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Detection of *Phytophthora nicotianae* and *P. citrophthora* in citrus roots and soils by nested PCR

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Abstract

The polymerase chain reaction (PCR) was used for the specific detection of *Phytophthora nicotianae* and *P. citrophthora* in citrus roots and soils. Primers were based on the nucleotide sequences of the internal transcribed space regions (ITS1 and ITS2) of 16 different species of *Phytophthora*. Two primer pairs, Pn5B–Pn6 and Pc2B–Pc7, were designed specifically to amplify DNA from *P. nicotianae* and *P. citrophthora*, respectively. Another primer pair (Ph2–ITS4) was designed to amplify DNA from many *Phytophthora* species. All primer pairs were assessed for specificity and absence of cross-reactivity, using DNA from 118 isolates of *Phytophthora* and 82 of other common soil fungi. In conventional PCR, with a 10-fold dilution series of template DNA, the limit of detection was of 1 pg μl^{-1} DNA for all the primer pairs (Ph2–ITS4, Pn5B–Pn6, and Pc2B–Pc7). In nested PCR, with primers Ph2–ITS4 in the first round, the detection limit was of 1 fg μl^{-1} for both the primer sets (Pn5B–Pn6 and Pc2B–Pc7). Simple, inexpensive and rapid procedures for direct extraction of DNA from soil and roots were developed. The method yielded DNA of a purity and quality suitable for PCR within 2–3 h. DNA extracted from soil and roots was amplified by nested PCR utilizing primers Ph2–ITS4 in the first round. In the second round the primer pairs Pn5B–Pn6 and Pc2B–Pc7 were utilized to detect *P. nicotianae* and *P. citrophthora,* respectively. Comparison between the molecular method and pathogen isolation by means of a selective medium did not show any significant differences in sensitivity.

Abbreviations: CTAB – cetyltrimethylammonium bromide; dNTPs – deoxynucleoside triphosphates; DMSO – Dimethyl sulfoxide; ITS – internal transcribed spacer; PCR – polymerase chain reaction.

Introduction

Phytophthora root rot, caused by *P. nicotianae* van Breda de Haan [syn. *P. n.* van Breda de Haan var. *parasitica* (Dast) Watherhouse] and *P. citrophthora* (Sm. *et* Sm.) Leonian, is the most important disease of citrus in the world (Menge and Nemec, 1997). Control of *Phytophthora* root rot of citrus begins with the production of disease-free seedlings, since most *Phytophthora* associated with citrus root rot in the field probably originates from infested nursery stock (Menge and Nemec, 1997). Other means of control (chemical control, soil fumigation, cultural practices, and biological control) are expensive and not always effective. Therefore, disease-free propagating material planted into pathogen-free soils is a basic measure for preventing disease. To produce sound material and determine the absence of pathogens in soils, it is essential to develop rapid and sensitive methods for the detection of the pathogens. Current methods for the detection and identification of *Phytophthora* species involve baiting from soil or isolation by means

of selective media from infected plant tissue or soil, followed by pure-culturing the organism and identification based on morphological characteristics. These procedures are time and labour-consuming and preclude the handling of large number of samples (Tsao, 1983; Rahimian and Mitchell, 1988; Larkin et al., 1995). In addition, identification of *Phytophthora* at the species level on the basis of morphology is often complicated by the lack of diagnostic morphological characters. In order to improve efficiency and accuracy for the detection of *Phytophthora,* alternative methods based on enzyme immuno-assays (ELISA) have been developed and tested (Gabor et al., 1993; Devergne et al., 1994). The ELISA systems for *Phytophthora* species have proved to be highly sensitive, however it was not possible to identify the pathogen at the species level and the results were not always consistent with commonly used selective media (Timmer et al., 1993). Species-specific oligonucleotide hybridization probes have been developed in order to identify several species of *Phytophthora* (Goodwin et al., 1990a,b; Lee et al., 1993b; Judelson and Messenger-Routh, 1996). Other methods based on molecular analysis and in particular the polymerase chain reaction (PCR) are much more promising and have been widely applied to identify different fungal and Oomycete plant pathogens (Lacourt and Duncan, 1997; Bonants et al., 1997; Schena et al., 2002a,b). These methods have the advantage that they are highly specific, sensitive, and rapid, with potential to be automated (Taylor et al., 2001). Regarding the two species that attack citrus, *P. nicotianae* and *P. citrophthora*, specific primers for their identification have been developed (Èrsek et al., 1994; Lacourt and Duncan, 1997; Grote et al., 2002). However, no specific studies were conducted to detect *P. nicotianae* and *P. citrophthora* directly from citrus roots and soils.

In this paper, we describe the development of PCR primers from internal transcribed space regions (ITS1 and ITS2) (Cooke and Duncan, 1997) and their use in a nested PCR assay to detect *P. nicotianae* and *P. citrophthora* in citrus roots and soils.

Materials and methods

Source of isolates and DNA extraction

Phytophthora isolates were collected in Southern Italy mainly from diseased citrus plants and soils from 1990 to 2001; additional isolates were donated by various individuals or purchased from international culture collections (Table 1). Other fungal and Oomycete species (Table 2) were available from the collection of the Department of Plant Protection and Applied Microbiology (University of Bari, Italy) or purchased from international culture collection. All isolates were stored on potato dextrose agar (PDA) slants at 20 °C (*Phytophthora* isolates) or 5 °C (other fungi).

To extract total DNA, all isolates were incubated on malt extract agar (MEA) covered with sterile cellophane sheets before inoculation, to facilitate collection of the mycelium. Between 50 and 100 mg of mycelia per Petri dish, collected after 5–15 days of incubation at 24 °C, were suspended in 400 µl of lysis buffer (Hoffman and Winston, 1987) and extracted with 400 µl of phenol/chloroform/isoamyl alcohol (25 : 24 : 1), in the presence of 25 mg of acid-washed glass beads (425–600 µm diameter) and two sterile 5 mm stainless steel ball bearings. This mixture was vortexed at 3000 rpm for 10 min, and centrifuged for 15 min at 13,000 × *g*. The aqueous phase was collected, extracted with an equal volume of

