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A combination of baiting and PCR techniques for the detection of *Phytophthora quercina* and *P. citricola* in soil samples from oak stands

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Summary

A description is given of the use of a combination of polymerase chain reaction (PCR) and baiting techniques for the specific detection of *Phytophthora quercina* and *Phytophthora citricola* from soil around declining oak trees. The soil was flooded with water and subjected to a specific baiting procedure using *Quercus robur* leaflets as baits. Single round or nested PCR, respectively, with species-specific primers allowed the detection of *P. quercina* and *P. citricola* in infected oak leaflets used as baits and in the water from the same bait tests. PCR detection of both fungi was also possible after soil samples had been thoroughly mixed with water and the floating organic debris had been collected. *Phytophthora quercina* and *P. citricola* could be readily detected in almost every case in the water from these tests by PCR but less frequently in the organic debris. The identities of *P. quercina* and *P. citricola* were confirmed by restriction digests of the corresponding PCR amplicons. The presence of both fungi was also confirmed in parallel in soil samples tested by baiting with oak leaflets. Nested PCR with the primers used allowed the detection of as few as five zoospores of *P. citricola* and 300 zoospores of *P. quercina* in a volume of 100 µl. The methods presented here allow detection and identification of species of *Phytophthora* in soil without the need for direct extraction of soil samples, and without specific knowledge of the morphological characteristics of the genus.

1 Introduction

Since the early 1980s, pedunculate oak (*Quercus robur* L.) and sessile oak (*Quercus petraea* L.) in Central Europe have been affected by a chronic decline. There have been extensive investigations of above-ground symptoms but only a few on the root systems of declining trees. Histopathological changes in roots of declining trees were associated with invasion by *Phytophthora* species (Blaschke 1994; Jung and Blaschke 1996). In a long-term field study at 27 sites in Germany, Switzerland, Hungary, Italy and Slovenia, several *Phytophthora* species, including *Phytophthora citricola* Sawada, *Phytophthora cactorum* (Lebert and Cohn) Schroeter, *Phytophthora cambivora* (Petri) Buisman, *Phytophthora gonapodyides* (Petersen) Buisman and *Pythium undulatum* Petersen, were isolated from the rhizosphere soil and from roots of declining oaks (Jung et al. 1996). Most frequently isolated was the newly described species, *Phytophthora quercina* (Jung et al. 1999). Molecular data also supported *P. quercina* as a new species (Cooke et al. 1999). Soil infestation experiments showed that *P. quercina* was the most aggressive *Phytophthora* species on oak seedlings (Jung et al. 1999).

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Due to the significance of *Phytophthora* spp. as the primary cause of many root-rot diseases, the detection of propagules of these fungi in seeds, roots and soil is an important component of effective disease management. However, *Phytophthora* is recognized as a difficult pathogen both to isolate and identify at the species level (TSAO 1990). Within the last 10 years rapid techniques have been developed to detect propagules of these fungi (for summaries see WERRES and STEFFENS 1994; MILLER 1996). Initially, serological techniques were used, with polyclonal or monoclonal antibodies in enzyme-linked immunosorbent assay (ELISA) (ELLIS and MILLER 1993; TIMMER et al. 1993; MILLER et al. 1994; WERRES et al. 1997). As part of this method, flotation techniques were developed for the simple and effective separation of the organic matter or debris from the mineral fraction of soil (TIMMER et al. 1993; MILLER et al. 1994).

More recently, the polymerase chain reaction (PCR) has been utilized for the detection of *Phytophthora* and other fungi (summarized by HENSON and FRENCH 1993; MILLER 1996; EDEL 1998). As the internal transcribed spacer (ITS) regions of genes encoding ribosomal RNA (rDNA) are highly variable among morphologically distinct species (LEE and TAYLOR 1992), mutate at a rate suitable for species discrimination and are present in many copies, they are frequently used as a target region for the design of species-specific PCR primers (WHITE et al. 1990). Such primers are capable of distinguishing among closely related species. Polymerase chain reaction diagnostics (PCRDs), based on ITS differences and other sequences, have been used simultaneously to detect and identify fungal infection of different host tissues (JOHANSON and JEGER 1993; ERSEK et al. 1994; BONANTS et al. 1997; TOOLEY et al. 1997; LIEW et al. 1998; AKHTER and ANTONOVICS 1999). However, with roots and, more so, soils, DNA purification is a problem. Extraction protocols release organic soil components, particularly humic acids and phenolics, which are known inhibitors of *Taq* DNA polymerase. Purification of DNA is therefore a critical step. Recently there have been reports of methods to overcome these problems and to clean DNA extracts from these substances (PICARD et al. 1992; JOHNSTON and AUST 1994; VOLOSSIOUK et al. 1995; BERTHELET et al. 1996; ZHOU et al. 1996; CULLEN and HIRSCH 1998). Some of these techniques are either time-consuming or employ hazardous chemicals (BAHNWEG et al. 1998). The use of commercial kits for DNA extraction (TOOLEY and CARRAS 1996; SCHUBERT et al. 1999) and purification (BONANTS et al. 1997) can partly avoid these limitations. However, low inoculum levels and uneven distribution of inoculum in the soil are additional factors that may contribute to negative PCR detection results. The aim of this study was to compare and combine traditional isolation methods with PCR-based systems to detect *Phytophthora* species from forest soil samples without the need for direct extraction of soil samples. Such methods will be valuable to forest research since they circumvent the need for lengthy soil DNA extraction protocols, isolation protocols and difficulties in pathogen identification. Accurate and rapid methods will allow more samples to be analysed thus facilitating more extensive forest surveys.

