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FULL RESEARCH PAPER

# Detection of *Phytophthora nicotianae* and *P. palmivora* in citrus roots using PCR-RFLP in comparison with other methods

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**Abstract** *Phytophthora nicotianae* and *P. palmivora* are the most important soil-borne pathogens of citrus in Florida. These two species were detected and identified in singly and doubly infected plants using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of internal transcribed spacer (ITS) regions of ribosomal DNA. The sensitivity of the PCR-RFLP was analyzed and the usefulness of the method evaluated as an alternative or supplement to serological methods and recovery on semi-selective medium. In a semi-nested PCR with universal primers ITS4 and ITS6, the detection limit was 1 fg of fungal DNA, which made it 1000× more sensitive than a single-step PCR with primers ITS4 and DC6. The sensitivity of detection for *P. nicotianae* was shown to be ten-fold lower than for *P. palmivora*, limiting its detection with restriction profiles in plants infected by both fungal species. *Phytophthora nicotianae* was detected with species-specific primers in all samples inoculated with this species despite the absence of species-specific patterns in RFLP. In contrast, the incidence of detection of *P. palmivora* in the presence of *P. nicotianae* was considerably lower using plating and morphological detection methods. Due to its high sensitivity, PCR amplification of ribosomal ITS regions is a valuable tool for detecting and identifying *Phytophthora* spp. in citrus roots, provided a thorough knowledge of reaction conditions for the target species is established prior to the interpretation of data.

**Keywords** ITS regions · Phytophthora root rot

## Introduction

The genus *Phytophthora* causes some of the most serious soil-borne diseases of plants worldwide (Erwin and Ribeiro 1996). Together with the genus *Pythium* and the downy mildews, *Phytophthora* belongs to the class oomycetes. Although commonly regarded as fungi, oomycetes differ from the true fungi in various morphological, biochemical and molecular characteristics and are more closely related to the golden-brown and the heterokont algae (Cavalier-Smith 1986; Erwin and Ribeiro 1996; Govers 2001).

In Florida citrus, *Phytophthora* spp. cause economic losses from damping-off of seedlings in nurseries, foot rot of the trunk, brown rot of fruit, and fibrous root rot in groves leading to tree decline

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and yield losses (Timmer and Menge 1988; Graham and Menge 1999). The most important *Phytophthora* spp. affecting citrus worldwide are *P. nicotianae* (syn. *P. parasitica*), *P. palmivora*, and *P. citrophthora*. The latter species causes brown rot and gummosis on the trunks of trees in Mediterranean climates where winter rainfall is predominant, but *P. citrophthora* is usually not a serious problem on citrus trunks and roots in warm subtropical areas and has not been found in recent Florida surveys (Graham et al. 1998).

Rootstock resistance or tolerance is the principal tactic used to manage *Phytophthora* diseases, but commercially important rootstocks differ substantially in susceptibility or tolerance towards each *Phytophthora* species. Many hybrids of trifoliate orange (*Poncirus trifoliata*), such as the widely used rootstock Swingle citrumelo (*Citrus paradisi* × *P. trifoliata*), have previously been considered tolerant towards root rot caused by *P. nicotianae* (Graham 1995). However, it is now evident that this does not hold true for *P. palmivora* (Bowman et al. 2002; Graham et al. 2003; Grosser et al. 2003; Albrecht and Bowman 2004). Conversely, some other rootstocks not derived from *P. trifoliata*, like Cleopatra mandarin (*Citrus reticulata*), are relatively susceptible to *P. nicotianae*, but seem to possess a higher tolerance to *P. palmivora* than trifoliate orange (Graham et al. 2003).

Traditional methods for detection, identification and characterization of *Phytophthora* spp. involve the use of leaf and fruit baits (Grimm and Alexander 1973), plating onto semi-selective agar media (Tsao and Guy 1977; Timmer et al. 1988), isozyme analysis (Oudemans and Coffey 1991; Mchau and Coffey 1994; Graham et al. 1998), and serological methods (Timmer et al. 1993; Miller et al. 1997). The most common method of species identification is to use morphological criteria for colonies growing on media, which is time-consuming, requires expertise, and can be limited by interference from fast-growing secondary microflora (Tsao 1990; Nechwatal and Oßwald 2001). Serological techniques facilitate detection but are only useful at the genus level. Also, low sensitivity and cross-reactions with other species limit their extensive application (Mohan 1989; Miller 1996). In recent years, molecular methods have been developed to allow species identification based on restriction enzyme digest patterns of the internal transcribed spacer (ITS) regions of the ribosomal RNA genes (Cooke and Duncan 1997; Cooke et al. 2000). The internal transcribed spacer regions of ribosomal DNA are particularly useful for species discrimination of fungal taxa, because they evolve in a neutral manner at a rate that approximates the rate of speciation (White et al. 1990; Lee and Taylor 1992). Also, ribosomal RNA genes are present in multiple copies within the genome, increasing PCR sensitivity as compared with single-copy genes. The development of ITS methodology has lead to extensive application of PCR in various fields of research including studies on evolution and taxonomy (Cooke and Duncan 1997; Cooke et al. 2000), studies on intraspecific variation (Cohen et al. 2003) and species hybridization (Bonants et al. 2000), as well as identification of species responsible for disease outbreaks (Schubert et al. 1999; Nechwatal and Oßwald 2001; Vettraiano et al. 2001). Based on information derived from sequencing ribosomal ITS regions, a number of species-specific primers have also been developed, eliminating the need for generating restriction profiles (Bonants et al. 1997; Trout et al. 1997; Böhm et al. 1999; Schubert et al. 1999; Nechwatal et al. 2001; Grote et al. 2002).

Evaluating promising new rootstock candidates for their resistance or tolerance to *Phytophthora* diseases is one important objective of the citrus breeding programmes in Florida (Bowman et al. 2001, 2002, 2003; Grosser et al. 2003; Albrecht and Bowman 2004). An early step in the evaluation process is the screening of newly developed rootstocks in the greenhouse under controlled environmental conditions. The application of molecular detection methods may facilitate the rapid testing of large numbers of plants and the selection of new candidate rootstocks for their resistance or tolerance to *Phytophthora* diseases before proceeding with the more time-consuming examination under natural field conditions. A number of studies have been conducted using molecular techniques for detection of citrus phytophthoras (Zheng and Ward 1998; Cohen et al. 2003; Ippolito et al. 2002, 2004). Whereas the studies of Zheng and Ward (1998) and Cohen et al. (2003) focused on the taxonomic variation of *Phytophthora* spp. using cultured isolates from different geographic locations, Ippolito et al. (2002) tested detection sensitivity of *Phytophthora* spp. from soil and infected root material using species-specific primers. However, except for Ippolito et al. (2004) who used species-specific primers in a multiplex PCR assay,

detection procedures were limited to individual pathogens only and did not address complications which may arise when testing for multiple pathogenic organisms coexisting in the tissue.