Table 1. Isolates of *Phytophthora* used to screen the primer specificity

Species	Isolate code	Host	Source	Amplification with primer		
				Ph2–ITS4	Pn5B–Pn6	Pc2B–Pc7
P. nicotianae	Ph3	Citrus	DPPMA	+	+	−
P. nicotianae	Ph5	Citrus	DPPMA	+	+	−
P. nicotianae	Ph8	Citrus	DPPMA	+	+	−
P. nicotianae	Ph9	Citrus	DPPMA	+	+	−
P. nicotianae	Ph12	Tomato	G. Cristinzio	+	+	−
P. nicotianae	Ph15	Tomato	G. Cristinzio	+	+	−
P. nicotianae	Ph16	Citrus	DPPMA	+	+	−
P. nicotianae	Ph17	Citrus	R. D'Anna	+	+	−
P. nicotianae	Ph18	Citrus	DPPMA	+	+	−
P. nicotianae	Ph19	Tomato	F. Casulli	+	+	−
P. nicotianae	Ph20	Citrus	DPPMA	+	+	−

Table 1. (*Continued*)

Species	Isolate code	Host	Source	Amplification with primer		
				Ph2–ITS4	Pn5B–Pn6	Pc2B–Pc7
P. nicotianae	Ph24	Citrus	DPPMA	+	+	−
P. nicotianae	Ph25	Citrus	DPPMA	+	+	−
P. nicotianae	Ph26	Citrus	DPPMA	+	+	−
P. nicotianae	Ph32	Citrus	DPPMA	+	+	−
P. nicotianae	Ph43	Citrus	DPPMA	+	+	−
P. nicotianae	Ph44	Olive	DPPMA	+	+	−
P. nicotianae	Ph45	Citrus	DPPMA	+	+	−
P. nicotianae	Ph49	Olive	DPPMA	+	+	−
P. nicotianae	Ph51	Citrus	DPPMA	+	+	−
P. nicotianae	Ph53	Citrus	DPPMA	+	+	−
P. nicotianae	Ph55	Citrus	DPPMA	+	+	−
P. nicotianae	Ph56	Citrus	DPPMA	+	+	−
P. nicotianae	Ph58	Citrus	DPPMA	+	+	−
P. nicotianae	Ph61	Tomato	F. Casulli	+	+	−
P. nicotianae	Ph63	Tomato	F. Casulli	+	+	−
P. nicotianae	Ph70	Tomato	C. Cariddi	+	+	−
P. nicotianae	Ph73	Citrus	DPPMA	+	+	−
P. nicotianae	Ph74	Citrus	DPPMA	+	+	−
P. nicotianae	Ph75	Citrus	DPPMA	+	+	−
P. nicotianae	Ph76	Citrus	DPPMA	+	+	−
P. nicotianae	Ph77	Citrus	DPPMA	+	+	−
P. nicotianae	Ph78	Citrus	DPPMA	+	+	−
P. nicotianae	Ph79	Citrus	DPPMA	+	+	−
P. nicotianae	Ph80	Citrus	DPPMA	+	+	−
P. nicotianae	Ph81	Citrus	DPPMA	+	+	−
P. nicotianae	Ph82	Citrus	DPPMA	+	+	−
P. nicotianae	Ph86	Citrus	DPPMA	+	+	−
P. nicotianae	Ph87	Citrus	DPPMA	+	+	−
P. nicotianae	Ph90	Citrus	DPPMA	+	+	−
P. nicotianae	Ph91	Citrus	INRA Antibes	+	+	−
P. nicotianae	Ph92	Not known	G. Magnano S.L.	+	+	−
P. citrophthora	Ph27	Citrus	DPPMA	+	−	+
P. citrophthora	Ph33	Olive	DPPMA	+	−	+
P. citrophthora	Ph34	Olive	DPPMA	+	−	+
P. citrophthora	Ph50	Olive	DPPMA	+	−	+
P. citrophthora	Ph52	Citrus	DPPMA	+	−	+
P. citrophthora	Ph60	Not known	INRA Antibes	+	−	+
P. citrophthora	Ph64	Citrus	DPPMA	+	−	+
P. citrophthora	Ph65	Citrus	DPPMA	+	−	+
P. citrophthora	Ph66	Citrus	DPPMA	+	−	+
P. citrophthora	Ph67	Citrus	DPPMA	+	−	+
P. citrophthora	Ph68	Citrus	G. Cutuli	+	−	+
P. citrophthora	Ph71	Citrus	DPPMA	+	−	+
P. citrophthora	Ph72	Citrus	DPPMA	+	−	+
P. citrophthora	Ph88	Citrus	G. Cutuli	+	−	+
P. citrophthora	Ph93	Citrus	DPPMA	+	−	+
P. citrophthora	Ph94	Citrus	DPPMA	+	−	+
P. citrophthora	Ph95	Citrus	DPPMA	+	−	+
P. citrophthora	Ph96	Citrus	DPPMA	+	−	+
P. citrophthora	Ph97	Citrus	DPPMA	+	−	+
P. citrophthora	Ph98	Citrus	DPPMA	+	−	+
P. citrophthora	Ph100	Citrus	DPPMA	+	−	+
P. citrophthora	Ph110	Citrus	INRA Antibes	+	−	+
P. citrophthora	Ph115	Citrus	INRA Antibes	+	−	+
P. citrophthora	Ph117	Citrus	INRA Antibes	+	−	+

Table 1. (*Continued*)

