which they have recently been excluded because of their cytological and biochemical characteristics. However, due to behavioural similarities to the true fungi (Cooke *et al.*, 2000a), they are still on occasion referred to as fungi and are considered as such in this study. The distribution of *P. cactorum* is worldwide with a very wide host range (Erwin and Ribeiro, 1996); it can be found on every continent except the Polar Regions, and can attack up to 160 herbaceous and woody plant species belonging to 60 families and 150 genera (Tucker, 1933; Nienhaus, 1960).

P. cactorum is well adapted to temperate areas where it can cause serious diseases, such as seedling blight and root and collar rot in nurseries (Werres, 1995; Lilja *et al.*, 1996; Belisario *et al.*, 1997; Lilja *et al.*, 1998), orchards, horticultural and ornamental crops as well as in a wide range of forest species (Nienhaus, 1960; Jung *et al.*, 1996; Anselmi *et al.*, 1999). In the diseased plant the aerial part shows non-specific symptoms, such as chlorosis, stunted terminal growth, cankers and dieback of twigs and branches that normally only become evident when underground rot has developed considerably. It is thus impossible to make a timely and reliable symptom-based diagnosis, and the presence of *P. cactorum* must be confirmed by pathogen isolation from diseased tissues, followed by morphological and physiological characterization of the isolated mycelia. All these techniques are time-consuming, labour-intensive and very complicated, in particular for morphological characterization (Waterhouse, 1963; Stamps *et al.*, 1990; Liew *et al.*, 1991; Erwin and Ribeiro, 1996; Liew *et al.*, 1998; Cooke *et al.*, 2000b) and require specialised staff to be correctly performed.

To improve efficiency and accuracy of the diagnostic methods used to distinguish different *Phytophthora* species, serological methods (Förster *et al.*, 1989; Nygaard *et al.*, 1989; Oudemans and Coffey, 1991a,b) and a range of molecular techniques (Panabieres *et al.*, 1989; Liew *et al.*, 1991; Lee and Taylor, 1992; Whisson *et al.*, 1992; Tyler *et al.*, 1995) have been developed. Among these techniques PCR-based diagnostics have in recent years assumed a dominant role and for some species of *Phytophthora* PCR-based tests have been developed (Lee *et al.*, 1993; Dobrowolski, 1998; Bonants *et al.*, 1997;

Loucourt *et al.*, 1997; Ristaino *et al.*, 1998; Meng *et al.*, 1999; Schubert *et al.*, 1999; Cooke *et al.*, 2000b; Drenth and Irwin, 2001). However, for many *Phytophthora* species, *P. cactorum* included, a lack of specificity of the primers proposed in the literature is also reported. These primers, in fact, can give rise to cross-reactions with other species of *Phytophthora* and with other very similar fungi, for example those of the *Pythium* or *Pero­nospora* groups (Loucourt *et al.*, 1997; Tooley *et al.*, 1997; Trout *et al.*, 1997; Lindqvist *et al.*, 1998; Schubert *et al.*, 1999). Schubert *et al.* (1999) ascribed this lack of specificity to the method employed to construct the PCR primers that are routinely derived from ITS regions of ribosomal RNA genes. The ITS sequences in *Phytophthora* species may not be the best source for development of species-specific PCR primers, because interspecific differences in this part of the sequence are too small (Cooke *et al.*, 1996, 2000b). The same authors suggest that the use of RAPD-derived fragments could be a valid alternative, just when it was found that attempts to overcome the problems of specificity, caused by small differences in the ITS regions, did not give satisfactory results.

The aim of this study was to construct species-specific PCR primers for *P. cactorum* outside the intergenic sequences of ribosomal genes, using Sequence-Characterised Amplified Region (SCAR) markers (Hermosa *et al.*, 2001; Larsen *et al.*, 2002) in order to develop a reliable, simple and rapid diagnostic PCR for detecting the pathogen in infected plant material.

MATERIALS AND METHODS

Fungal material. Twenty-nine isolates of *Phytophthora* representing twelve species, together with nineteen isolates belonging to twelve other fungal genera were used to construct the primer sequences. Twelve new isolates of *P. cactorum* and two of *Phytophthora idaei* D.M. Kennedy were subsequently added and used together with the other fungi to screen the primers for specificity and efficiency. The origin and maintenance conditions (temperature, growth media, hosts and geographic origin) of all these isolates are listed in Table 1. The fungi were initially grown on potato dextrose agar (PDA) at 17°C [*Phytophthora infestans* (Montagne) de Bary], 20°C (*Phytophthora phaseoli* Thaxter) or 24°C (all other isolates) in Petri dishes, until the main diameter of the colony reached 6 cm. For each isolate, a mycelium suspension was produced by adding 5 ml of sterile water to the Petri dishes and gently scraping the surface of the colony. Two millilitres of this suspension were used to inoculate 50 ml of potato dextrose broth (PDB) in a 100 ml Erlenmeyer flask. After incubation in an orbital shaker for one week at 24°C, the mycelium was harvested from the broth through four layers of sterile cheese-cloth and rinsed in distilled water.

DNA preparation. To extract the DNA from fresh mycelium the method described by Henrion *et al.* (1994) was used with slight modifications. Approximately 200 mg (wet weight) of a fungal sample were crushed in liquid nitrogen using a pestle and mortar, and then immediately transferred into a micro-centrifuge tube and suspended in 1 ml of lysis buffer consisting of 100 mM Tris-HCl (pH 8), 20 mM EDTA (pH 8), 1.4 mM NaCl, 2% cetyltrimethylammonium bromide (CTAB), 1% polyvinylpyrrolidone (PVP), 1% mercaptoethanol and incubated for 60 minutes at 65°C. Proteins were denatured and removed with one or more extractions by gently shaking for one hour in ice with 1 ml chloroform/isoamyl alcohol (24:1, v/v) followed by centrifugation at 17,300 *g* for 10 min to separate the phases. The aqueous phase was carefully recovered and the DNA was precipitated by adding 2/3 volume of isopropanol and 1/10 volume of 3 M sodium acetate (pH 5.2) and maintaining the sample at -20°C for 20 min. The DNA was then pelleted by centrifugation at 17,300 *g* for 10 min, washed with 70% (v/v) ethanol, pelleted again and dried at room temperature. The dried pellet was resuspended in 100 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA) and stored at -20°C until use.

For the detection tests of *P. cactorum* in infected plant material, DNA was extracted from both healthy and infected tissues as reported above, with the addition of a further chloroform/isoamyl alcohol extraction to improve the DNA purification. The extracted DNA was diluted in water at a ratio of 1:100 to give a concentration ranging from 15 to 25 ng µl⁻¹ before being used as template in subsequent PCR amplification.