2 Material and Methods

2.1 Field soil sampling

Soil was collected in April 1999 from under six *Quercus robur* trees (60–100 years old) growing at Landau (Bavaria, Germany) on a Paternia soil (FAO Fluvisol). Three soil sections (approximately 20 cm × 20 cm wide × 30 cm deep) were collected with a spade from between the root buttresses of each tree about 80–100 cm from the base of the trunk. Oak roots within each soil section were carefully removed together with any adhering soil. The roots from all three sections around each tree were combined into one sample, then the

soil adhering to the roots was removed by shaking, mixed thoroughly and stored. Half of this 'rhizosphere' soil was used for baiting experiments and the other half for flotation experiments (see below).

2.2 Baiting experiments

An aliquot of each 'rhizosphere' soil from each tree sample at Landau was subjected to a bait test, in which young leaflets of *Quercus robur* seedlings were floated over the soil samples after flooding with distilled water (JUNG et al. 1996). Leaflets showing brown discoloration after approximately 1 week were examined microscopically for the presence of sporangia that were characteristic of *Phytophthora*. Infected leaflets were cut into small pieces and plated onto selective PARPNH agar (TSAO and GUY 1977). Colonies that developed on the agar were transferred to V8 agar plates for further identification and maintenance.

In a second set of baiting experiments, field soil samples known to be infested with either *P. citricola* or *P. quercina*, were used to generate infected oak leaflets as described above for subsequent DNA extraction and PCR amplification of fungal DNA (see below). *Phytophthora* infection was confirmed microscopically by examining sporangial shape. The water from the *P. citricola* bait tests was filtered through a 5 μm nitrocellulose membrane filter and the material retained on the filter was scraped off and DNA extracted from it (see below). Soil samples known to be free of *Phytophthora* were subjected to the same baiting procedure in order to produce discoloured (sometimes *Pythium*, but not *Phytophthora*-infected) leaflets and material to be retained on the membrane filter. These materials were used as negative controls for diagnostic PCR experiments. 'Baiting experiments' without oak leaflets were performed to check whether bait tissue was needed for successful detection in water from these tests.

2.3 Flotation experiments

The flotation technique (TIMMER et al. 1993; MILLER et al. 1994) was modified as follows: 50 g of air-dried soil were ground to release organic material, poured into 120 ml plastic bottles to which 80 ml of distilled water were added. The bottles were shaken vigorously for 1 min and incubated at 8°C for 24 h. After removing the floating organic debris (small root pieces) with forceps, the water was decanted and centrifuged for 10 min at 5000 g. DNA was extracted from the organic debris and the pellet obtained by centrifugation for PCR testing (see below).

2.4 Zoospores of *P. quercina* and *P. citricola*

Dilution series of *Phytophthora* zoospores were used to test the potential sensitivity of the PCR detection methods described here. Zoospores of *P. citricola* and *P. quercina* were produced by flooding 1-week-old V8 agar cultures in Petri dishes with 25–30 ml of distilled water. The dishes were incubated at 18°C in natural light with the water being replaced every 2 to 3 days. After 10 days, sporangial production was checked using a binocular microscope. Then the water was replaced again and the plates were placed at 4°C for 1 h to enhance zoospore release. The resulting zoospore suspensions were filtered through paper filters (Schleicher and Schuell, no. 595 1/2) to remove mycelial fragments and sporangia. After filtration, the zoospore suspension was shaken vigorously to induce encystment and zoospores were counted under the microscope using a haemocytometer. A dilution series of zoospores ranging from 3 to 3000 per 100 μl for *P. quercina* and from 5 to 50 000 per 100 μl for *P. citricola* was prepared in autoclaved distilled water.

2.5 DNA extraction for PCRDs

2.5.1 Infected oak leaflet baits

DNA was extracted from infected leaflets using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Each leaf was placed in a **2** ml Eppendorf tube and crushed thoroughly with a glass rod before extraction. The extracted DNA, which was usually light to dark brown in colour, was purified using the Wizard-Kit (Promega, Madison, WI, USA) (BONANTS et al. 1997). The same Qiagen extraction procedure was also used to extract DNA from pure *Phytophthora* cultures to provide control DNA.