This study investigates whether molecular methods based on ITS-fingerprinting can be effectively used for the detection and identification of *Phytophthora* spp. of citrus under controlled conditions in the greenhouse. The main objective was to apply existing molecular detection methods in addition to traditional procedures and to evaluate their value for breeding programmes. In contrast to most studies on plant pathogens, experimental treatments included the simultaneous infection of citrus seedlings by *P. nicotianae* and *P. palmivora*, since both are associated with citrus decline in Florida and are frequently isolated from the same location in the field. The sensitivity of molecular detection was tested and compared with results obtained from morphological analyses using selective plating techniques and serological assays.

## Materials and methods

### Pathogen strains

Isolates of *P. nicotianae* (Pn198) and *P. palmivora* (Pp99-59-1) were used in three experiments. *Phytophthora nicotianae* isolate Pn198 was isolated in 2000 from citrus roots in one of the greenhouses at the Citrus Research and Education Centre in Lake Alfred (Polk County, Florida), while *P. palmivora* isolate Pp99-59-1 was isolated in 1995 from citrus fruit in a field site in the same county. In addition, an isolate of *P. citrophthora* (M140), isolated in 1988 from soil under citrus trees in California, was used for molecular studies regarding the specificity of PCR reactions. Isolates were maintained on 20% clarified V8 juice (Campbell, Camden, NJ) agar at 18°C with annual transfers to fresh medium.

### Plant material

Seedlings of the three-rootstock genotypes: Cleopatra mandarin, sour orange (*Citrus aurantium*) and Swingle citrumelo were evaluated. The ages of seedlings upon initiation of the studies were 12 weeks (experiment 1), 17 weeks (experiment 2) and 14 weeks (experiment 3).

### Inoculation of plants

Inoculum of *Phytophthora* spp. was prepared by growing cultures on sterilized millet (*Panicum ramosum*) seeds. Seeds (Brown Top Millet, Seedland Inc., Wellborn, FL) were soaked overnight in deionized water (75 ml water/100 ml seeds), then autoclaved twice with 24 h between cycles. For experiments 1 and 2, seeds were inoculated by adding two agar discs of 5 mm diam from five day-old actively growing *P. nicotianae* or *P. palmivora* cultures per 100 ml of seeds. For dual inoculations one disc of each species per 100 ml of seeds was used. Millet seeds were incubated for 10–15 days at 23–25°C in the dark under sterile conditions. For the third experiment the amount of inoculum for *P. palmivora* was increased by 50% to compensate for the apparently slower growth of this species under the conditions used in this study. Millet seeds for control treatments were prepared in the same manner, but were not inoculated with *Phytophthora* spp.

A commercially available soil-less potting medium composed of steam-sterilized peat/perlite/vermiculite (Pro-Mix BX, Premier Horticulture Inc., Red Hill, PA) was filled into 63 cm × 40 cm × 22 cm drained plastic tubs to a depth of 15 cm. Millet seeds were incorporated at a rate of 20 ml inoculum per 1,000 ml potting medium, and tubs were filled to a final depth of 18 cm. Citrus seedlings of all three rootstock genotypes were planted into the tubs with five randomized replicates for each rootstock in each tub. One tub was prepared for each treatment in experiments 1 and 2, resulting in a total of 60 plants per experiment, and three tubs were prepared for each treatment in experiment 3, resulting in a total of 180 plants. Treatments were potting medium containing (1) non-inoculated millet seeds, (2) millet seeds inoculated with *P. nicotianae*, (3) millet seeds inoculated with *P. palmivora*, and (4) millet seeds inoculated with *P. nicotianae* and *P. palmivora*.

Tubs were placed into non-draining 75 cm × 50 cm × 15 cm plastic trays and arranged in a completely randomized order on the greenhouse benches. Seedlings were grown under natural light conditions in an enclosed greenhouse with a

maximum photosynthetic photon flux (PPF) of 1,200–1,300 µmol $s^{-1}$ $m^{-2}$. Potting medium was kept at near field capacity by maintaining water in the trays at a level of 2.5 cm above the bottom of the tubs. Tubs were irrigated with a water-soluble fertilizer mix, 20N-10P-20K (Peters Professional, The Scotts Company, Marysville, OH) once every week, applied at a rate of 500 mg N $l^{-1}$ water. Before each fertilizer application, water was allowed to drain from the tubs. Four weeks (experiment 1), 6 weeks (experiment 2) and 7 weeks (experiment 3) after initiating the experiment, plants were extracted from the medium. Roots were washed thoroughly with tap water to remove adhering particles and blotted dry. For the morphological detection of *Phytophthora* spp., seven root segments of 1–2 cm length were excised from each plant and plated onto pimaricin-ampicillin-rifampicin-pentachloronitrobenzene-hymexazol (PARPH) semi-selective agar medium (Timmer et al. 1988). Species were identified based on sporangium morphology and colony characteristics (Erwin and Ribeiro 1996). Fresh weight of shoots and roots was measured and roots were kept at −80°C until used for DNA extraction and enzyme-linked immunosorbent assays (ELISA). Samples of potting medium were taken from each tub and the propagules per $cm^3$ of medium determined by dilution-plating as described by Graham (1995).

## DNA extraction

### *Root tissue*

Citrus roots were ground in liquid nitrogen with a mortar and pestle. One hundred milligrams of ground tissue was used for DNA extraction. DNA was extracted using the Plant DNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. DNA was quantified at 260 nm using a Beckman Coulter DU 640 spectrophotometer.

### *Fungal tissue*

Fungal cultures were grown at room temperature in the dark in 100 ml 20% clarified V8 juice. After 7–10 days, mycelium was harvested by filtration, washed with sterile deionized water (sdw), freeze-dried and stored at −20°C. Fungal tissue was ground in liquid N with a mortar and pestle in preparation for DNA extraction. Twenty milligrams of tissue was used for extraction and quantification of DNA as described above.

## Primers

PCR amplification of ribosomal ITS regions as described by Cooke et al. (2000) was performed with the primers ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and DC6 (5′-GAGGGACTTTTGGGTAATCA-3′), which specifically amplify ribosomal DNA from the major pathogenic oomycete groups *Pythium*, *Phytophthora* and the downy mildews (Bonants et al. 1997). PCR products were then amplified in a second, semi-nested round, using universal primers ITS4 and ITS6 (5′-GAAGGTGAAGTCGTAACAAGG-3′). Second round PCR amplifications of the ITS regions specific for *P. nicotianae* were performed with primer pair PNIC1 (5′-CAATAGTTGGGGGTCTTATT-3′) and PNIC2 (5′-GTATACCGAAGTACACATTAAG-3′) developed by Grote et al. (2002).

All primers were purchased from MWG-Biotech (High Point, NC).

## PCR conditions

PCR reactions were performed in a total volume of 25 µl using the Platinum SuperMix (Invitrogen, Carlsbad, CA) composed of 22 mM Tris–HCl (pH 8.4), 55 mM KCl, 1.65 mM $MgCl_2$, 220 µM dGTP, 220 µM dATP, 220 µM dTTP, 220 µM dCTP, and 22 U/ml recombinant *Taq* DNA polymerase providing an automatic 'hot start'. Each reaction included 1 µl of DNA template, equivalent to 20–30 ng of total DNA, and 1 µl of each primer at a final concentration of 0.5 µM each. Amplification was carried out in a PTC-100 or PTC-200 Thermal Cycler (MJ Research, Reno, NV) using 0.2 ml tubes. An initial denaturation step at 94°C for 3 min was followed by 35 cycles of annealing at 60°C for 30 s, extension at 72°C for 1 min and denaturation at 94°C for 30 s before a final extension step at 72°C for 10 min. In nested PCR, 1 µl of the first round amplification product was used for the second PCR round. Five µl of PCR products were separated by

electrophoresis in 2% agarose gels (Amresco) for 120 min at 5–6 V $cm^{-1}$, stained with ethidium bromide and visualized under UV light (Fluor S Imaging System, Biorad, Hercules, CA). All PCR reactions included multiple negative controls using sdw in place of DNA template.