Screening for RAPD markers and detection of SCAR. Eleven 10-mer RAPD primers of the OPA series (OPA-1 – OPA-11) (Operon Technologies Inc., Alameda, CA, USA) were tested using the DNA extracted from the fungal isolates listed in Table 1. The reactions were performed in 25 µl volumes with 10-50 ng of template DNA, 100 µM of each dNTP, 2.5 µl of 10 × buffer (200 mM Tris-HCl pH 9, 500 mM KCl, 1% Triton® X100), 2 mM MgCl₂, 0.2 µM of RAPD primer and 1 Unit of Taq DNA polymerase (Promega Corp., Madison, WI, USA). A drop of sterile mineral oil was overlaid on the reaction mix and negative controls, without the template DNA, were performed to check for DNA contamination of the reagents.

The Thermal Cycler (Cycler TM, Bio-Rad, Milano, Italy) was programmed as follows: initial denaturation at 94°C for 2 min and 30 sec, followed by 45 cycles of amplification (denaturation at 94°C for 30 sec, annealing at 38°C for 1 min, extension at 72°C for 2 min) with a final extension of 5 min at 72°C after cycling. The amplification products were separated at 90V in 0.5x TBE (45 mM Tris-borate, 1 mM EDTA) for two hours in 1.5% TBE agarose gels, stained with ethidium bromide

(Sambrook *et al.*, 1989) and photographed under UV illumination (302 nm).

The RAPD band specific for *P. cactorum* was directly eluted from the agarose gel using an Agarose gel DNA Extraction Kit (Boehringer Mannheim Corp, Indianapolis, IN, USA) and the purified DNA was ligated into a plasmid pGEM-T Vector System (Promega Corp., Madison, WI, USA) according to the manufacturer's recommendations. The plasmids were used to transform *Escherichia coli* strain JM 109 competent cells (Promega Corp., Madison, WI, USA) and the recombinant colonies were identified by blue-white colour selection after 12 h of growth at 37°C on LB agar medium (1.8% trypton-NaCl, 0.5% yeast extract, 1.6% agar-agar) containing ampicillin, IPTG (isopropyl β -D-1-thiogalactopyranoside) and X-Gal (5-Bromo-4-chloro-3-indolyl β -D-galactopyranoside) following the manufacturer's instructions.

Plasmids were purified from LB/ampicillin liquid cultures of the selected colonies using a High Pure Plasmid Isolation Kit (Boehringer Mannheim Corp, Indianapolis, IN, USA); the manufacturer's recommendations were also followed for this operation. The DNA sequence was obtained for double-stranded DNA by the di-deoxy terminator method and samples were prepared using the Taq Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). The sequencing reactions were run on an automatic sequencer 373A DNA Sequencer (Applied Biosystems Foster City, CA, USA) and the primers SP6/T7 were used.

Design and screening of SCAR primers and optimisation of PCR conditions. Based on the DNA sequence derived from the RAPD marker, following the guidelines outlined by Trower and Elgar (1997) and Kolmodin and Williams (1996), and using primer design software primer3 (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi), five primers (PC1, PC2, PC3, PC4, PC5) were designed for the conversion of the selected RAPD marker to a SCAR primer pair (Table 2).

Specificity of the PCR for *P. cactorum* using the five PC primers, (paired in the combinations PC1/PC2, PC1/PC3, PC3/PC4, PC4/PC5), was tested on all the fungi listed in Table 1. The PCR conditions were optimised for annealing temperature, number of amplification cycles and DNA dilutions; the final profile employed was: initial denaturation for 3 min at 94°C, followed by 30 cycles of amplification (denaturation 30 sec at 94°C, annealing 30 sec at 61°C, extension 1 min at 72°C) with a final extension of 7 min at 72°C after cycling. All the PCRs were performed in a 25 μ l reaction containing 15-25 ng of template DNA, 200 μ M of each dNTP, 2.5 μ l of 10x buffer (100 mM Tris-HCl pH 9, 1.5 mM MgCl₂, 500 mM KCl), 0.5 μ M of each primer and 1 Unit of Taq DNA polymerase (Amersham Biosciences,

Cologno Monzese, MI, Italy). A drop of sterile mineral oil was overlaid on the reaction mix and a negative control was performed without the template DNA to check for DNA contamination of the reagents. With these conditions the cross reactivity with the DNA of the six *P. cactorum* host plants listed in Table 3 was also tested.

All the amplification products were separated and visualised as described above for RAPD reactions.

Sensitivity of PCR amplification. The sensitivity of the improved PCR profile was determined by using a series of dilutions (1:10; 1:100; 1:1,000; 1:10,000 and 1:100,000) of *P. cactorum* genomic DNA as template. The sensitivity was also determined by using mixtures of *P. cactorum* DNA with DNA extracted from tomato (*Lycopersicon esculentum*) and downy oak (*Quercus pubescens*). The DNA extracted from each host plant was mixed with *P. cactorum* DNA as follows: the amount of *P. cactorum* DNA was maintained constant (5 ng) while plant DNA was increased, giving for each host 5 mixtures with a pathogen/host DNA ratio of 1:10,000, 1:1,000, 1:700, 1:350 and 1:10; 2 μ l of each mixture were used as template in the PCR reactions. The amplification products were size-fractionated in agarose gel and visualised as previously described.

Artificial host inoculation and detection of *P. cactorum* in plant tissues. The F101 isolate, randomly selected from the fourteen *P. cactorum* isolates listed in Table 1 was used in this trial. Samples were collected from the plant parts of the six host plants listed in Table 3, rinsed in sterile distilled water, briefly surface-disinfected by immersion for 1 min in 15% H₂O₂, rinsed again in sterile distilled water and laid in sterile moist chambers arranged on Petri dishes (\varnothing 15 cm) containing wet filter paper. After gentle scraping, the surfaces of the samples were inoculated with a 2-mm² mycelium plug, taken from two-week-old colonies actively growing on PDA at 24°C in the dark. As a control, moist chambers containing samples inoculated with sterile PDA were also produced. Four replicates were performed for each treatment.

All the moist chambers were incubated at room temperature in natural daylight, until lesions become evident in the inoculated samples. *P. cactorum* F101 was also inoculated in two-month-old tomato plants, grown in Top Green[®] mould (Fertil s.r.l. Calcinate, BG, Italy) in 10 l pots and in a greenhouse (24 \pm 2°C, 80% RH, natural daylight). At 3-4 cm from the collar, the stem was surface disinfected with absolute ethanol and aseptically wounded to produce a small pocket without completely removing the incised tissues. A *P. cactorum* inoculum of 2-mm² agar plug, produced as previously described, was placed into the wound, and the pocket was closed by pressing the partially removed external tissues against the stem by a cover of autoclaved wet filter paper maintained in position with Parafilm[®]. Controls were inocu-

Table 1. Fungal isolates included in molecular study.