2.5.2 Residuals from membrane filter

DNA was extracted using the DNeasy Plant Mini Kit (Qiagen). 150 mg of glass beads (diameter 0.5–1 mm) and 400 µl of the Qiagen lysis buffer were added, and the material was disrupted in a dentist amalgam shaker (Espe Cap-Mix, no. 443009, Seefeld, Germany) for 30 s at highest setting. Afterwards extraction was carried out according to the manufacturer's instructions. Extracted DNA was purified using the Wizard-Kit (Promega).

2.5.3 Pellet from flotation experiments

After centrifugation of the water from flotation experiments the resultant pellet was extracted using the DNeasy Plant Mini Kit according to the manufacturer's instructions, and the extracted DNA was purified using the Wizard Kit.

2.5.4 Organic debris from flotation experiments

The organic debris, which had been removed with forceps, was subjected to three successive cycles of freeze–thawing in liquid nitrogen in a **2** ml Eppendorf tube and heated to 70°C for 5 min (CULLEN and HIRSCH 1998). Glass beads (150 mg of diameter 0.5–1 mm) and 400 µl of the Qiagen lysis buffer were added and the organic debris was disrupted in a dentist amalgam shaker (Espe Cap-Mix) for 1min at highest setting. The DNA was extracted using the DNeasy Plant Maxi Kit and purified using the Wizard Kit.

2.5.5 Zoospores

Zoospore suspensions were placed in the freezer at –70°C in order to break zoospore walls. After thawing 250 µl of each suspension was extracted directly using the DNeasy Plant Mini Kit. All extracted DNA samples were stored at 4°C prior to PCR amplification.

2.6 PCR amplification

Nested PCR was used to detect *P. quercina* and semi-nested PCR to identify *P. citricola*. Primer sequences and sources are listed in Table 1. None of the primer pairs showed any cross-reactions with a collection of isolates including other *Phytophthora* and *Pythium* species (SCHUBERT et al. 1999; unpublished data).

The primer combinations were as follows:

Phytophthora quercina: first round: QUERC1 and QUERC2; second round: QUERC3 and QUERC4.

Phytophthora citricola: first round: DC6 and CITR2; second round: CITR1 and CITR2.

For the second round of PCR in nested or semi-nested PCR, 1 µl of the first round PCR product was added to the second round reaction mixture. The PCR reaction was performed in 0.5 ml PP tubes in a gene cycler (BioRad No. 170–6701, Herkules, CA, USA). The 50 µl reaction mixture contained 5 µl PCR-buffer amended with 15 mM $MgCl_2$ (Qiagen), 5 µl bovine serum albumin (10 mg/ml), 1 µl of each dNTP (100 µM

Table 1. **Nucleotide sequences of the PCR primers used in this study**

Primer	Sequence	Source
CITR1	5′ TCTTGCTTTTTTTGCGAGCC 3′	SCHUBERT et al. 1999
CITR2	5′ CGCACCGAGGTGCACACAAA 3′	SCHUBERT et al. 1999
DC6	5′ GAGGGACTTTTGGGTAATCA 3′	BONANTS et al. 1997
QUERC1	5′ GTGATCGCAGGAGTGCTCTT 3′	SCHUBERT et al. 1999
QUERC2	5′ GTGATCGCAGTAAGAAATGAGT 3′	SCHUBERT et al. 1999
QUERC3	5′ GAGTGCTCTTTAGTGTCGAC 3′	SCHUBERT, personal communication
QUERC4	5′ GAAATGAGTGTGATCCATTCCA 3′	SCHUBERT, personal communication

each), 1 µl of each primer (0.2 µM each), 0.5 µl (2.5 units) HotStar *Taq* (Qiagen) and 1 µl template DNA. The PCR conditions for nested or semi-nested PCR with each species were as follows:

Phytophthora quercina: first round (primer pair QUERC1 and QUERC2): First cycle: denaturation and *Taq* activation 15 min 95°C; annealing 2 min 62°C; extension 2 min 72°C; next 10 cycles: denaturation 30 s 94°C; annealing 1 min 62°C; extension 1 min 72°C; next 10 cycles: denaturation 30 s 94°C; annealing 1 min 56°C; extension 1 min 72°C; next 20 cycles: denaturation 30 s 94°C; annealing 1 min 50°C; extension 1 min 72°C; final primer extension 5 min 72°C. Compared to the original protocol by SCHUBERT et al. (1999), this is an empirically optimized protocol that proved to give better results with the PCRDs described here.

