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Detection and identification of *Phytophthora fragariae* Hickman by the polymerase chain reaction

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Abstract

Phytophthora fragariae Hickman, which causes strawberry red stele and raspberry root rot, is a quarantine organism for which specific and sensitive detection methods are required to test the health of planting material. Sequences of the internal transcribed spacer regions of the ribosomal gene repeat (rDNA) were used to develop primers for *P. fragariae* in a nested Polymerase Chain Reaction (PCR). The fungus was readily detected in infected but symptomless roots by nested, but not single-round, PCR. It was also detected in infested water samples obtained from the Dutch General Inspection Service by nested PCR. Detection of PCR products was at least 10-fold more sensitive by PCR-ELISA than by conventional visualisation on agarose gels.

Introduction

Phytophthora fragariae Hickman exists as two varieties, *fragariae* and *rubi*, which respectively cause the root rot diseases, red core of strawberry and raspberry root rot (Hickman, 1940). Although spread can occur locally in soil and in water movement from one field to another, most new outbreaks are thought to be due to the planting of infected stock. This is almost certainly the way in which red stele spread across N. Europe in the late 1970's and raspberry root rot in the 1980's (Duncan, 1990).

Infection of both strawberry and raspberry roots by *P. fragariae* can be difficult to detect, especially in summer when the fungus is largely inactive and is present principally as oospores. Severely infected strawberry plants are often badly stunted with bluish-green foliage, likewise badly diseased raspberry plants show a variety of characteristic and often dramatic symptoms. Low levels of infection are not so easily spotted and the planting of runners or young canes from such plants could easily occur. By EPPO and European Union 'Plant Passport' rules, the fungus is a quarantine organism on which a 'nil tolerance' is placed, i.e. no level of infection is tolerated in propagation stocks. However, this is not the case for other *Phytophthora* spp. such as *P. cactorum* which causes crown rot in strawberry; tolerance levels of typically 0.5–1.0% infection are allowed for this disease (Cleave, 1989). Raspberries in particular, can be infected by a large number of commonly occurring *Phytophthora* spp. (Wilcox, 1989).

There is a need therefore, for highly sensitive and discriminatory tests that can be used to detect low levels of infection in strawberry and raspberry by *P. fragariae*. The root tip bait test for strawberry red stele (Duncan, 1980) has been used with some success in Scotland, the Netherlands, Switzerland and Sweden to detect and eliminate infected propagation stocks of strawberry. It is highly sensitive and can detect $<1\%$ infection levels but it is time-consuming (5–6 weeks), requires the tester to have mycological expertise, taxonomic experience and must be done at about 12 °C. More recently, a similar test has been used in Scotland to test the health of raspberry plants (Duncan, unpublished results; Chard et al., Scottish Agricultural

Science Agency, Edinburgh, personal communication) but the same restrictions apply to it as to the strawberry test.

Other methods of detecting *P. fragariae* have been developed. All are based on ELISA but in general the results have lacked specificity or sensitivity (Werres and Casper, 1987; Werres, 1988; Amouzou-Alladaye et al., 1988; Olsson, 1995).

The in vitro amplification of DNA using the polymerase chain reaction (PCR) (Mullis and Faloona, 1987) has been utilised for the detection of many pathogens, e.g. for *Verticillium* (Nazar et al., 1991), *Gaeumannomyces* (Henson et al., 1993) and *Phoma* (Rollo et al., 1990). PCR primers appear to be sufficiently sensitive and specific to allow detection in field samples. Screening random clones from *P. fragariae* var. *rubi*, produced a probe which hybridised specifically with *P. fragariae* and PCR primers developed from this probe detected *P. fragariae* var. *rubi* in infected raspberry samples from the field (Stammler et al., 1993; Stammler and Seemüller, 1993).

By chance the probe contained the small sub-unit of the ribosomal RNA gene repeat (rRNA) of *P. fragariae.* The utility of this sub-unit and, more particularly, the internal transcribed spacer (ITS) regions that flank either side of it, in distinguishing among species of *Phytophthora*, has since been recognized (Lee and Taylor, 1992; Cooke and Duncan, 1997). ITS regions contain appropriate interspecific variation for detection by PCR (Cooke and Duncan, 1997; Cooke et al., 1996; Crawford et al., 1996) or hybridisation (Lee et al., 1993). The teams at IPO-DLO and SCRI using rDNA as a source of sequences have developed PCR primers for detecting a range of *Phytophthora* species in plant material including *P. fragariae*. The various probes and primers developed in each laboratory have proved useful in studying *P. fragariae*, in particular for developing a method for detecting small amounts of infection of plant material by *P. fragariae* and small numbers of zoospores in samples of water. It is these developments that are reported here.

Materials and methods

Techniques used in both laboratories were largely identical or very similar but where sufficiently different to warrant mention, the locations have been entered in parentheses.

Fungal material and culturing conditions

Phytophthora species used in this study are detailed in Table 1. All species were maintained on V8 oatmeal agar containing 50 ppm vancomycin (IPO-DLO) or French bean agar (SCRI) at 4 °C. Cultures of liquid V8 oatmeal were inoculated with discs of the fungus growing on V8 oatmeal agar, and incubated for 3–4 weeks at 22 °C (IPO-DLO) or in still cultures of a sucrose/asparagine/mineral salts broth containing 30 mg ml^{-1} β-sitosterol (Cooke et al., 1996). Mycelium was harvested by vacuum filtration, washed with sterile water and freeze dried for extended storage at −20 °C.