Genus	Species	Strain	Source ^a	Growth conditions		Host ^b	Geographic origin	Strains used in RAPD analysis
				°C	Media			
<i>Phytophthora</i>	<i>cactorum</i>	FI01	Unifi	24	V8A, PDA, CMA	Oak	Italy	*
<i>Phytophthora</i>	<i>cactorum</i>	435.64	C.B.S.	24	V8A, PDA, CMA	Apple	Netherlands	*
<i>Phytophthora</i>	<i>cambivora</i>		Unifi	24	V8A, PDA, CMA	Chestnut	Italy	*
<i>Phytophthora</i>	<i>cambivora</i>	356.78	C.B.S.	24	V8A, PDA, CMA	Lawson Cypress	Belgium	*
<i>Phytophthora</i>	<i>cambivora</i>	248.60	C.B.S.	24	V8A, PDA, CMA	Chestnut	France	*
<i>Phytophthora</i>	<i>cinnamomi</i>		Unifi	24	V8A, PDA	Oak	Italy	*
<i>Phytophthora</i>	<i>citrophthora</i>		Unifi	24	V8A, PDA	Chestnut	Italy	*
<i>Phytophthora</i>	<i>cryptogea</i>		Unifi	24	V8A, PDA	Chestnut	Italy	*
<i>Phytophthora</i>	<i>drechsleri</i>	359.52	C.B.S.	24	V8A, PDA, OA	Potato	Argentina	*
<i>Phytophthora</i>	<i>infestans</i>	431.90	C.B.S.	17	V8A, PDA, CMA	Potato	Netherlands	*
<i>Phytophthora</i>	<i>infestans</i>	366.51	C.B.S.	17	V8A, PDA, CMA	Potato	Netherlands	*
<i>Phytophthora</i>	<i>megasperma</i> var. <i>sojae</i>	274.89	C.B.S.	24	V8A, PDA, OA	Garden Asparagus	Netherlands	*
<i>Phytophthora</i>	<i>nicotianae</i>		Unifi	24	V8A, PDA	Tobacco	Italy	*
<i>Phytophthora</i>	<i>phaseoli</i>	556.88	C.B.S.	20	V8A, PDA, OA	Lima bean	Unknown	*
<i>Phytophthora</i>	<i>pseudotsugae</i>	444.84	C.B.S.	24	V8A, PDA, OA	Douglas fir	USA, Oregon	*
<i>Pythium</i>	<i>debaryanum</i>	752.96	C.B.S.	24	PDA, CMA	Garden tulip	UK	*
<i>Pythium</i>	<i>ultimum</i>	807.95	C.B.S.	24	PDA, CMA	Pea	Canada, Alberta	*
<i>Pythium</i>	sp.		Unipd	24	PDA, CMA	Oak seedling	Italy	*
<i>Alternaria</i>	sp.		Unipd	24	PDA	Tomato	Italy	*
<i>Botritis</i>	<i>cinerea</i>		Unipd	24	PDA	Grape	Italy	*
<i>Coniothyrium</i>	<i>minitans</i>	C102	C.B.S.	24	PDA	N.p.	Italy	*
<i>Coniothyrium</i>	<i>minitans</i>	Z ₁	Unipi	24	PDA	N.p.	Italy	*
<i>Cryphonectria</i>	<i>parasitica</i>	EC5V19	Unipd	24	PDA	Chestnut	Italy	*
<i>Cryphonectria</i>	<i>parasitica</i>	20.90V	Unipd	24	PDA	Chestnut	Italy	*
<i>Fusarium</i>	sp.		Unipd	24	PDA	Asparagus	Italy	*
<i>Fusarium</i>	sp.		Unipd	24	PDA	Wheat	Italy	*
<i>Pleurotus</i>	<i>ostreatus</i>		Unipd	24	PDA	N.p.	Italy	*
<i>Rhizopus</i>	sp.		Unipd	24	PDA	Strawberry	Italy	*
<i>Sclerotinia</i>	<i>minor</i>		Unipd	24	PDA	Lettuce	Italy	*

<i>Sclerotium</i>	<i>coepivorum</i>		Unipd	24	PDA	Garlic	Italy	*
<i>Trichoderma</i>	<i>hartianum</i>	T ₁ Q	Unipd	24	PDA	N.p.	Italy	*
<i>Trichoderma</i>	<i>viridae</i>	TV ₁ NE	Unipd	24	PDA	N.p.	Italy	*
<i>Trichoderma</i>	sp.		Unipd	24	PDA	N.p.	Italy	*
<i>Verticillium</i>	sp.		Unipd	24	PDA	Grapevine	Italy	*
<i>Phytophthora</i>	<i>cactorum</i>	PC1	Unipd	24	V8A, PDA, CMA	Strawberry	France	
<i>Phytophthora</i>	<i>cactorum</i>	PC2	Unipd	24	V8A, PDA, CMA	Oak	France	
<i>Phytophthora</i>	<i>cactorum</i>	PC3	Unipd	24	V8A, PDA, CMA	Oak	France	
<i>Phytophthora</i>	<i>cactorum</i>	PC5	Unipd	24	V8A, PDA, CMA	Strawberry	France	
<i>Phytophthora</i>	<i>cactorum</i>	PC6	Unipd	24	V8A, PDA, CMA	Apple	France	
<i>Phytophthora</i>	<i>cactorum</i>	PC7	Unipd	24	V8A, PDA, CMA	Apple	France	
<i>Phytophthora</i>	<i>cactorum</i>	PC8	Unipd	24	V8A, PDA, CMA	Apple	France	
<i>Phytophthora</i>	<i>cactorum</i>	PCF5	Unipd	24	V8A, PDA, CMA	Strawberry	Netherlands	
<i>Phytophthora</i>	<i>cactorum</i>	PCF7	Unipd	24	V8A, PDA, CMA	Strawberry	Netherlands	
<i>Phytophthora</i>	<i>cactorum</i>	PCF18	Unipd	24	V8A, PDA, CMA	Strawberry	Netherlands	
<i>Phytophthora</i>	<i>cactorum</i>	PCF77	Unipd	24	V8A, PDA, CMA	Strawberry	Netherlands	
<i>Phytophthora</i>	<i>cactorum</i>	PQ12	Unipd	24	V8A, PDA, CMA	Oak	Germany	
<i>Phytophthora</i>	<i>idaei</i>	968.95	C.B.S.	24	V8A, PDA, OA	Raspberry	UK, Scotland	
<i>Phytophthora</i>	<i>idaei</i>	971.95	C.B.S.	24	V8A, PDA, OA	Raspberry	UK, Scotland	

^a C.B.S. = Centraalbureau voor Schimmelcultures, Utrecht, NL; Unipd = Dipartimento Territorio e Sistemi Agroforestali, Università di Padova, Italy; Unifi = Dipartimento Biotecnologie Agrarie, Università di Firenze, Italy; Unipi = Dipartimento Coltivazione e Difesa delle Specie Legnose, Università di Pisa, Italy.

^b N.p. = non pathogenic.

Table 2. Sequence and orientation of PC primers.

Primer	Nucleotide Sequence	Orientation	T _m °C	% CG
PC1	5'GAAACGGGTGTTGATATCGGAC3'	forward	66	50
PC2	5'GTTTCGGGTGCTGCCAAAACT3'	reverse	66	50
PC3	5'GGATTCAGTATGTCGAAGTAGCT3'	reverse	66	43,5
PC4	5'GCGACTGGCTGCTGTTTTTAAAC3'	forward	68	47,8
PC5	5'TCTCACATACATGTACCTGTAGC3'	reverse	66	43,5

lated with sterile PDA plugs and three replicates, each of three tomato plants, were performed for each treatment. All plants were maintained in the greenhouse (24±2°C, 80% RH, natural daylight) until lesions became visible.

