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Real-time detection of *Phytophthora nicotianae* and *P. citrophthora* in citrus roots and soil

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Abstract

Two primers, specific for *Phytophthora nicotianae* (Pn6) and *P. citrophthora* (Pc2B), were modified to obtain Scorpion primers for real-time identification and detection of both pathogens in citrus nursery soils and roots. Multiplex PCR with dual-labelled fluorogenic probes allowed concurrent identification of both species of *Phytophthora* among 150 fungal isolates, including 14 species of *Phytophthora*. Using *P. nicotianae* specific primers a delayed and lower fluorescence increase was also obtained from *P. cactorum* DNA. However, in separate real-time amplifications, the aspecific increase of fluorescence from *P. cactorum* was avoided by increasing the annealing temperature. In multiplex PCR, with a series of 10-fold DNA dilutions, the detection limit was 10 pg μl^{-1} for *P. nicotianae* and 100 pg μl^{-1} for *P. citrophthora*, whereas in separate reaction DNA up to 1 pg μl^{-1} was detected for both pathogens.

Simple and rapid procedures for direct DNA extraction from soil and roots were utilised to yield DNA whose purity and quality was suitable for PCR assays. By combining these protocols with a double amplification (nested Scorpion-PCR) using primers Ph2-ITS4 amplifying DNA from the main *Phytophthora* species (first round) and PnB5-Pn6 Scorpion and Pc2B Scorpion-Pc7 (second round), it was possible to achieve real-time detection of *P. nicotianae* and *P. citrophthora* from roots and soil. The degree of sensitivity was similar to that of traditional detection methods based on the use of selective media. The analyses of artificially and naturally infested soil showed a high and significant correlation between the concentration of pathogen propagules and the real-time PCR cycle threshold.

Introduction

Root rot caused by *Phytophthora nicotianae* (syn. *P. n.* var. *parasitica*) and *P. citrophthora* is one of the major soilborne diseases of citrus in all growing areas of the world. Control begins with the production of *Phytophthora*-free seedlings since much of the inoculum originates from infested nursery stock (Menge and Nemec, 1997). Quantification of *Phytophthora* in the field can help to determine the necessity for and the extent of control measures, because a noticeable yield loss usually occurs only when a high population of the pathogen is present (Menge, 1986). Appropriate phytosanitary provisions have been issued by the European and Mediterranean Plant Protection Organization (EPPO) to prevent the spread of dangerous pathogens such as *P. nicotianae* and *P. citrophthora* (Anonymous, 2002). In particular, EPPO activities encourage the development of detection methods effective and suitable for large-scale analyses. The early, rapid, and accurate identification and detection of pathogens either in the plant, in soil, or water is essential in order to limit their diffusion and improve prophylaxis, especially when pathogens have a wide host range

but are not equally severe in the disease that they cause.

Current methods used for detection and identification of *P. nicotianae* and *P. citrophthora* involve baiting or isolation by means of selective media from infected plant tissue, soil, or water, followed by a subculturing on nutrient media and an examination of the morphology of the fungal colonies, sporangia, oospores, etc. These procedures are time and labour-consuming and require skilled expertise to identify the pathogen at the species level. Recently, conventional PCR-based techniques have contributed to the alleviation of some of the issues associated with the detection, identification, control, and spread of plant pathogens (Bonants et al., 1997; Lacourt and Duncan, 1997; Bridge and Spooner, 2001; Ippolito et al., 2002; Schena et al., 2002b; Schena and Ippolito, 2003). Nevertheless, technical limitations related to post-amplification procedures (gel electrophoresis and ethidium bromide staining) and cross contamination still limit large-scale applications of PCR for plant pathogen diagnosis. These difficulties may be overcome to a certain extent by real-time PCR, a recent technology which combines the sensitivity of conventional PCR with the generation of a specific real-time fluorescent signal throughout the reaction. Real-time PCR enables automation of the technique and is suitable for large-scale sample processing. Among the PCR-based real-time techniques, Taq-Man (Lee et al.,1993), Molecular beacons (Tyagi and Kramer, 1996), and Scorpion-PCR (Whitcombe et al., 1999) are the most promising with increasing reports regarding their application in plant pathology (Bates et al.,2001; Cullen et al.,2001; Schena et al., 2002a, in press). Scorpion-PCR seems to be more rapid and sensitive (Thelwell et al.,2000) and it has been utilised both in human pathology to detect HIV virus (Saha et al.,2001) and in plant pathology to identify and detect antagonistic (Schena et al., 2002a) or pathogenic fungi (Ippolito et al.,2000; Bates and Taylor, 2001; Nigro et al., 2002) and viruses (Finetti Sialer et al., 2000). Moreover, real-time-PCR has the potential for an accurate quantification of target DNA (Schmittgen, 2001). Quantitative analyses are of basic importance for development of predictive diagnostic test to identify high-risk fields where pathogens inoculum is above threshold values (Cullen et al., 2001). In the present work, primers specific for *P. nicotianae* (Pn5B-Pn6) and *P. citrophthora* (Pc2B-Pc7) (Ippolito et al., 2002) were modified to develop a Scorpion-PCR-based detection system. Real-time PCR was combined with a simple, rapid procedure for DNA extraction directly from soil and roots to develop specific, sensitive detection systems suitable for large-scale analyses of *P. nicotianae* and *P. citrophthora* in citrus soils and roots.

