European Journal of Plant Pathology **110:** 689–702, 2004.

A combination of baiting and different PCR formats, including measurement of real-time quantitative fluorescence, for the detection of *Phytophthora fragariae* in strawberry plants

Peter J.M. Bonants[1], Marga P.E. van Gent-Pelzer[1], Rien Hooftman[2], David E.L. Cooke[3], Dave C. Guy[3] and Jim M. Duncan[3]
[1]*Plant Research International, P.O. Box 16, 6700 AA Wageningen, The Netherlands (Phone: +31 317 467213; Fax: +31 317 418094; E-mail: peter.bonants@wur.nl);* [2]*Naktuinbouw, P.O. Box 135, 2370 AC Roelofarendsveen, The Netherlands;* [3]*Scottish Crop Research Institute, Dundee DD2 5DA, Scotland, UK*

Accepted 15 December 2003

Key words: nested PCR, TaqMan, Molecular Beacon, PCR–ELISA, DIAPOPS, internal PCR control

Abstract

Phytophthora fragariae, the cause of strawberry red stele disease, is a quarantine pathogen in Europe. Detecting low levels of infection requires sensitive and specific methods. In the past, Dutch and English inspection services have used bait plants to test strawberry propagation stocks destined for export. Increasingly though, PCR is being incorporated into these testing procedures in an effort to increase sensitivity and speed. Various combinations of baiting and PCR assays were compared with existing testing procedures. Water and root samples from the bait test were screened by nested PCR and the PCR amplicon was detected by several methods, including fluorescent labelled probes (TaqMan™ and Molecular Beacon™). PCR amplification was monitored in real-time and semi-quantitative detection was possible. Because PCR reactions are sensitive to inhibitors present in extracted DNA samples, an internal control containing the primer sequences specific for *P. fragariae* was developed to avoid false negatives.

Introduction

Detection of plant pathogens in propagation material is an important issue for plant health, especially if there is 'nil tolerance' for the pathogen. High sensitivity, specificity and reliability are prerequisites of any detection system. Propagators, shippers, growers and those enforcing plant health legislation prefer rapid results. For detection of *Phytophthora* spp. in plant material, many PCR methods have been developed based upon internal transcribed spacer (ITS) sequences (Böhm et al., 1999; Bonants et al., 1997; Cooke and Duncan, 1997; Lacourt et al., 1997; Niepold and Schober-Butin, 1997; Ristaino et al., 1998; Schubert et al., 1999). Nested PCR has been found to increase assay sensitivity (Bonants et al., 1997; Lacourt et al., 1997; Stark et al., 1998).

Phytophthora fragariae var. *fragariae*, which is pathogenic only to strawberry, causes the serious root rot disease, red stele. It is an European and Mediterranean Plant Protection Organisation (EPPO) quarantine organism with 'A2' status, i.e., the pathogen is recognised to be present in Europe but its further spread should be prevented. As such, a 'nil tolerance' is placed on the disease for propagation material; if detected in a stock at any level of propagation, the stock must be rejected (Anonymous, 1994). The existing detection method is a bait test, developed by Duncan (Duncan, 1980, 1985; Duncan et al., 1985; Duncan and Kennedy, 1994). Although sensitive and specific, it has the serious drawback of taking 5–6 weeks to complete; too long for practical use. Previous research has shown that the fungus can be detected in root samples within a day by a specific and sensitive nested-PCR assay (Bonants et al., 1997; Lacourt et al., 1997) but it is time consuming to extract suitable DNA from large numbers of root samples. However, the same nested-PCR assay produces the expected DNA fragment, without the need

for a long DNA extraction protocol, with water samples and zoospore suspensions (Bonants et al., 1997). The assay detects as few as 10 zoospores, about the same number as are needed to infect bait plants under ideal conditions (Duncan and Kennedy, 1994).

In the Netherlands, the Inspection Service, Naktuinbouw (Roelofarendsveen; formerly NAKB) tests a wide range of plant material. For *P. fragariae* in strawberries, Naktuinbouw, Central Science Lab (York, Great Britain) and the Scottish Agricultural Science Agency (Edinburgh, Scotland) have used Duncan's bait test since the 1980s, but continue to seek a more rapid alternative with similar sensitivity and specificity. This paper describes experiments at Plant Research International (PRI; formerly IPO-DLO), the Scottish Crop Research Institute (SCRI) and Naktuinbouw to create an improved test in which baiting and a nested PCR procedure are combined.

To avoid time consuming and potentially hazardous gel electrophoresis and to minimise the risks of cross-contamination, new methods of detecting the PCR amplicon were developed and tested: PCR–ELISA (Bonants et al., 1997; Grimm and Geisen, 1998; Loffler et al., 1998), DIAPOPS (Casademont et al., 2000; Chevrier et al., 1993; Nielsen et al., 1998; Niessen et al., 1997; Rasmussen et al., 1994) and fluorescent methods using TaqMan™ (Böhm et al., 1999; Brandt et al., 1998; Livak et al., 1995; Zhang et al., 1999) or Molecular Beacon™ probes (Giesendorf et al., 1998; Leone et al., 1998; Marras et al., 1999; Tyagi and Kramer, 1996). In the last two methods, real-time detection in a closed-tube during PCR amplification permits quantification. A comparison of all the above methods was made and the advantages and disadvantages of each assessed. PCR is often inhibited by polysaccharides, polyphenolics, humic acids and other substances present in the isolated DNA sample (De Boer et al., 1995; Jobes et al., 1995). For this reason an internal control was developed for single and nested PCR to reduce false negatives. Internal controls contain specific primer sequences developed for the pathogen (Courtney et al., 1999).

Materials and methods

Fungal isolates, plants and DNA isolation

Phytophthora fragariae, American race A9 (Bonants et al., 1997) was maintained at 11 °C on V8 agar. Zoospore suspensions were prepared (Bonants et al., 1997). DNA of *Phytophthora infestans* isolate 97 11.3 (obtained from Wilbert Flier, PRI) was used in the preparation of an internal control for nested PCR. Plants of the alpine strawberry *Fragaria semperflorens* cv. 'Baron Solemacher', grown from seed and the woodland strawberry *Fragariae vesca* cv. 'White Vesca', grown from runners in sterile soil in the greenhouse, were used as bait plants and are both highly susceptible to all isolates of the fungus (Duncan, 1980). Fungal DNA was extracted from mycelium, water from bait experiments, zoospore suspensions and infected strawberry roots as described previously (Bonants et al., 1997). DNA concentrations were determined using Precision Molecular Mass standard (BioRad, Veenendaal, the Netherlands) on a 1% agarose gel in 0.5× TBE buffer. The PCR product amplified from *P. fragariae* DNA using the universal primers, ITS1 and ITS4 (White et al., 1990), was cloned into the pGEM-T vector (Promega) (Sambrook et al., 1989) and termed pF1.