Second round (primer pair QUERC3 and QUERC4): First cycle: denaturation and *Taq* activation 15 min 95°C; annealing 30 s 58°C; extension 1 min 72°C; next 30 cycles: denaturation 30 s 94°C; annealing 30 s 58°C; extension 1 min 72°C; final primer extension 5 min 72°C.

Phytophthora citricola: first round (primer pair DC6 and CITR2): First cycle: denaturation and *Taq* activation 15 min 95°C; annealing 30 s 58°C; extension 1 min 72°C; next 30 cycles: denaturation 30 s 94°C; annealing 30 s 58°C, extension 1 min 72°C; final extension 5 min 72°C.

Second round (primer pair CITR1 and CITR2): First cycle: denaturation and *Taq* activation 15 min 95°C; annealing 30 s 62°C; extension 1 min 72°C; next 30 cycles: denaturation 30 s 94°C; annealing 30 s 62°C, extension 1 min 72°C; final extension 5 min 72°C.

In some experiments, only single-round PCR was performed. In these cases, *P. quercina* and *P. citricola* were detected with the primer pairs QUERC1/QUERC2 and CITR1/CITR2, respectively, using the second-round conditions from the nested and semi-nested PCRs described above.

PCR products were separated by gel electrophoresis in 1.5% agarose gels (Gibco, Life Technologies, Karlsruhe, Germany) in TBE buffer and visualized under UV light after ethidium bromide staining. Tubes containing the reaction mixture without any template DNA served as a negative control; *Phytophthora* DNA was a positive control. DNA from discoloured leaflets and from material retained on filter membranes of soil samples known to be free of *Phytophthora* was used as negative controls.

2.7 DNA digest experiments

Amplified DNA was digested for 3 h at 37°C with 15 units (1 µl) of *Msp*I (Amersham Pharmacia, Uppsala, Sweden) and 5 units (1 µl) of *Alu*I (Amersham Pharmacia), respectively. Reaction mixtures were set up according to the manufacturer's instructions in a total of 20 µl, containing 10 µl of the amplification product. The DNA fragments were separated on 2.5% NuSieve GTG agarose gels (FMC BioProducts, Rockland, ME, USA) in TBE buffer and were visualized under UV light after ethidium bromide staining.

3 Results

3.1 Detection of *P. quercina* and *P. citricola* in soil samples by baiting and flotation experiments

Phytophthora quercina was detected in all six soil samples by conventional baiting with oak leaves. It was also detected by nested PCR in all six pellets prepared from the water in the flotation test but only in three of the six samples of organic debris from the same test (Table 2). Likewise, *P. citricola* was detected by baiting in five of the six soil samples and in four of the water pellet samples by semi-nested PCR, but only in one sample of organic debris (Table 2).

The original gels from the PCR tests are shown in Fig. 1a,b. The amplicons obtained in the PCR tests for *P. quercina* and *P. citricola* samples were digested separately with the restriction enzymes *Alu*I and *Msp*I. All resultant digest patterns matched the restriction digest patterns of pure DNA from *P. quercina* or *P. citricola*, respectively (data not shown).

3.2 Detection of *P. quercina* and *P. citricola* in infected bait leaves by PCR

Both *P. quercina* and *P. citricola* were detected by PCR in infected leaves from bait tests. Even single-round PCR, using the primer pairs QUERC1/QUERC2 and CITR1/CITR2 readily detected the pathogens (Fig. 2). Cleaning DNA with the Wizard-Kit increased signal intensity (Fig. 2a). The water controls and DNA from non-infected and *Pythium*-infected leaflets did not give any signals.

Table 2. **Comparison of PCR and baiting results for the detection of *P. quercina* and *P. citricola* from soil samples taken at the stand Landau**

Results for *Phytophthora quercina*			
Tree no.	PCR (organic debris)[a]	PCR (water)[b]	Bait test
1	–	+	+
2	–	+	+
3	+	+	+
4	–	+	+
5	+	+	+
6	+	+	+
Results for *Phytophthora citricola*			
Tree no.	PCR (organic debris)[c]	PCR (water)[d]	Bait test
1	–	+	+
2	–	+	+
3	–	–	+
4	+	+	+
5	–	+	+
6	–	–	–

[a] Lanes 3–8 in Figure 1a; [b] Lanes 9–14 in Figure 1a; [c] Lanes 9–14 in Figure 1b; [d] Lanes 3–8 in Figure 1b.