Zoospore production

Zoospores were produced by incubating small agar discs from the edge of a 2 week-old culture of *P. fragariae* in a compost extract at 14 °C. The extract was changed daily for three days after which it was replaced with distilled water. The plates were subsequently incubated overnight at 4 °C and then for 4 h at room temperature to release zoospores.

DNA isolation

Mycelium. DNA from fungal mycelium was isolated by grinding freeze-dried mycelium in liquid nitrogen with a mortar and pestle, followed by extraction in buffer (50 mM Tris/HCl pH 8.0, 10 mM EDTA, 1% SDS, 0.15 M NaCl), extraction with phenol/chloroform, precipitation with ethanol, treatment with RNase, extraction with chloroform and precipitation with ethanol (IPO-DLO) or using a Nucleon DNA extraction kit (Scotlab, Coatbridge, Scotland) according to the manufacturers recommendations (SCRI).

Strawberry roots. Strawberry roots were rinsed with tap water to remove soil particles, dried on paper tissues, ground in a mortar and pestle under liquid nitrogen, extracted with CTAB extraction buffer (0.1 M Tris/HCl pH 7.0, 2% (w/v) CTAB, 1.4 m NaCl, 0.2% β-mercapto-ethanol, 1% PVP), extracted with phenol/chloroform, chloroform and precipitated with ethanol. After RNase treatment the sample was extracted with chloroform and again precipitated with ethanol. Finally the DNA samples were cleaned using the 'Wizard'-kit (Promega, Leiden, The Netherlands).

Table 1. Details of *Phytophthora* spp. isolates used in this study

	Species	Isolate name	Source	Host
1.	*P. fragariae* var. *fragariae*	A1		Strawberry
2.	*P. fragariae* var. *fragariae*	A2		"
3.	*P. fragariae* var. *fragariae*	A3		"
4.	*P. fragariae* var. *fragariae*	A4		"
5.	*P. fragariae* var. *fragariae*	A5		"
6.	*P. fragariae* var. *fragariae*	A6		"
7.	*P. fragariae* var. *fragariae*	A7		"
8.	*P. fragariae* var. *fragariae*	A8		"
9.	*P. fragariae* var. *fragariae*	A9		"
10.	*P. fragariae* var. *fragariae*	A10		"
11.	*P. fragariae* var. *fragariae*	NS1		"
12.	*P. fragariae* var. *fragariae*	NS2		"
13.	*P. fragariae* var. *fragariae*	NS3		"
14.	*P. fragariae* var. *fragariae*	NS4		"
15.	*P. fragariae* var. *fragariae*	FVF 7	SCRI 168	"
16.	*P. fragariae* var. *fragariae*	FVF 9	SCRI 171	"
17.	*P. fragariae* var. *fragariae*	FVF 18	SCRI 365	"
18.	*P. fragariae* var. *fragariae*	FVF 19	SCRI 372	"
19.	*P. fragariae* var. *fragariae*	FVF 36	SCRI 499/1	"
20.	*P. fragariae* var. *fragariae*	FVF 50	SCRI 512	"
21.	*P. fragariae* var. *fragariae*	PD 94/960	SCRI 168	"
22.	*P. fragariae* var. *rubi*	PD 94/958	SCRI R49-type	Raspberry
23.	*P. fragariae* var. *rubi*	PD 94/961	SCRI/JPM	"
24	*P. fragariae* var. *rubi*	FVR 11	SCRI R49	"
25.	*P. fragariae* var. *rubi*	FVR 61	SCRI R188*	"
26.	*P. fragariae* var. *rubi*	FVR 67	SCRI R200	"
27.	*P. cryptogea*	PD 92/25		ex *Cichorium intybus*
28.	*P. cryptogea*	PD 91/1906		ex *Cichorium intybus*
29.	*P. cryptogea*	PD 91/2009		ex *Cichorium intybus*
30.	*P. cryptogea*	PD 94/1345		ex *Cichorium intybus*
31.	*P. cactorum*	PD 88/415		ex Fragaria
32.	*P. cambivora*	PD 92/1471		ex Alnus
33.	*P. capsici A1*	PD 92/989		ex Cyclamen
34.	*P. capsici A2*	PD 94/355		CBS 370.72
35.	*P. cinnamomi*	PD 93/1389		ex Calluna
36.	*P. citricola*	PD 88/671		ex Azalea
37.	*P. citrophthora*	PD 94/353		CBS 274.32
38.	*P. drechsleri*	PD 95/5473		ex Spathiphyllum
39.	*P. erythroseptica*	PD 92/133		ex *Lycopersicon esculentum*
40.	*P. idaei*	PD 94/959		SCRI R77 ex *Rubus idaeus*
41.	*P. ilicis*	PD 91/595		ex Ilex
42.	*P. iranica*	PD 95/9148		CBS 374.72
43.	*P. megasperma*	PD 94/118		ex *Rubus idaeus*
44.	*P. nicotianae A1*	PD 94/357		CBS 310.62
45.	*P. nicotianae A2*	PD 94/358		CBS 311.62
46.	*P. palmivora MF1*	PD 93/56		ex Rhododendron
47.	*P. porri*	PD 92/214		ex *Allium porrum*
48.	*P. pseudotsugae*	PD 95/9141		CBS 444.84
49.	*P. syringae*	PD 92/502		ex Pyrus

Obtained from SCRI, Invergowrie, Dundee, Scotland — Isolates 15–20 and 24–26

SCRI cultures have been renamed. Both the old and new names are included to allow comparison with previous publications.