PCR methods

Nested PCR. Primer and probe sequences for all PCR techniques are provided in Table 1 and their locations in the ribosomal DNA are shown in Figure 1. PCR conditions were as described (Bonants et al., 1997). The primers for single-round PCR were DC1/B5 (DC1 in forward sense and B5 in reverse) and for nested PCR, DC6/ITS4 were used in the first round and DC1/B5 in the second (Bonants et al., 1997) (Table 1). The reaction mixture (25 µl) for both single-round and nested PCR was: 10 mM Tris–HCl pH 8.0, 50 mM KCl, 1.5 mM $MgCl_2$ (PCR buffer: Roche, Mannheim, Germany); dNTPs 60 µM each; 0.6 µM of each primer; 1 U of Taq polymerase (Roche). Conditions for the first round of nested PCR were 2 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 57 °C, 60 s at 72 °C and a final 10 min at 72 °C. For the second round of nested PCR, 5 µl of 1 : 20 diluted (in Milli-Q water) first-round PCR amplicon was used as template. The conditions for this, and for single-round PCR, were 2 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 65 °C, 60 s at 72 °C and a final 10 min at 72 °C. PCR products were separated by gel electrophoresis in 1% agarose in 0.5× TBE buffer and bands were visualised by ethidium bromide staining and UV illumination.

DIAPOPS. NucleoLink microtitre plates were coated with primer 10T-B5 as recommended by the

Table 1. Primers and probes used in this study for the detection of *P. fragariae*

Name	Primer/probe sequence	Test
DC6	5′-GAGGGACTTTTGGGTAATCA-3′	First-round primer for nested PCR (Bonants et al., 1997)
ITS4	5′-TCCTCCGCTTATTGATATGC-3′	Universal primer (White et al., 1990)
DC1	5′-ACTTAGTTGGGGGCCTGTCT-3′	Second-round primer nested PCR (Bonants et al., 1997)
B5	5′-TGAGATCCACCCGCAGCA-3′	Second-round primer nested PCR (Bonants et al., 1997)
Biot-B5	5′-Biotin-TGAGATCCACCCGCAGCA-3′	Second-round primer nested PCR (Bonants et al., 1997)
10T-B5	5′-TTTTTTTTTTTGAGATCCACCCGCAGCA-3′	Second-round primer nested DIAPOPS
MP3	5′-GGGCTACTGGCTCAGTTCCC-3′	Second-round primer nested Mol Beacon
MP5	5′-AAAAGGGCTACTGGCTCAGTTC-3′	Second-round primer nested TaqMan
CPB2	5′-Biotin-GCCCTTTTCTTTTAAAC-3′	Capture probe PCR–ELISA (Bonants et al., 1997)
FTAQ1	5′-FAM-AGGACCCAAACGCTCGCCATGATA-TAMRA-3′	TaqMan™ probe
MBPfrag	5′-FAM-GCGAGCCTGTGTGTCGGGCCCTATCAGCTCGC-Dabcyl-3′	Molecular Beacon™ probe

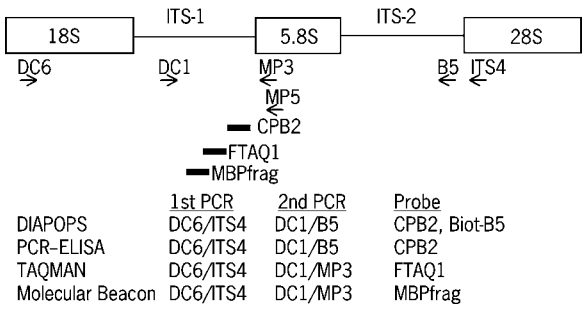

Figure 1. Localisation of primers and probes on the ribosomal DNA for the detection of *P. fragariae*.

manufacturer (NUNC A/S., Roskilde, Denmark). Forty-five microlitre of PCR mix and 5 µl of template (1 : 20 diluted (in Milli-Q water) first-round PCR amplicon) was added to each well with the final concentrations as follows: 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.1% gelatine, 0.1% Tween-20, 5% DMSO, 200 µM of each dNTP, 0.5 µM primer DC1 and 0.06 µM primer B5, 1 U of Taq polymerase (Roche). PCR conditions were: 3 min at 94 °C, 40 cycles of 10 s at 94 °C, 15 s at 65 °C, 10 s at 72 °C and a final 5 min at 72 °C. PCR reactions were run in a PE 9600 thermocycler (Perkin-Elmer) and PCR amplicons were detected by hybridisation with biotinylated probes (50 nM biot-B5 and 50 nM CPB2). One hundred microlitre of alkaline phosphatase-conjugated streptavidin (1 : 3000 diluted) were added and 100 µl of 1 mM 4-methylumbelliferyl phosphate substrate in 1 M diethanolamine pH 9.8 and 1 mM $MgCl_2$. Fluorescence was measured after 30 min of incubation at 50 °C in a fluorescence plate reader (excitation 355 nm, emission 460 nm).

PCR–ELISA. DIG-dUTP (Roche) was added to PCR reactions and PCR–ELISA was performed as described previously (Bonants et al., 1997).

TaqMan™. Forty-five microlitre of PCR mix and 5 µl of template (1 : 20 diluted first-round amplicon) was added to each tube with final concentrations: TaqMan™ buffer (10 mM Tris–HCl pH 8.3, 50 mM KCl, 4 mM $MgCl_2$), 60 µM dNTP, 0.6 µM of each primer (DC1/MP5), 0.2 µM TaqMan™ probe (Perkin-Elmer; Nieuwerkerk aan de IJssel, the Netherlands) and 2.5 U of AmpliTaq Gold polymerase (Perkin-Elmer). Conditions for PCR were: 10 min at 95 °C, 35 cycles of 15 s at 95 °C, 1.5 min at 60 °C. Fluorescence was measured in an ABI 7700 (Perkin-Elmer).

Molecular Beacon™. Twenty microlitre of PCR mix and 5 µl of template (1 : 20 diluted first-round amplicon) were added to each tube with final concentrations: PCR buffer (10 mM Tris–HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), 60 µM dNTP, 0.6 µM of each primer (DC1/MP3), 0.2 µM Molecular Beacon™ (Isogen,

Maarssen, the Netherlands), 0.75 μM ROX (5- and 6-carboxy-X Rhodamine), 1 U of Taq polymerase (Roche, Mannheim, Germany). Conditions for PCR were: 2 min at 95 °C, 35 cycles of 30 s at 95 °C, 1 min at 60 °C, 1 min at 72 °C. Fluorescence was measured in an ABI 7700 (Perkin-Elmer).

Bait test with controlled levels of infection

A bait experiment was set up in Weiss climate chambers at 12 °C, 75% relative humidity and 16 h light. Ten-gram mixtures of fragmented roots from artificially infected and healthy plants of *F. semperflorens* cv. Baron Solemacher were immersed in tap water (800 ml) in 1000-ml buckets. The treatments were: (1) 10 g of roots from healthy strawberry plants; (2) 9 g of roots from healthy with 1 g of roots from diseased plants; (3) 10 g of diseased roots; (4) 10 g of diseased roots. The roots of a single intact bait plant (ca. 4-weeks-old) were immersed in the water of treatments 1, 2 and 3 and held in place by the cover of the bucket. No bait plant was used in treatment 4. Water samples (100 ml) were collected and replaced with the same volume of tap water 1 h, 1 day, 1 week, 2 and 5 weeks after establishment. DNA was extracted from 50 ml of the water sample and analysed by nested PCR as described above. The remaining 50 ml of water was poured onto a fresh healthy strawberry bait plant (*F. semperflorens* cv. Baron Solemacher) growing in soil in pots, which was then kept for 5 weeks in the same incubator under the same conditions. After 5 weeks, the bait plants from the water and those in soil onto which the water samples had been poured were scored for disease index from healthy to severe wilting. Roots of the bait plants were washed, inspected for the presence of red steles and oospores typical of *P. fragariae* and weighed. DNA was extracted from ~25 mg of roots for testing by nested PCR and the other PCR methods as described above.