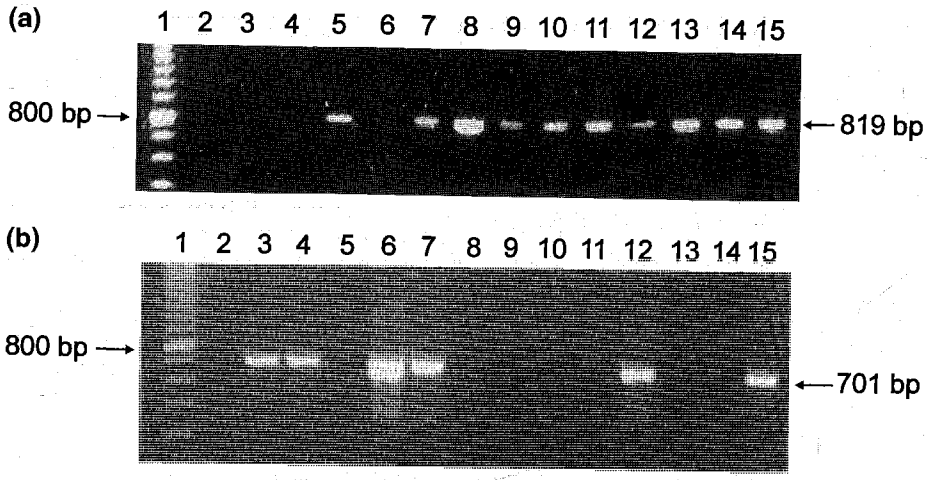

Fig. 1. (a) PCR-based detection of *Phytophthora quercina* in soil samples of the stand Landau. *Phytophthora quercina*: nested PCR, primer pairs QUERC1/QUERC2, then QUERC3/QUERC4; lane 1: 100 bp ladder; lane 2: water control (without template); lanes 3–8: organic debris of soil samples of trees no. 1–6; lanes 9–14: flotation water of soil samples of trees no. 1–6; lane 15: *P. quercina* DNA. (b) PCR-based detection of *Phytophthora citricola* in soil samples of the stand Landau. Semi-nested PCR, primer pairs DC6/CITR2, then CITR1/CITR2; lane 1: 100 bp ladder; lane 2: water control (without template); lanes 3–8: flotation water of soil samples of trees no. 1–6; lanes 9–14: organic debris of soil samples of trees no. 1–6; lane 15: *P. citricola* DNA

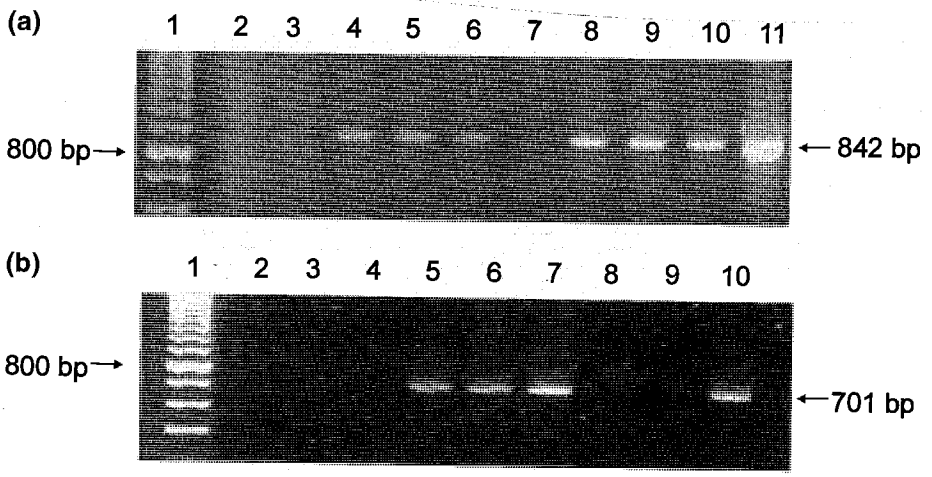

Fig. 2. Gel electrophoresis of amplification products after PCR with DNA extracted from (a) *P. quercina*- and (b) *P. citricola*-infected baiting leaflets. (a) Amplification products of *P. quercina*-infected baiting leaflets. Single round PCR, primer pair QUERC1/QUERC2: Lane 1: 100 bp ladder; lane 2: water control (without template DNA); lane 3: uninfected oak leaflets; lanes 4–6: leaflets infected with *P. quercina*; lanes 7–10 correspond to lanes 3–6 after cleaning with the Wizard-Kit; lane 11: *P. quercina* DNA. (b) Amplification products of *P. citricola*-infected baiting leaflets. Single round PCR, primer pair CITR1/CITR 2. All DNA samples were cleaned with the Wizard-Kit. Lane 1: 100 bp ladder; lane 2: water control (without template DNA); lane 3: uninfected oak leaflets; lane 4: oak leaflets infected with *Pythium* sp.; lanes 5–7: leaflets infected with *P. citricola*; lanes 8 and 9: leaflets showing discoloration, harvested after baiting of soil not contaminated with *P. citricola*; lane 10: *P. citricola* DNA

3.3 Detection of *P. citricola* in water samples from bait tests

Phytophthora citricola was readily detected in water from leaf bait tests of field soil samples after filtration (Fig. 3). Semi-nested PCR, using the above-mentioned primer pairs, was needed for detection of the pathogen. *P. citricola* was not detected without the inclusion of bait leaves in the tests (data not shown).