Species	Isolate code	Host	Source	Amplification with primer		
				Ph2–ITS4	Pn5B–Pn6	Pc2B–Pc7
P. citrophthora	Ph119	Citrus	INRA Antibes	+	−	+
P. boehmeriae	Ph131	Ficus sp.	INRA Antibes	+	−	−
P. cactorum	Ph2	Walnut-tree	S.O. Cacciola	+	−	−
P. cactorum	Ph23	Chestnut	N. Anselmi	+	−	−
P. cactorum	Ph48	Olive	DPPMA	+	−	−
P. cactorum	Ph84	Chestnut	N. Anselmi	+	−	−
P. cactorum	Ph89	Olive	DPPMA	+	−	−
P. cactorum	Ph103	Strawberry	INRA Antibes	+	−	−
P. cactorum	Ph109	Apple	INRA Antibes	+	−	−
P. cactorum	Ph118	Pear	INRA Antibes	+	−	−
P. cambivora	Ph83	Chestnut	N. Anselmi	+	−	−
P. cambivora	Ph107	Chestnut-tree	INRA Antibes	+	−	−
P. capsici	Ph1	Pepper	S.O. Cacciola	+	−	−
P. capsici	Ph108	Pepper	DPPMA	+	−	−
P. cinnamoni	Ph4	Not known	S.O. Cacciola	+	−	−
P. cinnamomi	Ph102	Not known	INRA Antibes	+	−	−
P. cinnamoni	Ph112	Walnut-tree	INRA Antibes	+	−	−
P. cinnamoni	Ph113	Chestnut-tree	INRA Antibes	+	−	−
P. citricola	Ph30	Chestnut	N. Anselmi	+	−	−
P. citricola	Ph31	Olive	DPPMA	+	−	−
P. citricola	Ph54	Not known	DPPMA	+	−	−
P. citricola	Ph85	Chestnut	N. Anselmi	+	−	−
P. citricola	Ph111	Not known	INRA Antibes	+	−	−
P. cryptogea	Ph6	Not known	S.O. Cacciola	+	−	−
P. cryptogea	Ph29	Tomato	S.O. Cacciola	+	−	−
P. cryptogea	Ph105	Not known	INRA Antibes	+	−	−
P. cryptogea	Ph116	Not known	INRA Antibes	+	−	−
P. drechsleri	Ph114	Almond	INRA Antibes	+	−	−
P. heveae	Ph99	Avocado	INRA Antibes	+	−	−
P. palmivora	Ph7	Olive	DPPMA	+	−	−
P. palmivora	Ph21	Olive	DPPMA	+	−	−
P. palmivora	Ph35	Olive	DPPMA	+	−	−
P. palmivora	Ph38	Olive	DPPMA	+	−	−
P. palmivora	Ph40	Olive	DPPMA	+	−	−
P. palmivora	Ph42	Olive	DPPMA	+	−	−
P. palmivora	Ph46	Olive	DPPMA	+	−	−
P. palmivora	Ph47	Olive	DPPMA	+	−	−
P. palmivora	Ph57	Citrus	DPPMA	+	−	−
P. palmivora	Ph69	Olive	DPPMA	+	−	−
P. palmivora	Ph104	Fig	INRA Antibes	+	−	−
P. erythroseptica	Ph10	Not known	S.O. Cacciola	+	−	−
P. megasperma	Ph101	Wistaria	INRA Antibes	+	−	−
P. megasperma	Ph106	Wistaria	INRA Antibes	+	−	−
Phytophthora sp.	Ph11	Pepper	G. Cristinzio	+	−	−
Phytophthora sp.	Ph13	Slipperwort	G. Cristinzio	+	−	−
Phytophthora sp.	Ph14	Olive	DPPMA	+	−	−
Phytophthora sp.	Ph22	Not known	S.O. Cacciola	+	−	−
Phytophthora sp.	Ph28	Not known	S.O. Cacciola	+	−	−
Phytophthora sp.	Ph36	Olive	DPPMA	+	−	−
Phytophthora sp.	Ph37	Olive	DPPMA	+	−	−
Phytophthora sp.	Ph39	Olive	DPPMA	+	−	−
Phytophthora sp.	Ph62	Olive	DPPMA	+	−	−

INRA Antibes = isolates provided by Frank Panabieres and Paul Venard, INRA, Antibes, F; DPPMA = isolates present in the collection of 'Dipartimento di Protezione delle Piante e Microbiologia Applicata', University of Bari, Italy.

Table 2. Fungal isolates used to screen the primer specificity

Fungus	Isolate code	Hosts
Alternaria brassicicola	Fv359	Cauliflower
Alternaria citri	Fv33	Lemon
Alternaria sp.	Fv167, Fv169, Fv232, OL67, F5, F20	Pistachio, Parsley, Cycas, Olive, Chestnut-tree, Sweet cherry
Aspergillus niger	Asp3	Grapevine
Aspergillus sp.	F15	Sweet cherry
Botryosphaeria ribis	Fv20, Fv360	Currant, Orange
Botryosphaeria sp.	A4	Grapevine
Botrytis cinerea	Fv69, Fv132, Fv349	Grapevine, Kiwifruit, Grapevine
Camarosporium sp.	Fv253	Orange
Cephalosporium sp.	Fv61	Cotton
Cladosporium sp.	Fv267	Not known
Colletotrichum sp.	Fv68	Clementine
Cylindrocarpon sp.	Fv128	Kiwifruit
Cytospora sp.	Fv196	Sweet cherry
Endothia parasitica	Fv46	Chestnut-tree
Euypa lata	A5	Grapevine
Fomitiporia puntata	A1	Lemon
Fusarium roseum	F4	Almond
Fusarium sp.	Fv27, Fv183, Fv231, F11, F14, F17, F18	Kiwifruit, Cycas, Sweet cherry
Fusicoccum amygdali	Fv342	Sweet cherry
Gliocladium roseum	Fv39	Not known
Gliocladium sp.	Fv114, Fv303	Kiwifruit, Potato
Gloeosporium sp.	Fv94	Apple
Macrophomina sp.	Fv313	Potato
Mycocentrospora cladosporioides	OL177	Olive
Myrothecium roridum	Fv50	From soil
Penicillium digitatum	Fv51, F7	Orange, Almond
Penicillium funiculosum	Fv273	From air
Penicillium italicum	Fv99	Mandarin
Penicillium sp.	F16	Sweet cherry
Phaemoniella chlamydospora	A2	Grapevine
Phialophora sp.	Fv266	Sweet cherry
Phialophora parasitica	OL25	Olive
Phoma sp.	Fv156	Clementine
Phomopsis diospyri	Fv205	Japanese persimmon
Phomopsis viticola	A3	Grape vine
Phyllosticta arbuti	Fv265	Strawberry tree
Pleurotus ostreatus	Fv32	Not known
Pythium sp.	F1, F2, F3	Almond
Rhizoctonia solani	F10	Sweet cherry
Rosellinia necatrix	R12, R24	Almond, Sweet cherry
Rosellinia aquila	R31	Not known
Rosellinia limoniispora	R33	Wheat
Rosellinia mammiformis	R34	Ash
Rosellinia millegrana	R35	Wine cork
Rosellinia reticulispora	R40	Wheat
Rosellinia sanguinolenta	R41	Twig
Sclerotinia sp.	Fv75	Lemon
Septoria tritici	Fv95	Wheat
Stemphylium sp.	Fv123	Pistachio
Trichoderma coningii	Fv283	Not known
Trichoderma pseudoconingii	Fv154	Clementine
Trichoderma harzianum	F12	Sweet cherry

Table 2. (*Continued*)