Direct versus *indirect detection by PCR*

Strawberry root samples (~60 g) collected during routine nursery field testing by Naktuinbouw were divided in half. One-half was sent to SCRI for direct detection, where DNA was isolated from the roots and tested by nested PCR with primers DC6/ITS4 and DC1/B5 and gel electrophoresis. The other half was kept at Naktuinbouw and incubated in tap water (1000 ml per bucket) in a greenhouse at 14 °C for 5 weeks with three bait plants (cv. Baron Solemacher). After 10 days incubation, DNA was extracted from a 50-ml water sample and tested by nested PCR with primers DC6/ITS4 and DC1/B5 and gel electrophoresis. After 5 weeks the bait plant roots were inspected for the presence of red steles and oospores typical of *P. fragariae*.

Molecular Beacon™ experiments

The Molecular Beacon™ and TaqMan™ formats were compared against a dilution series of pure *P. fragariae* target DNA. In single-round PCR, primers DC1/MP3 or DC1/MP5 were used at an annealing temperature of 60 °C. In nested PCR, first-round primers were DC6/ITS4 followed by DC1/MP3 or DC1/MP5 (Table 1). The Molecular Beacon™ method was also tested by nested PCR of a zoospore dilution series and water and root samples from the bait tests with controlled levels of infection.

Development of an internal control for nested PCR

DNA of *P. infestans* was amplified at low stringency (annealing temperature of 45 °C) with primers DC1/B5 (Table 1). A band (~850 bp) was cut from a low-melting agarose gel and amplified in two successive PCR reactions at 65 °C annealing temperature with primers DC1/B5. The resultant fragment was cloned into pGEM-T vector as described above and used as an internal control in single-round PCR.

For nested PCR, the 850-bp fragment, containing at its outer ends the DC1 and B5 primer sequences, in turn was amplified with the combined primers DC6-DC1 (40-mer) and B5-ITS4 (38-mer) (see Table 1) giving a fragment of 890 bp, which was cloned into the pGEM-T vector for use as an internal control in nested PCR.

Results

Relative sensitivity of different methods

Single-round and nested PCR of *P. fragariae* were compared using a dilution series of pure DNA of *P. fragariae* or pF1 plasmid DNA (Table 2). The amplicons in each case were detected by gel electrophoresis with ethidium bromide staining, DIAPOPS, PCR–ELISA or TaqMan™. As little as

Table 2. Results of single and nested PCR experiments of a dilution series of *P. fragariae* DNA and plasmid DNA (pF1) with various methods to detect the PCR amplicon

Amount of DNA	Single-round PCR								Nested (two-round) PCR							
	Agarose gel[a]		DIAPOPS (fluorescence)[b]		PCR–ELISA (A_{405})[c]		TaqMan™ (C_t-value)[d]		Agarose gel[a]		DIAPOPS (Fluorescence)[b]		PCR–ELISA (A_{405})[c]		TaqMan™ (C_t-value)[d]	
	DNA[e]	pF1[f]	DNA	pF1	DNA	pF1	DNA	pF1	DNA	pF1	DNA	pF1	DNA	pF1	DNA	pF1
10 ng	+++	+++	**3182**	**2777**	**1.36**	**1.44**	**14.9**	**9.5**	++	+++	**3280**	**3491**	**2.23**	**1.33**	**8.9**	**9.8**
1 ng	+++	+++	**2089**	**2603**	**1.21**	**1.32**	**17.3**	**11.0**	++	+++	**4079**	**3631**	**2.03**	**1.44**	**8.8**	**9.1**
500 pg	+++	nt	**1441**	nt	**1.13**	nt	**19.8**	nt	++	nt	**3992**	nt	**2.28**	nt	**9.0**	nt
100 pg	+++	+++	759	**1457**	**1.01**	**1.19**	**22.0**	**13.2**	++	+++	**3876**	**4495**	**2.28**	**1.30**	**8.9**	**10.1**
50 pg	+++	nt	365	nt	**0.98**	nt	**22.0**	nt	++	nt	**3836**	nt	**2.20**	nt	**8.9**	nt
10 pg	++	+++	378	721	**0.59**	**0.95**	**24.2**	**16.1**	++	+++	**3917**	**3836**	**2.14**	**1.36**	**9.0**	**9.9**
1 pg	+	+++	318	803	**0.26**	**1.07**	**27.2**	**26.1**	++	+++	**3162**	**2803**	**2.21**	**1.38**	**10.0**	**11.9**
100 fg	−	++	557	466	0.09	**0.69**	35.0	35	++	+++	**3102**	**1717**	**1.92**	**1.14**	**10.9**	**15.9**
10 fg	−	++	611	799	0.08	**0.43**	35.0	33.2	++	++	**2035**	609	**2.09**	**1.09**	**13.3**	**20.5**
1 fg	−	+	739	634	0.08	0.15	35.0	35	++	++	965	396	**1.85**	**0.87**	**16.6**	**24.1**
100 ag	−	−	637	608	0.07	0.08	35.0	35	+	+	821	735	**1.41**	**0.74**	**18.3**	**25.3**
10 ag	−	−	683	460	0.07	0.82	35.0	35	−	−	868	871	0.11	0.08	35.0	35.0
1 ag	−	−	737	616	0.08	0.82	35.0	35	−	−	928	911	0.09	0.08	34.2	35.0
10^{-1} ag	−	−	591	578	0.07	0.81	35.0	35	−	−	904	715	0.08	0.07	34.4	32.2
Milli-Q water	−	−	557	465	0.07	0.82	35.0	35	−	−	747	846	0.08	0.07	35.0	35.0

Positive samples are indicated in bold. nt: not tested.

[a]PCR products are visualised by staining with ethidium bromide and illumination by UV (agarose gel). +++: strong band, ++: moderate band, +: weak band, −: no band.

[b]PCR products are visualised by fluorescence signal value (DIAPOPS).

[c]PCR products are visualised by absorbance value at 405 nm (PCR–ELISA).

[d]PCR products are visualised by C_t-value: number of cycles in PCR when fluorescence increases above background values (TAqMan–PCR).

[e]DNA: pure DNA from *P. fragariae*.

[f]pF1: DNA from plasmid pF1.

1 pg of *P. fragariae* DNA (equivalent to ~10 uninucleate zoospores) was detected by single-round PCR and 100 ag by nested PCR (Table 2). The sensitivity of PCR–ELISA and TaqMan™ were equivalent to gel electrophoresis and ethidium bromide gel staining, but DIAPOPS was not as sensitive, requiring 500 pg (single PCR) and 10 fg (nested PCR) for detection. TaqMan™ assays using the reverse primer (MP5) (Table 1), designed to yield smaller (120-bp) amplicons, increased sensitivity over the standard primers, which yielded longer (750-bp) amplicons (data not shown). The nested procedure was 1000–10,000 times more sensitive compared to single-round PCR (Table 2).