Obtained from Ir. W.E. van der Weg, CPRO-DLO, Wageningen, The Netherlands — Isolates 1–14

Obtained from Plant Protection Service, Wageningen, The Netherlands — Isolates 21–23 and 27–49

CBS: Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands

Zoospores and water samples. A 50 μl sample of zoospores was mixed with 200 μl of Instagene (6% solution; BioRad, Veenendaal, The Netherlands). The mixture was incubated for 30 min at 56 °C, boiled for 8 min, vortexed and centrifuged for 3 min at 12,000 rpm. The supernatant was used in PCR: in the case of the water samples it was cleaned further using the 'Wizard'-kit before PCR.

Sequences of probe PS3003 and of ITS regions of Phytophthora *spp. isolates*

The nucleotide sequence of PS3003 was described by Stammler (Stammler, 1992). Sequences of ITS1 and ITS2 regions of several *Phytophthora* species and isolates have also been described and determined (Cooke et al., 1996) using universal primers (White et al., 1990; Lee and Taylor, 1992).

PCR amplification

All primers used in this study were based on DNA sequences of various parts of the rDNA. Sequence and location of the primers are shown in Table 2 and Figure 1.

Primers ITS1, ITS2, ITS3, ITS4 are located in the 28S, 18S and 5.8S subunits flanking the ITS regions of rDNA and have been described previously (White et al., 1990). DC6 (forward) primer (SCRI) was designed after comparing the DNA sequences of 18S subunits of rDNA from a wide range of plants and fungi. When used with reverse primer ITS4, DC6 gives a product only with genera, including *Phytophthora*, in the orders Pythiales and Peronosporales of the Oomycota (Hawksworth et al., 1995). P2 (forward) and P1 (reverse) were designed by Stammler (Stammler and Seemüller, 1993) from the end sequences of the specific probe, PS3003. B5 (reverse) primer (IPO-DLO) was also prepared from the sequence of the rDNA probe PS3003. DC1 (forward) and DC5 (reverse) (SCRI) were based on DNA sequences of the ITS regions of *P. fragariae*, after sequencing and comparing the ITS regions of over twenty species of *Phytophthora* (Lee and Taylor, 1992; Cooke and Duncan, 1997), including both varieties of *P. fragariae.*

Conditions for using primers P1 and P2 were as follows: Buffer (20 mM Tris/HCl pH 8.4, 50 mM KCl); 200 μm of each deoxyribonucleotide (dATP, dCTP, dGTP, dTTP); 2 units of Taq-polymerase (Gibco-BRL, Breda, The Netherlands); 0.05% W1 (Gibco-BRL); 0.2 μm of each primer. 10 ng of fungal DNA was added in a final reaction volume of 50 μl. Mixtures were overlaid with Sigma mineral oil and run in PCR with conditions as follows: after 2 min at 94 °C, 35 cycles of 40 sec 94 °C, 1 min 55 °C, 2 min 30 sec at 72 °C with a final extension of 5 min at 72 °C before cooling to 4 °C.

For the other primers, the above conditions were only varied with P1 and P2, were as follows: 60 μM of each deoxyribonucleotide; 0.6 μM of each primer and conditions: after 2 min at 94 °C, 35 cycles of 1 min 94 °C, 1 min 62 °C, 2 min 72 °C with a final extension of 10 min at 72 °C before cooling to 4 °C.

In nested PCR, 1 μl of the first PCR round was added to the second PCR reaction mixture. All PCR experiments were all done in 500 μl tubes (Biozym, Landgraaf, The Netherlands) in a Perkin Elmer 480 apparatus. Products (5 μl) were separated on 0.7, 1.0 or 1.2% agarose gels, 0.5 × TBE (10 × TBE = 0.9 M Tris/HCl, 0.9 M Boric acid, 10 mm EDTA pH 8.0) with 1 kb markers or the 123 bp markerset of Gibco-BRL and gels were stained with ethidium bromide.

PCR-ELISA

DIG-labelled dUTPs were used in PCR reactions according to the protocol of Boehringer (Mannheim, Germany) and for PCR-ELISA, the kit of Boehringer was used. PCR product (5 μl) was incubated with 20 μl of alkaline denaturation solution for 10 min at room temperature. Then 225 μl of hybridisation solution (50 ng of capture probe ml^{-1} of hybridisation buffer) was added. The capture probe (CPB2, Table 2) was derived from ITS 1 sequences and was biotinylated at the 5′ site. 220 μl of the mixture was pipetted into a streptavidin-coated well of a microtiterplate and incubated at 42 °C with gentle shaking for 30 min. After washing, incubation with anti-DIG-POD conjugate for 60 min at 37 °C, washing again and incubation with substrate solution for 60 min, the absorbance was measured at 405 nm in a microtiterplate reader.