From both infected and uninfected moist chamber samples and infected and uninfected tomato plants, re-isolations on PDA were made to verify the presence (or absence) of the pathogen.

Extraction of total DNA from both healthy and infected tissues, collected at least 5 mm from the point of inoculation, was performed as described above and PCRs were undertaken to detect *P. cactorum* presence. The amplification protocol was repeated twice with a dilution step between the two reactions. The first PCR round was performed using as template the DNA extracts diluted 1:100, the second one using as template the products of the first round diluted 1:10, 1:50, and 1:100. In all the trials 15-25 ng of pure *P. cactorum* DNA were used as a positive control; a negative control, without the template DNA, was also performed to check for DNA contamination of the reagents. All PCRs were performed using the improved PCR profile and primer combination that gave the best results in the above described trials. All the amplification products were separated and visualised as reported above for RAPD technique.

RESULTS

Nucleotide sequence of RAPD marker and SCAR primers design. Among the eleven 10-mer RAPD primers tested, ten showed amplifications and only one (OPA-6) gave no band. The best results were obtained using OPA-7 that gave a distinct RAPD marker for only *P. cactorum* isolates at approximately 450 bp (Fig. 1). Cloning and sequencing in both directions of this band gave the 444-nucleotide sequence, as shown in Fig. 2 and the five primers listed in Table 2 were designed, based on this SCAR sequence. Their positions inside the sequence are underlined in Fig. 2. The primers had a GC content ranging from 43 to 50% and a melting temperature (T_m) ranging from 66 and 68°C (Table 2).

Screening of primers and optimisation of diagnostic-PCR parameters. Among the four combinations tested, the primer pair PC1/PC2 gave the best results. These primers specifically amplified a single SCAR of approximately 450 bp only from DNA extracted from the isolates of *P. cactorum*; no amplifications were observed in the DNA of either of the other *Phytophthora* species or the fungi of different genera listed in Table 1 (Fig. 3 and 4). No amplicons were derived from the DNA extracted from healthy tissues of tomato, potato, strawberry and pea plants, downy oak, walnut and pear trees. The re-

Table 3. Plant hosts used for detection of *P. cactorum* in infected tissue.

Host plant	Sample	Source	Growth conditions
Tomato (<i>Lycopersicon esculentum</i>)	Stem portions	University greenhouse	24 ± 2°C, 80% UR, natural daylight
Potato (<i>Solanum tuberosum</i>)	Stem portions	University farm of Legnaro	open ground
Strawberry (<i>Fragaria vesca</i>)	Basal part of stems and fruits	University greenhouse	24 ± 2°C, 80% UR, natural daylight
Walnut (<i>Juglans regia</i>)	Leaves and twig portions	University farm of Legnaro	open ground
Pear tree (<i>Pirus communis</i>)	Leaves and twig portions	University farm of Legnaro	open ground
Pea (<i>Pisum sativum</i>)	Leaves and stem portions	University farm of Legnaro	open ground

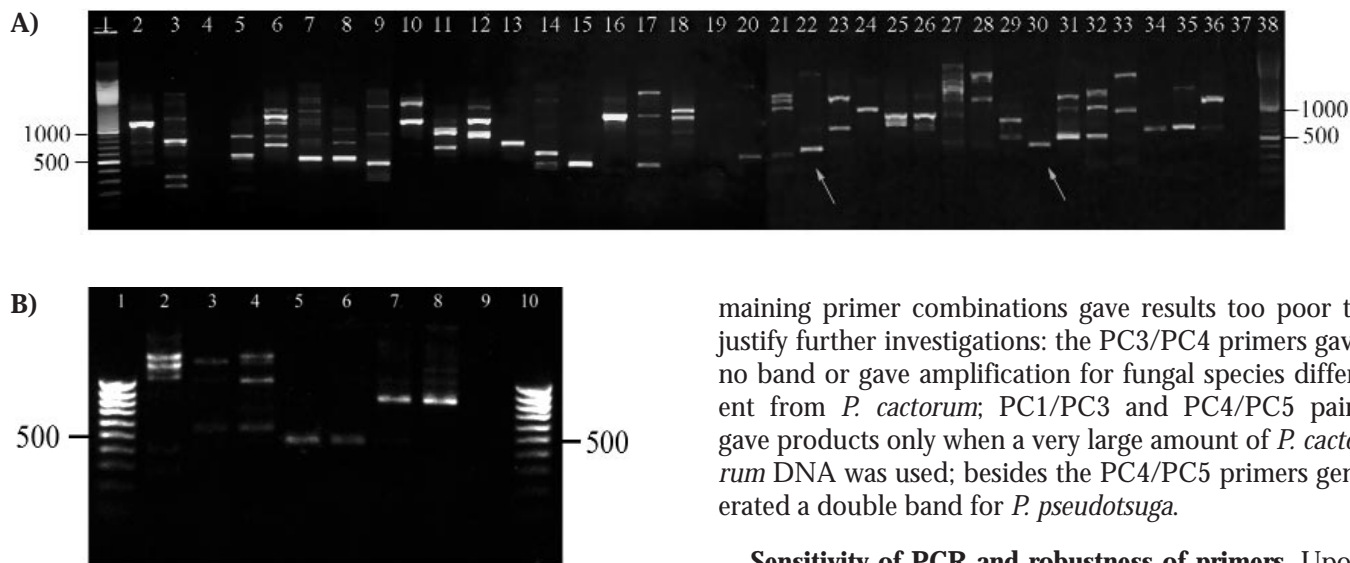


Fig. 1. RAPD patterns produced from fungal isolates using OPA-7. **(A):** Lane 1 marker 100 bp DNA Ladder (Promega); Lane 2 *C. parasitica* EC5V19; Lane 3 *Fusarium* sp.; Lane 4 *P. ostreatus*; Lane 5 *C. minitans* Z₁; Lane 6 *T. hartianum* T₁Q; Lane 7 *T. viridae* TV₁NE; Lane 8 *Alternaria* sp.; Lane 9 *B. cinerea*; Lane 10 *Trichoderma* sp.; Lane 11 *Rhizopus* sp.; Lane 12 *Fusarium* sp.; Lane 13 *C. minitans* C102; Lane 14 *B. cinerea*; Lane 15 *S. coepivorum*; Lane 16 *C. parasitica* 20.90V; Lane 17 *S. minor*; Lane 18 *P. ostreatus*; Lane 19 negative control; Lane 20 *Verticillium* sp.; Lane 21 *P. cambivora*; Lane 22 *P. cactorum* FI01; Lane 23 *P. nicotianae*; Lane 24 *P. cryptogea*; Lane 25 *P. citrophthora*; Lane 26 *P. cinnamomi*; Lane 27 *P. megasperma* var. *sojae* 247.89; Lane 28 *P. pseudotsuga* 444.84; Lane 29 *P. drechsleri* 359.52; Lane 30 *P. cactorum* 435.64; Lane 31 *P. cambivora* 356.78; Lane 32 *P. cambivora* 248.60; Lane 33 *P. phaseoli* 556.88; Lane 34 *P. ultimum* 807.95; Lane 35 *P. debaryanum* 752.96; Lane 36 *Pythium* sp.; Lane 37 negative control; Lane 38 marker 100 bp DNA Ladder (Promega). **(B):** Lane 1 marker 100 bp DNA Ladder (Genenco); Lane 2 *P. cambivora*; Lane 3 *P. cambivora* 356.78; Lane 4 *P. cambivora* 248.60; Lane 5 *P. cactorum*; Lane 6 *P. cactorum* 345.64; Lane 7 *P. infestans* 431.90; Lane 8 *P. infestans* 366.51; Lane 9 negative control; Lane 10 marker 100 bp DNA Ladder (Genenco).