Material and methods

Primers design, specificity and sensitivity

Two specific primer pairs, amplifying portions of ITS2 region from *P. nicotianae* (Pn5B-Pn6) and *P. citrophthora* (Pc2B-Pc7) (Ippolito et al., 2002) were modified according to Whitcombe et al. (1999) to synthesize primer Pn6-Scorpion and Pc2B-Scorpion (Figure 1) for amplicon detection

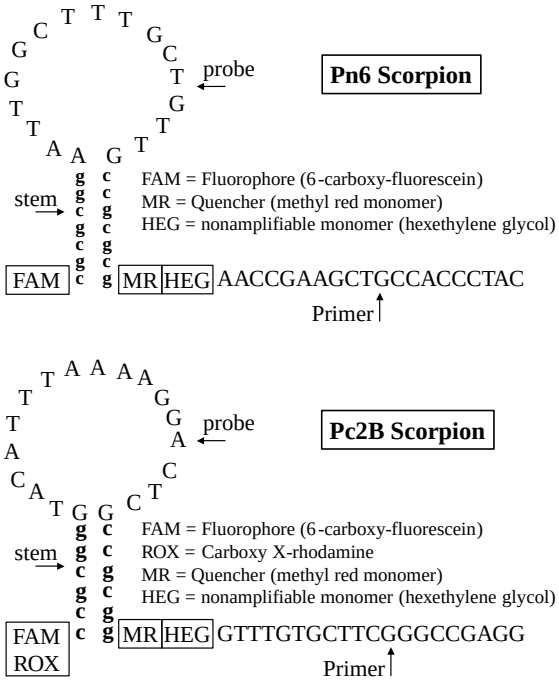

Figure 1. Schematic representation of *P. nicotianae* (Pn6 Scorpion) and *P. citrophthora* (Pc2B Scorpion) specific probes. Scorpion primers are composed of a primer element, a non-amplifiable monomer (HEG), a quencher (MR), a closed stem, a probe element and a fluorophore (FAM or ROX) (Whitcombe et al., 1999).

by fluorescence emitted by a fluorophore through a self-probing reaction (Oswel Research Products Ltd., Southampton, UK). Primer Pc2B Scorpion was labelled with carboxy fluorescein (FAM) or, alternatively, with carboxy X-rhodamine (ROX), whereas primer Pn6-Scorpion was labelled only with FAM.

Primer specificity was preliminarily assessed using a large number of *Phytophthora* isolates, collected in Southern Italy from 1990 to 2001, and additional isolates donated by various individuals (Table 1). Other fungal isolates (Table 2) were made available by the collection of the Department of Plant Protection and Applied Microbiology (DPPMA), University of Bari, Italy. All fungal isolates were stored on potato dextrose agar (PDA) slants at 20 °C (*Phytophthora* isolates) or 5 °C (other fungi). Total DNA was extracted from all isolates as described by Schena et al. (2002b), diluted to the final concentration of 100 ng μl^{-1} and stored at −20 °C for PCR amplifications.

In multiplex Scorpion-PCR, reactions were performed in a 25 µl mixture containing 100 ng of genomic DNA, 10 mM Tris–HCl (pH 9), 50 mM KCl, 0.1% Triton X-100, 100 µM each dNTPs, 1 mM $MgCl_2$, 1 unit of *Taq* polymerase, 0.3 µM of primer Pn6-Scorpion (FAM-labelled), 0.3 µM of primer Pc2B Scorpion (ROX-labelled) and 1 µM of each conventional primer (Pn5B and Pc7). Amplifications consisted of an initial denaturing step at 94 °C for 5 min followed by 40 cycles, each consisting of 45 s of denaturation at 94 °C and 45 s of annealing extension at 50 °C.

In separate Scorpion PCR reactions, the amplification mixture contained only one FAM-labelled primer pair and annealing extension was conducted at 50 and 62 °C for primer Pc2B Scorpion-Pc7 and primer Pn6 Scorpion- Pn7, respectively.

To compare the sensitivity of multiplex and separate Scorpion-PCR reactions, the total DNA extracted from pure cultures of *P. nicotianae* and *P. citrophthora* was serially diluted in the PCR reaction mixture to the final concentrations of 1 ng μl^{-1}, 100, 10, 1 pg μl^{-1} and 100 fg μl^{-1}. Water was used as a negative control to replace template DNA. Amplification conditions were those previously described.