Results

Single round PCR with primers P1, P2

Primers P1 and P2 (Stammler and Seemüller, 1993) developed originally for the detection of *P. fragariae* var. *rubi* in raspberry were used in this study to detect *P. fragariae* var. *fragariae* in strawberry. With pure fungal DNA, they produced a clear 3000 bp PCR

Table 2. PCR primers used in this study, their sequences, origins, specificities and uses

Primer	Sense	Sequence	Detects	Source
ITS1	Forward	5′-TCCGTAGGTGAACCTGCGG	Eukaryotes	18S rDNA
ITS2	Reverse	5′-GCTGCGTTCTTCATCGATGC	Eukaryotes	5.8S rDNA
ITS3	Forward	5′-GCATCGATGAAGAACGCAGC	Eukaryotes	5.8S rDNA
ITS4	Reverse	5′-TCCTCCGCTTATTGATATGC	Eukaryotes	28S rDNA
DC6*	Forward	5′-GAGGGACTTTTGGGTAATCA	Peronosporales	18S rDNA
P1	Forward	5′-CCGTTACTAGGGGAATCCTT	*P. fragariae*	pPS3003
P2	Reverse	5′-TTCATTTTCGGATAGAACCG	*P. fragariae*	pPS3003
B5	Reverse	5′-TGAGATGCCACCCGCAGCA	*P. fragariae*	ITS2 rDNA
DC1*	Forward	5′-ACTTAGTTGGGGGCCTGTCT	*P. fragariae*	ITS1 rDNA
DC5*	Reverse	5′-CGCCGACTGGCCACACAG	*P. fragariae*	ITS2 rDNA
CPB2	Forward	5′-BGCCCTTTTCTTTTAAAC	*P. fragariae*	ITS1 rDNA

* Primers DC1, DC5 and DC6 are protected under Brittish Patent Application number 9509112.0.

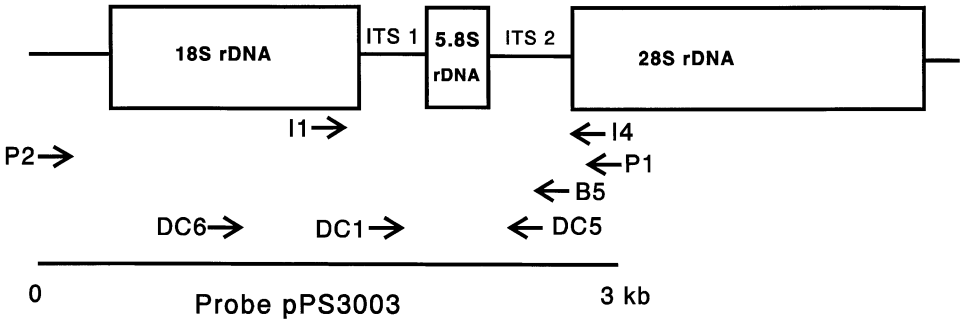

Figure 1. Locations of ITS regions, probe PS3003 and PCR primers within rRNA gene repeat of *Phytophthora fragariae.*

product from all 8 isolates of *P. fragariae* (Figure 2) and from all other isolates of *P. fragariae* tested (Table 1) (data not shown). However, *P. citrophthora*, *P. nicotianae* A1 and *P. capsici*, which had not been tested previously (Stammler and Seemüller, 1993), also gave products of the same size (Figure 2). After isolation of DNA from lightly, moderately, severely and uninfected strawberry roots (based on visual and microscopical assessment) and performing PCR experiments with primers P1 and P2, we could clearly detect *P. fragariae* in heavily infected strawberry roots (Figure 3). In moderately infected roots a faint band was observed. Moreover, in lightly infected roots, even when oospores were visible microscopically, the PCR product was hardly visible (Figure 3).

Development of nested primers

Because of this lack of specificity and sensitivity nested primers were developed based on sequence information of the ITS regions of rDNA (Table 2, Figure 1). Specificity of the internal primers DC1 and B5 was tested on the same isolates as in Figure 2 (Figure 4). Only the *P. fragariae* isolates resulted in the clear amplification of the predicted 750 bp PCR product. A 2300 bp PCR product was amplified from *P. porri* DNA but no product was produced from primers P1 and P2 for this species. Other Oomycetes didn't react either (data not shown). Primers DC1 and B5 (or DC5) generally lacked great sensitivity in single round PCR and gave similar results as with primers P1 and P2. Therefore a nested PCR was developed both at IPO-DLO and at SCRI.

Nested PCR was used at IPO-DLO to increase specificity and sensitivity of the single-round PCR described above. As the sequences that matched the DC1 and B5 primers were internal to those matching P1 and P2, a nested PCR was developed using primers P1, P2 in the first round and primers DC1, B5 in the second. In nested PCR at SCRI, primers DC6 and ITS4 were used in the first round to amplify DNA from any fungus belonging to the Pythiales or Peronospo-