maining primer combinations gave results too poor to justify further investigations: the PC3/PC4 primers gave no band or gave amplification for fungal species different from *P. cactorum*; PC1/PC3 and PC4/PC5 pairs gave products only when a very large amount of *P. cactorum* DNA was used; besides the PC4/PC5 primers generated a double band for *P. pseudotsuga*.

Sensitivity of PCR and robustness of primers. Upon the performance of the PCR, with the DNA extracted from pure mycelium of *P. cactorum* as template, amplification was still discernible at an amount of DNA as low as 6 pg. When the *P. cactorum* and tomato DNA were mixed and used as DNA template in PCR reactions, *P. cactorum* DNA was still detected at a pathogen/host ratio of 1:1,000 and, as expected, an increase of DNA host concentration determined a decreasing intensity of the PCR bands obtained. With the DNA mixture of *P. cactorum* and downy oak, amplification was obtained only at ratio of 1:10 and the resulting band had a lower intensity than the ones obtained with the same DNA mixture ratio of *P. cactorum* and tomato. As previously found no amplification was obtained from the DNA of the host plant.

The primer robustness (i.e. the ability to detect different isolates of *P. cactorum*) was demonstrated by the amplifications obtained from all the fourteen *P. cactorum* isolates used in this work (Table 1).

Detection of *P. cactorum* from infected plant tissues. In the detection assays, the second round of the double PCR was always necessary to detect *P. cactorum*; the best results were obtained when the second round am-

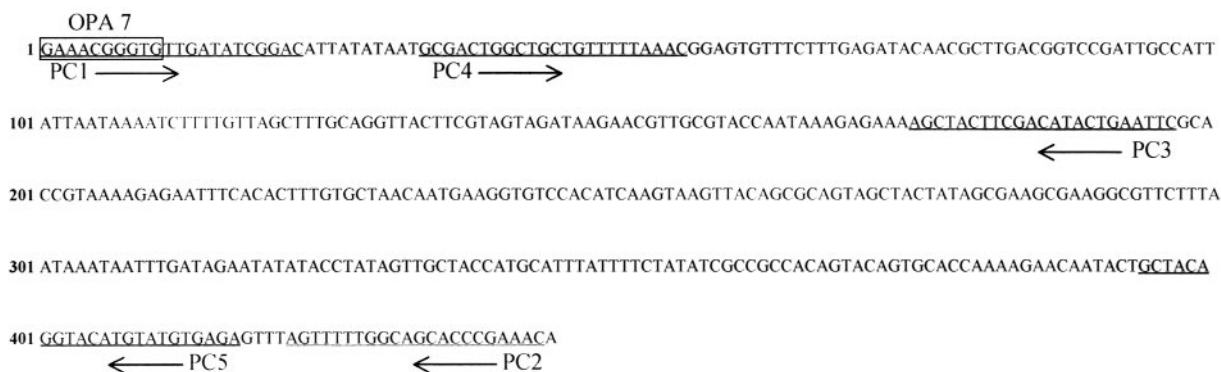


Fig. 2. Sequence of *P. cactorum* RAPD fragment; the positions of the primers PC are indicated in this sequence from the underlined nucleotide sequences.



Fig. 3. Amplification products of *Phytophthora* and *Pythium* species by using specific primers PC1/ PC2: Lane 1 100 Base-pair Ladder (Amersham Biosciences); Lane 2 *P. cactorum* FI01; Lane 3 *P. cactorum* 435.64; Lane 4 *P. cambivora*; Lane 5 *P. cambivora* 356.78; Lane 6 *P. cambivora* 248.60; Lane 7 *P. cinnamomi*; Lane 8 *P. citrophthora*; Lane 9 *P. cryptogea*; Lane 10 *P. drechsleri* 359.52; Lane 11 *P. infestans* 431.90; Lane 12 *P. infestans* 366.51; Lane 13 *P. megasperma* var. *sojae* 247.89; Lane 14 *P. nicotianae*; Lane 15 *P. phaseoli* 556.88; Lane 16 *P. pseudotsuga* 444.84; Lane 17 *P. debaryanum* 752.96; Lane 18 *P. ultimum* 807.95; Lane 19 *Pythium* sp.; Lane 20 negative control.



Fig. 4. Amplification products of *Phytophthora* species by using specific primers PC1/ PC2: Lane 1 marker 100 Base-pair Ladder (Amersham Biosciences); Lane 2 *P. cactorum* FI01; Lane 3 *P. cactorum* 435.64; Lane 5 *P. cactorum* PC1; Lane 6 *P. cactorum* PC2; Lane 7 *P. cactorum* PC3; Lane 8 *P. cactorum* PC5; Lane 9 *P. cactorum* PC6; Lane 10 *P. cactorum* PC7; Lane 11 *P. cactorum* PC8; Lane 12 *P. cactorum* PCF5; Lane 13 *P. cactorum* PCF7; Lane 14 *P. cactorum* PCF18; Lane 15 *P. cactorum* PCF77; Lane 16 *P. cactorum* PQ12; Lane 17 *P. idaei* 968.95; Lane 18 *P. idaei* 971.95; Lane 19 negative control; Lane 20 100 Base-pair Ladder (Amersham Biosciences).

plification was performed using the 1:100 diluted product of the first round as DNA template. The described double PCR reactions carried out with DNA extracted from both the infected host samples incubated in the moist chamber and infected tomato plants, gave a single product, of approximately 450 bp, having the same size of the amplicons derived from the pure *P. cactorum* DNA. No PCR band was obtained from DNA extracted from healthy tissues (Fig. 5). *P. cactorum* isolation from altered tissues was successfully achieved both in the infected tissues incubated in moist chambers and tomato plants. Pathogen isolation from healthy tissues was never obtained.