Fungus	Isolate code	Hosts
Trichoderma sp.	F8, F9, F13, F19	Sweet cherry
Trichoderma viridae	Fv181	Not known
Trichothecium sp.	Fv236	Strawberry tree
Ulocladium sp.	Fv263	Sow thistle
Verticillium albo-atrum	Fv336	Tomato
Verticillium dahliae	Fv330, OL61	Olive

Table 3. Sequences, amplified fragment sizes, and specificity of primers utilized to identify and detect *P. nicotianae* and *P. citrophthora*

Forward primers (5′–3′)	Reverse primers (5′–3′)	Fragment size (bp)	Specificity
Ph2	ITS4	700	*Phytophthora* spp.
ATACTGTGGGGACGAAAGTC	TCCTCCGCTTATTGATATGC		
Pn5B	Pn6	120	*P. nicotianae*
GAACAATGCAACTTATTGGACGTTT	AACCGAAGCTGCCACCCTAC		
Pc2B	Pc	160	*P. citrophthora*
GTTTGTGCTTCGGGCCGAGG	GCAGAAAAGCATACAATAAGCGCCTGT		

chloroform/isoamyl alcohol (24 : 1) and precipitated with two volumes of 100% cold (−20 °C) ethanol. The precipitated DNA was washed with 70% cold ethanol, dissolved in 50 µl of TE buffer (10 mM Tris–HCl, 0.1 mM EDTA, pH 7.6), quantified using a spectrophotometer and diluted to 50 ng μl^{-1}.

Primers design and amplification

To ensure the quality of template DNA, all extracts were amplified by PCR with universal primers ITS5 and ITS4 (White et al., 1990). PCR reactions were performed in a total volume of 25 µl containing 100 ng of genomic DNA, 10 mM Tris–HCl (pH 9), 50 mM KCl, 0.1% Triton X-100, 100 µM dNTPs, 1 mM $MgCl_2$, 1 unit of *Taq* polymerase (Taq DNA polymerase, Promega Corporation, WI, USA) and 2 µM of ITS4 and ITS5 primers. The PCR reaction was incubated in a programmable thermal cycler (PCR express, Hybaid, UK) starting with 5 min denaturation at 95 °C, followed by 35 cycles at 95 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 1 min. A negative control (no template DNA present in PCR reaction) was included in all experiments. Amplicons were analysed by electrophoresis in 2% agarose gels in TAE buffer (Sambrook et al., 1989) and visualized by staining with ethidium bromide (2 µg ml^{-1}).

Two specific primers for *P. nicotianae* (Pn5B–Pn6) and *P. citrophthora* (Pc2B–Pc7) were designed by comparison of the internal transcribed space regions of 16 different species: *P. cactorum, P. idaei, P. infestans, P. nicotianae, P. megakarya, P. palmivora, P. citrophthora, P. citricola, P. pseudotsugae, P. capsici, P. megasperma, P. cryptogea, P. fragariae* var. *fragariae, P. fragariae* var. *rubi, P. cambivora,* and *P. cinnamomi* (Cooke and Duncan, 1997). An additional primer (Ph2) with a nucleotide sequence common to the 16 species of *Phytophthora* was designed in the ITS1 region and combined with the universal primer ITS4 (White et al., 1990) to amplify DNA from many *Phytophthora* species. Primer sequences and their schematic localization in the internal transcribed space regions are shown in Table 3 and Figure 1, respectively.

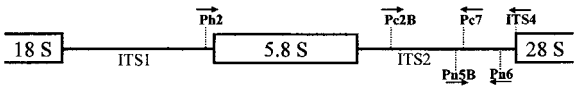

Figure 1. Schematic diagram of ITS1, ITS2, and 5.8S ribosomal genes of *Phytophthora* and location of primers utilized to amplify DNA from *Phytophthora* species (Ph2–ITS4) and to identify *P. nicotianae* (Pn5B–Pn6) and *P. citrophthora* (Pc2B–Pc7).

Specificity of all primer pairs was assessed against 118 isolates of *Phytophthora* (Table 1) and 82 isolates of other species (Table 2). PCR amplifications were conducted as described for the universal primers ITS5–ITS4, except for the addition of DMSO (5%) in the reaction mixture with primers Pn5B–Pn6 and the annealing temperature, which was 51 °C (primers Ph2–ITS4) or 55 °C (primers Pn5B–Pn6 and Pc2B–Pc7).

Sensitivity of conventional and nested PCR

To assess sensitivity of the PCR reaction total DNA extracted from a pure culture of *P. nicotianae* (isolate Ph16) and *P. citrophthora* (isolate Ph33) was serially diluted 10-fold to give the final concentrations in the reaction mixture of 100 ng μl^{-1} to 100 ag μl^{-1}. Water was used as a negative control to replace template DNA in the PCR reaction. DNA dilutions were amplified with the primer pairs Pn5B–Pn6 and Pc2B–Pc7, specific for *P. nicotianae* and *P. citrophthora*, respectively. Moreover, to increase the sensitivity of the detection a nested PCR was applied, using primers Ph2–ITS4 (amplifying a DNA fragment of approximately 700 bp from *Phytophthora* species) in the first round and primers Pn5B–Pn6 (specific for *P. nicotianae*) and Pc2B–Pc7 (specific for *P. citrophthora*) in the second round. Specific primers were located in the internal transcribed space region amplified by Ph2–ITS4 primers (Figure 1). Reactions were carried out as described above.

Detection of P. nicotianae *and* P. citrophthora *in soil*

Tests were done in February and June using soils collected from 2-year-old sour orange seedlings grown in 5-litre pots. In February, samples were collected from five nurseries (JP, LC, FF, B, DM). In each nursery two-soil mixtures, obtained by mixing soils from five different pots, were analysed. In June, samples were collected in triplicate from nurseries B (four samples) and DM (five samples) and from the rhizosphere of 20-year-old clementine plants (samples LS1 and LS2), grafted on sour orange rootstock, showing symptoms of decline. One healthy soil (samples LS3) collected from a wheat field was used as a negative control. Soil samples were collected with a drill at a depth of 15–20 cm.