All reactions were conducted in sealed tubes in a 96-well microtiter plate (Bio-Rad, Hercules, CA, USA) and fluorescence was monitored using a spectrofluorometric thermal cycler (iCycler Thermal Cycler, Bio-Rad, Hercules, CA, USA) for real-time data collection during the annealing-extension

Table 1. Phytophthora spp. isolates utilised to evaluate primer specificity

Species	Number of isolates	Geographic origin	Hosts
P. nicotianae	42	Southern Italy (41), California (1)	Citrus (33), Tomato (6), Olive (2), not known (1)
P. citrophthora	25	Southern Italy (20), France (3), Cyprus (1), Morocco (1)	Citrus (21), Olive (3), not known (1)
P. cactorum	8	Southern Italy (5), France (3)	Olive (2), Chestnut (2), Walnut (1), Strawberry (1), Apple (1), Pear (1)
P. cambivora	2	Southern Italy (1), France (1)	Chestnut
P. capsici	2	Southern Italy	Pepper
P. cinnamomi	4	Southern Italy (1), France (3)	Chestnut (1), Walnut (1), not known (2)
P. citricola	4	Southern Italy (3), France (1)	Chestnut (2), Olive (1), not known (1)
P. cryptogea	4	Southern Italy (2), France (2)	Tomato (1), not known (3)
P. drechsleri	1	Greece	Almond
P. heveae	1	USA	Rhododendron
P. palmivora	11	Southern Italy (10), Venezuela (1)	Olive (9), Citrus (1), Fig (1)
P. erythroseptica	1	Southern Italy	Not known
P. megasperma	2	France	Wistaria
Phytophthora spp.	9	Southern Italy	Olive (5), Pepper (1), Slipperwort (1), not known (2)

Per each species, number in parenthesis indicates the number of isolates from each geographic origin and host.

Table 2. Fungal species utilised to evaluate primer specificity

Alternaria spp. (6)	*Gliocladium* spp. (2)	*Rosellinia aquila* (1)
Alternaria brassicicola (1)	*Gliocladium roseum* (1)	*Rosellinia limoniispora* (1)
Alternaria citri (1)	*Gloeosporium* sp. (1)	*Rosellinia mammiformis* (1)
Aspergillus spp. (2)	*Macrophomina* sp. (1)	*Rosellinia millegrana* (1)
Aspergillus niger (1)	*Mycocentrospora cladosporioides* (1)	*Rosellinia necatrix* (5)
Botryosphaeria sp. (1)	*Myrothecium roridum* (1)	*Rosellinia reticulispora* (1)
Botryosphaeria ribis (2)	*Penicillium* sp. (1)	*Rosellinia sanguinolenta* (1)
Botrytis cinerea (3)	*Penicillium funiculosum* (1)	*Sclerotinia* spp. (2)
Camarosporium sp. (1)	*Penicillium digitatum* (2)	*Septoria tritici* (1)
Cephalosporium sp. (1)	*Penicillium italicum* (1)	*Stemphylium* sp. (1)
Cladosporium sp. (1)	*Phaemoniella chlamydospora* (1)	*Trichoderma* spp. (4)
Colletotrichum sp. (1)	*Phialophora* sp. (1)	*Trichoderma harzianum* (1)
Cylindrocarpon sp. (1)	*Phialophora parasitica* (1)	*Trichoderma koningii* (1)
Cytospora sp. (1)	*Phoma* sp. (1)	*Trichoderma pseudokoningii* (1)
Endothia parasitica (1)	*Phomopsis diospyri* (1)	*Trichoderma viride* (1)
Eutypa lata (1)	*Phomopsis viticola* (1)	*Trichothecium* sp. (1)
Fomitiporia punctata (1)	*Phyllosticta* sp. (1)	*Ulocladium* sp. (1)
Fusarium spp. (7)	*Pleurotus ostreatus* (1)	*Verticillium albo-atrum* (1)
Fusarium roseum (1)	*Pythium* spp. (3)	*Verticillium dahliae* (2)
Fusicoccum amygdali (1)	*Rhizoctonia solani* (1)	

Number in parenthesis refers to the number of isolates analysed.

step. Relative normalised fluorescence (ΔRn) was plotted and utilised to calculate the average background fluorescence emission in the initial PCR cycles before fluorescence increased. Threshold fluorescence intensity was established at a level 10 times higher than the standard deviation in the initial PCR cycles and any sample that reached a fluorescence value exceeding the fluorescence threshold value was considered positive. The PCR cycle at which fluorescence exceeded the threshold was defined as the cycle threshold (Ct). Therefore, data from molecular analyses of root and soil samples were recorded as Ct.

Detection of P. nicotianae *in artificially infested soils*

A soil artificially infested with *P. nicotianae* (isolate Ph 78 from the collection of DPPMA) obtained as described by Roiger and Jeffer (1991), was serially diluted with a healthy soil to have 8 soil mixtures containing a progressively lower pathogen concentration (Figure 4A). Undiluted healthy soil served as a negative control. Each soil mixture ($\cong$1 kg) was accurately crumbled, mixed, and sieved before analyses.

Three soil sample replicates of 0.5 g were collected from each dilution and processed to extract DNA as described by Schena et al. (2002b) for molecular analyses. One microlitre of purified genomic DNA (Bramwell et al., 1995) was amplified by Scorpion-PCR and nested Scorpion-PCR. In the former, amplifications were carried out with primers Pn5B-Pn6 Scorpion; in nested Scorpion-PCR, 1 µl of amplified product obtained with primers Ph2-ITS4 (Ippolito et al., 2002) was utilized as a template for the primer pair Pn5B-Pn6 Scorpion. DNA from pure cultures of *P. nicotianae* was used as a positive control, whereas water (no template DNA in PCR reaction) was used as a negative control.