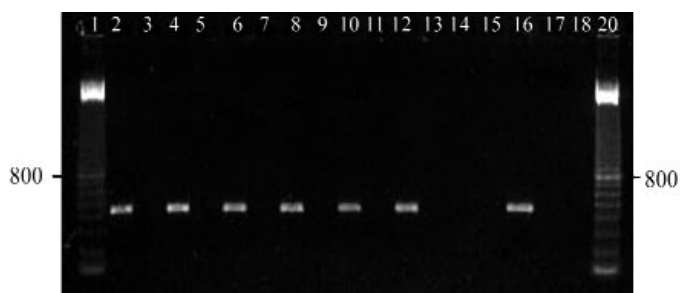


Fig. 5. Detection of *P. cactorum* in tissues of various host plants: Lane 1 100 Base-pair Ladder (Amersham Biosciences); Lane 2 DNA from inoculated *Lycopersicon esculentum*; Lane 3 DNA from uninoculated *Lycopersicon esculentum*; Lane 4 DNA from inoculated *Solanum tuberosum*; Lane 5 DNA from uninoculated *Solanum tuberosum*; Lane 6 DNA from inoculated *Fragaria vesca*; Lane 7 DNA from uninoculated *Fragaria vesca*; Lane 8 DNA from inoculated *Juglans regia*; Lane 9 DNA from uninoculated *Juglans regia*; Lane 10 DNA from inoculated *Pirus communis*; Lane 11 DNA from uninoculated *Pirus communis*; Lane 12 DNA from inoculated *Pisum sativum*; Lane 13 DNA from uninoculated *Pisum sativum*; Lane 14 empty; Lane 15 empty; Lane 16 *P.cactorum* PCF7; Lane 18 negative control; Lane 19 100 Base-pair Ladder (Amersham Biosciences).

DISCUSSION

The PCR carried out with the SCAR primers derived from the sequence of the approximately 450 bp RAPD band, produced a single diagnostic DNA amplification product that easily distinguished *P. cactorum* from the other eleven *Phytophthora* species, three *Pythium* species, eleven other fungal genera and from the DNA of six host plants. These primers also permitted the detection of the pathogen in infected tissues of tomato, potato, strawberry and pea plants, and walnut and pear trees.

In testing for specificity of the PCR based diagnostic protocol suggested in this paper, twenty-three species other than *P. cactorum* were considered; the absence of cross reactions with all these fungi and particularly with *P. pseudotsugae* and *P. idaei* can be considered as an indicator of a satisfactory degree of specificity. In fact, *P. pseudotsugae* and *P. idaei* were found to be very similar genetically to *P. cactorum*, both in RAPD pattern and ITS region analysis (Cooke *et al.*, 1996; Cooke *et al.*, 2000a,b). This genetic similarity is so pronounced that the same authors (Cooke *et al.*, 1996; Cooke *et al.*, 2000b) considered *P. cactorum*, *P. pseudotsugae* and *P. idaei* much more closely related to one another than to any other *Phytophthora* species. This aspect represents a large obstacle in the development of species-specific PCR primers for *P. cactorum*; in fact the primers ADF1 and ADR1 that are commonly considered specific for this pathogen, cannot distinguish between *P. cactorum* and *P. idaei* (Loucourt *et al.*, 1997). This restricted lack of specificity is usually accepted as the ADF1 and ADR1

primers were, until now, the best available solution to detect *P. cactorum* by PCR and the distinction between the two *Phytophthora* species was achieved by carrying out a restriction of the amplified fragment (Loucourt *et al.*, 1997). The PC1 and PC2 primers, here proposed, overcome this need and improve the molecular diagnosis of *P. cactorum* as they give no amplification for both *P. idaei* and *P. pseudotsugae*; furthermore a simple diagnostic PCR able to distinguish *P. idaei* from *P. cactorum* is useful since this two pathogens have been found on the same host (raspberry) (Kennedy and Duncan, 1995).

Nevertheless, it is questionable whether the primers developed using the sequence of a RAPD derived fragment could be much too specific and have a lack of robustness; i.e. these primers could be sensitive to intraspecific variability and give no reaction with some *P. cactorum* isolates. On the other hand, Dobrowolski (1998) reported that many of the products generated by RAPD-PCR are derived from repetitive DNA sequences and are frequently species-specific; in fact, in recent years the SCAR markers were successfully used to produce species-specific probes and PCR primers in *Phytophthora* (Dobrowolski, 1998; Schubert *et al.*, 1999) and in other fungal species (Hermosa *et al.*, 2001; Mercado-Blanco *et al.*, 2001; Taylor *et al.*, 2001; Larsen *et al.*, 2002).

Furthermore, the construction of the PCR primers based on the SCAR marker, as proposed in this paper, was the best alternative possible, since, as already reported, the use of ITS regions, that are considered of superior reliability compared to the random non-defined primers (Liew *et al.*, 1998), can not be considered the best way to construct PCR primers in *Phytophthora* species. In fact, in ITS regions many *Phytophthora* species have relatively low sequence diversities (Schubert *et al.*, 1999) and in particular the cited low differences among *P. cactorum*, *P. pseudotsugae* and *P. idaei* involved less than 1% of nucleotides and cannot be used to separate *P. cactorum* from these closely related *Phytophthora* species (Cooke *et al.*, 1996; Cooke *et al.*, 2000a,b).

Moreover, Schubert *et al.* (1999) demonstrated that in two *Phytophthora* species (*P. cambivora* and *P. quercina*) the sequence derived from RAPD bands allows species-specific primers to be designed, just when the attempts to design such primers on the basis of ITS sequence were unsuccessful. Consequently, it must be considered unprofitable to look for primers derived from ITS. The PC1/PC2 primers developed in this work were tested on fourteen different isolates of *P. cactorum* and always gave amplification. Nevertheless, the specificity and the robustness of PC1/PC2 should be verified before they are used to detect non-European *P. cactorum* isolates when they are known to have genetic differences to the European ones (Hantula *et al.*, 1997), as for example, the North American strains of *P. cactorum* isolated from the strawberry plant (Hantula *et al.*, 2000).

The sensitivity of the PCR protocol proposed gave suitable results since 6 pg of template DNA were easily detected when the DNA was extracted from pure culture of *P. cactorum*. According to Schubert *et al.* (1999) who considered the amount of DNA per nuclei comparable among the various *Phytophthora* species, it could be assumed that 6 pg of DNA are approximately equivalent to 6-12 nuclei. This sensitivity level is similar to that reported for PCR carried out with a primer pair derived from ITS sequences (Tooley *et al.*, 1997; Liew *et al.*, 1998; Schubert *et al.*, 1999). Therefore, according to Schubert *et al.* (1999) it can be assumed that the threshold target copy number of the marker here considered is not significantly lower than the high threshold target copy number of the repetitively organized ITS sequences indicating that the copy number of the cloned sub genomic RAPD fragment is likely highly repetitive.

The detection limit of *P. cactorum* DNA greatly decreased when it was mixed with the DNA of tomato or downy oak and the negative effect of oak was greater than those of tomato. As previously discussed, a low target copy number of markers could be excluded; therefore, an inhibitory effect due to contaminants in the host DNA, already observed by the authors for PCR carried out to characterise ectomycorrhizae of oak root, could be hypothesized. This, in accordance with other authors (Wyss and Bonfante, 1993; Wilson, 1997; Schubert *et al.*, 1999; Amicucci *et al.*, 2001; Grote *et al.*, 2002; Larsen *et al.*, 2002), suggests that PCR failure could result from the presence of inhibitors such as phenols, polysaccharides and salts.