All soil samples (approximately 1 kg each) were dried at room temperature for five days, carefully crumbled, mixed and sieved with a 2 mm mesh and the inoculum level of *Phytophthora* assessed using a selective media (Masago et al., 1977) as described by Ippolito et al. (1992) with minor modifications. Briefly, 10 g of sieved soil were suspended in 100 ml of water in an Erlenmeyer flask, and vigorously shaken, using a magnetic stirrer. One millilitre of soil suspension was transferred with a wide mouth pipette to the surface of the selective media and spread by shaking the dish. After 24 h of incubation at 20 °C, soil was removed from the plates with a gentle stream of running water and incubated again at 20 °C for other three days. Colonies showing the characteristic morphology of *P. nicotianae* and *P. citrophthora* were counted to give the propagules per gram (ppg) of dry soil. Soil moisture was determined by desiccating 20 g of soil for 24 h at 110 °C.

For each sample, DNA extraction was performed in triplicate by using the protocol described by Cullen et al. (2001) with minor modifications. Soil (0.5 g) was suspended in 700 µl of extraction buffer (0.12 M Na_2HPO_4, 1.5 M NaCl, 2% CTAB) in the presence of two 5 mm stainless steel ball bearings and 0.5 g acid-washed glass beads (425–600 µm diameter). The extraction mixture was vortexed at 3000 rpm for 10 min and centrifuged at 13,000 × *g* for 5 min at room temperature. The upper phase was extracted with 750 µl of chloroform, precipitated with two volumes of isopropanol, washed with 70% cold ethanol, dried and resuspended in 50 µl of sterile distilled water. Before amplification, DNA was purified with Sepharose CL-6B (Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany) in a spin column (Bramwell et al., 1995).

One microlitre of purified genomic DNA was amplified using both conventional and nested PCR. In the former, amplifications were carried out with primers Pn5B–Pn6 and Pc2B–Pc7; in nested PCR, 1 µl of amplified product obtained with primers Ph2–ITS4 was utilized as template for the primer sets Pn5B–Pn6 and Pc2B–Pc7. Reactions were carried out as described above. DNA from pure cultures of *P. nicotianae* and *P. citrophthora* were used as a positive control, whereas water (no template DNA present in PCR reaction) was used as a negative control. Amplifications were analysed as described before.

Detection of P. nicotianae *and* P. citrophthora *from roots*

Tests were conducted in October on roots collected from 2-year-old sour orange seedlings grown in 5-litre pots. Samples were collected from 12 different seedlings showing various symptoms of canopy decline. Roots collected from 3-month-old seedling grown in an healthy soil collected from a wheat field were used as a negative control. All rootlet samples were washed with tap water, dried with blotting-paper, cut with shears into segments approximately 1 cm long, which were divided in two equivalent parts for

traditional and molecular analyses. Traditional analysis was conducted by plating, for each sample, 300 root segments in 15 Petri dishes containing a selective media (Masago et al., 1977). The colonies grown from the roots after three days of incubation at 20 °C were identified on the basis of their morphology and used to assess the degree of root infection, expressed as percentage of segments infected.

For molecular analysis, rootlets (2–3 g) were grounded with liquid nitrogen using a mortar and pestle to produce a powder. Triplicate 0.1 g samples were transferred to 2-ml screw-cap tubes containing an equal volume of PVP (polyvinylpyrrolidone), two 5 mm stainless steel ball bearings, 0.5 g acid-washed glass beads (425–600 µm diameter), and 1.5 ml of extraction buffer (200 mM Tris–HCl, pH 7.7), 250 mM NaCl, 25 mM EDTA, 0.5% SDS). The extraction mixture was vortexed at 3000 rpm for 5 min and centrifuged at 13,000 × *g* for 5 min at 4 °C. The upper phase (approximately 800 µl) was extracted two times with 1 ml of phenol/chloroform (1 : 1) and 700 µl of chloroform, and precipitated with an equal volume of isopropanol for 1 h at 5 °C. DNA was washed with cold ethanol (70%), purified with Sepharose and amplified by means of conventional and nested PCR as described before for soils.

Results

DNA extraction and primer specificity

The protocol for extracting DNA from pure cultures gave 50–150 ng ml^{-1} of DNA per culture. Template DNA was suitable for PCR amplifications, as demonstrated by the amplification with the universal primer ITS5–ITS4 of single fragments (including both ITS regions and the 5.8S rDNA) from all *Phytophthora* species and other fungal isolates (data not shown).

Primer specificity was assessed against 118 different isolates of *Phytophthora* and 82 isolates of other fungal and Oomycete species. Primer pair Ph2–ITS4

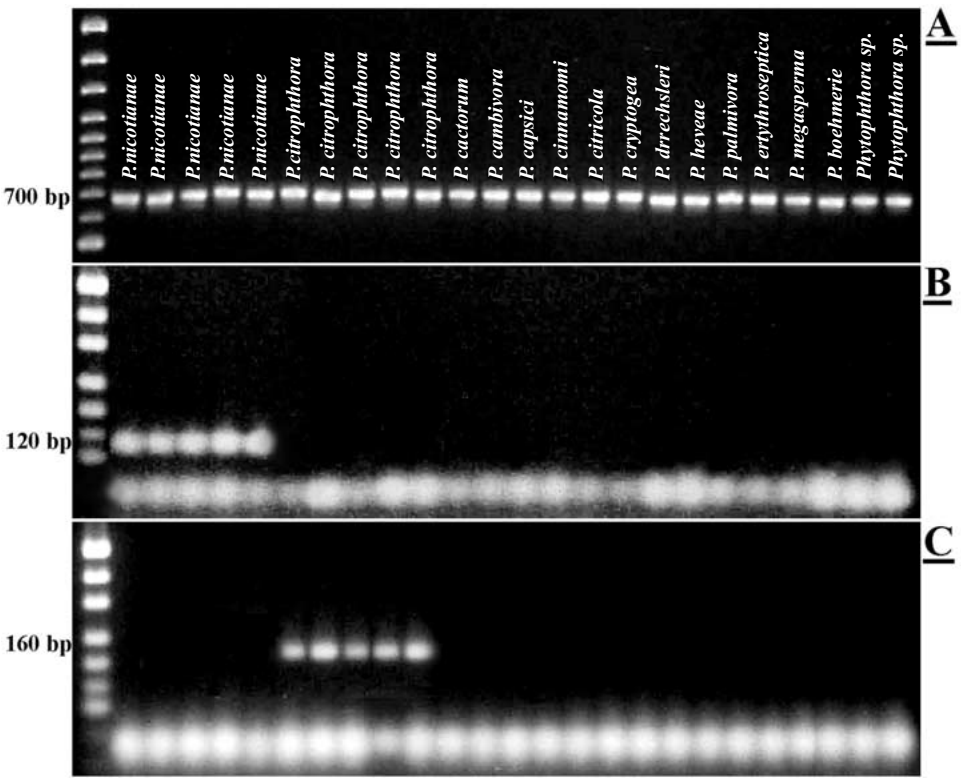

Figure 2. Agarose gel electrophoresis of polymerase chain reaction products from genomic DNA of various isolates of *Phytophthora* belonging to 14 different species with primers Ph2–ITS4 (A), Pn6–Pn5B (B) and Pc2B–Pc7 (C). Markers were loaded in the first lane on the left; in (A) 100-bp DNA ladder and in (B, C) pUC19 DNA/*Msp*I marker (MBI Fermentas GmbH, Germany).