3.4 Detection of zoospores of *P. quercina* and *P. citricola* by PCR

Nested PCR detected minimum values of 300 and five zoospores of *P. quercina* and *P. citricola*, respectively, in a volume of 100 µl (Fig. 4). The cut-off point between detection and non-detection of *P. quercina* zoospores appeared to be very sharp with a strong signal generated by 300 zoospores and no signal with 100. In contrast, with fewer and fewer zoospores of *P. citricola*, there was steady decline in the strength of the PCR signal (Fig. 4).

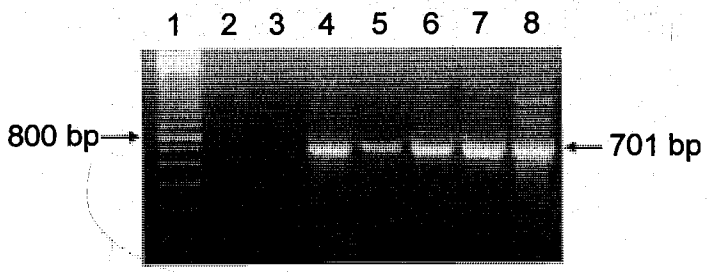

Fig. 3. Gel electrophoresis of amplification products of *P. citricola* zoospores of baiting water samples after semi-nested PCR, primer pairs DC6/CITR2, then CITR1/CITR2; lane 1: 100 bp ladder; lane 2: water control (without template); lane 3: soil without *P. citricola* contamination; lane 4: field soil sample a; lane 5: field soil sample b; lane 6: field soil sample c; lane 7: field soil sample d; lane 8: *P. citricola* DNA

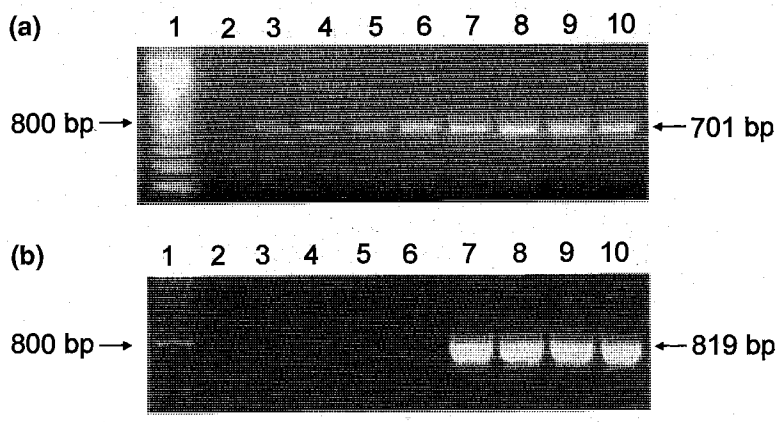

Fig. 4. Gel electrophoresis of amplification products after PCR with DNA from zoospores of *Phytophthora citricola* and *Phytophthora quercina*. (a) *Phytophthora citricola*: semi-nested PCR, primer pairs DC6/CITR2, then CITR1/CITR2; lane 1: 100 bp ladder; lane 2: reaction mixture without template DNA; lanes 3–10: zoospore concentrations of 5; 10; 50; 500; 5000; 17 000; 25 000; 50 000 zoospores per 100 µl. (b) *Phytophthora quercina*:Nested PCR, primer pairs QUERC1/QUERC2, then QUERC3/QUERC4; lane 1: 100 bp ladder; lane 2: reaction mixture without template DNA, lanes 3–10: zoospore concentrations of 3, 10, 30, 100, 300, 750, 1500 and 3000 zoospores per 100 µl

These results were confirmed in several replicate experiments with different, freshly prepared zoospore suspensions.

4 Discussion

Recently PCRDs have been developed for several fungal plant pathogens. Given their frequent presence in infested planting material (JEFFERS and ALDWINCKLE 1988; DUNCAN 1990) and their ability to survive for long periods in soil (ZENTMYER and ERWIN 1970; DUNCAN and COWAN 1980), *Phytophthora* sp. are particularly important targets for PCRDs (STAMMLER and SEEMÜLLER 1993; BONANTS et al. 1997; LACOURT and DUNCAN 1997; LIEW et al. 1998). To date most PCRDs are designed for use with plant material, seeds, roots or other vegetative propagating materials. When investigations on the distribution of species of *Phytophthora* are made, their detection in soil samples is of particular interest. However, co-extraction of humic acids and phenolics from soil samples, inhibiting *Taq* polymerase, can negatively influence PCR reactions and prevent successful detection. Within the last years various organisms have been detected in soil samples by PCR, employing different extraction protocols, whereas *Phytophthora* species have only rarely been the target of such detection experiments. Apart from problems with DNA purity PCR detection of *Phytophthora* sp. in soil samples strongly depends on inoculum densities in the soil. Since inoculum levels may be very low at periods with unfavourable soil conditions (MITCHELL and KANNWISCHER-MITCHELL 1992) and, at that, inoculum may be unevenly distributed in the soil (MITCHELL and KANNWISCHER-MITCHELL 1992), direct extraction from small soil samples may not result in successful detection by PCR even when the pathogen is present in the rhizosphere of an affected tree.