Detection of the plasmid (pF1) containing the *P. fragariae* PCR product was also very sensitive: the limit in single PCR was 1 fg (~230 copies of the plasmid) and 100 ag in nested PCR.

Bait test with controlled levels of infection

DNA samples from water and roots from the incubator bait experiment were tested with the same PCR formats as described above. No infection was detected at any time in treatment 1 (healthy roots only), but *P. fragariae* was detected in water samples from treatments 2, 3 and 4 at different time points after the start of the experiment (Table 3). Detection in water generally agreed well with the presence of typical disease symptoms on the bait plants. Only water sample 2-2 was negative whereas corresponding roots of bait plants in soil were positive. The condition of the bait plant, fresh root weight and presence of oospores generally agreed with the results of PCR tests on water samples from the bait tests. However, no symptoms were visible on root sample 4-3 of the bait plant in soil, while corresponding water sample 4-4 was positive. There was little difference in detection efficiency between treatment 2, in which 1 g of diseased roots in the sample was incubated, and treatment 3 with 10 g of diseased roots. Water samples from treatments 2 and 3 tested positive by PCR within a short time after the start of the experiment: 1 h and 1 day for treatments 2 and 3, respectively. The pathogen was also detected in the roots of the floated bait plants (baits 2 and 3: Table 3). The PCR detection efficiency was marginally higher on roots watered with the bait water than in tests directly on the bait water (Table 3).

Similarly, the results of different methods of detecting amplicons agreed well with each other. DIAPOPS again was less sensitive than the other methods. Taqman™ and PCR–ELISA were slightly more sensitive than gel electrophoresis. No positive samples were identified with single-round PCR (data not shown).

Molecular Beacon™ probe

A Molecular Beacon™ is an oligonucleotide probe, with a central region complementary to the target amplicon and a 6–7 bp sequence (one endlabelled with a quencher and the other with a fluorescent dye) that complement each other at the 3′ and 5′ ends. Such a probe forms a stem-loop structure in which fluorescence is prevented by the close proximity of the 3′ and 5′ ends. The parting of the quencher and fluorescent tags that occurs when the probe binds to the target PCR product results in an increase in fluorescence during PCR and thus quantification (Tyagi and Kramer, 1996).

A Molecular Beacon™ probe (Table 1) was developed to detect *P. fragariae* PCR amplicons in a quantitative manner similar to TaqMan™. The real-time fluorescence measurements (Figure 2) indicate that *P. fragariae* was detected in DNA samples using the Molecular Beacon™ probe. A plot of C_t-value against log *P. fragariae* genomic DNA concentration was linear over four orders of magnitude from 100 ag to 1 pg (Figure 3). In a comparative study, the sensitivities of the Molecular Beacon™ and the TaqMan™ probe against a dilution series of *P. fragariae* genomic DNA were similar (Figure 3). Molecular Beacons™ were studied in more detail because of their low cost and because they are not degraded by the 5′-nuclease activity of Taq polymerase.

Use of Molecular Beacon™ probe against experimental samples and zoospores

The Molecular Beacon™ probe detected amplicons in samples with as little as 100 ag of genomic DNA and as few as 25 zoospores of *P. fragariae* in nested PCR (Table 4). The pathogen was also detected in DNA from water and root samples (Table 4) from the bait test with controlled levels of infection. *P. fragariae* was detected with Molecular Beacon™ probes in single-round and nested PCR with C_t values comparable to those obtained using TaqMan™ (Table 3). As before, nested PCR was more sensitive.

Direct versus *indirect detection*

Results of nested PCR (primer pairs DC6/ITS4 and DC1/B5) on DNA extracted directly from roots and

Table 3. Results of morphological and nested-PCR tests in various formats for *P. fragariae* in water and strawberry root samples from bait plant experiments

Treatment[a]	Time (days) from start of expt.	Bait plant in soil at end of test			Nested PCR on							
		Condition[b]	Roots[c] (g)	Red stele[d]/ oospores[e]	Water from bait tests				Roots from bait plant in soil and inoculated with 50 ml water from bait tests			
					PCR format				PCR format			
					Agarose gel[f]	DIAPOPS fluoresc.[g]	PCR–ELISA A^{h}_{405}	TaqMan C_t-value[i]	Agarose gel[f]	DIAPOPS fluoresc.[g]	PCR–ELISA A^{h}_{405}	TaqMan C_t-value[i]
1-0	1 h	+	27.0	−/−	−	416	0.11	35.0	−	516	0.085	34.2
1-1	1 d	+	25.0	−/−	−	396	0.08	35.0	−	442	0.094	32.9
1-2	7 d	−	35.0	−/−	−	462	0.08	35.0	−	450	0.083	33.6
1-3	14 d	−	28.0	−/−	−	416	0.08	35.0	−	362	0.089	33.6
1-4	35 d	−	30.0	−/−	−	291	0.19	35.0	−	332	0.094	32.9
2-0	1 h	+	13.5	+/+	++	312	**1.58**	**24.5**	−	**2433**	**1.982**	**11.3**
2-1	1 d	++	7.5	+/++	++	**2035**	**2.08**	**21.3**	+++	**819**	**2.189**	**13.4**
2-2	7 d	++	9.0	+/++	−	300	0.08	35.0	−	360	**1.838**	**22.2**
2-3	14 d	+	26.0	+/++	+++	**1676**	**2.11**	**19.1**	+++	**1064**	**1.807**	**13.2**
2-4	35 d	+	33.0	+/−	+++	504	**1.18**	**17.9**	+++	**681**	**1.826**	**14.0**
3-0	1 h	−	42.0	−/−	−	464	0.09	35.0	−	337	**0.373**	30.4
3-1	1 d	++	5.5	++/++	++	427	**1.61**	**21.5**	+++	**3142**	**1.929**	**11.3**
3-2	7 d	++	18.0	++/++	+++	**1387**	**1.99**	**19.2**	+++	**1851**	**2.247**	**11.6**
3-3	14 d	±	42.0	+/−	+++	**1020**	**1.86**	**19.4**	+++	**1887**	**2.421**	**11.4**
3-4	35 d	−	52.0	−/−	−	345	0.08	35.0	−	349	0.100	32.6
4-0	1 h	−	54.0	−/−	−	358	0.09	35.0	−	468	0.130	**18.1**
4-1	1 d	±	40.0	−/−	−	334	0.08	35.0	−	499	0.100	33.5
4-2	7 d	++	14.0	+/−	++	**1365**	**1.25**	**25.0**	+++	**526**	**1.801**	**18.4**
4-3	14 d	−	60.0	−/−	++	**1086**	**1.13**	**25.3**	−	513	**0.219**	30.7
4-4	35 d	−	46.0	−/−	−	591	0.10	35.0	−	337	0.077	34.3
Bait 1 in water		±	ND	−/−					−	338	0.082	34.0
Bait 2 in water		++	ND	++/++					+++	539	**2.004**	**14.0**
Bait 3 in water		++	ND	++/++					+	**839**	**1.981**	**12.6**
Positive control					+++	**1946**	**2.13**	**11.0**	+++	**2379**	**1.914**	**21.0**
Negative control					−	288	0.07	35.0	−	486	0.078	35.0

Positive samples are indicated in bold.