amplified a unique DNA fragment of approximately 700 bp (Figure 2A) from all *Phytophthora* species (Table 1). Primer pair Pn5B–Pn6 was specific for *P. nicotianae* amplifying a fragment of approximately 120 bp (Figure 2B) from all the isolates of *P. nicotianae* but not from the other tested species of *Phytophthora* (Table 1). Similarly, primer pair Pc2B–Pc7 amplified a fragment of approximately 160 bp (Figure 2C) from all the isolates of *P. citrophthora,* but not from other species of *Phytophthora* (Table 1). No amplification was achieved with any of the three primer pairs with any of the other species (Table 2).

Sensitivity of conventional and nested PCR

Sensitivity of all primer pairs was assessed using serial dilutions of total DNA extracted from *P. nicotianae* (Pn5B–Pn6), *P. citrophthora* (Pc2B–Pc7) or from both fungi (Ph2–ITS4). In conventional PCR distinct DNA bands were generated by all primer pairs (Ph2–ITS4, Pn5B–Pn6, and Pc2B–Pc7) with as a little as 1 pg μl^{-1} of DNA (Figure 3A and B). In nested PCR, after first round amplification with primers Ph2–ITS4, the detection limit was 1 fg μl^{-1} for both Pn5B–Pn6 and Pc2B–Pc7 primer pairs (Figure 3C).

Detection of P. nicotianae *and* P. citrophthora *in soil*

Inoculum level of *Phytophthora* in the soil was preliminarily assessed using the selective medium. In the first test carried out in February, soils gave levels of infections ranging from 0 (LC1–5 and B6–10) to 320 propagules of the pathogen per gram of dry soil (Table 4). In particular, in soil mixture JP 1–5 propagules of both species were found. In the second series of tests (June), five soils (DM1, DM2, DM6, LS1, and LS2) had varying numbers of *P. nicotianae* propagules and one (B6) was infested by *P. citrophthora* (Table 4). The remaining soils were free of both pathogens. *P. nicotianae* and *P. citrophthora* were absent in wheat-cultivated soil LS3 (negative control).

The protocol used to extract DNA from soils enabled the extraction of genomic DNA suitable for PCR amplification in approximately 2 h. In all tests, no amplified fragments were visible after the first amplification with all the primer pairs. However, a fragment of the expected size was achieved from the positive controls, i.e. genomic DNA from a pure culture of the pathogens (data not shown). In the first experiment, specific fragments were amplified in nested PCR from all the

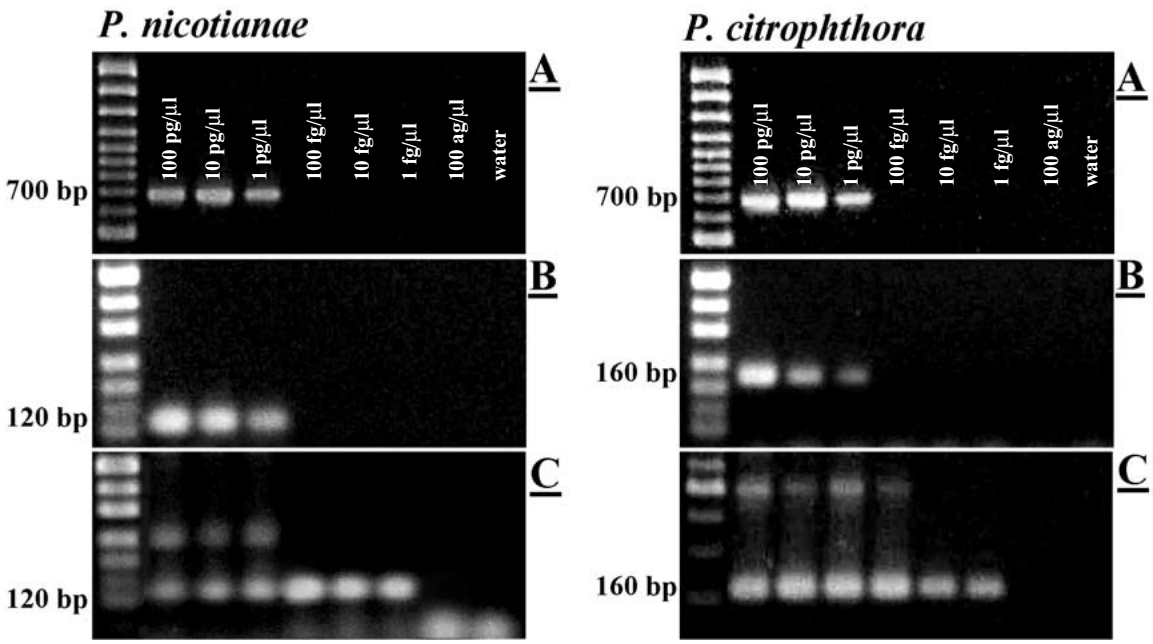

Figure 3. Sensitivity of polymerase chain reaction using a series of 10-fold dilutions of *P. nicotianae* and *P. citrophthora* DNA. For both pathogens a specific DNA fragment was amplified up to 1 pg μl^{-1} after conventional PCR with primer Ph2–ITS4 (A) and the specific primers for *P. nicotianae* and *P. citrophthora* (B). After nested PCR target DNA was amplified up to 1 fg μl^{-1} (C). Markers were loaded in the first lane on the left; in (A) 100-bp DNA ladder and in (B, C) pUC19 DNA/*Msp*I marker (MBI Fermentas GmbH, Germany).