Baiting with different host tissues that are infected by the pathogen's zoospores still is the most common way to detect and isolate *Phytophthora* species from infested soil samples (RIBEIRO 1978). A modified baiting technique for the isolation of *Phytophthora* species involved in root rot of oak has been developed (JUNG et al. 1996). However, subsequent isolation of *Phytophthora* from infected leaflets requires time (several days), special techniques, selective agar media, and considerable knowledge of the genus. Furthermore the presence of fast growing fungi such as *Pythium* in the baits can make isolation and identification of *Phytophthora* difficult or even impossible (TSAO 1990).

The limitations of both PCR and baiting can be overcome by combining standard baiting methods and PCRDs. In this paper, we describe such combinations enabling us to detect the root rot fungi *P. quercina* and *P. citricola* in soil samples collected from under declining oak trees. Employing the techniques described here, followed by DNA extraction and PCR reactions, allowed specific detection of the pathogens and confirmation of the presence of the species. Most of the PCRDs employed (semi)nested PCR and highly specific primers. Furthermore, identity of the species was confirmed by restriction enzyme digests. The digest patterns of the PCR amplicons from both fungi confirmed that the PCR signals were those of the target pathogens.

Most of the PCR detection methods described here are based upon classical baiting techniques stimulating and resulting in the production of infectious zoospores. These can be collected by centrifugation, filtration or via the infection of bait tissues. Flotation as well as filtration techniques were first developed to detect *Phytophthora* and *Pythium* sp. in soil from citrus orchards, pepper and cucurbit crops, and in irrigation water using ELISA-kits (ALI-SHTAYEH et al. 1991; TIMMER et al. 1993; MILLER et al. 1994; WAKEHAM et al. 1997). Using flotation and filtration techniques in combination with PCR, both *P. quercina* and *P. citricola* were detected in field soil samples from beneath declining oaks. Apart from the stimulation of zoospore production an additional advantage of the flotation technique is the organic debris floating on top of the water meniscus. This material can be easily collected and allows

detection of resting spores in decaying rootlets even when the pathogen is in an inactive phase of its life cycle (e.g. as oospores). This dormancy is often hard to break (ZENTMYER and ERWIN 1970; TSAO 1990) and baiting experiments, either alone or in combination with PCRDs, would yield negative results. However, focusing on the detection of zoospores induced in baiting or flotation water gave more reliable results, whereas the use of the organic debris from such tests was much less reliable with these pathogens.

When infected baiting leaflets are extracted and used for PCRDs the inoculum present in a given soil sample is not only concentrated on the leaf but also multiplied many times. Presumably because of the growth of the pathogen on leaves, subsequent sporulation and infection of further leaflets, *P. citricola* and *P. quercina* were easily detected in infected oak leaflets from bait tests even after only single-round PCR. For the same reason, detection in water samples generated without baiting leaflets gave negative results (data not shown). Co-infection with other organisms (e.g. *Pythium* sp.) did not affect the procedure since specific primer pairs not showing any cross-reactions with various soil fungi (SCHUBERT et al. 1999; unpublished data) were used. Purifying the DNA using the Wizard-Kit (BONANTS et al. 1997) is not necessarily required but is recommended as it increased signal intensity (see Fig. 2a). Oak leaflets are suitable baits since they were shown to be susceptible to many *Phytophthora* species present in European forest soils (JUNG et al. 1996).

Although PCR increases the precision and speed of bait tests, time is still required for the fungus to infect and develop on the oak leaves. The flotation technique which focuses on water or organic material fraction of a soil–water mixture offers an attractive alternative which is almost as sensitive and much more rapid. Both fungi could be detected in the water pellet in less than 2 days.

By combining baiting and PCRDs the disadvantages of extracting DNA from soil have been avoided. No co-extraction of humic acids, present in soil and known to inhibit *Taq* DNA polymerase, therefore occurs, and there is no longer the need to extract the whole soil sample in order to detect fungal contamination. Furthermore, identification of a *Phytophthora* species isolated from a soil sample by baiting is facilitated since it is no longer dependent on morphological characteristics of the isolates.