[a]Treatment (1) 10 g of healthy roots; (2) 9 g of healthy and 1 g of diseased roots; (3,4) 10 g of diseased roots. (1,2,3) with bait plant on top of bucket; (4) without bait plant on top of bucket. [b]Condition of bait plant in soil: −: healthy plant; ±: slight wilting; +: moderate wilting; ++: severe wilting.

[c]Roots (g): fresh weight of roots from bait plant grown in soil. ND: not determined.

[d]Red stele: −: no red cylinder visible; +: faint red cylinder visible; ++: clear red cylinder visible.

[e]Oospore: −: no oospores visible; +: few oospores visible; ++: many oospores visible.

[f]PCR products are visualised by staining with ethidium bromide and illumination by UV (agarose gel). −: no band; +: weak band; ++: moderate band; +++: strong band.

[g]PCR products are visualised by fluorescence signal value (DIAPOPS).

[h]PCR products are visualised by absorbance value at 405 nm (PCR–ELISA).

[i]PCR products are visualised by C_t-value: number of cycles in PCR when fluorescence increases above background values (TaqMan–PCR).

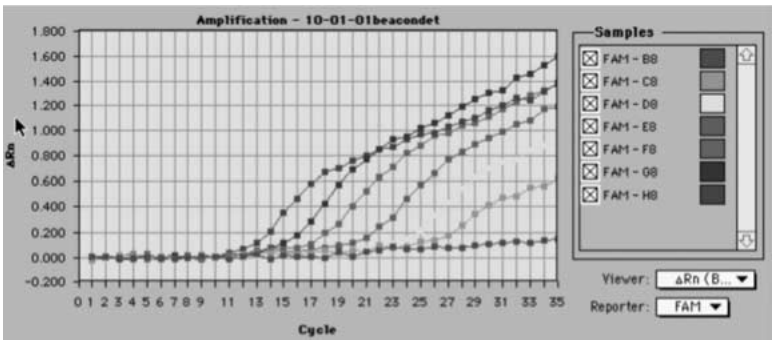

Figure 2. Real-time fluorescence with Molecular Beacon™ during PCR for six random DNA samples of *P. fragariae*.

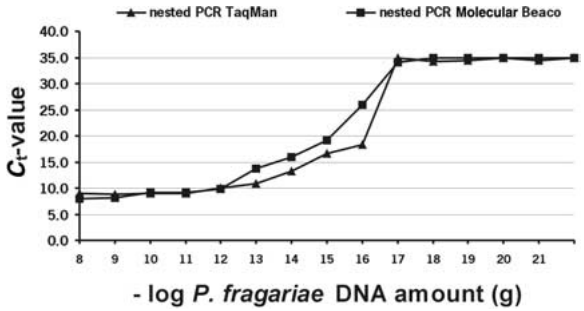

Figure 3. Results of a comparison study between TaqMan™ and Molecular Beacon™ in nested PCR for a dilution series of *P. fragariae* DNA. Plot of C_t-value *versus* log template concentration. C_t-value = number of cycles in PCR when fluorescence increases above background values.

from water, in which roots were incubated with bait plants, were similar, but not identical (Table 5). For one sample (no. 652) the Naktuinbouw bait test was positive but both PCR tests were negative. In another sample (no. 672) the water bait PCR test proved positive when all other results were negative.

Water and root samples were also investigated for the presence of *Phytophthora cactorum*, which causes strawberry crown rot, an increasingly important disease in Europe. Several positive samples were found using *P. cactorum* primers (data not shown). In contrast, samples positive in the first-round PCR directly on DNA extracted from roots were not always positive in the second round, in which the specific *P. fragariae* primers were used. Given the universality of the first-round primers (DC6/ITS4) for Peronosporales (Bonants et al., 1997), it is likely that they were amplifying DNA from other *Phytophthora* or *Pythium* species.

Internal control for (nested) PCR

By fusing the inner primers with the outer primer sequence, the developed internal control can be used in nested PCR. The results of an experiment in which these primers were used with zoospores and root and water samples (Figure 4) show the larger internal control fragment (890 bp) easily distinguishable from the 750-bp *P. fragariae* fragment in positive samples. In negative samples, only the internal control was present (Figure 4, lanes 2–4). Failure of amplification due to inhibition of the PCR reaction results in no amplification of the internal control (Figure 4, lane 17). Dilution of the same template resulted in a positive PCR reaction (Figure 4, lane 16).

Discussion

Diagnostic tests based on molecular biology have been developed for many plant pathogens. Usually, this involves testing the specificity and sensitivity of the tests under optimised laboratory conditions and some testing on infected plant material. Unfortunately, that is as far as many tests progress; they remain as research tools rather than being utilised effectively by the agricultural/horticultural industry. This study aimed to develop the existing red core diagnosis based on bait plants grown in mixtures of roots and compost (Duncan, 1980), into a more rapid and simple test suitable for practical plant health monitoring.

Phytophthora fragariae var. *fragariae* is present in most European countries (Smith et al., 1997). Nevertheless, as an EPPO 'A2' quarantine organism, its further spread must be prevented (Council Directive 2000/29 EC). Because infected planting material

Table 4. Results of PCR for the detection of *P. fragariae* with Molecular Beacon™ for several DNA samples

Target	Sample	C_t-value[a]		Target	Sample	C_t-value[a]	
		Single-round PCR	Nested PCR			Single-round PCR	Nested PCR
DNA (amount)	10 ng	15	ND	Water (number)	3-2	35	11.9
	1 ng	16.6	ND		3-3	35	23.6
	100 pg	18.7	10		3-4	35	23.4
	10 pg	22.1	9.7		4-0	35	33.8
	1 pg	24.7	10.4		4-1	35	32
	100 fg	27.7	11.3		4-2	35	15.1
	10 fg	ND	13.2		4-3	35	35
	1 fg	ND	19		4-4	35	35
	100 ag	ND	26				
	10 ag	ND	34	Root of bait plant in soil (number)	1-3	ND	35
	1 ag	ND	35		2-0	24.6	10.3
					2-1	33	12.1
Zoospore-suspension (number)	25000	27.2	11.9		2-2	35	19.5
	2500	35	17		2-3	31.5	13.2
	250	ND	16.7		2-4	32.9	14.6
	25	ND	18.7		3-0	35	20.4
	2.5	ND	35		3-1	26.1	11
	0.25	ND	35		3-2	25.8	11.3
					3-3	25.9	10.2
Water (number)	1-3	ND	35		3-4	35	35
	2-0	35	20.3		4-0	35	34.4
	2-1	35	15.2		4-1	35	35
	2-2	35	19		4-2	30.2	17.1
	2-3	35	28.6		4-3	34.8	21.5
	2-4	35	29.3		4-4	35	35
	3-0	35	26				
	3-1	35	13.3	Water control		35	35

[a] C_t-value = number of cycles in PCR when fluorescence increases above background values. The codes of water and root samples 2-0, 2-1, 2-2, 2-3, 2-4, etc, refer to the samples used in bait test with controlled levels of infection (Table 3) and time points −0 (1 h), −1 (1 d), −2 (7 d), −3 (14 d) and −4 (35 d), respectively.
ND: not determined.

is the principal means of long-distance dispersal, there is a clear need for a sensitive and specific test to detect low levels of this pathogen in plants, particularly in roots. Speed is also essential to meet the exigencies of national and international trade in plants that supply an annual requirement of 500–750 million new plants per annum (J.M. Duncan, unpubl. estimate). Many, if not most, of these strawberry plants are not produced in the country where they will be planted. For example, a large proportion of the runners planted in the European Union comes from countries that are not yet members of the EU, e.g., Poland and Hungary.