Soil mixtures were also analysed to assess the inoculum density (ID) of *P. nicotianae* using a selective medium (Masago et al.,1977), as described by Ippolito et al. (2002). The ID was expressed as the number of propagules of the pathogen per gram of dry soil.

Data from molecular analyses and selective medium were subjected to first degree regression analysis (Snedecor and Cochran, 1980) to evaluate the correlation between ID (Log scale) and Ct values.

Detection of P. nicotianae *and* P. citrophthora *in naturally infested soils*

Tests were repeated in two different periods (March and July) utilizing soils collected from 2-

year-old sour orange seedlings grown in 5-l pots. In March, samples were collected from four nurseries H (1 samples), B (4 samples), DM (5 sample), and S (2 samples). In July, samples were collected from nurseries JP (3 samples), DM (4 samples), B (3 samples), and FF (2 samples). In both experiments three healthy soils (LS3, LS4, and LS5) collected from a wheat field were used as negative controls. Soil samples were collected with a drill at a depth of 15–20 cm.

All soils were analysed using the selective medium and by Scorpion- and nested Scorpion-PCR, as described before. Amplifications were conducted in separate reactions for *P. nicotianae* and *P. citrophthora* with FAM-labelled primers.

The data collected by means of molecular analyses and selective medium were utilised to evaluate the correlation between ID and Ct value, as previously described.

Detection of P. nicotianae *and* P. citrophthora *on naturally infected roots*

Tests were conducted in October utilizing feeder roots from 2-year-old sour orange seedlings grown in 5-l pots. Samples were collected from 10 different seedlings showing various symptoms of canopy decline. Roots collected from 3-month-old seedlings grown in a healthy soil collected from a wheat field were utilized as negative controls. All rootlet samples were rinsed with tap water, dried with blotting-paper, cut with shears into approximately 1 cm long segments and divided in two equal parts for traditional (selective medium) and molecular analyses. For each sample, traditional analysis was conducted by plating 300 feeder root segments in 15 Petri dishes containing a selective medium (Masago et al., 1977). After 3 days of incubation at 20 °C, the colonies grown from the roots were identified on the basis of their morphology and utilized to assess the degree of root infection, expressed as the percentage of infected segments.

For molecular analysis, DNA was extracted in triplicate from 2 to 3 g of rootlets, as described by Schena and Ippolito (2003). Nucleic acids were purified with Sepharose (Bramwell et al., 1995) and amplified, as described before.

The data collected by means of molecular analyses and selective medium were utilised to evaluate the correlation between the degree of root infection and Ct value, as previously described.

Results

Primer specificity and sensitivity

The specificity of primers Pn5B-Pn6 Scorpion and Pc2B Scorpion-Pc7 was evaluated using a large number of *Phytophthora* species (Table 1), both in multiplex and separate Scorpion-PCR reactions. In multiplex Scorpion-PCR, primers Pc2B Scorpion-Pc7 were specific, yielding a significant increase of ROX fluorescence only for *P. citrophthora* DNA (Figure 2). Primers Pn5B-Pn6 Scorpion were less specific, causing a significant increase in FAM fluorescence for all *P. nicotianae* isolates, but also from *P. cactorum* DNA (Figure 2); however, in the latter, delayed

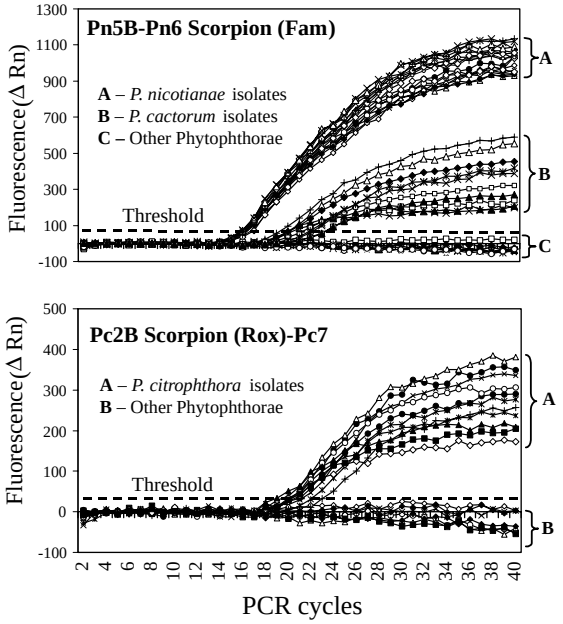

Figure 2. Real-time identification of *P. nicotianae* and *P. citrophthora* by multiplex Scorpion-PCR using primer Pn6 Scorpion labelled with FAM (top) and primer Pc2B Scorpion labelled with ROX (down). Primers Pc2B Scorpion-Pc7 were specific, yielding a significant increase in ROX fluorescence only for *P. citrophthora* (down), whereas primers Pn5B-Pn6 Scorpion caused a significant increase in FAM fluorescence for all isolates of *P. nicotianae,* but also from *P. cactorum.*