### Restriction digests

Ten μl of the amplification products generated with primer pair ITS4 and ITS6 were digested with restriction enzymes *Alu*I, *Msp*I and *Taq*I (New England BioLabs, Beverly, MA) in a total volume of 20 μl according to the manufacturer's instructions. Reaction products were analyzed by electrophoresis in 2.5% agarose gels (Amresco) for 2–3 h at 5–6 V $cm^{-1}$. Restriction fragment patterns were compared with data provided by Cooke et al. at www.phytid.org/list.asp.

### Sensitivity of detection

To determine the sensitivity of PCR detection for each *Phytophthora* sp., ten-fold serial dilutions ranging from 1 ng to 0.1 fg of fungal genomic DNA were prepared in sdw or DNA extracts from healthy citrus roots at a concentration of 10 ng $\mu l^{-1}$. Nested PCR was performed as described above. To determine the sensitivity of simultaneous detection of two *Phytophthora* spp. with RFLP, samples were prepared using different ratios of DNA of all three *Phytophthora* spp. Ratios tested were 99:1, 49:1, 9:1, 1:1; 1:9, 1:49, and 1:99 for all combinations of species. DNA stocks of each species were adjusted previously to 1 ng $\mu l^{-1}$ of DNA. Nested PCR and digests were performed as described above.

### Enzyme-linked immunosorbent assay (ELISA)

For the quantification of *Phytophthora* spp., a multiwell-test system (Agdia Incorporated, Elkhart, IN) using a polyclonal *Phytophthora* antibody and a monoclonal alkaline phosphatase-conjugated secondary antibody was used. For each reaction, 1 ml of extraction buffer GEB2 was added to 100 mg of root tissue, previously ground with liquid N using a mortar and pestle. Samples were mixed by vortexing for 10 s, incubated at room temperature for 15 min and centrifuged at 20,800 *g* for 5 min. One-hundred microliters of supernatant was used for each well; all samples were tested in duplicate. Linearity of the test system and detection threshold were determined using ten-fold serial dilutions of extracts derived from freeze-dried mycelia of *P. palmivora* and *P. nicotianae* ranging from 1 pg to 10 mg of fungal tissue. All assays were performed according to the manufacturer's instructions. Absorbance was measured at 405 nm using a Spectra Max Pro 190 Microplate Spectrophotometer (Molecular Devices Corp., Sunnyvale, CA) in combination with the software Soft Max Pro, Version 2.6.

### Statistical analysis

Growth data and immunological data were tested by analysis of variance using Statistica version 6.0 (StatSoft, Tulsa, OK). Students–Newman–Keuls (SNK) test was used for mean comparison when the $F$-test was significant at $P < 0.05$.

## Results

### Identification of *Phytophthora* species

#### *PCR*

First round amplification of DNA from *P. nicotianae, P. palmivora*, or *P. citrophthora* with primer pair ITS4 and DC6 typically generated a PCR product of about 1,300 bp (Fig. 1a). After second round amplification with the universal primers ITS4 and ITS6, a PCR product of about 900 bp was obtained, which is in the range of band sizes typically observed for *Phytophthora* (Fig. 1b).

#### *Species-specific primers*

Nested PCR of DNA from *P. nicotianae* with primer pair PNIC1 and PNIC2 produced an amplification product of about 750 bp (Fig. 1c). A few non-specific bands were observed when template concentrations were high. No visible product specific for *P.*

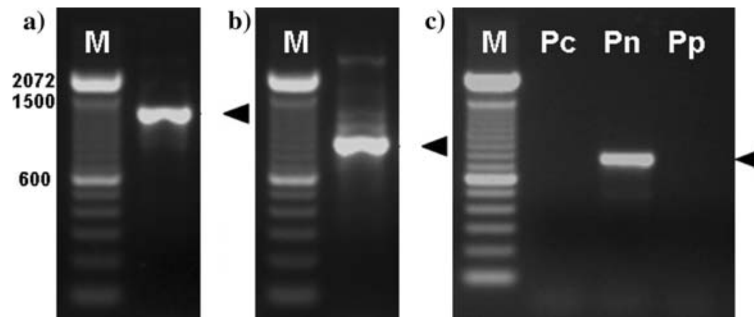


**Fig. 1** (**a**) Amplification product of DNA from *P. palmivora* using primers ITS4 and DC6 (◀). (**b**) Amplification product of DNA from *P. palmivora* after nested PCR with primers ITS4 and ITS6 (◀). (**c**) Amplification product of DNA from *P. nicotianae* (Pn) after nested PCR with primers PNIC1 and PNIC2 (◀); no product was obtained with DNA from *P. citrophthora* (Pc) and *P. palmivora* (Pp). M, 100 bp ladder; molecular weights in bp are indicated on the left

*nicotianae* was obtained for DNA from *P. palmivora*, and *P. citrophthora*.

### *RFLP*

*Phytophthora nicotianae* was identified by the presence of restriction fragments of 745 bp, 117 bp and 52 bp after digest with *Alu*I and fragments of 404 bp, 390 bp and 120 bp after digest with *Msp*I (Fig. 2).

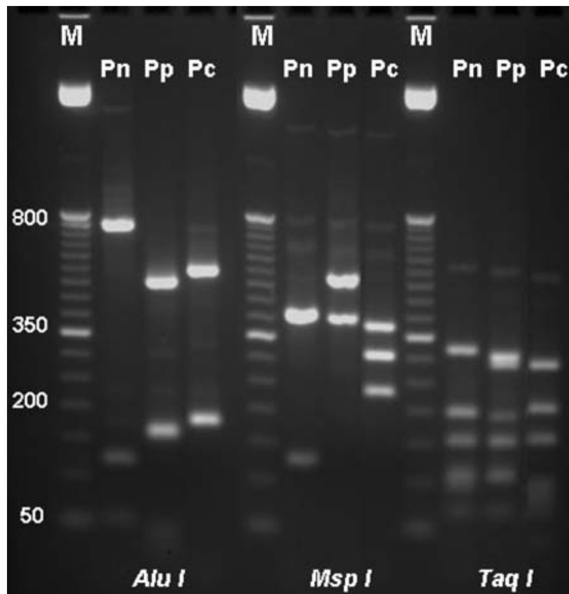


**Fig. 2** Restriction profiles of *Phytophthora* species obtained after digestion of ITS4/ITS6-amplification products with *Alu*I, *Msp*I, and *Taq*I. Pn, *P. nicotianae*; Pp, *P. palmivora*; Pc, *P. citrophthora*. M, 50 bp ladder; molecular weights in bp are indicated on the left

The two bands of 404 bp and 390 bp after *Msp*I digest generally appeared as one broad band under the electrophoresis conditions used in this study. *Phytophthora palmivora* was identified by the presence of restriction fragments of 501 bp, 160 bp, and 157 bp after digest with *Alu*I, with the latter two bands appearing as one broad band, and fragments at 508 bp and 389 bp after digest with *Msp*I. Restriction patterns obtained after digest with *Taq*I revealed profiles typical for both pathogens but did not aid in species discrimination and were therefore not used routinely.