Nested PCR was consistently more sensitive than single-round PCR and is therefore recommended. In the second round of nested PCR, DC1 should always be used as the forward primer, for it determines the specificity of the reaction (Bonants et al., 1997). The use of other primers depends on the PCR format and the method of amplicon detection. The sensitivity of traditional gel electrophoresis and staining with ethidium bromide was very good and similar to PCR–ELISA, TaqMan™ and Molecular Beacon™. However, the latter methods are less hazardous and amenable to automation and, thus, increased throughput. DIAPOPS was less sensitive than all other formats tested with a poor correlation between the final fluorescence readings and the amount of template DNA. Perhaps the amplicon was formed in solution and was not consistently bound to the wall of the microtitre plate well. A drawback of PCR–ELISA is the need to transfer samples to a microtitre plate, which slows the procedure. TaqMan™ and Molecular Beacon™ worked well with

Table 5. Results of a comparison study between direct and indirect PCR detection for the presence of *P. fragariae* on strawberry root samples

Sample no.	Bait test Naktuinbouw Red stele/ oospores[a]	Direct PCR, Root (SCRI)		Indirect PCR Water (PRI)
		First-round[b] agarose gel[c]	Second-round[b] agarose gel[c]	Second-round[b] agarose gel[c]
651	−	−	−	−
652	+	−	−	−
653	+	+++	+++	+++
654	−	−	−	−
655	−	+	−	−
656	−	−	−	−
657	−	−	−	−
658	−	−	−	−
659	−	−	−	−
660	−	−	−	−
661	−	−	−	−
662	+	+++	+++	+++
663	−	−	−	−
664	−	++	−	−
665	−	+	−	−
666	−	++	−	−
667	−	−	−	−
668	−	−	−	−
669	−	++	−	−
670	−	−	−	−
671	−	+	−	−
672	−	−	−	+
673	+	+++	+++	+++
674	−	−	−	−
675	−	+	−	−
Negative control		−	−	−
Positive control		+++	+++	+++

Roots were split in two. PCRs were performed on DNA from half of the roots at SCRI. Bait tests were performed at Naktuinbouw on the other half of the roots. PCRs were performed on DNA from water samples from the same bait tests at PRI.

[a]Red stele/oospores: −: no red cylinder and oospores visible; +: clear red cylinder and many oospores visible.

[b]First/second-round PCR: Universal primers for first-round PCR were: DC6/ITS4; *P. fragariae*-specific primers for second-round PCR were: DC1/B5.

[c]Agarose gel: staining with ethidium bromide and UV illumination, +++: strong band, ++: moderate band, +: weak band, −: no band.

the original longer (750-bp) amplicons but sensitivity was consistently improved with the reverse primers (MP3/MP5) designed to yield shorter (120-bp) amplicons. Another advantage of using a detection probe is that it matches the sequence in *P. fragariae* DNA, thus providing a safeguard against any non-specific amplification. However, such amplification has never been observed with second-round primers.

To date, Molecular Beacon probes have been used most frequently for detecting single-stranded (ss) DNA or ssRNA and rarely for double-stranded DNA. Although in PCR there is competition between the complementary strand target and Molecular Beacon™, our work demonstrates that Molecular Beacon™ can give results as good as TaqMan™ in PCR. Both TaqMan™ and Molecular Beacon™ offer 'real-time' measurements in a closed-tube system on the ABI 7700. Both can quantify the target DNA with C_t values giving a good indication of the amount of *P. fragariae* present. Quantification may not be important for *P. fragariae* with its 'nil tolerance', but it will be of importance if a diagnostic test based on PCR (PCRD)

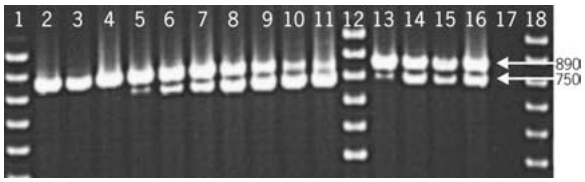

Figure 4. Nested-PCR results of several DNA samples and internal control DNA as template. Lanes 1, 12, 18: MW marker; lane 2: MQ water; lanes 3–11: dilution series of *P. fragariae* DNA (1 ag–100 pg in increments of 10×); lanes 13, 14: 25 and 25,000 zoospores of *P. fragariae*; lane 15: water sample 2-1; lane 16, root sample 2-1 diluted 1 : 20; lane 17, root sample 2-1 (undiluted). The codes of water and root samples 2-1, refer to the samples used in the bait test with controlled levels of infection (Table 3) and time points −1 (1 day).

for *P. cactorum* (Cooke and Duncan, pers. comm.) is to be developed further. This damaging and increasingly important pathogen is tolerated at low levels in strawberry propagation; typically 0.5–1% of plants have visible symptoms. Plants with visible symptoms may represent a minority of all infected plants and currently there is really no satisfactory test for this disease at such levels. PCR could provide an effective method of detection but under present rules it would have to be quantitative, a definite advantage of TaqMan™ or Molecular Beacon™ formats.

In numerous single-round or nested PCR experiments, the specificity of primers DC1 and B5 for *P. fragariae* has been confirmed (Bonants et al., 1997; Lacourt et al., 1997); no amplification of DNA from any *Phytophthora* species other than *P. fragariae* was demonstrated (Bonants et al., 1997). Therefore, the nested PCR has the necessary properties to fulfil the requirements of an effective detection system.

The original Duncan bait test is very sensitive; it consistently detected <1% infected roots among healthy roots (Duncan, 1980) and probably can detect even lower levels, because 10 zoospores (less than the number produced by one sporangium) were enough to give clear symptoms on bait plants within 5 weeks of inoculation (Duncan and Kennedy, 1994). It is also quite specific in that the symptoms and signs, red steles and oospores, seen on the plants are very characteristic of infection by *P. fragariae*; isolations from bait plants showing such symptoms consistently yielded typical cultures of *P. fragariae* (Duncan, 1980, 1985; Duncan and Kennedy, 1994). However, in its existing format, the process is too lengthy (5–6 weeks) for modern commerce. The diagnostic test based on PCR (a PCRD) shown in this study is a more rapid alternative to Duncan's bait test while still maintaining its sensitivity and specificity. In this study, nested PCR, in various formats, detected 100 ag (10^{-16} g) of pure DNA of *P. fragariae*. This is equivalent to ∼1/60 part of one nucleus, assuming that the genome size of *P. fragariae* is similar to that of the closely related *Phytophthora sojae*, ∼62 Mb (Mao and Tyler, 1991). Such sensitivity is possible because rDNA is a multicopy gene (Russel et al., 1984). In practice, nested PCR has consistently detected between 5 and 10 zoospores of the pathogen (Bonants et al., 1997; Duncan, unpubl.), similar to the number required for infection of bait plants (Duncan and Kennedy, 1994). Sensitivity for detection of other *Phytophthora* species ranged from 100 to 200 pg for *P. citrophthora* (Ersek et al. 1994), 10 pg for *P. infestans* (Wangsoonboondee and Ristaino, 2002) to pg quantities for *P. citricola*, *P. cambivora* and *P. quercina* (Schubert et al., 1999) and even down to femtogram quantities for *P. infestans* (Judelson and Tooley, 2000) depending on which multi- or single-copy sequences were used and/or nested or single PCR was performed. Femtogram quantities was more or less in the same range as we observed for *P. fragariae*.