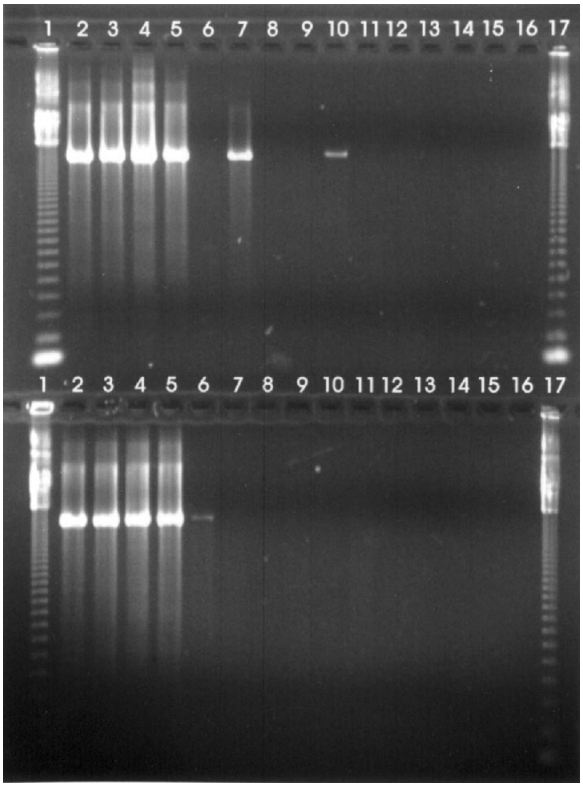

Figure 2. Gel electrophoresis of DNA products after PCR with primers P1, P2: (Upper gel) lane 1, 17: 123 bp molecular markers; lane 2–5: DNA from *P. fragariae* isolates 1, 3, 19 and 24; lane 6–15: DNA from *P. palmivora, P. citrophthora, P. citricola, P. erythroseptica, P. nicotianae* A1, *P. cryptogea, P. ilicis, P. syringae, P. cinnamomi, P. idaei*, respectively; lane 16: negative control (water). (Lower gel) lane 1, 17: 123 bp molecular markers; lane 2–5: DNA from *P. fragariae* isolates 6, 16, 26 and 23; lane 6–15: DNA from *P. capsici* A1, *P. megasperma, P. drechsleri, P. cactorum, P. capsici* A2, *P. cambivora, P. porri, P. nicotianae* A2, *P. pseudotsugae, P. iranica*, respectively; lane 16: negative control (water).

rales, thereby improving sensitivity, followed by a second round with primers DC1 and DC5 to achieve the desired specificity.

Detection in root material

An aliquot of the product from the P1/P2 PCR shown in Figure 3 was amplified using DC1 and B5 as the inner primers. Sensitivity was greatly improved over single round PCR with P1/P2 primers alone, with lightly, moderately and heavily infected roots all giving clear products of the correct size (Figure 5). Healthy roots gave a very faint band, which can be scored as negative. Similar results were achieved at SCRI using DC6 and I4 as the first round primers and DC1 and DC5 in the second round.

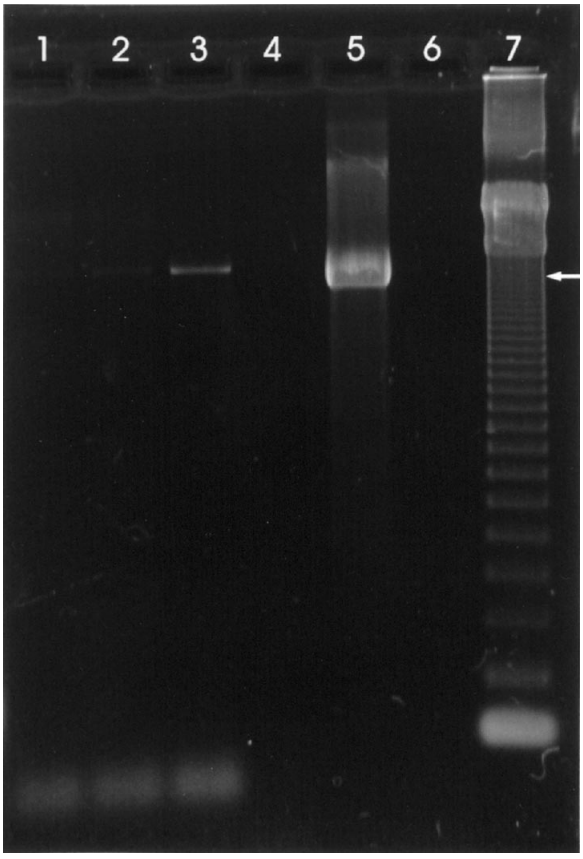

Figure 3. Gel electrophoresis of DNA products after PCR with primers P1, P2: lane 1–4: DNA from lightly, moderately, heavily and uninfected strawberry roots, respectively; lane 5: positive control (10 ng of DNA from *P. fragariae* race A3); lane 6: negative control (water); lane 7: 123 bp molecular markers. Arrow indicates the expected 3000 bp PCR product.

Detection of zoospores and in water samples

The protocol for extracting DNA from zoospores worked well and when different numbers of zoospores were tested in nested PCR as few as twenty were detected at both IPO-DLO (Figure 6) and SCRI.

Water samples were collected from conventional bait tests for red stele disease, 10 days after establishment of the tests. They were tested in single round, and nested PCR at IPO-DLO. Most samples were dirty and turbid and consistent amplification was not obtained until purification removed the substances inhibitory to PCR. The fungus was readily detectable in such samples by nested PCR (Figure 7), but not by conventional PCR (data not shown). Results matched the bait tests, i.e. water from bait tests that subsequently proved positive also tested positive by nested PCR.

Table 3. Specificity of capture probe CPB2 in PCR ELISA: Absorbance measurements at 405 nm (A_{405}) on PCR products of DNA of different *Phytophthora* spp. with primers ITS1, ITS4 and with primers DC1, B5 and capture probe CPB2

	PCR product ITS1/ITS4 A_{405} (60 min)	PCR product DC1/B5 A_{405} (60 min)
P. fragariae var. *frag.* FVF 19	2.622	2.537
P. fragariae var. *frag.* A2	1.990	2.534
P. fragariae var. *rubi* FVR 94/961	2.495	2.489
P. fragariae var. *rubi* FVR 94/958	2.660	2.413
P. palmivora	0.142	0.124
P. citrophthora	0.113	0.141
P. citricola	0.157	0.139
P. erythroseptica	0.163	0.119
P. nicotianae A1	0.206	0.153
P. cryptogea	0.284	0.121
P. ilicis	0.189	0.137
P. syringae	0.120	0.131
P. cinnamomi	0.090	0.121
P. idaei	0.099	0.121
P. capsici A1	0.107	0.136
P. megasperma	0.151	0.117
P. drechsleri	0.102	0.142
P. cactorum	0.164	0.114
P. capsici A2	0.170	0.137
P. cambivora	0.211	0.147
P. porri	0.202	0.130
P. nicotianae A2	0.192	0.124
P. pseudotsugae	0.190	0.119
P. iranica	0.183	0.112
MQ water in PCR	0.107	0.118
MQ water in PCR-ELISA	0.115	0.111