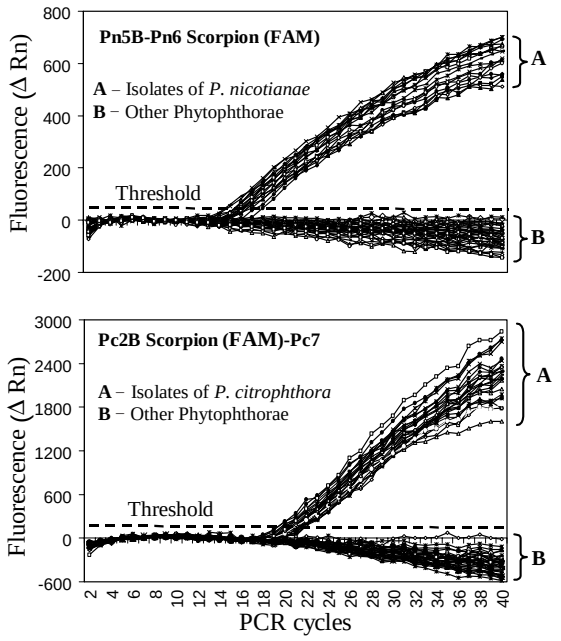

Figure 3. Specific identification of *P. nicotianae* (top) and *P. citrophthora* (down) by real-time PCR in separate reactions. Both Scorpion primers (Pn6 Scorpion and Pc2B Scorpion) were labelled with FAM.

fluorescence increases (higher Ct values) and lower relative normalised fluorescence were achieved.

In separate Scorpion-PCR reactions, the *P. citrophthora* specific primers (Pc2B Scorpion-Pc7), labelled with FAM, confirmed their specificity (Figure 3) and the relative normalised fluorescence was higher than ROX fluorescence. Similarly, with an annealing-extension temperature of 62.5 °C, instead of 50 °C, the primer pair Pn5B-Pn6 Scorpion specifically recognised *P. nicotianae* (Figure 3). No amplification was achieved with any of the two primer pairs with any of the other species (Table 2).

Serial dilutions of *P. nicotianae* and *P. citrophthora* template DNA were utilised to compare the sensitivity of multiplex and separate Scorpion-PCR reactions. In multiplex Scorpion-PCR, the detection limit was 10 pg μl^{-1} for primer Pn5B-Pn6 Scorpion (*P. nicotianae*) and 100 pg μl^{-1} for primer Pc2B Scorpion-Pc7 (*P. citrophthora*). In separate Scorpion-PCR, a higher degree of sensitivity was achieved with a detection limits of 1 pg μl^{-1} for both Scorpion primer pairs (data not shown).

Detection of P. nicotianae *in artificially infested soils*

Dilution series of an artificially infested soil with a healthy soil were utilised to develop a PCR-based quantitative detection method and to compare the sensitivity of molecular detection and selective medium. The protocol utilised to extract DNA from soil enabled the extraction of 1–2 μg of total nucleic acids per g of soil in a 2–3 h period. The detection limit of nested Scorpion-PCR was 1.56% of infested soil with an inoculum density of 1.7 propagules per g of soil and a Ct value ranging from 30 to 32 (Figure 4A); however, with the

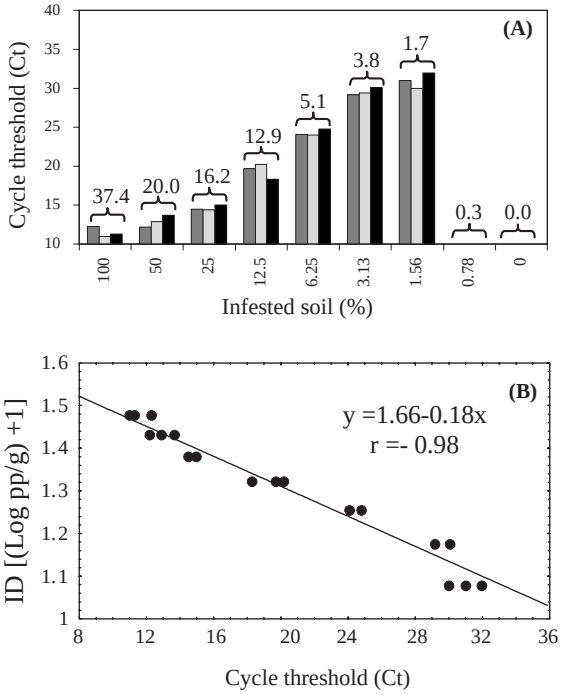

Figure 4. Detection of *Phytophthora nicotianae* in soil mixtures with different concentrations of the pathogen, obtained by mixing an artificially infested soil with a healthy soil (A). The histograms represent cycle threshold (Ct) values from different DNA extractions and amplifications (three per sample) after nested Scorpion-PCR. Samples in which fluorescence did not rise above the threshold level have no histogram. The average inoculum density (ID) of *P. nicotianae* (propagules per gram of soil) assessed with the selective medium for each soil mixture is reported at the top of the corresponding histograms. In B, the relationship between ID and the corresponding Ct value is reported. Linear regression equation of Ct (x) on Log (propagules per gram of soil + 1) (y) was: $y = 1.66–0.18x$; $r = -0.98$; correlation coefficient was significant at $P \leq 0.001$.

selective medium, the detection limit was 0.3 propagules in 0.78% of infested soil. A high and significant correlation value ($r = -0.98$; $P \leq 0.001$) was found between inoculum density and Ct values (Figure 4B).