### Sensitivity of PCR detection

First round amplification of decreasing concentrations of fungal DNA prepared in sdw resulted in a PCR product still visible at 1 pg of fungal DNA derived from *P. nicotianae* (Fig. 3a). PCR products obtained from DNA of *P. palmivora* and *P. citrophthora* were still detectable at 0.1 pg of fungal DNA. Lower amounts of template generally yielded amplification products of lesser intensity. After nested PCR with primer pair ITS4 and ITS6, *P. nicotianae* was detectable at 10 fg of DNA thus increasing sensitivity of detection by 100-fold (Fig. 3b). *Phytophthora palmivora* was detectable at 1 fg of DNA, 1/10th of the threshold for *P. nicotianae*. The detection limit for *P. citrophthora* was the same as that for *P. palmivora* (data not shown). Detection of *P. nicotianae* with species-specific primers PNIC1 and PNIC2 after nested PCR was possible down to 1 fg of fungal DNA (Fig. 4). Results obtained from serial dilutions

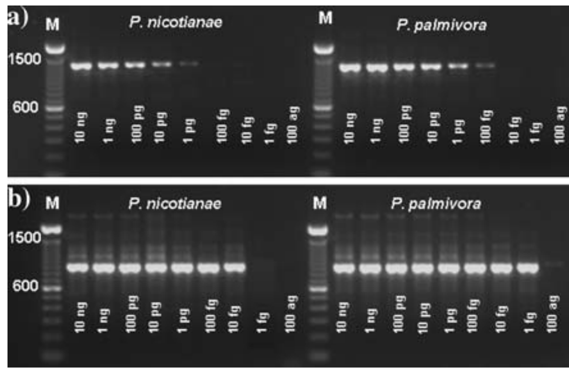


**Fig. 3** Sensitivity of simple and nested PCR for the detection of *P. nicotianae* and *P. palmivora* using tenfold serial dilutions of fungal DNA. (**a**) Simple PCR with primers ITS4 and DC6. (**b**) Nested PCR with primers ITS4 and ITS6. M, 100 bp ladder; molecular weights in bp are indicated on the left. DNA dilutions were prepared in sterile water

of fungal DNA prepared in citrus DNA extracts were identical to those obtained from dilutions prepared in sdw (data not shown).

When using restriction patterns to simultaneously identify two *Phytophthora* spp. in DNA preparations containing varying proportions of template, the following was observed. Due to the lower sensitivity of ITS primers, *P. nicotianae* was only detectable in mixed preparations when DNA was present in ratios equivalent to or above 9:1 (Fig. 5). In samples containing equal amounts of *P. nicotianae* and *P. palmivora* or *P. citrophthora* DNA, bands specific for *P. nicotianae* were never detected. Samples containing DNA of *P. palmivora* and *P. citrophthora* in equal proportions produced restriction patterns specific for both species. At ratios above 9:1, only bands

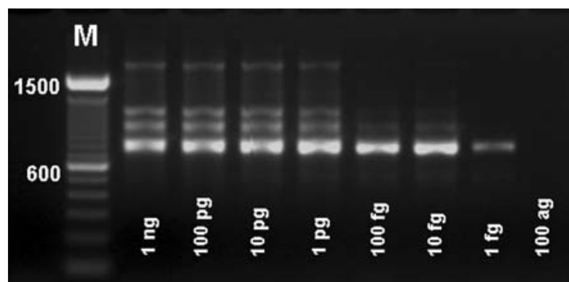


**Fig. 4** Sensitivity of nested PCR with primers PNIC1 and PNIC2 for the detection of *P. nicotianae* using ten-fold serial dilutions of fungal DNA. M, 100 bp ladder; molecular weights in bp are indicated on the left. DNA dilutions contained citrus DNA at a concentration of 10 ng $\mu l^{-1}$. With increasing template concentration non-specific amplification products appear

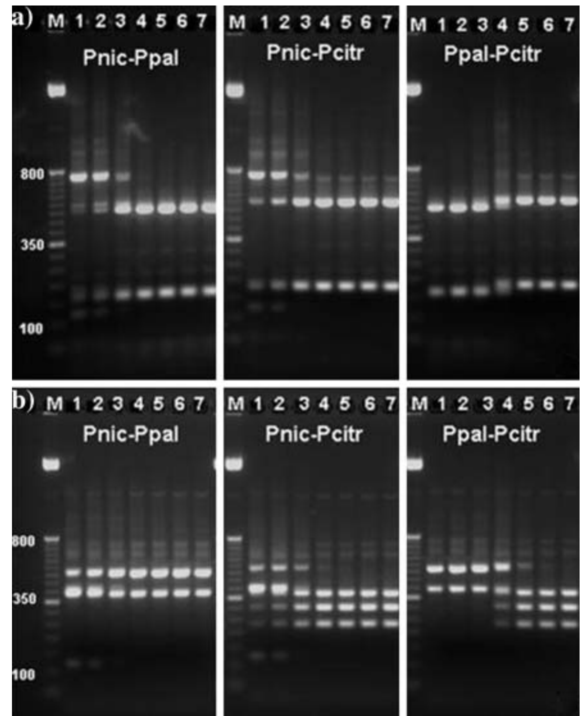


**Fig. 5** Restriction profiles obtained after digestion of nested ITS4/ITS6-amplification products from DNA preparations containing varying proportions of DNA from different *Phytophthora* species. (**a**) Restriction profiles after digest with *Alu*I. (**b**) Restriction profiles after digest with *Msp*I. DNA proportions were 99:1 (lane 1), 49:1 (lane 2), 9:1 (lane 3), 1:1 (lane 4), 1:9 (lane 5), 1:49 (lane 6), and 1:99 (lane 7). Pnic, *P. nicotianae*; Ppal, *P. palmivora*; Pcitr, *P. citrophthora*. M, 50 bp ladder; molecular weights in bp are indicated on the left

specific for the species in excess were clearly discernible.

## ELISA

Linearity of the assay was determined for absorbance values ranging from 0.1 to 1.8 corresponding to 1 ng to 1 mg of freeze-dried fungal tissue, with a detection threshold of approximately 0.1 ng of fungal tissue. No differences in sensitivity of the system were observed for the three *Phytophthora* spp. tested.