Direct identification on selective agar

The presence of *P. fragariae* var. *fragariae* in strawberry roots suspected as being infected and plated onto selective media, was confirmed at IPO-DLO and SCRI by removing small pieces of mycelia growing from the roots with a tooth pick and testing them in nested PCR (data not shown).

PCR-ELISA

Using digoxygenin-labelled dUTPs in the second round of nested PCR and specific labelling of the PCR product with the capture probe, CPB2, further enhanced specific detection of the fungus. The capture probe CPB2 was located in the ITS 1 region with maximum basepair differences with other *Phytophthora* spp. The specificity of this capture probe is shown in Table 3. When PCR products of DNA from several *Phytophthora* spp., obtained with the non-specific universal primers ITS1 and ITS4, were tested in the same way, only *P. fragariae* gave a strong reaction. Several other *Phytophthora* spp. gave a slight reaction, even though electrophoresis showed that the same amount of PCR product was formed with DNA from all *Phytophthora* spp. (Figure 8). However this slight reaction with some *Phytophthora* spp. was completely eliminated when the specific PCR primers (DC1 and B5) for *P. fragariae* were used (Table 3). Also the 2300 bp fragment amplified by *P. porri* DNA gave a negative signal with PCR-ELISA. So by introducing another specificity step (the capture probe) in PCR-ELISA one can eliminate false positives even more.

In a comparison of gel electrophoresis and PCR-ELISA, using a dilution series of PCR products of

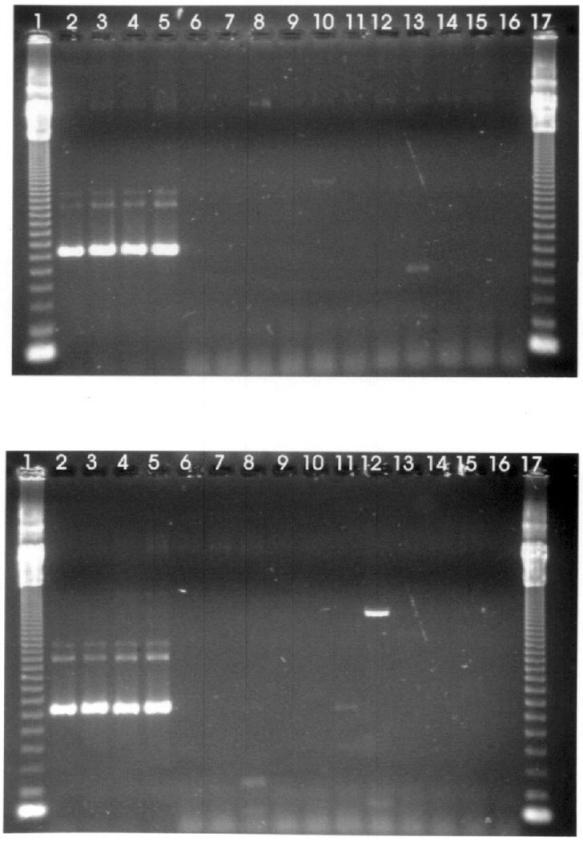

Figure 4. Gel electrophoresis of DNA products after PCR with primers DC1, B5: (Upper gel) lane 1, 17: 123 bp molecular markers; lane 2–5: DNA from *P. fragariae* isolates 1, 3, 19 and 24; lane 6–15: DNA from *P. palmivora, P. citrophthora, P. citricola, P. erythroseptica, P. nicotianae* A1, *P. cryptogea, P. ilicis, P. syringae, P. cinnamomi, P. idaei*, respectively; lane 16: negative control (water). (Lower gel) lane 1, 17: 123 bp molecular markers; lane 2–5: DNA from *P. fragariae* isolates 6, 16, 26 and 23; lane 6–15: DNA from *P. capsici* A1, *P. megasperma, P. drechsleri, P. cactorum, P. capsici* A2, *P. cambivora, P. porri, P. nicotianae* A2, *P. pseudotsugae, P. iranica*, respectively; lane 16: negative control (water).

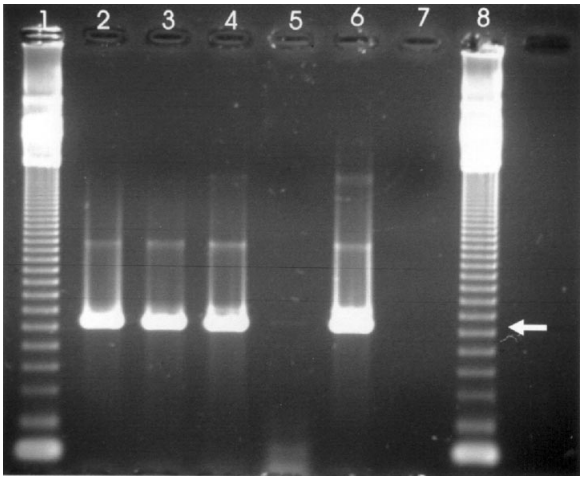

Figure 5. Gel electrophoresis of DNA products after nested PCR with primers P1, P2 in the first round and primers DC1, B5 in the second round: lane 1 and 8: 123 bp molecular markers; lane 2–5: DNA from lightly, moderately, heavily and uninfected strawberry roots, respectively; lane 6: positive control (10 ng of DNA from *P. fragariae* race A3); lane 7: negative control (water). Arrow indicates the expected 750 bp PCR product.