These final results outline the importance of the extraction method, mainly when the host tissues are known for their richness in pigments, complex polyphenolics and tannins as in the case of *Quercus* sp.. In other words, it may be necessary to modify the method of DNA extraction and purification to adapt it to the specific host and to the desired sensitivity. To overcome this complication, in accordance with some authors that suggest the use of dilution to minimize the negative effect of inhibitor in DNA extracts (Wyss and Bonfante, 1993; Trappe and Jumpponen, 1995; Larsen *et al.*, 2002), the strategy, successfully adopted in the present work, was to dilute the inhibitors that could be present in the DNA derived from host tissues. In order to avoid losses of sensitivity, this dilution was preceded by the initial PCR and then the diluted products of this PCR were used as a template DNA in the second reaction. To adopt a two-step PCR strategy can also lead to a second advantage as in this way small amounts of DNA template can be amplified, further improving the pathogen detection. However it will be profitable to check the results of the first PCR in order to avoid the dilution step and the second PCR round, if a suitable result was already obtained.

The detection system suggested in this paper could

be useful to distinguish *P. cactorum* mycelia from the other *Oomycetes*. The same protocol with a twice repeated PCR, when tested in more naturally infected hosts, could be proposed as a useful diagnostic tool to detect the pathogen directly in infected plant tissues, avoiding isolation, purification and culturing of the mycelium. Moreover, the problems due to morphological intraspecific variation and overlapping characters, that make an accurate identification of isolates difficult even for specialists (Loucourt *et al.*, 1997), are overcome and an easy rapid diagnosis can be performed by a single operator in 24 hours from the receipt of the samples.

ACKNOWLEDGEMENTS

Grateful thanks are expressed to Dr. Kelvin Hughes of Central Science Laboratory, Sand Hutton, York, UK, for critical reading of the manuscript.

REFERENCES

- Amicucci A., Zambonelli A., Guidi C., Stocchi V., 2001. Morphological and molecular characterization of *Pulvinula constellatio* ectomycorrhizae. *FEMS Microbiology Letters* **194**: 121-125.
- Anselmi N., Vannini A., Vettraino A.M., 1999. Specie di *Phytophthora* riscontrate sulle latifoglie forestali in Italia. *Informatore Fitopatologico* **49** (11): 53-58.
- Belisario A., Cacciola S.O., Magnano di San Lio G., 1997. *Phytophthora cactorum* on walnut seedlings in Italian nurseries. *European Journal of Forest Pathology* **27**: 137-146.
- Bonants P.J.M., Hagenaar-de Weerd M., van Gent-Pelzer M., Lacourt I., Cooke D., Duncan J., 1997. Detection and identification of *Phytophthora fragariae* Hickman by the polymerase chain reaction. *European Journal of Plant Pathology* **103**: 345-355.
- Cooke D.E.L., Drent A., Duncan J.M., Wagels G., Brasier C.M., 2000a. A molecular phylogeny of *Phytophthora* and related Oomycetes. *Fungal Genetics and Biology* **30**: 17-30.
- Cooke D.E.L., Duncan J.M., Williams N.A., Hagenaar-de Weerd M., Bonants P.J.M., 2000b. Identification of *Phytophthora* species on the basis of restriction enzyme fragment analysis of the internal transcribed spacer regions of ribosomal RNA. *EPPO Bulletin* **30**: 519-523.
- Cooke D.E.L., Kennedy D.M., Guy D.C., Russel J., Unkles S.E., Duncan J.M., 1996. Relatedness of group I species of *Phytophthora* as assessed by randomly amplified polymorphic DNA (RAPDs) and sequences of ribosomal DNA. *Mycological Research* **100**: 297-303.
- Dobrowolski M.P., 1998. Microsatellites in the mitochondrial genome of *Phytophthora cinnamomi* failed to provide highly polymorphic markers for population genetics. *FEMS Microbiology Letters* **163**: 243-248.
- Drenth A., Irwin J.A.G., 2001. Routine DNA based diagnostic test for *Phytophthora*. A report for Rural Industries Research and Development Corporation. RIRDC Publication No 01/36; RIRDC Project No UQ-68A.
- Erwin D.C., Ribeiro O.K., 1996. *Phytophthora* Disease Worldwide. American Phytopathological Society Press, St. Paul, Minnesota, USA.
- Förster H., Kinscherf T.G., Leong S.A., Maxwell D.P., 1989. Restriction fragment length polymorphisms of the mitochondrial DNA of *Phytophthora megasperma* isolated from soybean, alfalfa and fruit trees. *Canadian Journal of Botany* **67**: 529-537.
- Grote D., Olmos A., Kofoet J.J., Bertolini E., Cambra M., 2002. Specific and sensitive detection of *Phytophthora nicotianae* by simple and nested-PCR. *European Journal of Forest Pathology* **108**: 197-207.
- Hantula J., Lilja A., Parikka P., 1997. Genetic variation and host specificity of *Phytophthora cactorum* isolated in Europe. *Mycological Research* **101**: 565-572.
- Hantula J., Lilja A., Nuorteva H., Parikka P., Werres S., 2000. Pathogenicity, morphology and genetic variation of *Phytophthora cactorum* from strawberry, apple, rhododendron and silver birch. *Mycological Research* **104**: 1062-1068.
- Henrion B., Chevalier G., Martin F., 1994. Typing truffle by amplification of the ribosomal DNA spacers. *Mycological Research* **98**: 37-43.
- Hermosa M.R., Grondona I., Diaz-Minguez J.M., Iturriaga E.A., Monte E., 2001. Development of a strain-specific SCAR marker for the detection of *Trichoderma atroviride* 11, a biological agent against soil borne fungal plant pathogens. *Current Genetics* **38**: 343-350.
- Jung T., Blaschke H., Neumann P., 1996. Isolation, identification and pathogenicity of *Phytophthora* species from declining oak stands. *European Journal of Forest Pathology* **26**: 253-272.
- Kennedy D.M., Duncan J.M., 1995. A papillate *Phytophthora* species with specificity to *Rubus*. *Mycological Research* **99**: 57-68.
- Kolmodin L.A., Williams F., 1996. PCR, Basic Principles and Routine Practice. In: Methods in Molecular Biology. PCR Cloning Protocols: From Molecular Cloning to Genetic Engineering, pp. 3-15. B.A. White Humana Press Inc., Totowa, New Jersey, USA.
- Larsen R.C., Hollingsworth C.R., Vandemark G.J., Gritsenko M.A., Gray F.A., 2002. A rapid method using PCR-based SCAR markers for the detection and identification of *Phoma sclerotoides*: the cause of brown root rot disease of Alfalfa. *Plant Disease* **86**: 928-932.
- Lee S.B., Taylor J.W., 1992. Phylogeny of five fungus-like Protoctistan *Phytophthora* species, inferred from the internal transcribed spacers of ribosomal DNA. *Journal of Molecular Biology and Evolution* **9**: 636-653.
- Lee S.B., White T.J., Taylor J.W., 1993. Detection of *Phytophthora* species by oligonucleotide hybridization to amplified ribosomal DNA spacers. *Phytopathology* **83**: 177-181.
- Liew E.C.Y., Maclean D.J., Irwin J.A.G., 1998. Specific PCR based detection of *P. medicaginis* using the intergenic spacer region of ribosomal DNA. *Mycological Research* **102**: 73-80.
- Liew E.C.Y., Maclean D.J., Manners J.M., Dawson D., Irwin J.A.G., 1991. Use of restriction fragment length polymorphisms to study genetic relationships between Australian