## Growth responses of citrus rootstocks

The average fresh weights of roots and shoots from all healthy plants ranged from 2.7 g to 10.1 g and from 8.7 g to 24.5 g, respectively (Table 1). Results

**Table 1** Average fresh weights of roots and shoots from non-infected seedlings of three citrus rootstocks

| Rootstock | Fresh weight (g) | | | |
|---|---|---|---|---|
| | Roots | | Shoots | |
| | Mean | SD[a] | Mean | SD |
| *Experiment 1* | | | | |
| Cleopatra mandarin | 2.74 | 0.21 | 8.69 | 0.51 |
| Sour orange | 4.31 | 0.49 | 9.32 | 0.66 |
| Swingle citrumelo | 4.74 | 0.35 | 10.88 | 1.34 |
| *Experiment 2* | | | | |
| Cleopatra mandarin | 4.30 | 0.36 | 15.87 | 0.81 |
| Sour orange | 10.00 | 1.46 | 24.46 | 2.44 |
| Swingle citrumelo | 9.06 | 1.27 | 22.03 | 2.71 |
| *Experiment 3* | | | | |
| Cleopatra mandarin | 4.49 | 0.27 | 15.75 | 0.75 |
| Sour orange | 7.11 | 0.54 | 21.01 | 1.18 |
| Swingle citrumelo | 10.05 | 0.55 | 22.03 | 1.09 |

[a] Standard deviation

of MANOVA for reductions of root mass, shoot mass and ELISA with rootstock, fungal species and experiment as main effects were highly significant ($P < 0.001$) as were interactions between rootstock × fungal species, rootstock × experiment, and fungal species × experiment (Table 2). MANOVA performed for each experiment produced highly significant ($P < 0.001$) results for rootstock and fungal species (Table 3). A significant ($P < 0.05$) interaction between rootstock and fungal species were observed only in experiment 3.

Univariate results showed significant ($P < 0.05$) differences of root mass reductions for rootstock and fungal species effect in all three experiments, but no significant interactions. Shoot mass reductions were significant for both effects in experiment 1 ($P < 0.05$), for fungal species in experiment 2 ($P < 0.001$) and for rootstock in experiment 3 ($P < 0.0001$). No significant interaction between rootstock and fungal species was observed.

Infected plants from all three experiments exhibited growth reductions from 16% to over 70% (Table 4). Rootmass reductions after infection with *P. nicotianae* were generally smallest (25–51%) for seedlings of Swingle citrumelo as were shoot mass reductions which ranged from 30% to 45%. Growth reductions for Cleopatra mandarin and sour orange were larger with up to 73% in sour orange after infection with this pathogen. Similarly, lowest root mass reductions from 28% to 38% were observed for Swingle citrumelo after dual infection of roots with *P. nicotianae* and *P. palmivora*. Root mass reductions for Cleopatra and sour orange were considerably larger and ranged from 30% to 64%. Results for shoot mass reductions after dual infection varied little between Swingle and sour orange (21–49%) and were largest for Cleopatra (43–58%) with the exception of experiment 2. Infection with *P. palmivora* caused only small root mass reductions (17–21%) for Swingle citrumelo. Shoot mass reductions after infection with this species were between 16% and 37% for Swingle and sour orange and were generally larger for Cleopatra mandarin, reaching up to 50%.

Univariate results for ELISA showed significant differences for fungal species effect and rootstock × fungal species interaction in experiment 1 ($P < 0.05$). Significant results were observed for fungal species in experiment 2 ($P < 0.0001$) and for both effects and their interaction in experiment 3 ($P < 0.01$).

The amount of pathogen detected in roots using ELISA was considerably larger in all citrus seedlings inoculated with *P. palmivora*, particularly in experiments 1 and 2 where absorption values ranged from 0.6 to 1.0 compared with 0.3 to 0.5 for roots infected

**Table 2** Results of MANOVA for root mass reductions, shoot mass reductions and ELISA across all three experiments

| Effect | Wilks Lambda | *F* | Effect df | Error df | *P* |
|---|---|---|---|---|---|
| Rootstock | 0.668 | 14.59 | 6 | 392.00 | 0.00000 |
| Fungal Species | 0.332 | 48.04 | 6 | 392.00 | 0.00000 |
| Experiment (Exp) | 0.370 | 42.14 | 6 | 392.00 | 0.00000 |
| Rootstock × Fungal Species | 0.840 | 2.94 | 12 | 518.86 | 0.00058 |
| Rootstock × Exp | 0.729 | 5.49 | 12 | 518.86 | 0.00000 |
| Fungal Species × Exp | 0.674 | 6.94 | 12 | 518.86 | 0.00000 |
| Rootstock × Fungal Species × Exp | 0.907 | 0.81 | 24 | 569.06 | 0.72711 |

**Table 3** Results of MANOVA for root mass reductions, shoot mass reductions and ELISA comparing experiments 1–3

| Effect | Wilks Lambda | *F* | Effect df | Error df | *P* |
|---|---|---|---|---|---|
| *Experiment 1* | | | | | |
| Rootstock | 0.269 | 10.52 | 6 | 68.00 | 0.00000 |
| Fungal Species | 0.228 | 12.39 | 6 | 68.00 | 0.00000 |
| Rootstock x Fungal Species | 0.681 | 1.18 | 12 | 90.25 | 0.31146 |
| *Experiment 2* | | | | | |
| Rootstock | 0.468 | 5.23 | 6 | 68.00 | 0.00018 |
| Fungal Species | 0.152 | 17.69 | 6 | 68.00 | 0.00000 |
| Rootstock × Fungal Species | 0.707 | 1.05 | 12 | 90.25 | 0.41019 |
| *Experiment 3* | | | | | |
| Rootstock | 0.703 | 7.96 | 6 | 248.00 | 0.00000 |
| Fungal Species | 0.552 | 14.30 | 6 | 248.00 | 0.00000 |
| Rootstock × Fungal Species | 0.843 | 1.83 | 12 | 328.36 | 0.04309 |

with *P. nicotianae* or with both species simultaneously (Table 4). Absorption values obtained for experiment 3 were generally lower but indicated the same trend. However, no clear relationship between the growth reductions observed for the different rootstock genotypes and the amount of pathogen detected in the roots was observed.

## Detection of *P. nicotianae* and *P. palmivora* in citrus roots

In all plants from inoculated treatments, an amplification product typical for the genus *Phytophthora* was obtained after nested PCR with primers ITS4 and ITS6 (Fig. 6). No phytophthora-specific amplification product was detected in non-inoculated plants, though DNA fragments of different size were observed occasionally. Sequence analysis confirmed that these fragments did not derive from *Phytophthora* or other plant pathogenic fungi. The presence or absence of reaction products after first round amplification with primer pair ITS4/DC6 was inconsistent after repeated analysis and appeared to be affected by the thermal cycler model used for the assay.

Restriction profiles obtained with *Alu*I and *Msp*I were specific for *P. nicotianae* in all root samples inoculated with *P. nicotianae* (Fig. 7 and Table 5). Nested PCR with species-specific primers PNIC1 and PNIC2 revealed a *P. nicotianae*-specific reaction product in all samples (Fig. 6). Morphological analyses after plating on semi-selective agar identified *P. nicotianae* in all plants from this treatment; no *P. palmivora* was detected in any of the root segments (Table 5). Propagule levels in potting medium inoculated with *P. nicotianae* were high with an average of 4580 $cm^{-3}$ of medium for all treatments; no *P. palmivora* propagules were detected (Table 6).