Why then consider the more time consuming and expensive combination of a bait testing and PCR? In essence, a bait test is a biological amplification (growth of the pathogen on the host) and PCR is a biochemical amplification. Combining them should increase the sensitivity and prove faster than bait testing alone, which was indeed shown in our experiments. Bait tests are also more appropriate for larger samples of roots, an important consideration when detection of a low level of disease is being sought in samples collected from a large field. To test more material, the number of plant pots (for soil baiting) or containers (for water baiting as in this paper) could simply be increased accordingly. Similarly, more water can be collected from the water bait containers with subsequent centrifugation to concentrate fungal propagules. The growth of the pathogen on the roots of the bait plants will be followed by the release of zoospores into the surrounding water within days rather than weeks, from where they can be concentrated easily by filtration or centrifugation. Preliminary experiments (Duncan, unpubl.) have shown that concentrating zoospores by filtration is possible and that pieces of the filter can be used as the template in PCR. In contrast, although techniques have been developed for extracting DNA directly from large amounts of roots, as yet only relatively small amounts of that DNA

can be tested in PCR. Moreover, DNA extraction from water is simpler than from roots and less prone to failure as a result of PCR inhibition. Wangsoonboondee and Ristaino (2002) described several DNA extraction methods to be used for different kind of propagules of *P. infestans*. Similar methods could be incorporated for *P. fragariae* as well and enhance the described method even more. The value of including a bait plant to amplify the infection is evidenced by the lower frequency of detection and lower amounts of fungal DNA in infected samples without a bait plant.

Results of direct testing (PCR of DNA extracted directly from the root sample under test) were comparable to those obtained by indirect detection (PCR of DNA extracted from water from bait tests) of *P. fragariae*. Such an approach allows the same DNA sample to be used for a *P. cactorum* test. However, where large numbers of root samples require testing, the manual grinding of root material in liquid nitrogen makes the DNA extraction process a lengthy one with the risk of cross-contamination via mortar and pestles. This problem might be overcome by using robots and simpler extraction procedures but until it is, a bait test in water followed by PCR detection in water samples is the favoured approach. Using PCR we detected the pathogen in water samples from 1 to 14 days after start of the bait experiments, meaning that the duration of bait tests can be shortened to meet the practical requirements of commercial trade. Nevertheless, it may be advisable to wait 14 days before performing PCR. Unless infection in the root samples is in an active mycelial form, the fungus will exist as oospores. These must be induced to germinate and form zoosporangia and release zoospores within this period. It would be valuable to have at least one round of secondary zoosporangia and infection on the roots of the baits before attempting detection by PCR. One possible limitation of this type of analysis is that zoospore release into the bait water probably occurs in phases and the timing of sampling is therefore critical (Duncan, unpubl.). Sampling at regular intervals and pooling of samples for a PCR test after 14 days is therefore a safer approach.

In the incubator bait test, there was no difference in the efficiency of detection between 1 and 10 g of diseased roots. Bait tests were repeated many times in different set ups showing that much lower levels of infection ($<1\%$) have been detected consistently by PCR in water from those bait tests (Duncan, unpubl.).

To be used in official testing schemes, PCRDs will require appropriate controls. Controls that protect against false positives and negatives, particularly the latter will be important. An internal control, containing the sequences of all the primers used in nested PCR, has provided an important check for inhibition of the PCR reaction. Its use should highlight any false negatives. Other simple procedures can improve PCR results. Dilution of the template can alleviate PCR inhibition but may also result in a loss of sensitivity. Using PVPP columns to remove inhibitors from DNA extracts is a good alternative (unpubl. results) as well as silica-based column chromatography. Combining several water or root samples would increase the chance to detect the pathogen.

The storage and condition of infected root material before testing was not investigated in this paper. However, oospores lose germinability and viability at temperatures above 20 °C (Duncan, 1985), so it is advisable to store roots at refrigerator temperatures before processing. Interestingly, temperature and duration of storage did not affect direct detection in roots by PCR. The fungus was readily detectable after 12 weeks of storage at all temperatures tested from 0 to 20 °C (Duncan and Guy, SCRI, unpubl.), even though the roots held at the higher temperatures dried out during storage. However, the tests were carried out directly on root DNA and the oospores did not have to be viable to be detected. In contrast, for the combined bait/PCR test to work, oospores must germinate; therefore, good storage conditions for roots are essential.

Acknowledgements

This work has been performed as part of the European Union Standards, Measurement and Testing Project SMT-PL96-1321 'Development of Diagnostic Protocols for Red Core Disease of strawberry (*P. fragariae*)' – short title 'REDCORE', also as part of DWK program 337 of the Dutch Ministry of Agriculture, Nature Management and Fisheries, and with the support of the Scottish Executive Environment and Rural Affairs Department and Horticultural Development Council.

The authors also wish to thank Dr. Henrik Rasmussen and co-workers (Nunc A/S, Roskilde) for their help with the DIAPOPS analysis.

References

Anonymous (1994) Certification scheme for strawberry. Bulletin OEPP/EPPO Bulletin 24: 857–889

Böhm J, Hahn A, Schubert R, Bahnweg G, Adler N, Nechwatal J, Oehlmann R and Oßwald W (1999) Real-time quantitative PCR: DNA determination in isolated spores of the mycorrhizal fungus *Glomus mossae* and monitoring of *Phytophthora infestans* and *Phytophthora citricola* in their respective host plants. Journal of Phytopathology 147: 409–416

Bonants PJM, Hagenaar-de Weerdt M, van Gent-Pelzer MPE, Lacourt I, Cooke DEL and Duncan JM (1997) Detection and identification of *Phytophthora fragariae* Hickman by the polymerase chain reaction. European Journal of Plant Pathology 103: 345–355

Brandt ME, Padhye AA, Mayer LW and Holloway BP (1998) Utility of random amplified polymorphic DNA PCR and TaqMan automated detection in molecular identification of *Aspergillus fumigatus*. Journal of Clinical Microbiology 36: 2057–2062

Casademont I, Bizet C, Chevrier D and Guesdon JL (2000) Rapid detection of *Campylobacter fetus* by polymerase chain reaction combined with non-radioactive hybridization using an oligonucleotide covalently bound to microwells. Molecular and Cellular Probes 14: 233–240

Chevrier D, Rasmussen SR and Guesdon JL (1993) PCR product quantification by non-radioactive hybridization procedures using an oligonucleotide covalently bound to microwells. Molecular and Cellular Probes 7: 187–197