- and Japanese isolates of *Phytophthora vignae*. *Australian Journal of Botany* **39**: 335-346.
- Lilja A., Karjalainen R., Parikka P., Kammiovirta K., Nuorteva H., 1998. Pathogenicity and genetic variation of *Phytophthora cactorum* from silver birch and strawberry. *European Journal of Plant Pathology* **104**: 529-535.
- Lilja A., Rikala R., Parikka P., Hietala A., Heinonen R., 1996. Stem lesions on *Betula pendula* seedlings in Finnish forest nurseries and the pathogenicity of *Phytophthora cactorum*. *European Journal of Plant Pathology* **26**: 89-96.
- Lindqvist H., Koponen H., Valkonen J.P.T., 1998. *Peronospora sparsa* on cultivated *Rubus articus* and its detection by PCR based on ITS sequences. *Plant Disease* **82**: 1304-1311.
- Loucourt I., Bonants P.J.M., Van Gent-Pelzer M.P., Cooke D.E.L., Hagenaar-De Weerd M., Surplus L., Duncan J.M., 1997. The use of nested primers in the polymerase chain reaction for the detection of *Phytophthora fragariae* and *Phytophthora cactorum* in strawberry. In: *Proceedings of the 3rd international Strawberry Symposium, Veldhoven 1996*, 165-167.
- Meng X.Q., Shoemaker R.C., Yang X.B., 1999. Analysis of pathogenicity and genetic variation among *Phytophthora sojae* isolates using RAPD. *Mycological Research* **103**: 173-178.
- Mercado-Blanco J., Rodriguez-Jurado D., Jiménez-Díaz R.M., 2001. Detection of the nondefoliating pathotype of *Verticillium daliae* in infected olive plants by nested PCR. *Plant Pathology* **50**: 609-619.
- Nienhaus F., 1960. Das Wirtsspektrum von *Phytophthora cactorum* (Leb. et Cohn) Schröet. *Phytopathologische Zeitschrift* **38**: 33-68.
- Nygaard S.L., Elliot C.K., Cannon S.J., Maxwell D.P., 1989. Isozyme variability among isolate of *Phytophthora megasperma*. *Phytopathology* **79**: 773-779.
- Oudemans P., Coffey M.D., 1991a. Isozyme comparison within and among worldwide sources of three morphologically distinct species of *Phytophthora*. *Mycological Research* **95**: 19-30.
- Oudemans P., Coffey M.D., 1991b. A revised systematic of twelve papillate *Phytophthora* species based on isozyme analysis. *Mycological Research* **95**: 1025-1046.
- Panabieres F., Marais A., Trentin F., Bonnet P., Ricci P., 1989. Repetitive DNA polymorphism analysis as a tool for identifying *Phytophthora* species. *Phytopathology* **79**: 1105-1109.
- Ristaino J.B., Madritch M., Trout C.L., Parra G., 1998. PCR amplification of ribosomal DNA for species identification in the plant pathogen genus *Phytophthora*. *Applied and Environmental Microbiology* **64**: 948-954.
- Sambrook J., Fritsch E.F., Maniatis T., 1989. *Molecular cloning a laboratory manual* (2nd edition). Cold Spring Harbor Laboratory Press, Plainview, New York, USA.
- Schubert R., Bahnweng G., Nechwall G.J., Jung T., Cooke D.E.L., Duncan J.M., Müller-Stark G., Langebartels C., Sandermann H. Jr., Oßwald W., 1999. Detection and quantification of *Phytophthora* species, which are associated with root-rot disease in European deciduous forests by species-specific Polymerase Chain Reaction. *European Journal of Forest Pathology* **29**: 169-188.
- Stamps D.J., Waterhouse G.M., Newhook F.J., Hall G.S., 1990. Revised Tabular key to the species of *Phytophthora*. 2nd Ed. Mycological Paper No. 162, Commonwealth Mycological Institute, Kew, UK.
- Taylor E.J.A., Stevens E.A., Bates J.A., Morreal G., Lee D., Kenyon D.M., Thomas J.E., 2001. Rapid-cycle PCR detection of *Pyrenophora graminea* from barley seed. *Plant Pathology* **50**: 347-355.
- Tooley P.W., Bunyard B.A., Carras M.M., Hatziloukas E., 1997. Development from internal transcribed spacer region 2 for detection of *Phytophthora* species infecting potatoes. *Applied and Environmental Microbiology* **63**: 1467-1475.
- Trappe J.M., Jumpponen A., 1995. Taxonomy of ectomycorrhizal fungi: a starting point for their biotechnology. In: Stocchi V. (ed.). *Biotechnology of Ectomycorrhizae*, pp. 25-33. Plenum Press, New York, USA.
- Trout C.L., Ristaino J.B., Madritch M., Wangsoomboondee T., 1997. Rapid detection of *Phytophthora infestans* in late blight infected potatoes and tomatoes using PCR. *Plant Disease* **81**: 1042-1048.
- Trower M.K., Elgar G.S., 1997. Cloning PCR Products Using T-Vectors. In: Harwood A. (ed.). *Methods in Molecular Biology: Basic DNA and RNA Protocols*, pp. 313-324. Humana Press Inc., Totowa, New Jersey, USA.
- Tucker C.M., 1933. Distribution of genus *Phytophthora*. University of Missouri Agricultural Experiment Station Research Bulletin N. 184.
- Tyler B.M., Forster H., Coffey M.D., 1995. Inheritance of avirulence factors and restriction fragment length polymorphism markers in outcrosses of the oomycete *Phytophthora sojae*. *Molecular Plant-Microbe Interactions* **8**: 515-523.
- Waterhouse G.M., 1963. Key to the species of *Phytophthora* de Bary. Mycological Paper No. 92. Commonwealth Mycological Institute, Kew, U.K..
- Werres S., 1995. Influence of the *Phytophthora* isolate and the seed source on the development of beech (*Fagus sylvatica*) seedling blight. *European Journal of Forest Pathology* **25**: 381-390.
- Whisson S.C., Maclean D.J., Manners J.M., Irwin J.A.G., 1992. Genetic relationships among Australian and North America isolates of *Phytophthora megasperma* f.sp. *Glycinea* by multi-copy DNA probes. *Phytopathology* **82**: 863-868.
- Wilson I.G., 1997. Inhibition and facilitation of nucleic acid amplification. *Applied and Environmental Microbiology* **63**: 3741-3751.
- Wyss P., Bonfante P., 1993. Amplification of genomic DNA of arbuscular-mycorrhizal (AM) fungi by PCR using short arbitrary primers. *Mycological Research* **97**: 1351-1357.