Detection of P. nicotianae *and* P. citrophthora *in naturally infested soils*

The inoculum density of *Phytophthora* in the soil was preliminarily assessed using the selective medium. In the first experiment carried out in March, soils showed different concentrations of pathogen propagules per gram of dry soil, ranging from 0 (H, B1, B2, B6) to 100 (B2) (Figure 5). Most of the colonies obtained on selective media were identified as *P. nicotianae* although two soil samples (DM6 and DM8) contained propagules of both species. In the second series of experiments (July), 10 soil samples (JP1, JP5, JP10, DM5, DM9, DM10, B8, B10, FF1, FF10) had varying numbers of *P. nicotianae* propagules, and no one was infested with *P. citrophthora* (Figure 6). Two samples (B3 and DM4) were free of pathogen propagules. At both sampling times (March and July), *P. nicotianae* and *P. citrophthora* were absent from the wheat-cultivated soils (LS3, LS4, and LS5) utilized as negative controls (Figures 5 and 6).

In Scorpion PCR, a fluorescence increase was achieved with primer Pn5B-Pn6 Scorpion from three samples (DM1 and DM2, sampled in March, and JP5, sampled in July) with a very high level of pathogen propagules and from the positive controls (genomic DNA from pure cultures) (data not shown). Higher levels of sensitivity were achieved in nested Scorpion-PCR. In the first experiment (March), a significant increase in fluorescence was

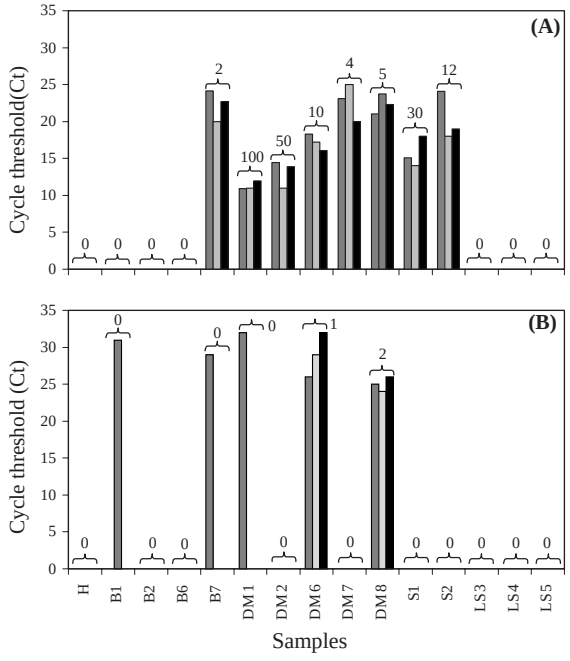

Figure 5. Detection of *P. nicotianae* (A) and *P. citrophthora* (B) on 12 potentially infested and 3 healthy soils (LS3, LS4, and LS5), as assessed by nested Scorpion-PCR and selective medium. Tests were conducted in March. For each sample, columns report cycle threshold (Ct) values from three different DNA extractions and amplifications; the average inoculum density (propagules per gram of soil) of pathogens per soil mixture is reported at the top of the corresponding histogram. Samples in which fluorescence did not rise above the threshold level have no histogram.

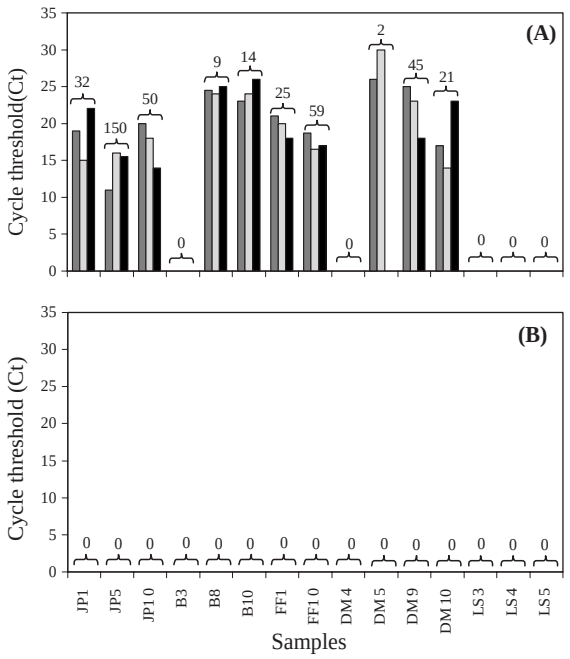

Figure 6. Detection of *P. nicotianae* (A) and *P. citrophthora* (B) on 12 potentially infested and 3 healthy soils (LS3, LS4, and LS5), as assessed by nested Scorpion-PCR and selective medium. Tests were conducted in July. For each sample, columns report cycle threshold (Ct) values from three different DNA extractions and amplifications; the average inoculum density (propagules per gram of soil) of pathogens per soil mixture is reported at the top of the corresponding histogram. Samples in which fluorescence did not rise above the threshold level have no histogram.

achieved with *P. nicotianae* specific primers from all infested soil samples (B7, DM1, DM2, DM6, DM7, DM8, S1 and S2). Ct values ranged from 11 to 25 (Figure 5A) and were inversely correlated with the inoculum density of *P. nicotianae* assessed on selective medium ($r = -0.88$; $P < 0.05$) (data not shown). Molecular detection of *P. citrophthora* confirmed the presence of the pathogen in the two soils (DM6 and DM8) from which the pathogen was also isolated on the selective media (Figure 5B). However, fluorescence also rose above the fixed threshold value in one of the three replications of soils B1, B7, and DM1, apparently free of propagules, as assessed by the selective medium (Figure 5B).