All root samples inoculated with *P. palmivora* showed banding patterns specific for *P. palmivora* after restriction digest (Fig. 7 and Table 5). No amplification products were obtained after nested PCR with species-specific primers PNIC1 and PNIC2 (Fig. 6). Morphological analyses revealed the presence of *P. palmivora* in all root samples from experiments 2 and 3; no *P. nicotianae* was detected in any of the root segments (Table 5). However, plate tests of samples from experiment 1 indicated a strong concurrent infection of plants with *P. nicotianae*, as well as *P. palmivora*. Mean propagule levels of potting medium inoculated with *P. palmivora* were 4020 $cm^{-3}$. No *P. nicotianae* was detected with the exception of medium from experiment 1, which appeared to be moderately contaminated with this species (Table 6).

Restriction patterns obtained for all plants from medium inoculated with both *Phytophthora* spp. were specific for *P. palmivora* (Fig. 7). *Phytophthora nicotianae*-specific restriction fragments were detected as faint bands in a few root samples from experiment 1. However, nested PCR with primers PNIC1 and PNIC 2 resulted in *P. nicotianae*-specific

**Table 4** Comparison of growth response of seedlings of three citrus rootstocks and amount of pathogen detected in roots after inoculation with *Phytophthora* spp

| Rootstock | Fungal species | *N* | Growth reductions (%) | | Amount of pathogen |
|---|---|---|---|---|---|
| | | | RM Red (%) | SM Red (%) | ELISA[a] |
| *Experiment 1* | | | | | |
| Cleopatra mandarin | Pnic | 5 | 43.50 a-c | 49.81 ab | 0.361 c |
| Sour orange | Pnic | 5 | 52.99 ab | 45.45 ab | 0.415 cd |
| Swingle citrumelo | Pnic | 5 | 25.23 bc | 44.56 ab | 0.352 c |
| Cleopatra mandarin | Pnic + Ppal | 5 | 64.30 a | 58.20 a | 0.501 cd |
| Sour orange | Pnic + Ppal | 5 | 61.16 a | 44.36 ab | 0.510 cd |
| Swingle citrumelo | Pnic + Ppal | 5 | 27.98 bc | 48.77 ab | 0.496 cd |
| Cleopatra mandarin | Ppal | 5 | 52.85 ab | 50.03 ab | 1.005 a |
| Sour orange | Ppal | 5 | 37.12 a–c | 27.34 b | 0.654 bc |
| Swingle citrumelo | Ppal | 5 | 16.88 b | 37.30 ab | 0.839 ab |
| Total | | 45 | 42.45 | 45.09 | 0.570 |
| *Experiment 2* | | | | | |
| Cleopatra mandarin | Pnic | 5 | 53.49 ab | 49.31 ab | 0.303 b |
| Sour orange | Pnic | 5 | 72.62 a | 54.37 a | 0.325 b |
| Swingle citrumelo | Pnic | 5 | 51.30 a–c | 40.07 ab | 0.395 b |
| Cleopatra mandarin | Pnic + Ppal | 5 | 63.26 ab | 43.14 ab | 0.398 b |
| Sour orange | Pnic + Ppal | 5 | 62.42 ab | 48.85 ab | 0.293 b |
| Swingle citrumelo | Pnic + Ppal | 5 | 37.53 bc | 45.07 ab | 0.397 b |
| Cleopatra mandarin | Ppal | 5 | 45.39 a–c | 34.14 ab | 0.697 a |
| Sour orange | Ppal | 5 | 47.34 a–c | 30.16 ab | 0.611 a |
| Swingle citrumelo | Ppal | 5 | 21.28 c | 24.95 b | 0.637 a |
| Total | | 45 | 50.51 | 41.12 | 0.451 |
| *Experiment 3* | | | | | |
| Cleopatra mandarin | Pnic | 15 | 47.42 a | 47.86 a | 0.253 c |
| Sour orange | Pnic | 15 | 44.21 a | 30.79 c | 0.246 c |
| Swingle citrumelo | Pnic | 15 | 43.51 a | 29.68 bc | 0.220 c |
| Cleopatra mandarin | Pnic + Ppal | 15 | 45.23 a | 43.74 ab | 0.283 c |
| Sour orange | Pnic + Ppal | 15 | 29.35 ab | 21.12 c | 0.261 c |
| Swingle citrumelo | Pnic + Ppal | 15 | 33.78 ab | 23.94 c | 0.282 bc |
| Cleopatra mandarin | Ppal | 15 | 45.17 a | 46.65 a | 0.459 a |
| Sour orange | Ppal | 15 | 20.77 b | 20.28 c | 0.291 c |
| Swingle citrumelo | Ppal | 15 | 19.40 b | 16.18 c | 0.364 b |
| Total | | 135 | 36.54 | 31.14 | 0.295 |

Comparison was by ANOVA. Different letters within columns indicate significant differences between means according to the Student-Newman-Keuls test for $P < 0.05$

[a] Mean absorbance values (405 nm) of assays performed in duplicate are presented. Pnic, *P. nicotianae*; Ppal, *P. palmivora*; RM Red, root mass reduction; SM Red, shoot mass reduction

amplification products in all samples from this treatment (Fig. 6; Table 5). Morphological analyses indicated the presence of *P. nicotianae* in all samples inoculated with both fungal species (Table 5). Only one root segment of all samples from experiment 1 exhibited the presence of *P. palmivora* during the plate tests. In experiment 2, *P. palmivora* was detected in only very few root samples from this

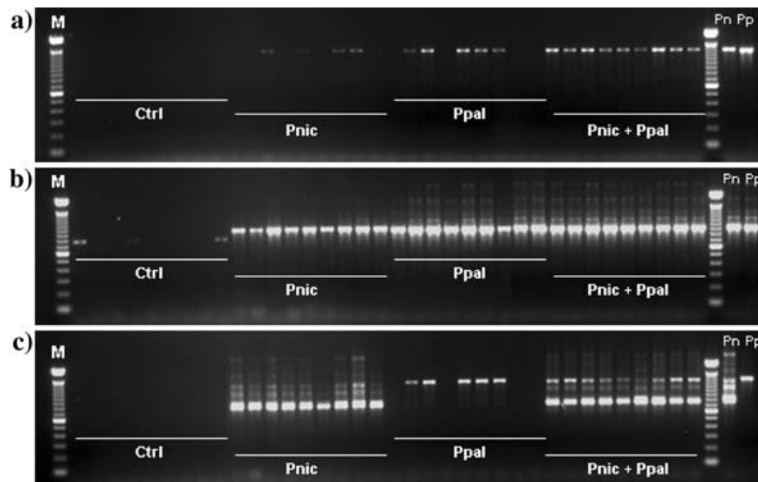


**Fig. 6** Amplification of DNA from non-infected and infected citrus roots. (**a**) Simple PCR with primers ITS4 and DC6. (**b**) Nested PCR with primers ITS4 and ITS6; non-specific amplification products are seen in some of the control plants. (**c**) Nested PCR with primers PNIC1 and PNIC2. Ctrl, non-inoculated control plants; Pnic, plants inoculated with *P. nicotianae*; Ppal, plants inoculated with *P. palmivora*; Pnic + Ppal, plants inoculated with *P. nicotianae* and *P. palmivora*. Pn, control DNA from *P. nicotianae*; Pp, control DNA from *P. palmivora*. M, 100 bp ladder