Table 4. Population of *P. nicotianae* and *P. citrophthora* in soils collected in February from five nurseries (JP, LC, FF, B, and DM) and in June from two nurseries (B and DM), and from the rhizosphere of 20-year-old clementine plants (LS1 and LS2). One healthy soil (LS3, sampled in June) collected from a wheat field was used as a negative control. Propagules were assessed using a selective medium in conjunction with the dilution-plate method

February			June		
Soil sample	*P. nicotianae* ppg/g	*P. citrophthora* ppg/g	Soil sample	*P. nicotianae* ppg/g	*P. citrophthora* ppg/g
JP1–5	76	30	B1	0	0
JP6–10	50	0	B2	0	0
LC1–5	0	0	B6	0	52
LC6–10	170	0	B7	0	0
FF1–5	36	0	DM1	96	0
FF6–10	12	0	DM2	39	0
B1–5	10	0	DM6	89	0
B6–10	0	0	DM7	0	0
DM1–5	12	0	DM8	0	0
DM6–10	320	0	LS1	15	0
			LS2	8	0
			LS3	0	0

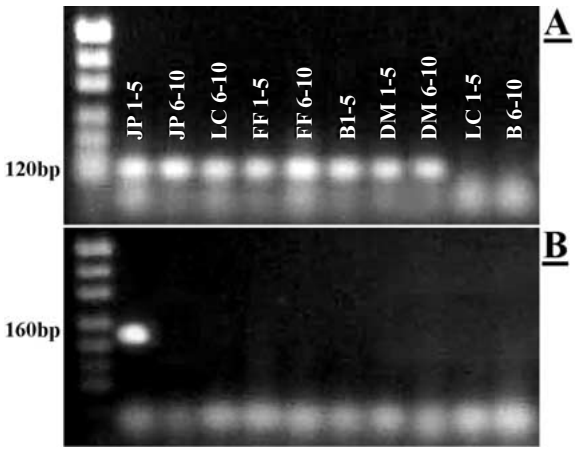

Figure 4. Detection of *P. nicotianae* and *P. citrophthora* by nested PCR in soils collected in February from five nurseries in Southern Italy. One microlitre of amplified product obtained with primers Ph2–ITS4 was amplified with primers Pn5B–Pn6 to detect *P. nicotianae* (A) and Pc2B–Pc7 to detect *P. citrophthora* (B). Markers were loaded in the first lane on the left; pUC19 DNA/*Msp*I marker (MBI Fermentas GmbH, Germany).

infected soil samples. In particular, a DNA fragment of approximately 120 bp, specific for *P. nicotianae*, was amplified with primer pair Pn5B–Pn6 from the samples JP1–5, JP6–10, LC6–10, FF1–5, FF6–10, B1–5, DM1–5, and DM6–10 (Figure 4A). A fragment of approximately 160 bp, specific for *P. citrophthora*, was obtained with primer pairs Pc2B–Pc7 from the sample JP1–5 (Figure 4B). No amplifications were achieved from the remaining soils.

Similar results were obtained in the second series of experiments. Specific DNA fragments were detected after nested PCR from the soils containing *P. nicotianae* or *P. citrophthora* propagules (Figure 5A and B). However, a specific DNA fragment of approximately 160 bp was also obtained with primer pair Pc2B–Pc7 from one of the three replications of the soils B2, DM1, and DM2 apparently free of propagules, as assessed by selective medium. No unexpected amplifications were achieved with primers Pn5b–Pn6.

Detection of P. nicotianae *and* P. citrophthora *from roots*

The presence of *Phytophthora* on feeder roots was assessed using the selective medium and the conventional and nested PCR. All tests excluded the presence of *P. citrophthora* (data not shown). The selective medium indicated the presence of variable levels of *P. nicotianae* infections ranging from 0% to 49% of infected feeder roots (Table 5). The protocol for extracting DNA from roots enabled the extraction of genomic DNA suitable for PCR amplification in approximately three hours. In conventional PCR a specific fragment for *P. nicotianae* of 120 bp was achieved from all roots having a degree of root infection of 11 or higher, whereas the detection limit for nested PCR was 0.3% of infected feeder roots (Table 5). No amplifications were obtained after conventional and nested PCR for negative controls and for samples (nos 10, 11, 12) that were healthy as assessed by the selective medium.

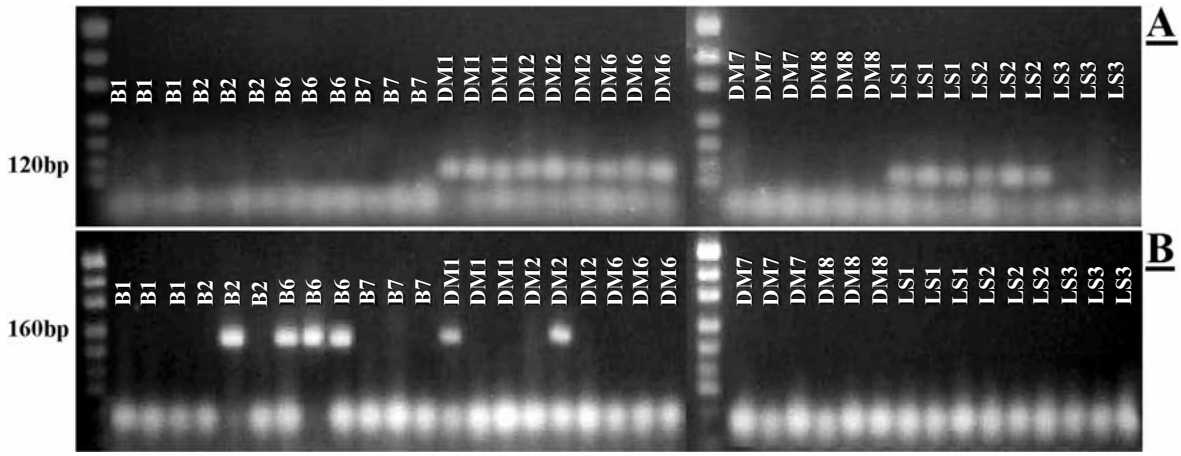

Figure 5. Detection of *P. nicotianae* and *P. citrophthora* by nested PCR in soils collected in June from two nurseries (B and DM) and from the rhizosphere of 20-year-old clementine plants (LS1 and LS2) in Southern Italy. One healthy soil (sample LS3) collected from a wheat field was used as a negative control. One microlitre of amplified product obtained with a primer pair specific for *Phytophthora* species (Ph2–ITS4), was amplified with primers Pn5B–Pn6 to detect *P. nicotianae* (A) and with primers Pc2B–Pc7 to detect *P. citrophthora* (B). Lanes 1 and 23 contain pUC19 DNA/*Msp*I marker DNA (MBI Fermentas GmbH, Germany).