Similar results were achieved in the second series of experiments. In nested Scorpion-PCR, fluorescence rose above the fixed threshold value from all soils containing propagules of *P. nicotianae*, whereas no fluorescence increase was achieved for *P. citrophthora* (Figure 6A and B). Ct values ranged from 11 to 30 (Figure 6A) and were inversely correlated with the inoculum density of *P. nicotianae* assessed on selective medium ($r = -0.77$; $P < 0.05$) for *P. nicotianae* infested soils (data not shown).

At both sampling times (March and July), no fluorescence increase was achieved in the wheat soils (LS3, LS4, and LS5) utilized as negative controls (Figures 5 and 6).

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

The presence of *Phytophthora* on feeder roots was assessed using the selective medium and the molecular approach. All tests ruled out the presence of *P. citrophthora* (data not shown). By contrast, the selective medium indicated the presence of variable levels of *P. nicotianae* infections, ranging from 0% to 52% of infected feeder roots (Figure 7).

The protocol utilized to extract DNA from roots enabled the extraction of genomic DNA suitable for PCR amplification in 3–4 h. DNA yields ranged from 2 to 3 μg per g of plant material. In Scorpion-PCR, a specific fluorescence increase was achieved from all roots having a degree of root infection of 6 or higher (samples H–N), whereas in nested PCR the presence of the pathogen was also ascertained in those samples (E–G)

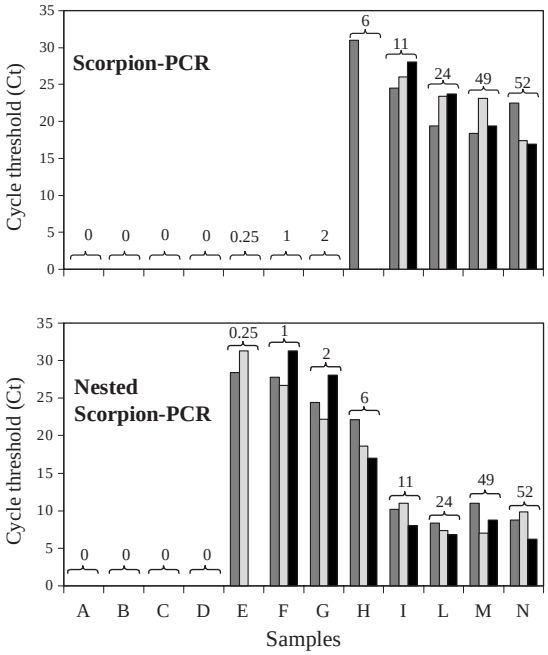

Figure 7. Detection of *Phytophthora nicotianae* from citrus roots with different degrees of infection. For each sample, columns report Ct values from the different DNA extractions and amplifications after Scorpion-PCR (top) and nested Scorpion-PCR (bottom); the average percentage of infected root segments as assessed by isolation on selective medium per sample is reported at the top of the corresponding histogram. Samples in which fluorescence did not rise above the threshold level have no histogram.

with a very low level of infection (0.25–2%). Ct values ranged from 16.9 to 31 in Scorpion PCR and from 6.2 to 31.3 in nested Scorpion PCR (Figure 7). Significant correlation ($P < 0.05$) values were found between the percentage of infected feeder roots and Ct values after Scorpion PCR ($r = -0.86$) and nested Scorpion PCR ($r = -0.93$) (not shown).

No amplification was achieved after Scorpion and nested Scorpion PCR for samples A and B (negative controls) and samples C and D (healthy feeder roots), as assessed by the selective medium (Figure 7).

Discussion

This research was undertaken to develop a sensitive and effective method based on real-time PCR to identify and detect *P. nicotianae* and *P. citrophthora* in citrus roots and soil. Several molec-

ular approaches have been proposed to identify and detect *P. nicotianae* and *P. citrophthora* (Érsek et al., 1994; Lacourt and Duncan, 1997; Ristaino et al., 1998; Grote et al., 2002; Ippolito et al., 2002; Kong et al., 2003); however, to our knowledge, this is the first report on the real-time PCR identification and detection of these two pathogens.