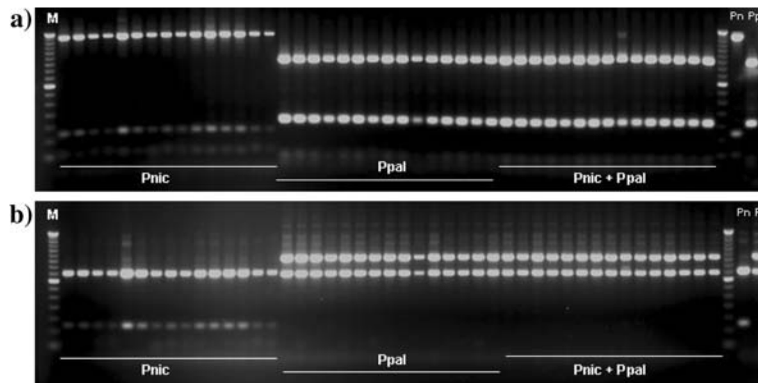


**Fig. 7** Restriction profiles obtained after digestion of ITS4/ITS6-amplification products from infected citrus roots with (**a**) *Alu*I and (**b**) *Msp*I. Pnic, plants inoculated with *P. nicotianae*; Ppal, plants inoculated with *P. palmivora*; Pnic + Ppal, plants inoculated with *P. nicotianae* and *P. palmivora*. Pn, control DNA from *P. nicotianae*; Pp, control DNA from *P. palmivora*. M, 50 bp ladder

treatment. Each of the two *Phytophthora* sp. was detected in many samples from experiment 3, though *P. nicotianae* appeared to be the dominant species. The average propagule numbers in potting medium inoculated with both *Phytophthora* spp. was more than twice as high for *P. nicotianae* as compared with *P. palmivora* in experiment 1 and 2. Propagule levels in experiment 3 were similar for both spp. (Table 6).

No *Phytophthora* spp. was detected from roots of non-inoculated plants using immunological assays, plating of root segments or PCR techniques (Fig. 6). With the exception of experiment 2, no *Phytophthora* spp. was detected in potting medium containing non-inoculated millet seeds (Table 6).

## Discussion

Infection of citrus roots by *P. nicotianae* and *P. palmivora* substantially reduced root and shoot mass of seedlings of the three rootstocks, depending on which *Phytophthora* spp. was present. Growth of

**Table 5** Molecular analysis and recovery of *Phytophthora* spp. from seedlings of three citrus rootstocks

| Rootstock | Fungal species | *N* | Molecular analysis[a] | | Plate tests[d] | | |
|---|---|---|---|---|---|---|---|
| | | | RFLP[b] | PNIC1/2[c] | Pnic | Ppal | Pnic + Ppal |
| *Experiment 1* | | | | | | | |
| Cleopatra mandarin | Pnic | 5 | Pnic | + | 32 | 0 | 0 |
| Sour orange | Pnic | 5 | Pnic | + | 32 | 0 | 0 |
| Swingle citrumelo | Pnic | 5 | Pnic | + | 34 | 0 | 0 |
| Cleopatra mandarin | Pnic + Ppal | 5 | Ppal | + | 31 | 1 | 0 |
| Sour orange | Pnic + Ppal | 5 | Ppal | + | 31 | 0 | 0 |
| Swingle citrumelo | Pnic + Ppal | 5 | Ppal | + | 30 | 0 | 0 |
| Cleopatra mandarin | Ppal | 5 | Ppal | – | 21 | 5 | 1 |
| Sour orange | Ppal | 5 | Ppal | – | 13 | 17 | 0 |
| Swingle citrumelo | Ppal | 5 | Ppal | – | 13 | 12 | 0 |
| Total | | 45 | | | 237 | 35 | 1 |
| *Experiment 2* | | | | | | | |
| Cleopatra mandarin | Pnic | 5 | Pnic | + | 35 | 0 | 0 |
| Sour orange | Pnic | 5 | Pnic | + | 30 | 0 | 0 |
| Swingle citrumelo | Pnic | 5 | Pnic | + | 33 | 0 | 0 |
| Cleopatra mandarin | Pnic + Ppal | 5 | Ppal | + | 29 | 2 | 1 |
| Sour orange | Pnic + Ppal | 5 | Ppal | + | 26 | 0 | 5 |
| Swingle citrumelo | Pnic + Ppal | 5 | Ppal | + | 26 | 4 | 2 |
| Cleopatra mandarin | Ppal | 5 | Ppal | – | 0 | 32 | 0 |
| Sour orange | Ppal | 5 | Ppal | – | 0 | 34 | 0 |
| Swingle citrumelo | Ppal | 5 | Ppal | – | 0 | 27 | 0 |
| Total | | 45 | | | 179 | 99 | 8 |
| *Experiment 3* | | | | | | | |
| Cleopatra mandarin | Pnic | 15 | Pnic | + | 94 | 0 | 0 |
| Sour orange | Pnic | 15 | Pnic | + | 89 | 0 | 0 |
| Swingle citrumelo | Pnic | 15 | Pnic | + | 95 | 0 | 0 |
| Cleopatra mandarin | Pnic + Ppal | 15 | Ppal | + | 61 | 20 | 13 |
| Sour orange | Pnic + Ppal | 15 | Ppal | + | 48 | 28 | 4 |
| Swingle citrumelo | Pnic + Ppal | 15 | Ppal | + | 50 | 21 | 11 |
| Cleopatra mandarin | Ppal | 15 | Ppal | – | 0 | 92 | 0 |
| Sour orange | Ppal | 15 | Ppal | – | 0 | 77 | 0 |
| Swingle citrumelo | Ppal | 15 | Ppal | – | 0 | 88 | 0 |
| Total | | 135 | | | 437 | 326 | 28 |

[a] Results are representative for all five replicated samples per rootstock and treatment

[b] Species-specific restriction fragment patterns obtained after digest of nested ITS4/ITS6 products with *Alu*I and *Msp*I

[c] Nested PCR with ITS4/DC6 amplification products

[d] Data denote the number of root segments of a total of 35 per rootstock in which *Phytophthora* was detected. Pnic, *P. nicotianae*; Ppal, *P. palmivora*. '+', PCR product present; '–', PCR product absent

Cleopatra mandarin and sour orange was reduced severely, whereas that of Swingle citrumelo seedlings was much less affected. These results are in marked contrast to previous field trials and greenhouse assays performed in this laboratory, which demonstrated better performance of Cleopatra mandarin in

**Table 6** Propagule density of *Phytophthora* spp. in potting medium from experiments 1–3

| Treatment | Propagules $cm^{-3}$ potting medium | |
|---|---|---|
| | *P. nicotianae* | *P. palmivora* |
| *Experiment 1* | | |
| Non-inoculated control | 0 | 0 |
| Inoculation with *P. nicotianae* | 4420 | 0 |
| Inoculation with *P. palmivora* | 160 | 4460 |
| Inoculation with both species | 4460 | 1880 |
| *Experiment 2* | | |
| Non-inoculated control | 80 | 0 |
| Inoculation with *P. nicotianae* | 4480 | 0 |
| Inoculation with *P. palmivora* | 0 | 3860 |
| Inoculation with both species | 4280 | 1620 |
| *Experiment 3* [a] | | |
| Non-inoculated control | 0 | 0 |
| Inoculation with *P. nicotianae* | 4850 | 0 |
| Inoculation with *P. palmivora* | 0 | 3730 |
| Inoculation with both species | 2980 | 2570 |

[a] Data presented are averages from three replicated treatments

comparison with Swingle citrumelo after inoculation with *P. palmivora* (Bowman et al. 2002 and 2003; Albrecht and Bowman 2004). This contrasting result may be related to the source of inoculum. In the earlier field study and greenhouse tests, plants were inoculated with infected field roots, whereas this study utilized cultured pure isolates of the two *Phytophthora* spp. grown on millet seeds. Experiments, currently in progress at our laboratory, suggest that differences in pathogenicity of field and cultured *Phytophthora* spp. may be at least partially responsible for the observed differences (data not shown). Also, other microorganisms included in the field root inoculum may have been involved in the pathogenic effect.