As was shown in repeated zoospore dilution experiments, the PCRDs for *Phytophthora quercina* were not as sensitive as those for *Phytophthora citricola* when standard zoospore dilutions were compared. A possible partial explanation is that the *P. citricola* primers were designed to amplify genomic rDNA, thought to be present as tandem repeats and therefore multicopy, whereas the primers for *P. quercina* are based on the sequence of a single RAPD (randomly amplified polymorphic DNA) band, probably present as a single copy only. This is in agreement with the results of JOHNSTON and AUST (1994) who compared primer pairs with different target regions for the detection of *Phanerochaete chrysosporium* in soil. However, both primer pairs can be considered sufficiently sensitive when taking into account the fact that a single zoosporangium of *Phytophthora* sp. can release up to 30 zoospores into the surrounding medium (ZENTMYER 1980).

Valuable information on the existence and activity of propagules of *Phytophthora* can be obtained, if soil samples are analysed simultaneously by the described PCR techniques and standard baiting. An extension of this work would be to quantify soil inoculum levels by combining the modified flotation technique with real-time quantitative PCR, which has recently been tested successfully with *Phytophthora* sp. (BÖHM et al. 1999). Furthermore, the development of primer pairs that are specific for other *Phytophthora* species would be desirable.

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Résumé

Détection de Phytophthora quercina et de P. citricola dans le sol de chênaies par une méthode combinant le piégeage et la PCR

L'article décrit la détection spécifique de *Phytophthora quercina* et de *P. citricola* dans le sol prélevé autour de chênes dépérissants, par une méthode combinant les techniques de piégeage et de PCR. Le sol a été immergé dans l'eau et soumis à la procédure du piégeage avec de très jeunes feuilles de *Quercus robur*. Une amplification par PCR simple ou par PCR gigogne, respectivement, avec des amorces spécifiques des espèces ont permis de détecter *P. quercina* et *P. citricola* dans les feuilles-piège infectées, et dans l'eau de piégeage. La détection des deux champignons par PCR a aussi été possible après que les échantillons de sol aient été soigneusement mélangés à de l'eau et que les débris organiques aient été collectés. *Phytophthora quercina* et *P. citricola* ont pu être facilement détectés par PCR dans presque tous les cas dans l'eau de piégeage, mais moins fréquemment dans les débris organiques. L'identité de *P. quercina* et de *P. citricola* a été confirmée par les profils de restriction des amplifiats obtenus. La présence des deux champignons a aussi été confirmée en parallèle dans des échantillons de sol par piégeage. L'amplification par PCR gigogne avec les amorces utilisées a permis la détection de seulement 5 zoospores de *P. citricola* et 300 zoospores de *P. quercina*, dans un volume de 100 µl. Les méthodes présentées ici permettent la détection et l'identification des espèces de *Phytophthora* dans le sol en évitant l'extraction directe d'ADN du sol, et sans connaissances spécifiques sur les caractéristiques morphologiques du genre.

Zusammenfassung

Eine Kombination von Köder- und PCR-Techniken zum Nachweis von Phytophthora quercina und P. citricola in Bodenproben von Eichenstandorten

Es wird der spezifische Nachweis von *Phytophthora quercina* und *P. citricola* in Bodenproben von absterbenden Eichen mit Hilfe einer Kombination von PCR- und Baiting-Methoden beschrieben. Die Bodenproben wurden mit Wasser geflutet und Baiting-Tests unterzogen, bei denen junge Blättchen von *Quercus robur* als Köder zum Einsatz kamen. Einfache oder nested PCR-Reaktionen mit artspezifischen Primern erlaubten den Nachweis von *P. quercina* und *P. citricola* in den infizierten Eichenblättchen aus diesen Tests und im jeweiligen 'Baiting-Wasser'. Der PCR-Nachweis beider Erreger war auch möglich, wenn Bodenproben gründlich mit Wasser gemischt wurden, das aufgeschwemmte organische Material abgesammelt und das Wasser abgenommen wurde. *P. quercina* und *P. citricola* wurden dabei in nahezu allen Fällen im Wasser, jedoch weniger regelmäßig im organischen Material nachgewiesen. Die Identität der betreffenden Arten wurde zusätzlich durch Restriktions-Analysen der entsprechenden Amplicons bestätigt. Außerdem wurde die Anwesenheit beider Arten in den untersuchten Bodenproben durch klassische Baiting-Methoden nachgewiesen. Nested PCR mit den verwendeten Primerpaaren erlaubte den Nachweis von nur 5 Zoosporen von *P. citricola* und 300 Zoosporen von *P. quercina* in einem Gesamt-Volumen von 100 µl. Die beschriebenen Methoden ermöglichen Nachweis und Identifizierung von Phytophthora-Arten in Bodenproben, ohne die Notwendigkeit einer direkten Extraktion des Bodens und ohne weitreichende Kenntnis der morphologischen Merkmale der Arten dieser Gattung.

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