In the present work, two Scorpion primers labelled with FAM and ROX fluorophores enabled the simultaneous identification of *P. nicotianae* and *P. citrophthora* (multiplex-PCR), although an aspecific increase of FAM fluorescence was also achieved by *P. cactorum* DNA. This signal was probably caused by the low annealing-extension temperature (50 °C) required by *P. citrophthora* specific primers. Furthermore, in multiplex Scorpion-PCR, a significant decrease in sensitivity was achieved for *P. nicotianae* (10 pg) and *P. citrophthora* (100 pg), compared to the level of sensitivity obtained for both species (1 pg) in separate reactions. However, providing an equal concentration of template DNA for both species, *P. nicotianae* always had a lower Ct value and higher relative normalised fluorescence than *P. cactorum*. Therefore, the multiplex Scorpion-PCR approach developed in the present work could be utilised to identify *P. nicotianae* and *P. citrophthora* isolates after their isolation and DNA extraction and quantification. The procedure described is accurate since in pure culture the concentration of DNA is not a major limitation and Scorpion-PCR proved to be effective in identifying single nucleotide polymorphisms (SNP) (Thelwell et al., 2000; Bates and Taylor, 2001). *P. nicotianae* and *P. cactorum* differ for one base in the target sequence of the Scorpion probe element (loop) and for three bases in both Pn5B and Pn6 target sequences (Cook and Duncan, 1997). In contrast, our multiplex-PCR would be unsuitable to detect *P. nicotianae* and *P. citrophthora* in naturally infested soils and in naturally infected roots in which very high levels of sensitivity are required. An increase in FAM fluorescence may be misinterpreted because of the unknown pathogen DNA concentration.

Scorpion primers in separate reactions enabled specific identification of target pathogens (*P. nicotianae* and *P. citrophthora*) among a large number of other Phytophthorae (116 isolates from 14 species) and fungi (86 isolates from 57 species) isolated mainly from the rhizosphere. Although a high level of sensitivity was achieved in separate PCR reactions (1 pg μl^{-1}), data from natural infested soils and natural infected roots indicate that a nested Scorpion-PCR approach is required to reach reasonable levels of sensitivity. A single amplification with Scorpion primer enabled the detection of the pathogen only in samples with a very high level of infection. By contrast, combining a first amplification with primer Ph2-ITS4 (Ippolito et al., 2002) and a second amplification with Scorpion primers specific for *P. nicotianae* (Pn5B-Pn6 Scorpion) and *P. citrophthora* (Pc2B Scorpion-Pc7), it was possible to detect the pathogens with a very high level of sensitivity, typical of a nested PCR. The degree of sensitivity of nested Scorpion-PCR is more than enough to detect *P. nicotianae* and *P. citrophthora* in soil at a population level below those causing yield loss and/or severe damage on bearing citrus plants, namely 15–20 propagules per gram of dry soil (ppg) for susceptible rootstocks (Menge and Nemec, 1997; Magnano di San Lio et al., 1988) and around 30 ppg for resistant ones (Ippolito et al., 1991). Moreover, the method is sufficiently sensitive to rule out the presence of the pathogens in plant propagative material, in soil mixes, and in field soil before planting.

The effectiveness of the entire molecular procedure (DNA extraction and amplification) was confirmed by the comparison of the results from molecular analysis with the traditional isolation method on selective medium. The two analyses gave similar results, but the traditional method required much more time and expertise to identify *P. nicotianae* and *P. citrophthora* after isolation. In fact, the use of rapid protocols to extract DNA from roots and soils enabled the analysis of natural infected sample in only one working day (3–4 h to extract DNA, 2 h for first round amplification and 2 h for second round amplification). In the second series of tests with soil, few replications were positive to *P. citrophthora* in PCR analysis, although the 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 PCR detection of the resting spores of the pathogen, such as chlamydospores, abundantly produced in summer (Magnano di San Lio et al., 1988) and not detectable by the selective media (Tsao, 1983). In consideration of the fact that PCR can also amplify dead organisms, the

detection of DNA from non-viable propagules cannot be ruled out. However, the rate of breakdown of DNA from dead fungi in soil should be fairly high due to the high microbial activity (England et al.,1998; Schena and Ippolito, 2003); therefore, the amplification of DNA from dead cells may be a minor problem.

A substantial advantage of real-time PCR when compared with conventional PCR is that the early PCR cycles can be recorded and, apart from the plateau effect, very subtle changes in detection (i.e. changes in Ct) can be monitored, enabling quantitative analyses (Bell et al., 1999; Ginzinger, 2002). In the present work which utilised serial dilutions of infested and healthy soils, a high and significant correlation ($r = -0.98$; $P \leq 0.001$) was found between inoculum density and Ct values. In tests with naturally infected roots and soils, a significant correlation was also found, although with lower correlation coefficients. The lower level of correlation obtained with naturally infected samples was expected since they obviously had different characteristics (composition, tissue texture, etc.) and pathogen propagule ratios (mycelia, zoospores, oospores, etc.) that can affect both culture and molecular detection methods (Bridge and Spooner, 2001). In a previous report, Bates et al. (2001) converted the DNA quantification results into the percentage of *Pyrenophora teres* infected seed by correlating real-time PCR data with agar culture tests; in this experiment, similarly to our results, a higher correlation coefficient was obtained using a constructed seed infection level gradient compared to randomly picked seed samples. Our data indicate that Ct values have a potential to be utilised for the quantitative detection of *P. nicotianae* and *P. citrophthora* propagules in soil and on roots. These results are of a basic importance for Phytophthora root rot where the threshold level of pathogen propagules trigger application of chemicals or other control means (Lutz and Menge, 1986).

In conclusion, the real-time PCR approach developed in the present work is sensitive, rapid, reliable, versatile and could enable quantitative analyses. Therefore, it has potential for the study of pathogen biology, ecology, host-pathogen interactions and generally speaking for addressing vital issues regarding Phytophthora root rot of citrus.

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