Cooke DEL and Duncan JM (1997) Phylogenetic analysis of *Phytophthora* species based on ITS1 and ITS2 sequences of the ribosomal RNA gene repeat. Mycological Research 101: 667–677

Courtney BC, Smith MM and Henchal EA (1999) Development of internal controls for probe-based nucleic acid diagnostic assays. Analytical Biochemistry 270: 249–256

De Boer SH, Ward LJ, Li X and Chittaranjan S (1995) Attenuation of PCR inhibition in the presence of plant compounds by addition of BLOTTO. Nucleic Acids Research 23: 2567–2568

Duncan JM (1980) A technique for detecting red stele (*Phytophthora fragariae*) infection of strawberry stocks before planting. Plant Disease 64: 1023–1025

Duncan JM (1985) Effect of temperature and other factors on *in vitro* germination of *Phytophthora fragariae* oospores. Transactions of the British Mycological Society 85: 455–462

Duncan JM, Fordyce W, Harper PC and Rankin PA (1985) Eliminating red core (*Phytophthora fragariae*) from Scottish certified stock strawberries. Research and Development in Agriculture 3: 43–46

Duncan JM and Kennedy DM (1994) Effect of temperature and host genotype on the production of inoculum by *Phytophthora fragariae* var. *fragariae* from the roots of infected strawberry plants. Plant Pathology 44: 10–21

Ersek T, Schoelz JE and English JT (1994) PCR amplification of species-specific DNA sequences can distinguish among *Phytophthora* species. Applied and Environmental Microbiology 60: 2616–2621

Giesendorf BAJ, Vet JAM, Tyagi S, Mensink EJMG, Trijbels FJM and Blom HJ (1998) Molecular beacons: A new approach for semiautomated mutation analysis. Clinical Chemistry 44: 482–486

Grimm C and Geisen R (1998) A PCR–ELISA for the detection of potential fumonisin producing *Fusarium* species. Letters in Applied Microbiology 26: 456–462

Jobes DV, Hurley DL and Thien LB (1995) Plant DNA isolation: A method to efficiently remove polyphenolics, polysaccharides, and RNA. Taxon 44: 379–386

Judelson HS and Tooley PW (2000) Enhanced polymerase chain reaction methods for detecting and quantifying *Phytophthora infestans* in plants. Phytopathology 90: 1112–1119

Lacourt I, Bonants PJM, van Gent-Pelzer MPE, Cooke DEL, Hagenaar-de Weerdt M, Surplus L and Duncan JM (1997) The use of nested primers in the polymerase chain reaction for the detection of *Phytophthora fragariae* and *P. cactorum* in strawberry. Proceedings of the third International Strawberry Symposium, Veldhoven, Netherlands, 29 April–4 May, 1996. Van der Scheer HAT, Lieten F and Dijkstra J (eds), Acta Horticul. 439: 829–838

Leone G, van Schijndel H, van Gemen B, Kramer FR and Schoen CD (1998) Molecular beacon probes combined with amplification by NASBA enable homogeneous, real-time detection of RNA. Nucleic Acids Research 26: 2150–2155

Livak KJ, Flood SJA, Marmaro J, Giusti W and Deetz K (1995) Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. PCR Methods and Applications 4: 357–362

Loffler J, Hebart H, Sepe S, Schumacher U, Klingebiel T and Einsele H (1998) Detection of PCR-amplified fungal DNA by using a PCR-ELISA system. Medical Mycology 36: 275–279

Mao Y and Tyler B (1991) Genome organization of *Phytophthora megasperma* f. sp. *glycinea*. Experimental Mycology 15: 283–293

Marras SAE, Kramer FR and Tyagi S (1999) Multiplex detection of single-nucleotide variations using molecular beacons. Genetic Analysis of Biomolecular Engineering 14: 151–156

Nielsen SL, Husted K and Rasmussen HN (1998) Detection of potato potyvirus Y in dormant tubers by a PCR-based method DIAPOPS (detection of immobilised amplified product in a one phase system). Fifteenth Danish Plant Protection Conference. Pests and Diseases. DJF-Rapport, Markbrug. No. 3: 73–80

Niepold F and Schober-Butin B (1997) Application of the one-tube PCR technique in combination with a fast DNA extraction procedure for detecting *Phytophthora infestans* in infected potato tubers. Microbiological Research 152: 345–351

Niessen ML, Klusmann J and Vogel RF (1997) Quantitative estimation of *Fusarium graminearum* using a novel solid phase PCR-assay. In: Dehne HW, Adam G, Diekmann M, Frahm J, Mauler-Machnik A and van Halteren P (eds), *Diagnosis and Identification of Plant Pathogens*. Proceedings of the Fourth International Symposium of the European Foundation for Plant Pathology, Bonn, Germany, 9–12 September 1996. Kluwer Academic Publishers, Dordrecht, the Netherlands, pp. 207–211

Rasmussen SR, Rasmussen HB, Larsen MR, Hoff-Jorgensen R and Cano RJ, 1994, Combined polymerase chain reaction-hybridization microplate assay used to detect Bovine Leukemia Virus and *Salmonella*. Clinical Chemistry 40: 200–205

Ristaino JB, Madritch M, Trout CL and Parra G (1998) PCR amplification of ribosomal DNA for species identification

in the plant pathogen genus *Phytophthora*. Applied and Environmental Microbiology 64: 948–954

Russel PJ, Wagner S, Rodland KD, Feinbaum RL, Russel JP, Bret-Harte MS, Free SJ and Metzenberg RL (1984) Organisation of the ribosomal ribonucleic acid genes in various wild-type strains and wild-collected strains of *Neurospora*. Molecular and General Genetics 196: 275–282

Sambrook J, Fritsch EF and Maniatis T (eds) (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. CSHL Press, Cold Spring Harbor, New York

Schubert R, Bahnweg G, Nechwatal J, Jung T, Cooke DEL, Duncan JM, Müller-Starck G, Langebartels C, Sandermann H Jr and Oßwald W (1999) Detection and quantification of *Phytophthora* species which are associated with root-rot diseases in European deciduous forests by species specific polymerase chain reaction. European Journal of Forest Pathology 29: 169–187

Smith IM, McNamara DG, Scott PR and Holderness M (eds) (1997) *Quarantine Pests for Europe*, 2nd edn. CAB International (in association with EPPO), Wallingford Oxon, p. 1425

Stark KD, Nicolet Y and Frey Y (1998) Detection of *Mycoplasma hypopneumoniae* by air sampling with a nested-PCR assay. Applied and Environmental Microbiology 63: 543–548

Tyagi S and Kramer FR (1996) Molecular beacons: Probes that fluoresce upon hybridization. Nature Biotechnology 14: 303–308

Wangsomboondee T and Ristaino JB (2002) Optimization of sample size and DNA extraction methods to improve PCR detection of different propagules of *Phytophthora infestans*. Plant Disease 86: 247–253

White TJ, Bruns T, Lee SB and Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ and White TJ (eds), *PCR Protocols: A Guide to Methods and Applications*. Academic Press, San Diego, pp. 315–322

Zhang AW, Hartman GL, Curio-Penny B, Pedersen WL and Becker KB (1999) Molecular detection of *Diaporthe phaseolorum* and *Phomopsis longicolla* from soybean seeds. Phytopathology 89: 796–804