Table 5. Detection of *P. nicotianae* on sour orange feeder roots by means of selective medium, conventional PCR, and nested PCR

Samples analysed	Infected feeder roots (%)	Amplification after conventional PCR			Amplification after nested PCR		
		1st rep.	2nd rep.	3rd rep.	1st rep.	2nd rep.	3rd rep.
Control	0	−	−	−	−	−	−
1	49	+	+	+	+	+	+
2	35	+	+	+	+	+	+
3	30	+	+	+	+	+	+
4	24	+	+	+	+	+	+
5	12	+	+	+	+	+	+
6	11	+	−	+	+	+	+
7	6	−	−	−	+	+	+
8	2	−	−	−	+	+	+
9	0.3	−	−	−	+	+	−
10	0	−	−	−	−	−	−
11	0	−	−	−	−	−	−
12	0	−	−	−	−	−	−

Discussion

The main goal was the development of a sensitive and effective method to identify and detect *P. nicotianae* and *P. citrophthora* in citrus roots and soil. Molecular detection methods (Goodwin et al., 1990a,b; Lee et al., 1993b; Bonants et al., 1997; Ristaino et al., 1998; Schubert et al., 1999), and, in particular, primer sequences which specifically amplify DNA from *P. nicotianae* and *P. citrophthora* have been reported (Érsek et al., 1994; Lacourt and Duncan, 1997; Grote et al., 2002). However, in this paper new primers, amplifying a very short and specific rDNA fragment are reported and their practical application is tested.

Combining primer Ph2 with the universal primer ITS4 (White et al., 1990) it was possible to amplify DNA from 14 species of *Phytophthora*. These primer pairs amplified a fragment of 700 bp that included 5.8S rDNA gene and the ITS2 region, in which target DNA of primer pairs Pn5B–Pn6 (specific for *P. nicotianae*), and Pc2B–Pc7 (specific for *P. citrophthora*) are located (Figure 1). This primer location enabled the use of amplified products from Ph2–ITS4 as a common template for nested PCR to

detect *P. nicotianae* and *P. citrophthora* with a very high level of sensitivity, reducing the number of reactions needed to detect both pathogens. Moreover, the small fragments amplified by the specific primers Pn5B–Pn6 (120 bp) and Pc2B–Pc7 (160 bp), open new possible applications, since they are suitable to develop real time PCRs such as Scorpion-PCR (Whitcombe et al., 1999), Molecular Beacons (Tyagi and Kramer, 1996) and Taq-Man (Lee et al., 1993a). These techniques strongly reduce times and risks of PCR-based detection systems, enabling their use for large scale analyses.

Primers proposed in the present paper are specific and sensitive. Specificity was verified by the absence of cross-reactivity with DNA from a large number of isolates: 118 isolates of *Phytophthora* (14 species) and 82 isolates of other fungal and Oomycete species (59 species) mainly isolated from the rhizosphere. Furthermore, the absence of amplification from the *Phytophthora* non-infested soils and from healthy roots removes any doubt about primer specificity. Regarding sensitivity, using DNA extracted from pure cultures a detectable amplification product was achieved up to 1 pg μl^{-1} of template DNA in conventional PCR and 1 fg μl^{-1} in nested PCR. Similar levels of sensitivity are reported for other phytopathogenic fungi (Faggian et al., 1999; Liew et al., 1998), and are essential to detect pathogens directly in soil or in infected tissues (Schena et al., 2002b; Grote et al., 2002). Also, considering that with conventional PCR *P. nicotianae* was detected only on roots with a very high degree of infection (above 11%) and both pathogens were not detected in soils, the use of nested PCR to reach reasonable sensitivity seems to be essential (Bonants et al., 1997).

Independently from the amplification system, the success and reliability of any PCR-based detection system largely depend on obtaining high yields of target DNA from samples (Cullen et al., 2001). In the present work, we combined the high sensitivity of conventional and nested PCR with simple and rapid extraction methods. The extraction methods used have a small number of efficient lysis and purification steps but maximize the yield and quality of recovered DNA to allow rapid processing of many samples. The effectiveness of the entire procedure (DNA extraction and amplification) was confirmed by comparing the results from molecular analysis with the traditional isolation method on selective medium. The two analyses gave similar results, but traditional method required much more time and expertise to identify *P. nicotianae* and *P. citrophthora* after isolation. Moreover, in the second series of tests with soil, a few were positive for *P. citrophthora* in PCR, although selective medium did not yield any propagule of this species. This positive result could be ascribed to the high sensitivity of the technique and, probably, to the detection by PCR of resting spores of the pathogen, such as chlamydospores, produced in summer (Magnano di San Lio et al., 1988) and not detectable by the selective media (Tsao, 1983). Considering that PCR can also amplify DNA from dead organisms it cannot be excluded that non-viable propagules are detected. However, the rate of break down of DNA from dead organisms in soil should be fairly high due to the high microbial activity (Herdina et al., 1999); therefore, amplification of DNA from dead cells may be a minor problem.

In conclusion, a highly specific, sensitive, and rapid detection method has been developed for *P. nicotianae* and *P. citrophthora* attacking citrus. The sensitivity of the method is more than enough to detect *P. nicotianae* and *P. citrophthora* in soil at population levels below those causing yield loss and/or severe damage on bearing citrus plants, i.e. 15–20 ppg for susceptible rootstocks (Lutz and Menge, 1986; Magnano di San Lio et al., 1988) and around 30 ppg for resistant ones (Ippolito et al., 1991). Moreover, the sensitivity of the method is sufficiently high to exclude the presence of the pathogens in plant propagative material, in soil mixture and in the field soil before planting.

This protocol would improve diagnosis and prophylaxis and, considering the wide host range of the pathogens, particularly of *P. nicotianae*, it could be applied to other host–pathogen combinations. Moreover, other *Phytophthora* diseases could be detected in a similar way, designing specific primers for the second round of PCR. Finally, considering that most *Phytophthora* diseases are related to soil inoculum of the pathogens it could be interesting to develop a protocol able to estimate the propagule number of the pathogen. This approach is being actively pursued and new primers have been designed to apply Real time Scorpion-PCR (Whitcombe et al., 1999).

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