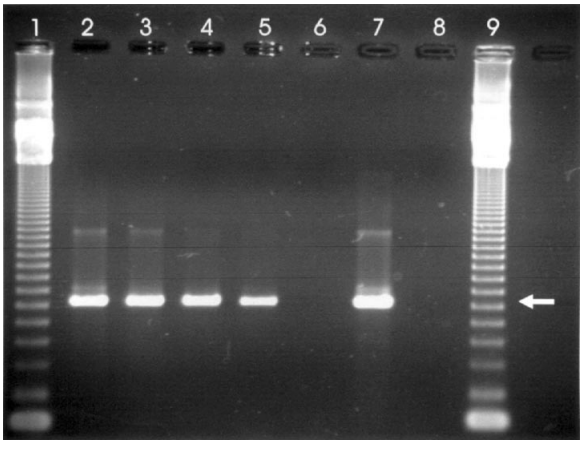

Figure 6. Gel electrophoresis of DNA products after nested PCR with primers P1 and P2 in the first round and primers DC1 and B5 in the second round: lane 1 and 9: 123 bp molecular markers; lanes 2–6: DNA from estimated 10,000, 2,000, 200, 20 and 2 zoospores of *P. fragariae* race A3, respectively; lane 7: positive control (10 ng of DNA from *P. fragariae* A3); lane 8: negative control (water). Arrow indicates the expected 750 bp PCR product.

P. fragariae, PCR-ELISA was at least 10 times more sensitive than gel electrophoresis (Figure 8, Table 4).

Discussion

Detecting fungi in plant material can be difficult, especially where the need is to detect very small amounts before evident symptom expression on the host, as is the case with plant health certification schemes for strawberry and other horticultural crops. Appropriate technology needs to be both sensitive and specific to avoid problems of cross-reaction with other fungi.

Immunological techniques based on polyclonal antisera are capable of considerable sensitivity but generally lack specificity (Mohan, 1989). Although monoclonal antibodies have been used as the basis of a highly specific dip-stick test to detect *P. cinnamomi* (Cahill and Hardham, 1994), similar tests for other

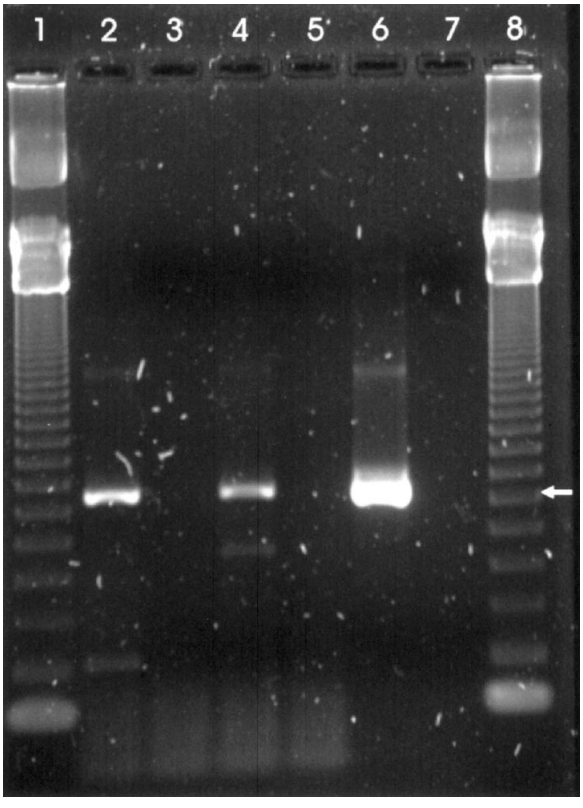

Figure 7. Gel electrophoresis of DNA products after nested PCR with primers P1, P2 in the first round and primers DC1, B5 in the second round: lanes 1 and 8: 123 bp molecular markers; lanes 2–5: DNA from different water samples from red stele bait tests provided by NAKB (Dutch General Inspection Service); lane 6: positive control (10 ng of DNA from *P. fragariae* A3); lane 7: negative control (water). Arrow indicates the expected 750 bp PCR product.

Table 4. Sensitivity of PCR-ELISA: Absorbance measurements at 405 nm (A_{405}) on different dilutions of *P. fragariae* PCR product with primers ITS1, ITS4 and capture probe CPB2

PCR product ITS1/ITS4	A_{405} (60 min)
P. fragariae var. *frag*. FVF 19	1.885
P. fragariae var. *frag*. FVF 19 (1:10)	0.278
P. fragariae var. *frag*. FVF 19 (1:100)	0.106
P. fragariae var. *frag*. FVF 19 (1:1000)	0.067
MQ water in PCR	0.052
MQ water in PCR-ELISA	0.065

Phytophthora species would require new monoclonal antibodies in turn, not a trivial exercise.