Despite the high *Phytophthora* damage levels, using *Phytophthora* reared on millet seeds may prove unsatisfactory as inoculum at the rate described here, since its incorporation into the potting medium was followed by the rapid development of unidentified secondary organisms. Nevertheless, *Phytophthora* was detected successfully in root samples from all inoculated treatments in all three experiments using PCR. Non-inoculated plants yielded negative results, and no cross-reaction with other organisms present in the medium was detected. In a single step PCR, detection limit was determined to be at 0.1 pg to 1 pg of purified fungal DNA. In nested PCR the pathogen was still detectable at concentrations down to 1 fg of DNA, hence increasing sensitivity by 100- to 1000-fold. Tooley and Therrien (1987) estimated the average genome size of diploid isolates of *P. infestans* to be 0.52 pg of DNA per nucleus. Assuming the genome size of *Phytophthora* spp. in this study is similar, only 0.002 nuclei were necessary to detect the pathogen, making nested PCR based on ribosomal ITS regions a very powerful detection method. These results are in agreement with other authors who reported sensitivity thresholds of 1 to 10 pg of DNA after simple PCR (Tooley et al. 1997; Ippolito et al. 2002) and of 0.1 fg (Judelson and Tooley, 2000), 1 fg (Ippolito et al. 2002), 60 fg (Grote et al. 2002) and as few as five zoospores (Nechwatal et al. 2001) after nested PCR with species-specific primers. Amplification of fungal DNA was not inhibited by citrus root DNA extracts in contrast to other studies (Schubert et al. 1999; Grote et al. 2002), which is probably due to the complete removal of possible inhibitory compounds of citrus roots by silica-gel-based DNA extraction and the use of a soil-less potting medium in the greenhouse.

Serological analyses using ELISA yielded positive results for all inoculated seedlings. No *Phytophthora* was detected in non-inoculated plants, which is in agreement with results obtained from molecular analyses. Interestingly, ELISA values were highest for all plants inoculated with *P. palmivora*, independent of the rootstock genotype. Similar observations were made by Widmer et al. (1998), who found a significantly higher colonization of root cells of both susceptible and tolerant citrus hosts with *P. palmivora* in comparison to *P. nicotianae*. Apparently this fungal species causes high infection rates, which are not always associated with plant decline.

Restriction digests of nested PCR products yielded profiles specific for *P. nicotianae* only in seedlings inoculated exclusively with this species. Roots inoculated with *P. palmivora* or with a combination of both pathogens generally exhibited *P. palmivora*-specific banding patterns due to the ten-fold lower sensitivity observed for DNA from *P. nicotianae* after amplification with ITS primers as compared to

*P. palmivora*. These results were confirmed by analyzing restriction patterns obtained from DNA samples containing varying proportions of purified fungal DNA from different species. Despite the reduced sensitivity of amplification and the negative results obtained with PCR-RFLP, *P. nicotianae* was detected in all samples inoculated with *P. nicotianae* individually, or in combination with *P. palmivora*, after nested PCR with species-specific primers PNIC1 and PNIC2. Species-specific DNA primers present an ideal alternative to PCR-RFLP technology, especially since the time-consuming steps of digestion and electrophoresis to separate fragments can be eliminated. However, the simple presence of an amplification product of specific molecular weight does not prove its identity, as opposed to RFLP patterns based on sequence variations. Also, design of species-specific primers derived from ribosomal ITS-regions can be problematic and cross-amplification with other closely-related species is often observed (Tooley et al. 1997; Schubert et al. 1999; Grote et al. 2002). Our attempts to design *P. palmivora*-specific primers based on ITS sequences have so far been unsuccessful due to cross-reactions with DNA from *P. nicotianae* (data not shown). Species-specific primers derived from random amplification of polymorphic DNA sequences (RAPDs) or from other DNA sequences are useful but do not always achieve the required sensitivity necessary for disease diagnosis prior to a noticeable expression of disease symptoms (Bonants et al. 1997; Lacourt and Duncan 1997; Schubert et al. 1999). Real-Time PCR, a recently developed highly sensitive methodology, may eliminate these problems and also permit the quantification of individual pathogens present in the infected tissue (Böhm et al. 1999; Schaad and Frederick 2002; Vandemark and Barker 2003; Ippolito et al. 2004).

Results from analyses of potting medium from all treatments with plating and morphological methods were generally in agreement with those obtained using PCR-RFLP, though slight contamination was detected in some of the soil samples. Selective agar-plating of infected root segments indicated the presence of *P. nicotianae* in several samples from experiment 1 which were inoculated with *P. palmivora* only and a predominance of *P. nicotianae* in all samples inoculated with both species in experiments 1 and 2. Since no *P. nicotianae* was detected in roots of seedlings exclusively inoculated with *P. palmivora* by PCR analysis with species-specific primers, contamination most likely occurred at some stage during the preparation or handling of the plates. The predominance of *P. nicotianae* in plating assays may be the result of much faster growth of this species in comparison to *P. palmivora* on PARPH medium, a trend that has also been observed with other strains of these species in continuing studies in this laboratory. Thus, the identification of *Phytophthora* spp. using semi-selective agar-plating may lead to inaccurate results in mixed infections.

The results described in this study confirm that molecular techniques based on ITS-fingerprinting provide powerful tools which can be applied to the detection and identification of *Phytophthora* species in screening programmes for disease resistance under controlled conditions in the greenhouse.

The high sensitivity of molecular methods allows the identification of less than a single cell of pathogen. Plants can thus be diagnosed at an early stage of infection when no visible symptoms are observed above-ground or are clouded by similar symptoms produced for other reasons. In addition, the convenience of being able to freeze tissue samples and to analyze them at a later stage is of great importance for our breeding programmes involving hundreds of plants, as opposed to most traditional methods where immediate processing of the samples is required. Despite its more limited sensitivity, serological assays using ELISA were shown to be very useful in detecting fungal pathogens at the genus-level and provide an important tool for estimating the amount of pathogen present in the plant tissue. The rearing of *Phytophthora* species on millet seed to be used for inoculation of potted plants appears efficient and reliable. In our continuing experiments with this method, we are using lower rates of millet seed inoculum in the pots to minimize the growth of other unwanted micro-organisms while obtaining the desired *Phytophthora* disease initiation.

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