In contrast, the polymerase chain reaction described here offers considerable flexibility, and high sensitivity and specificity. Because the lack of sensitivity and specificity of the primers used by Stammler (Stammler and Seemüller, 1993) new primers were developed. Nested PCR with the supra-generic primer (DC6) or species-specific primers in the first round followed by a second round with species-specific primers discriminated between *P. fragariae* and *P. cactorum* and from all other species tested to date, and detected minute amounts of infection by *P. fragariae* in roots of strawberry plants. As few as twenty zoospores of the fungus were readily detectable without amplification of DNA of any other species. Single-round PCR with highly specific primers was less sensitive and failed to detect light infections of roots and small numbers of zoospores of the fungus in watersamples from bait tests.

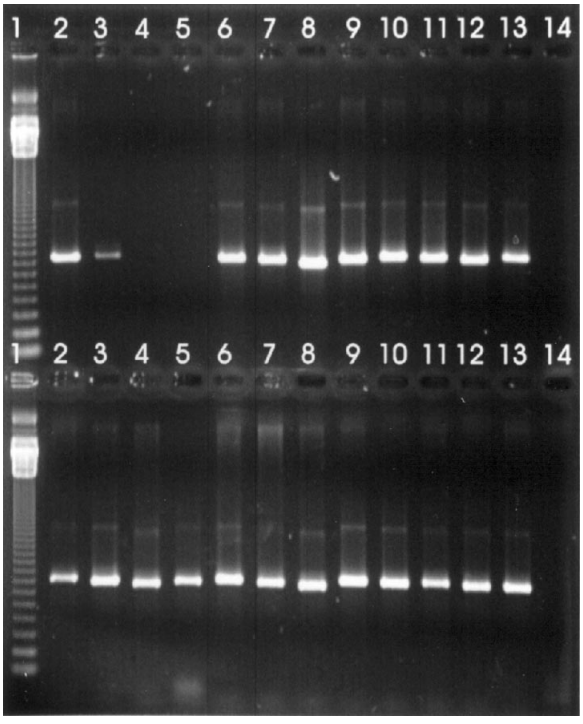

Figure 8. Gel electrophoresis of a dilution series of DNA products after PCR with DNA of *P. fragariae* (primers ITS1, ITS4) and with DNA of other *Phytophthora* spp.: (Upper gel) lane 1: 123 bp molecular markers; lane 2–5: dilution series of 5 μl PCR product *P. fragariae* var. *frag*. FVF 19 diluted 0, 10, 100 and 1000 fold, respectively; lane 6–13: 5 μl undiluted PCR product of *P. palmivora, P. citrophthora, P. citricola, P. erythroseptica, P. nicotianae A1, P. cryptogea, P. ilicis* and *P. syringae*, respectively; lane 14: negative control (water). (Lower gel) lane 1: 123 bp molecular markers; lane 2–13: 5 μl undiluted PCR product of *P. cinnamomi, P. idaei, P. capsici A1, P. megasperma, P. drechsleri, P. cactorum, P. capsici A2, P. cambivora, P. porri, P. nicotianae A2, P. pseudotsugae* and *P. iranica*, respectively; lane 14: negative control (water).

The present glasshouse-based bait test, and variants thereof, for detecting infection of strawberry plants by *P. fragariae* is robust, easily and quickly set up, requires little equipment, can handle large amounts of root material and is highly sensitive (Duncan, 1980). It has however, certain disadvantages: it requires five weeks to complete and expertise is needed for the diagnosis of the fungus in infected roots and for its isolation onto selective medium. However, large numbers of samples however, present a problem for a PCR-based test, as DNA extraction from roots is time consuming and laborious. One way of overcoming this problem would be to combine the advantages of both bait tests and PCR. Zoospores are readily detectable by PCR and were presumably the propagule of the fungus that was detected in water samples from bait tests within ten days of establishment, long before the manifestation of any infection as evident symptoms. The perfect match between the results of PCR and bait tests confirms the utility of such an approach, although more work would be needed before settling on a definitive protocol.

Further work should examine the need for bait plants in the test, e.g. would irrigation of root samples be sufficient to release zoospores without having to use bait plants, numbers of zoospores and timing of their release. On this latter point, about ten zoospores are needed to infect bait plants under ideal conditions (Duncan and Kennedy, 1994), very similar to the number of zoospores detected by PCR in this paper. It would appear therefore that the existing PCR can be at least as sensitive as the bait test without further modification.

Further improvements in a PCR-based technique are however possible and must involve simpler protocols for extracting DNA and for displaying and measuring the amounts of PCR product. PCR-ELISA is particularly promising for this latter purpose with its high sensitivity and complete lack of cross-reaction with specific primers. Moreover the specificity can be increased further by a specific capture probe. Its value in routine analysis would be increased by making it quantitative. Although quantification is not strictly necessary, since there is a 'nil' tolerance for *P. fragariae* in certification schemes, it would help set practical limits on the sensitivity of the procedure. One possibility is competitive PCR, in which a very similar but distinct DNA competes with target DNA for amplification. Already feasible for *P. cactorum* (Lacourt et al., 1996), it has yet to be developed for *P. fragariae*.

The present work has concentrated on *P. fragariae* in strawberry plants but nested PCR and the primers work without modification for *P. fragariae* var. *rubi* in raspberry plants. Other *Phytophthora* diseases could be detected in a similar way, given suitably specific primers for the second round of PCR. This approach is being actively pursued and already specific primers have been developed for six other species. The potential exists therefore for developing specific tests for almost any *Phytophthora* species affecting strawberry or any other horticultural crop.

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